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The interaction of adenosine-5'-monophosphate and fructose 2,6-bisphosphate with bovine liver fructose 1,6-bisphosphatase

Nancy Joan Ganson
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THE INTERACTION OF ADENOSINE-5'-MONOPHOSPHATE AND FRUCTOSE 2,6-BISPHOSPHATE WITH BOVINE LIVER FRUCTOSE 1,6-BISPHOSPHATASE

Iowa State University

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The interaction of adenosine-5'-monophosphate and fructose 2,6-bisphosphate with bovine liver fructose 1,6-bisphosphatase

by

Nancy Joan Ganson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of
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Iowa State University
Ames, Iowa
1985
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INTRODUCTION

Statement of the Problem

The discovery of fructose 2,6-bisphosphate (2,6FBP), an important regulator of glycolysis and gluconeogenesis, was reported in 1980 (1). Soon thereafter, 2,6FBP was found to be a potent inhibitor of fructose 1,6-bisphosphatase (EC 3.1.3.11) (FBPase), an enzyme important in the regulation of the gluconeogenesis pathway (2,3). Though numerous studies of the interaction of 2,6FBP and FBPase have appeared since the discovery of the regulator, the exact mechanism of inhibition is unclear.

This dissertation describes studies performed in order to elucidate the mode of interaction of 2,6FBP with bovine liver FBPase. The problem is approached primarily by using a nuclear magnetic resonance (NMR) method to calculate the distance from Mn²⁺ bound at the active site of FBPase to the phosphorus nuclei of a number of enzyme ligands known to bind at the active site of the enzyme. A comparison of these values with the analogous measurements for 2,6FBP will give an indication of the site of action of the regulator; that is, if the Mn²⁺-phosphorus distances obtained for 2,6FBP disagree with the measurements obtained for the active site ligands, then the regulator must occupy an allosteric site on the enzyme. However, an agreement between the appropriate distances will indicate that 2,6FBP interacts with FBPase at the same site as the other ligands; that is, the active site.
The NMR method used in these studies has been the subject of several reviews (4-6). Basically, the technique relates the relaxing effect a paramagnetic ion exerts on nearby nuclei to the distance between the paramagnetic center and the nucleus experiencing enhanced relaxation. The method provides a unique means to study the conformation and arrangement of individual atoms of a ligand bound to a macromolecule in solution.

Additional information on the interaction of 2,6FBP with FBPase is obtained by using classical kinetic techniques. The reverse (nonphysiological) reaction of FBPase can be monitored by using the appropriate coupling system (7). By observing the inhibition patterns produced by 2,6FBP in the reverse FBPase reaction, information on the site(s) of action of the inhibitor is obtained, certain kinetic constants are calculated, and knowledge of the kinetic mechanism of the enzyme is gained.

Finally we proposed to investigate the apparent synergism of the inhibition of FBPase activity which is observed with 2,6FBP and 5'AMP, an allosteric regulator of the enzyme (2,3). This problem was approached by using a method which monitors the binding of ligands to the enzyme, and the NMR method previously described.

To reiterate, studies were undertaken to clarify the mechanism of 2,6FBP inhibition of FBPase, with particular emphasis on determining whether the inhibitor acts at the active site of the enzyme, or at an allosteric site. The problem was approached by using an NMR method to map the active site of FBPase, and by using classical kinetic tech-
niques. In addition, the mechanism of inhibition of FBPase activity resulting from the simultaneous presence of 5'AMP and 2,6FBP is investigated by using both the NMR method and binding studies.

Literature Review of Fructose 1,6-Bisphosphatase

During early studies of glycolysis, it was noted that mammalian tissues could hydrolyze D-fructose 1,6-bisphosphate (FBP). Later, in 1943, Gomori (8) described the specific phosphatase which catalyzed the following reaction in the presence of Mg\(^{2+}\): D-fructose 1,6-bisphosphate + H\(_2\)O + D-fructose 6-phosphate + P\(_i\). It has since become quite apparent that the enzyme, fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose 1,6-bisphosphate 1-phosphohydrolase; FBPase), plays a key catalytic role in the gluconeogenic pathway, as well as functioning to regulate the futile cycle between FBP synthesis and degradation. There have been several recent reviews pertaining to the mechanism and regulation of FBPase (9-11).

FBPase has been isolated from a number of mammalian tissues, including rabbit liver (12), muscle (13), kidney (14), and intestine (15), as well as from bovine liver (16). The enzyme has also been reported in a number of bacteria, fungi, and plants; however, these FBPases have little in common with the mammalian enzyme (11).

Of all the FBPases isolated, the mammalian liver enzyme has been the subject of the most extensive studies over the past few decades; unfortunately, much of the work executed before 1971 was performed by using proteolytically altered rabbit liver FBPase, which has an alkaline
pH optimum for activity. More recent purification procedures produce an enzyme with a neutral pH optimum for activity.

The unproteolyzed mammalian FBPases seem to be very similar in molecular weight and subunit composition. For instance, FBPase from rabbit liver has a molecular weight of 140,000 (17) whereas the bovine liver enzyme has a molecular weight of 130,000 (16). Sodium dodecyl sulfate gel electrophoresis of mammalian FBPases from a number of sources has shown that the enzyme is a tetramer composed of four identical subunits with a molecular weight of approximately 35,000 (9). Using the technique of band centrifugation, Kolb has shown that the tetrameric form of a number of mammalian FBPases is essential for catalysis (18). However, FBPases from bovine (19) and rabbit (20) liver are reported to dissociate quite readily into the inactive subunits at low pH.

A comparison of the amino acid compositions determined for a number of mammalian FBPases, including the bovine liver enzyme indicates a large degree of homology among the enzymes from different species (19). A comparison of the amino acid composition of FBPases from different rabbit tissues indicates that the rabbit liver, intestinal and kidney enzymes are identical, though the muscle enzyme differs slightly in composition (15,21). Also, FBPases from beef, pig, rat, and sheep liver have been shown to be immunologically different from rabbit liver FBPase (22).

The bovine liver FBPase is unique with respect to amino acid composition, as it is reported to contain one mole of tryptophan per
mole of FBPase subunit, while this amino acid is absent from other mammalian FBPases (23).

The amino acid sequence has been determined for several fragments of rabbit liver FBPase (24-26). Horecker and co-workers have reported the sequences of the first 78 residues of the NH2-terminus of the enzyme (24,25) as well as the final 88 residues of the COOH-terminus (26). In addition, lysine residues near the active and AMP allosteric sites of the rabbit liver FBPase were tagged with pyridoxal 5'-phosphate, and short segments about these sites were sequenced (27,28).

Recently, the entire sequence of pig kidney FBPase has been determined (29). The NH2-terminal and COOH-terminal sequences are nearly identical to the analogous sequences in the rabbit liver enzyme, as were segments about the two pyridoxal 5'-phosphate-modified lysine residues. In addition, the sequence of a CNBr fragment of rat liver FBPase phosphorylated by cyclic AMP-dependent protein kinase has been determined (30). The peptide contains the last 43 amino acid residues of the COOH-terminus. Interestingly, the first 20 residues of the peptide are homologous with the COOH-terminus of pig kidney FBPase, which is not phosphorylated by cyclic AMP-dependent protein kinase, whereas the last 23 residues form a unique, proline rich region containing the phosphorylation site.

A common feature of mammalian FBPases is the presence of a single highly reactive sulfhydryl group per enzyme monomer (31-35). Reaction of pig kidney FBPase with [14C]iodoacetamide followed by cleavage and sequencing of the labeled peptide indicates that only Cys-128 reacts to
a significant extent with the chemical modifier (36). This residue, and
the sequence about it, was found to be highly conserved in a number of
mammalian FBPases.

To date, there have been no detailed X-ray crystallographic studies
of the FBPases. FBPase from avian liver crystallizes readily, but the
crystals suffer considerable damage during X-ray analysis (37,38).
However, FBPase from rabbit liver forms large crystals that are more
stable under analysis. Preliminary studies of the rabbit liver FBPase
at a resolution of 2.5 Å indicate the enzyme tetramer is roughly
spherical, with a diameter of 75 Å. High resolution studies are
reportedly in progress (39).

The dependence of FBPase catalytic activity upon Mg$^{2+}$ was first
reported by Gomori in 1943 (8). It was subsequently noted that Mg$^{2+}$
could be replaced by other divalent cations such as Mn$^{2+}$ (40),
Zn$^{2+}$ (40), or even Co$^{2+}$ (41). It is by now quite clear that FBPases
from all sources with the exception of brain (42), are metalloproteins
which may bind several different metal ions. However, the number of
metal ions bound and the sites occupied may differ depending upon
several variables, including the ion studied, the presence of substrate
or effectors, and the proteolysis state of the enzyme.

Direct studies of the binding of Mn$^{2+}$ and Zn$^{2+}$ to mammalian FBPases
have been performed. Libby et al. (43) monitored the binding of Mn$^{2+}$ to
neutral rabbit liver FBPase by using electron paramagnetic resonance
(EPR). In the absence of any effectors, four negatively cooperative
binding sites for Mn$^{2+}$ per enzyme tetramer were indicated. However, in
the presence of the substrate analog (α+β)methyl D-fructofuranoside 1,6-bisphosphate, a total of eight Mn$^{2+}$ binding sites were observed, and the negative cooperativity was lost. The results show the existence of four structural metal ion binding sites and four catalytic sites. Additional studies of rabbit liver FBPase (44) showed that the products, F6P and P$_i$, also induced the binding of Mn$^{2+}$ to the catalytic sites. Interestingly, the binding of only two equivalents of product or substrate analog to the enzyme tetramer was sufficient to induce all four of the catalytic Mn$^{2+}$-binding sites, indicating a positive cooperation among the sites (44).

Similar studies have been performed to monitor the binding of Zn$^{2+}$ to FBPase. Both rat (45) and rabbit (46) liver enzymes appear to bind a total of 12 Zn$^{2+}$ ions per enzyme tetramer when studied by using a gel filtration technique and $^{65}$Zn$^{2+}$. The binding of the first set of Zn$^{2+}$ ions is extremely tight; indeed, stringent steps must be taken to produce enzyme which is entirely free of Zn$^{2+}$ (47). The second set of Zn$^{2+}$ ions also binds tightly, with a dissociation constant of approximately 0.4 μM for both rabbit (46) and rat (45) liver FBPases.

The third set of Zn$^{2+}$ ions shows a lower affinity for FBPase, with dissociation constants of 1.5 μM and 2.5 μM for the rat (45) and rabbit (46) liver enzymes respectively. In both cases, the binding of Zn$^{2+}$ to the low affinity site is competitive with Mg$^{2+}$. In addition, the presence of substrate is necessary for the third set of Zn$^{2+}$ ions to bind to rabbit liver FBPase (46). These results suggest that the first two sites occupied by Zn$^{2+}$ are structural sites, whereas the third site
is the catalytic site. This postulate is supported by kinetic evidence, which shows that Zn$^{2+}$, which is inhibitory at very low concentrations (< 10 μM) activates FBPase at higher concentrations (45).

Neutral FBPase from a number of sources is very specific with respect to substrate. The rabbit liver enzyme has been tested for activity with a number of substrate analogs, including dihydroxyacetone phosphate, glycerol phosphate, and D-xylulose 1,5-bisphosphate. The substrate analogs were all hydrolyzed by FBPase to some extent, but the rate of the reaction was less than 5% of the rate of reaction with FBP (48).

Unlike other neutral mammalian FBPases, the bovine liver enzyme catalyzes significant β-glycerolphosphate hydrolysis at pH 9.0, with rates approaching 25% of the rate of hydrolysis of FBP at the same pH (49). The β-glycerolphosphatase activity also showed similar sensitivity to inhibition by the allosteric inhibitor 5'AMP. β-Glycerolphosphate was also a competitive inhibitor for FBP in the FBPase reaction at pH 9.0, indicating that the hydrolytic activity for both substrates arose from a common site, namely, the active site (49).

FBP in solution is known to exist as an equilibrium mixture of four forms in the following percentages: β-furanose, 90%; α-furanose, 10%; acyclic keto, 2%; and hydrated keto, 1.3% (50). The specificity of FBPase for the different forms of FBP has been investigated primarily through the use of substrate analogs which approximate the different forms of FBP in solution.
α-Methyl D-fructofuranoside 1,6-bisphosphate (αMeFBP) and β-methyl D-fructofuranoside 1,6-bisphosphate (βMeFBP) were found to be competitive inhibitors of rabbit liver FBPase at concentrations less than 100 μM, with inhibition constants of 7.2 μM and 1.7 μM, respectively (51). At higher concentrations, the β-analog, but not the α, inhibits in a noncompetitive fashion. These results suggest that the α anomer of FBP is the true substrate, and that the inhibition produced by high concentrations of βMeFBP may be a good model for the substrate inhibition of FBPase observed at high concentrations of FBP.

The inhibition of bovine liver FBPase by substrate analogs has also been investigated (52). 2,5-Anhydro-D-glucitol 1,6-bisphosphate, which is an analog of the α anomer of FBP, gave a $K_i$ of 0.55 μM, whereas 2,5-anhydro-D-mannitol 1,6-bisphosphate, an analog of the β anomer, gave a $K_i$ of 33 nM.

Additional information on the anomeric specificity of FBPase has been obtained through rapid-quench studies performed with rabbit liver FBPase (53). Pre-steady-state measurements indicate a rapid (5 ms) initial release of P$_i$, followed by a slower (150 ms) release of P$_i$ to complete the initial turnover, and finally, a steady-state rate which is dependent on the [αFBP]/[FBPase] ratio. Under conditions where FBP is limiting, the rate of the steady-state is controlled by the β → α solution anomerization. Though the β anomer of FBP does appear to bind to FBPase, these results suggest that anomerization to the α form must occur prior to catalysis; however, it is not clear if FBPase catalyzes the β → α anomerization. The results strongly indicate that the α
anomer of FBP is the true substrate of the FBPase reaction.

The binding of FBP to FBPase in the absence of divalent metal ions has been investigated by the use of a gel filtration method (43). The substrate binds to a total of four sites per enzyme tetramer, and the binding data yield nonlinear Scatchard plots that analyze in terms of increasing negative cooperativity. The dissociation constants for the binding of the first two substrate molecules are in the range of $10^{-5}$ M, whereas the dissociation constants for the occupation of the third and fourth sites are on the order of $10^{-4}$ M.

Binding of the products, F6P and $P_i$, to rabbit liver FBPase has also been investigated (44). F6P was found to bind to the enzyme in the absence of divalent metal ions, though the association is promoted by the presence of metal ion in the order $\text{Mn}^{2+} > \text{Zn}^{2+} > \text{Mg}^{2+}$. As with the substrate, FBP, a total of four sites were occupied per enzyme tetramer.

Similar studies of $P_i$ binding to FBPase (44) indicate an absolute requirement for the presence of a divalent metal ion, with $\text{Mn}^{2+}$ being more effective than $\text{Mg}^{2+}$. Again, a total of four sites were occupied by $P_i$ per enzyme tetramer.

The binding of both F6P and $P_i$ to FBPase exhibited negative cooperativity, with the first two product molecules binding more tightly to the enzyme than the final two molecules (44). Additional studies also indicated that F6P and $P_i$ could bind simultaneously to the enzyme, and that neither ligand affected the binding of the other in any fashion (44).
Finally, the binding of the nonhydrolyzable substrate analog (α+β)methyl D-fructofuranoside 1,6-bisphosphate to FBPase was monitored in the presence of Mn$^{2+}$, and found to strongly resemble the binding of FBP to the enzyme in the absence of metal ion (43,44). However, the binding constants for the substrate analog were on the average an order of magnitude smaller than the analogous values for the substrate; that is, the substrate analog in the presence of Mn$^{2+}$ bound to the enzyme less tightly than did the substrate in the absence of metal ion.

The kinetics of the bovine liver FBPase reaction have been investigated extensively (7,33,54-57). Various kinetic constants obtained from the studies are compiled in Table I. In the forward direction, the course of the reaction is Uni Bi if the participation of divalent cation is not considered; however, the amount of information obtainable from a one-substrate system is clearly limited, and several possible kinetic mechanisms for the reaction may be envisioned. Marcus et al. (56) have treated the forward reaction as a Bi Bi system by varying FBP and and Mg$^{2+}$ in initial rate studies; however, both reactants displayed substrate inhibition, thus complicating more detailed studies.

Though the FBPase reaction catalyzed by the rabbit liver enzyme has been reported to be irreversible (58), it is possible to study the bovine liver FBPase reaction in the nonphysiological direction (7). Because the back reaction is a two-substrate system, experiments could be carried out in greater depth and detail than had been previously accomplished. Studies of the reverse reaction using substrate and
### Table I. Kinetic Parameters for the FBPase Reaction

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Explanation</th>
<th>Source</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$</td>
<td>Michaelis constant for FBP</td>
<td>7</td>
<td>$(0.1\pm0.05) \times 10^{-6}$ M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>$(0.7\pm0.1) \times 10^{-6}$ M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
<td>$(0.25\pm0.08) \times 10^{-6}$ M</td>
</tr>
<tr>
<td>$K_q$</td>
<td>Michaelis constant for $P_i$</td>
<td>7</td>
<td>$(27.8\pm1.2) \times 10^{-3}$ M</td>
</tr>
<tr>
<td>$K_{iq}$</td>
<td>Dissociation constant for $P_i$</td>
<td>7</td>
<td>$(7.7\pm1.0) \times 10^{-3}$ M</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Michaelis constant for F6P</td>
<td>7</td>
<td>$(0.62\pm0.05) \times 10^{-3}$ M</td>
</tr>
<tr>
<td>$K_1$</td>
<td>$E + 2$ AMP $\rightleftharpoons E \cdot AMP^2$, forward reaction</td>
<td>55</td>
<td>$(3.14\pm0.03) \times 10^{-10}$ M$^2$</td>
</tr>
<tr>
<td>$K_1$</td>
<td>$E + 2$ AMP $\rightleftharpoons E \cdot AMP^2$, reverse reaction</td>
<td>55</td>
<td>$(5.65\pm0.31) \times 10^{-10}$ M$^2$</td>
</tr>
</tbody>
</table>
product analogs, along with studies in the forward direction using product inhibitors, have served to substantially narrow the possible kinetic mechanisms for the FBPase reaction.

Product inhibition studies in the forward direction showed F6P to be a slope-linear, intercept-linear, noncompetitive inhibitor with respect to FBP, whereas P_i was a competitive inhibitor for the substrate (Table II). These results were in conformity with either an ordered Uni Bi sequential mechanism (Scheme 1) or a Ping-Pong Uni-Bi mechanism (Scheme 2), as well as two rapid equilibrium sequential mechanisms (7). Because Casazza et al. (7) and Pontremoli et al. (12) have failed to demonstrate labeling of FBPase with [32P]P_i or [1-32P]FBP, the Ping-Pong mechanism, which would require the existence of a covalent enzyme phosphorus intermediate, was deemed the least likely.

\[
\begin{align*}
&\text{FBP} \\
&\downarrow \\
&E \\
&\quad \downarrow \\
&\quad \text{E-FBP} \\
&\quad \downarrow \\
&\quad \begin{pmatrix}
\text{E-F6P-P}_i \\
\downarrow \\
\text{E-FBP}
\end{pmatrix} \\
&\quad \uparrow \\
&\quad \text{E-P}_i \\
&\quad \uparrow \\
&\quad \downarrow \\
&\quad \text{E} \\
&\quad \uparrow \\
&\quad \text{P}_i \\
&\quad \uparrow \\
&\quad \downarrow \\
&\quad \text{F6P} \\
&\quad \uparrow \\
&\quad \text{H}_2\text{O} \\
&\quad \uparrow \\
&\quad \text{E-FBP} \\
&\quad \downarrow \\
&E \\
\end{align*}
\]

Scheme I
Table II. Inhibition Patterns for the FBPase Reaction

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Class</th>
<th>Source</th>
<th>Varied Substrate$^a$</th>
<th>Reference</th>
<th>FBP</th>
<th>F6P</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_i$</td>
<td>product inhibitor</td>
<td>7</td>
<td>Comp</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>F6P</td>
<td>product inhibitor</td>
<td>7</td>
<td>NC</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Phosphite</td>
<td>substrate analog</td>
<td>54</td>
<td>---</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>2-Deoxyribose 5-P</td>
<td>substrate analog</td>
<td>54</td>
<td>NC</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>2,5-Anhydromannitol-1,6-bisphosphate</td>
<td>substrate analog</td>
<td>54</td>
<td>Comp</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>AMP</td>
<td>allosteric</td>
<td>55</td>
<td>NC</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
</tbody>
</table>

$^a$The abbreviations are: NC (noncompetitive), U (uncompetitive), and Comp (competitive).
The classical ordered rapid-equilibrium mechanism is consistent with the kinetic results, if it is assumed that F6P binds at an allosteric inhibitory site in addition to the active site, and P_i binds at the active site. This mechanism was dismissed on the basis of data obtained in the back reaction, where both double reciprocal plots (1/v vs. 1/F6P and 1/v vs. 1/P_i) intersected in the third quadrant, rather than on the 1/v axis as is required for an ordered rapid-equilibrium mechanism. However, a unique, ordered rapid-equilibrium mechanism (Scheme 3) of the type proposed by Frieden (59), involving the formation of an inactive E·F6P complex was still a possibility, and will be discussed.
The rapid equilibrium random mechanism (Scheme 4) also was a possibility in light of the results of product inhibition studies if the formation of an inactive FBP·F6P·E complex is assumed. The existence of such a complex is one explanation for the substrate inhibition observed in the forward reaction with FBP concentrations greater than 10 μM (56). However, since P_i is strictly a competitive product inhibitor, the other abortive complex, P_i·FBP·E could not be included in the mechanism.

Unlike the case for the rapid-equilibrium ordered mechanism, no relationship exists to relate the intercepts of the double reciprocal plots for the reverse direction reaction to those obtained in the forward direction with product inhibitors. That is, the rapid equilibrium random mechanism could be neither verified nor eliminated by these experiments. The steady-state ordered mechanism also was consistent with the data. Because the off constant for P_i dissociation was much less than V_1, an isomerization of the E·P_i complex was proposed.
To differentiate between the steady state and rapid-equilibrium mechanisms, isotope partitioning studies using $[^{32}\text{P}]\text{P}_i$ were carried out in the reverse direction. The low level of isotope trapping strongly indicated a rapid equilibrium mechanism (54). These results served to decrease the likelihood of the iso-ordered Bi Uni mechanism, as well as lending additional support to the rejection of the Ping-Pong mechanism.

To further elucidate the kinetic mechanism of FBPase, phosphite and 2-deoxyribose-5-P were utilized as substrate analogs for $\text{P}_i$ and F6P, respectively. The use of substrate analogs to distinguish between ordered and random bireactant mechanisms has been detailed previously (60). For the reverse reaction of FBPase, phosphite was found to be a competitive inhibitor for $\text{P}_i$ and a noncompetitive inhibitor with respect to F6P, whereas 2-deoxyribose-5-P was a competitive inhibitor for F6P and a noncompetitive inhibitor for $\text{P}_i$ (54). The rate equations for inhibition of the reverse reaction by phosphite were similar for the steady-state ordered and rapid-equilibrium random mechanism; however, the two mechanisms were distinguishable on the basis of inhibition by the F6P analog, 2-deoxyribose-5-P, with respect to $\text{P}_i$. In the case of the ordered Bi Uni mechanism, the rate equation predicts 2-deoxyribose-5-P will cause uncompetitive inhibition with respect to $\text{P}_i$. Alternatively, for the rapid-equilibrium random mechanism, noncompetitive inhibition was predicted. The results of substrate analog studies for the reverse reaction were more closely in accord with the latter mechanism because double reciprocal plots gave clearly intersecting lines for 2-deoxyribose-5-P inhibition with respect to F6P.
The data therefore seemed in harmony with a rapid-equilibrium random Bi Uni mechanism, which included the formation of an abortive F6P•FBP•E complex to explain product inhibition by F6P in the forward direction (Scheme 4). However, a rapid equilibrium ordered mechanism of the type proposed by Frieden (59) involving the formation of an inactive E•F6P complex also was in harmony with the kinetic results (Scheme 3).

Several methods whereby the two mechanisms may be differentiated have been outlined (60). The method chosen in this case was the use of the multisubstrate analog, 2,5-anhydromannitol-1,6-bisphosphate (59). For the random mechanism, the product analog would be expected to bind each substrate site simultaneously and therefore be a competitive inhibitor for both F6P and P\textsubscript{i}. Because the analog presumably would bind only at the active site, only the enzyme term of the rate equation would be affected, producing linear competitive primary plots and slope-linear secondary plots.

On the other hand, with Frieden's mechanism, the analog would be expected to bind both the active site and the subsite for F6P. This would produce competitive primary plots for P\textsubscript{i} and noncompetitive plots for F6P. In addition, nonlinear secondary plots can be predicted on the basis of multiple binding of the analog to the enzyme, resulting in squared terms in the rate equation.

2,5-Anhydromannitol-1,6-bisphosphate gave linear competitive inhibition with respect to both F6P and P\textsubscript{i}. In addition, both secondary plots were slope linear. These results indicated the rapid-equilibrium Random Bi Uni mechanism (Scheme 4) to be more likely than the unique
ordered mechanism (Scheme 3) in the reverse reaction (59). However, if the forward direction is also rapid-equilibrium random, the formation of an abortive E·FBP·F6P complex must be proposed to explain the noncompetitive product inhibition observed with F6P. Although isotope partitioning studies indicate the reverse reaction approximates the rapid-equilibrium assumption, the faster forward reaction may be steady state; indeed, this seems to be the case for the muscle phosphofructokinase reaction (61). Assuming the steady-state condition in the forward reaction of FBPase, it is possible to generate a rate equation for product inhibition consistent with an ordered mechanism with nonproductive binding of F6P as shown in Scheme 3 (54).

To summarize, in light of the results of kinetic studies reviewed here, it is possible to select the rapid-equilibrium random Bi Uni mechanism as a likely possibility for FBPase in the reverse direction. If the release of products in the forward direction is also rapid-equilibrium, it is necessary to propose the formation of an abortive E·FBP·F6P complex. However, if product release is instead steady state, the abortive complex need not form, but F6P would be expected to form an inactive complex with the enzyme.

The overall equilibrium of the FBPase reaction is 227 M, determined at pH = 6.99 and no free Mg²⁺ (62). On the basis of rapid-quench studies (53), it has been calculated (9) that the equilibrium for FBP, F6P and Pₐ bound to the enzyme is approximately 100 M, similar to the value in solution. This differs from the results generally obtained with kinases, where the equilibrium constant in the bound state usually
is near unity (63).

Despite numerous protein modification studies performed on FBPases from a variety of mammalian sources, no single amino acid has conclusively been shown to participate in catalysis (9). For instance, reaction of rabbit liver FBPase with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide resulted in an 85% loss of enzyme activity, and the inactivation could be prevented by the presence of FBP (9). The number and type of residue modified were not determined; however, the results suggest a carboxyl group may be present at the active site of the enzyme.

Similarly, ethoxyformylation of rabbit liver FBPase causes a decrease in enzyme activity which can be prevented by the presence of substrate (35). In this case, it was determined that several histidine residues were modified, resulting in loss of catalytic activity. These results are particularly interesting because histidine residues are sometimes involved in metal ion binding sites. Possibly, FBPase histidine modification may disturb the binding of the catalytic metal ion.

As indicated earlier, the mammalian FBPases contain one very reactive sulfhydryl group per enzyme monomer. The result of modification of the bovine liver FBPase fast-reacting sulfhydryl groups has been studied extensively by two groups (31,33). Geller and Byrne (31) monitored the effect of modification of the enzyme by N-ethylmaleimide, 1-fluoro-2,4-dinitrobenzene and 5,5'-dithiobis-(2-nitrobenzoic acid) under various conditions of pH and metal ion. The results were complex,
in as much as the sulfhydryl reagents caused either an increase or a decrease in enzyme activity, depending on the conditions of the experiment. However, in all cases, the change in enzyme activity was less than 50%, indicating the modification occurs at a site remote from the catalytic site.

A similar study was performed by Nimmo and Tipton (33), in which bovine liver FBPase was reacted with p-chloromercuribenzoate and 5,5'-dithiobis(2-nitrobenzoate). Modification of the enzyme with p-chloromercuribenzoate caused either an increase or a decrease in enzyme activity, depending on the nature of the metal ion (Mn$^{2+}$ or Mg$^{2+}$) used in the assay; however, the change in activity was again less than 50%. Reaction of the four thiol groups with 5,5'-dithiobis-(2-nitrobenzoate) could be slowed by the presence of FBP. The reaction of FBPase with either reagent resulted in a decrease in the substrate inhibition observed at high FBP concentrations.

AMP is known to be an important regulator of FBPases from a variety of sources. The nucleotide is a specific noncompetitive inhibitor that acts at an allosteric site (9). The kinetics of AMP inhibition of the bovine liver FBPase reaction have been studied in detail by progress-curve analysis in the forward direction and initial rate kinetics in the reverse direction (55).

Because the $K_m$ for FBP is quite low (Table I), initial rate studies of the forward reaction using the coupled spectrophotometric assay are technically difficult. Therefore, progress-curve analysis was used to measure AMP inhibition for the forward reaction (55). Fitting of the
data to various rate equations indicated the inhibition by AMP was best described as noncompetitive slope-parabolic and inhibitor-parabolic.

In the reverse direction, AMP was again found to be a noncompetitive slope-parabolic, inhibitor-parabolic inhibitor with respect to both F6P and P_i. Kinetic constants obtained in these studies are listed in Table I. For both the forward and reverse reactions, terms lower or higher than AMP^2 were inconsequential in the rate equation, indicating the binding of AMP to FBPase is strongly cooperative. This observation agrees well with studies of the binding of AMP to the bovine liver FBPase (55). Scheme 5 summarizes the results obtained on the effect of AMP on FBPase.

\[
\begin{align*}
&\text{FBP} \\
&\rightarrow K_a' \underline{E \cdot (AMP)_2} \\
&\rightarrow E \cdot FBP \cdot (AMP)_2 \\
&\leftarrow 2 \text{ AMP} \rightarrow K_i \underline{E} \\
&\leftarrow K_{ii} 2 \text{ AMP} \\
&\leftarrow E \cdot FBP + + E + F6P + P_i \\
&\leftarrow K_a \underline{FBP}
\end{align*}
\]

Scheme 5

Nimmo and Tipton (57) originally reported bovine liver FBPase binds 2 mol AMP/mol FBPase, and exhibits positive cooperativity. Arneson et al. (64) later described the binding of AMP to FBPase as exhibiting
mixed cooperativity. In their experiments, 2 mol AMP/mol FBPase bound at 17 μM AMP, but it required 200 μM AMP to saturate four sites on the enzyme. In contrast to the rabbit liver FBPase, the substrate, FBP, does not affect the binding of AMP to the bovine liver FBPase (64).

One of the most exciting recent developments in the area of carbohydrate metabolism has been the discovery of 2,6FBP, a regulator of both glycolysis and gluconeogenesis. The activating effect of 2,6FBP on rat liver phosphofructokinase (PFK) was first reported (as a note added in proof) by Van Schaftingen et al. in 1980 (1). This was closely followed by a similar report by Pilkis et al. (65) indicating that 2,6FBP was an activator of rabbit muscle PFK. It was also reported that 2,6FBP was an inhibitor of rabbit muscle and rat liver FBPases (2,3,66).

The nature of 2,6FBP inhibition of FBPase was initially reported by Pilkis et al. (2) to be competitive with the substrate, whereas Van Schaftingen and Hers indicated that 2,6FBP changes the substrate saturation curve from hyperbolic to sigmoidal (66). Pilkis et al. were later able to confirm these results (3); however, Gottschalk et al. (67) have reported competitive inhibition exclusively for pig kidney FBPase. All reports (2,3,66) described the inhibition produced by 2,6FBP as being synergistic with AMP.

The binding of 2,6FBP to FBPases from rat liver (68) and rabbit liver (69) has been investigated. Rabbit liver FBPase was found to bind one mole of 2,6FBP per mole of enzyme monomer, with an estimated dissociation constant of $0.67 \times 10^{-6}$ M (69). A Scatchard plot of the binding data was linear, with no indication of cooperative interactions.
between enzyme and inhibitor. FBP, F6P, and P$_i$ all competed with the binding of the inhibitor, though AMP had no effect. High concentrations of Mg$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ inhibited the interaction of 2,6FBP with the enzyme.

Rat liver FBPase was also shown to bind one mole of 2,6FBP per mole of enzyme monomer (68); however, the binding exhibited marked cooperativity, with the intrinsic binding constants ranging from $K_1 = 2.0 \times 10^5$ M$^{-1}$ to $K_4 = 2.0 \times 10^4$ M$^{-1}$. Binding of 2,6FBP was dependent on the presence of a divalent metal ion (Mn$^{2+}$ or Mg$^{2+}$), and could be prevented by the substrate analogs aMeFBP and 8MeFBP. AMP did not affect the negative cooperativity or the total amount of 2,6FBP which bound to FBPase; however, AMP did enhance the binding of the first mole of 2,6FBP to the enzyme, increasing the association constant by approximately 50% (68).

Other mechanisms of FBPase regulation, in addition to inhibition by AMP and 2,6FBP, have been proposed. It is well-known that extensive proteolytic cleavage of mammalian FBPases will occur during purification if appropriate precautions are not observed. The resulting proteolytically altered enzymes are characterized by an alkaline pH optimum for activity, and a decreased sensitivity to inhibition by AMP (11). In vitro proteolysis of FBPase to the so-called "alkaline" form can be accomplished by treatment with a variety of proteases, including subtilisin and lysosomal preparations which contain cathepsins (70).

Cleavage of a number of mammalian FBPases by subtilisin has been characterized. Digestion of rabbit liver FBPase by subtilisin liberates
a 6500 molecular weight peptide from the amino terminus of the enzyme (71). Recently, the site of cleavage of pig kidney FBPase by subtilisin was investigated. In this case, the peptide contained 60 amino acids, and the entire sequence was determined (72). Though proteolysis resulted in decreased sensitivity to AMP inhibition, no AMP interaction site could be detected on the peptide.

A unique lysosomal protease which acts on both PFK and FBPase was purified from rabbit liver by Pontremoli et al. (73). The protease, designated cathepsin M, cleaves a small fragment from the carboxy-terminus of FBPase, leading to a decrease in enzyme activity. Interestingly, the inactivation can be reversed by the addition of cystamine, which probably forms a mixed disulfide derivative with the enzyme sulfhydryl groups (74).

The possibility that FBPase activity may be regulated by the phosphorylation state of the enzyme has also been investigated. Rat liver FBPase is a substrate for the catalytic subunit of cAMP-dependent protein kinase, which incorporates one mole of phosphorus per mole of FBPase monomer (75,76). In vivo phosphorylation of rat liver FBPase has also been shown to occur (75,76); indeed, purified FBPase exhibits multiple forms upon isoelectric focusing, which have been attributed to differing states of phosphorylation (77). The effect of phosphorylation on the activity of FBPase is unclear. Riou et al. reported a slight increase in phosphorylated FBPase activity (75), whereas Chatterjee et al. stated that enzyme activity is independent of the phosphorylation state (76). McGrane et al. have suggested that the discrepancy may be a
result of different degrees of phosphorylation of the enzyme, which subsequently causes differences in the regulation of FBPase activity (77).

Recently, it has been suggested that the oxidation state of FBPase thiol groups may play a role in the regulation of enzyme activity (78). Two forms of FBPase were isolated from rat liver in the presence (A) and absence (B) of dithiothreitol. Forms A and B had identical molecular weights and the same $K_m$ for FBP; however, they differed in their reactions with thiol reagents, and showed dissimilar sensitivity to AMP inhibition. Form A, the more reduced form of FBPase, showed 22.4 thiol groups per enzyme tetramer, 2.5 of these being the "fast-reacting" type. Form B, the more oxidized form of the enzyme, contained 19.2 thiol groups, 0.5 fast reacting. The $K_i$ for AMP inhibition was 140 $\mu$M for form A, and 370 $\mu$M for form B. However, form B regained sensitivity to AMP inhibition if 1 mM dithiothreitol was included in the assay. The effect of oxidation state on 2,6FDP inhibition was not investigated, but may prove interesting. In any case, the results suggest that the redox state of the enzyme may be important in controlling FBPase activity.

The hormonal regulation of gluconeogenesis has been intensively investigated, and several recent review articles pertaining to this topic have been published (79-81). Glucagon is known to increase the rate of gluconeogenesis in the liver while inhibiting glycolysis. Specifically, glucagon has been reported to raise the flux through FBPase, and to increase enzyme activity (82,83). The activities of PFK (82) and pyruvate kinase (84) are also affected by glucagon.
The effect of glucagon on pyruvate kinase activity clearly arises from phosphorylation of the enzyme by cAMP-dependent protein kinase, which results in a decreased affinity for the substrate, phosphoenolpyruvate. However, until recently, the mechanism of the glucagon effect on FFK and FBPase was unclear, as phosphorylation of both these enzymes only negligibly changes their activity.

It now appears the glucagon effect on both FFK and FBPase is mediated by 2,6FBP (85,86). Soon after the discovery of 2,6FBP, enzyme activities responsible for synthesis of the regulator from F6P and ATP (87-89), and degradation to F6P and P_i (90,91) were reported. Interestingly, a single protein is responsible for both activities (90,91). The enzyme (6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase), when phosphorylated by cAMP-dependent protein kinase in response to glucagon, catalyzes the degradation of 2,6FBP (90,91). However, the unphosphorylated enzyme catalyzes the synthesis of 2,6FBP. Therefore, glucagon potentiates FBPase activity in vivo by promoting the cAMP-dependent phosphorylation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase, thereby causing 2,6FBP to be degraded, and relieving the inhibition of FBPase (85,86).

In conclusion, FBPase plays a key role in the control of gluconeogenesis, and as such is highly regulated. The enzyme is regulated by the inhibitor 2,6FBP, which in turn is under the control of the hormone glucagon. AMP also is an important regulator of FBPase, though the mechanism causing AMP levels to vary is unclear. FBPase may also be regulated by phosphorylation, oxidation/reduction, or the presence of
metal ions or product; however, the effects produced are probably minor compared to the inhibition produced by 2,6FBP and AMP. Interestingly, no activators of FBPase have yet been reported.

Review of Paramagnetic Nuclear Relaxation Theory and Methods

A myriad of methods are available for studying the interaction of small compounds with macromolecules; however, NMR and X-ray crystallography are unique in permitting the "observation" of individual atoms within a macromolecular complex. A number of review articles (4-6, 92-96) and texts (97,98) describing the theory and various biochemical applications of NMR have been published.

When nuclei with spin $I=1/2$ (i.e., protons or phosphorus nuclei) are placed in a stable magnetic field, they will disperse between two energy levels corresponding to orientations of the spins parallel and antiparallel to the magnetic field. This may be visualized by picturing the dipoles aligned with the magnetic field as lying on the positive $z$ axis (+z), and those opposed to the magnetic field as lying on the negative $z$ axis (-z). The equilibrium population of the two energy levels is determined by the energy difference between them, and more dipoles are aligned in the +z direction, so that the vector sum of the magnetic moments of the nuclear spins (the total magnetization) is aligned in the direction of the applied magnetic field.
Just as in other types of spectroscopy, NMR arises from transitions in energy levels. In this case, the equilibrium of the system is perturbed by the absorption of energy from an applied electromagnetic field. The approach of the perturbed system back to equilibrium occurs exponentially with time, and is described by the time constants $T_1$ and $T_2$.

More exactly, the application of a radio-frequency pulse in the $y$-axis direction "flips" the total magnetization into the $x$-$y$ plane. Upon cessation of the pulse, the dipoles begin to precess about the $z$ axis direction. The precessing dipoles dephase in the $x$-$y$ plane because the precession frequencies of the individual dipoles differ; hence, the total magnetization decreases exponentially with a time constant, $T_2$.

Of course, tipping $M$ into the $x$-$y$ plane decreases the magnitude of the macroscopic magnetization vector in the $+z$ direction. At the same time that the dipoles precessing in the $x$-$y$ plane are losing phase coherence, they are also realigning with the applied magnetic field. Therefore, the magnetization in the $+z$ direction exponentially approaches the equilibrium value in a manner described by a time constant, $T_1$. Because realignment of the dipoles with the $z$-axis decreases the magnetization in the $x$-$y$ plane, $T_1$ is always greater than $T_2$, and any factors which affect $T_1$ will also affect $T_2$.

$T_1$ relaxation occurs because of the ability of the nuclear spin system to transfer excess energy to the surroundings; hence, $T_1$ is also known as the spin lattice relaxation time. In liquids, the rapid random motion of molecules results in fluctuating magnetic or electric fields
of a range of frequencies. That component of the frequency spectrum that is equal to the resonance frequency, called the Larmor frequency, \( \omega_0 \), is necessary for effective energy exchange leading to \( T_1 \) relaxation.

The magnitude of the component of the frequency spectrum at the resonance frequency depends upon several factors, including molecular motion, the magnitude of magnetic moments in the surrounding nuclei, and intermolecular and intramolecular interactions. The dependency upon molecular motion is expressed by a correlation function that defines the position of a molecule at time \( t \) relative to its position at an arbitrary initial time. The time required for the change of position is the correlation time, \( \tau_C \).

As mentioned, any factor which promotes \( T_1 \) relaxation will also lead to \( T_2 \) relaxation. However, additional contributions to \( T_2 \) relaxation will arise if the tumbling of the molecule in solution is insufficient to average out the magnetic fields generated by neighboring spins. In this case, energy can be exchanged between nuclei, giving rise to \( T_2 \), or spin-spin relaxation.

Obviously, relaxation by either the \( T_1 \) or \( T_2 \) process will only occur as a result of some specific interaction between the nucleus and its environment which results in an energy exchange. Several types of interactions have been identified, and include nuclear magnetic dipole-dipole interaction, chemical shift anisotropy, spin-rotation interaction, nuclear electric quadrupole interaction, scalar coupling effects, and the effect of paramagnetic species. The effect of paramagnetic species will be discussed in some detail below. Other
relaxation mechanisms are less pertinent to this discussion, and are
described in detail elsewhere (97-99).

Paramagnetic ions contain an unpaired electron, and therefore have
a large magnetic moment in comparison to dipolar nuclei. This results
in a strong, fluctuating magnetic field about the paramagnetic nucleus,
which may in turn provide an efficient relaxation mechanism for
neighboring nuclei.

A knowledge of the conformations and arrangements of ligands bound
to the active site of an enzyme in proximity to a paramagnetic ion can
be gained by observing the paramagnetic effect on the individual nuclei
of the ligands. The relaxing effect of an enzyme-bound paramagnetic ion
on nearby nuclei depends primarily on the following parameters: the
lifetime of the complex (τ_m), the relative stoichiometry of the
substrate and paramagnetic ion in the complex (q), the correlation time
for the electron-nuclear dipole interaction (τ_τ), and the distance (r)
between the paramagnetic ion and the nucleus in the complex. Thus, if
certain restrictions of the exchange rate are met, and τ_τ and q are
known, the value of r can be obtained.

The observed paramagnetic contribution to the relaxation rate of a
nucleus of a ligand in exchange with a paramagnet-enzyme complex
(1/T_P), is described by the Swift-Connick equation (100):

\[
\frac{1}{T_P} = f q / (\tau_m + T_{1m})
\]
where $f$ is the fraction of ligand bound to the paramagnet-enzyme complex, $\tau_m$ is the lifetime of the bound complex (and the reciprocal of the dissociation constant, $k_{diss}$), and $T_{1m}$ is the relaxation rate of the ligand nuclei in the bound state. An analogous equation describes the observed paramagnetic effect on $T_2$. Obviously, for $1/T_{1p}$ to be a good estimate of $fq/T_{1m}$, the lifetime of the ligand-enzyme-paramagnet complex should be short; that is, the ligand must be in fast exchange with the enzyme.

A number of methods are available to determine if a ligand-enzyme system is in fast, slow, or intermediate exchange. The simplest method involves determining the ratio of $T_{1p}/T_{2p}$. If exchange is slow, then $\tau_m$ will dominate $1/T_{1p}$, and hence $1/T_{2p}$; therefore, $T_{1p}$ will be approximately equal to $T_{2p}$, and the ratio will equal one. On the other hand, if the chemical exchange rate is fast, the two relaxation rates will not be equal, and $T_{1p}/T_{2p}$ will be greater than one.

Alternatively, the exchange mechanism may be determined by monitoring the effect of temperature on either $T_1$ or $T_2$. Because $T_2$ is related to the linewidth of the resonance arising from a nucleus by the relationship $1/T_2 = \pi$ (linewidth at half-height in hertz), it is usually simplest to monitor $T_2$ with changing temperature. If the system is in slow exchange, $T_{2m}$ will be less than $\tau_m$, and the linewidths will increase with increasing temperature, which decreases the lifetime of the bound state.

However, in the fast exchange limit, $\tau_m$ is less than $T_{2m}$. Because $1/T_{2m}$ is a function of the enzyme-ligand correlation time, $\tau_c$, the observed linewidth will narrow with increasing temperature, due to a
decrease in $\tau_c$. Finally, if intermediate exchange conditions prevail, changes in $\tau_m$ will counteract changes in $T_{2m}$, and little change in the linewidths will be observed.

If fast exchange conditions prevail for a particular system, the distance $r$ in angstroms between the enzyme-bound paramagnet and the nuclei of nearby ligands can be obtained by using the modified Solomon-Bloembergen equation (101):

$$r(\AA) = C[f \cdot T_{1p}(\frac{3\tau_c}{1 + \omega_1^2\tau_c^2})]$$

where $\omega_1$ is the Larmor precession frequency, and $C$ is a collection of constants for a specific paramagnet-nucleus interaction.

The use of the Solomon-Bloembergen equation to determine the distance between an enzyme-bound paramagnetic center and the nuclei of various ligands requires that a number of criteria be fulfilled. For instance, it must be shown that $T_{1p}$ is not exchange limited, as previously mentioned. It also is important that outer sphere contributions to the relaxation rate be small. For the interaction of paramagnetic ions and ligands free in solution, outer sphere effects may be significant. However, in the enzyme-bound complex, it is unlikely any ligand except that specifically bound to the enzyme in proximity to the paramagnetic center will experience enhanced relaxation. Also, any nonspecific interactions between the bound paramagnetic ion and ligand in solution are likely to take place over a large distance, and will contribute little to $T_{1p}$ relative to the ligand specifically bound in
close proximity to the paramagnetic center.

In addition, the hyperfine contact contributions to $1/T_{IP}$ should be small. This criterion is met if the paramagnetic ion has a visible EPR spectrum at room temperature, or if the ligand is not directly coordinated to the paramagnet.

As mentioned previously, the use of the Solomon-Bloembergen equation for determining paramagnet-ligand distances requires a knowledge of $\tau_c$, which is given by:

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m}$$

where $\tau_r$ is the rotational correlation time, $\tau_s$ is the electron-spin relaxation time, and $\tau_m$ is the residence time of the nuclear species in the bound state. Obviously, $\tau_c$ is dominated by whichever time is shortest: $\tau_r$, $\tau_s$ or $\tau_m$.

Several methods are available for the evaluation of $\tau_c$. The most accurate method measures $\tau_c$ of the nucleus for which the distance is desired by monitoring the frequency dependence of $T_{IP}$ of the ligand nuclei. Commonly, this measurement is made on separate instruments at different magnetic fields; however, if two magnetically active isotopes of the same nucleus are available (e.g., $^1H/^2H$, $^{14}N/^{15}N$) the frequency dependence study can be done at one magnetic field, because $\omega_1$ is different for each isotope (6). In any case, the method assumes that the ligand is in the fast exchange limit, and that the correlation time is frequency independent. The latter assumption is usually valid for
biological systems where \( t_C \) is in the range of \( 10^{-11} \text{ to } 10^{-9} \) seconds.

The correlation time may also be obtained from the ratio \( T_{1p}/T_{2p} \) for a particular nucleus. This method assumes the fast exchange limit, and that hyperfine and paramagnetic shift contributions to \( T_{2p} \) are negligible. Unfortunately, atoms heavier than protons have been reported to experience significant contact and shift contributions to \( T_{2p} \), resulting in an overestimation of \( t_C \) by as much as an order of magnitude when compared to \( t_C \) obtained by using other methods (102,103). Therefore, the \( T_{1p}/T_{2p} \) ratio method is most safely applied to those ligands which contain easily monitored protons.

A third method for obtaining \( t_C \) involves monitoring the relaxation rates of the solvent water in the system, assuming \( t_C \) for the water hydrogens in the first hydration sphere of the paramagnetic ion is equal to \( t_C \) for the other nuclei under observation. The correlation time may then be obtained either from the ratio \( T_{1p}/T_{2p} \) as previously described, or from the relaxation enhancement of the water protons when the paramagnetic enzyme-ligand complex is present, provided \( q \) is known (97). In either case, the method assumes that hyperfine contributions to \( 1/T_{1m} \) are small, and that the metal-water distance in the paramagnetic complex is the same as the value in pure aqueous solution.

The correlation time may also be estimated by measuring the linewidth of a single transition in the EPR spectrum of a solution of the paramagnetic complex. This method actually estimates \( t_s \), and therefore provides a lower limit for \( t_C \). The method is most useful in confirming other \( t_C \) determinations, and may help determine the relative
contribution of \( \tau_s \) to \( \tau_c \).

In addition, the Stokes-Einstein equation may be used to estimate \( \tau_r \), assuming the molecular weight of the complex is known. Because it is unlikely that any mechanism determining \( \tau_c \) will be longer than \( \tau_r \), this method provides an upper limit for \( \tau_c \).

The potential for obtaining structural information in biochemical systems from the relaxing effects of paramagnetic ions was first recognized by Eisinger, Shulman, and Szymanski in 1962 (104). In studying the binding of paramagnetic ions to DNA, this group noted that solvent water relaxation rates of the samples containing paramagnet-DNA complexes were increased significantly over the rates obtained for samples of the paramagnetic ion alone. Soon after this observation was made, a similar phenomenon was reported for Mn-enzyme and Mn-enzyme-ligand complexes by Cohn and Leigh (105).

Since 1962, the measurement of nuclear magnetic relaxation rates in the presence of paramagnetic probes has been used extensively to study the arrangement and conformation of enzyme-bound substrates. Early studies were performed primarily on water or other ligands with identifiable proton resonances. However, the development of high-field spectrometers with Fourier transformation capacity has increased sensitivity and allowed distance measurements to be performed on a number of other nuclei, including \( ^{31}\text{P}, ^{13}\text{C}, ^{15}\text{N}, ^{6}\text{Li}, \) and \( ^{19}\text{F} \).

In addition, a number of paramagnetic probes have been utilized in relaxation studies. The most commonly used probe is Mn(II), which is an activating divalent cation for a number of enzymes. In other cases,
zinc may be substituted with Co(II), which is paramagnetic. Even proteins such as immunoglobulins which do not require a metal ion for protein function may have specific binding sites for paramagnetic lanthanide ions such as Gd(II). Finally, if a protein does not specifically bind a paramagnetic species, it is sometimes possible to introduce a probe by using one of the following methods.

First, it may be possible to directly introduce a stable free radical probe in the form of a spin label. The most common spin labels contain a nitroxide moiety which has an unpaired electron. The nitroxide group is often covalently attached to some functional group which itself will react with amino acid residues of a protein; for instance, many spin labels contain an iodoacetamide group and therefore react primarily with sulfhydryl residues.

It is also possible to introduce a paramagnetic probe by using substrate or inhibitor analogs containing an unpaired electron. For instance, ATP and AMP analogs which contain a nitroxide group attached at N-6 of the adenine ring have been used to study nucleotide-binding enzymes (97). Similarly, substitution-inert Cr(III) derivatives of nucleotides have been developed to aid in the study of the metal-nucleotide environment of enzymes (6,106).

By monitoring the paramagnetic effect on the longitudinal relaxation rates of ligand nuclei, distances up to 24 Å from a paramagnetic center have been calculated (107). A comparison of distances obtained by the NMR method with the corresponding measurements obtained by using X-ray crystallography generally yields a good correlation between the two.
methods. This is especially true for smaller paramagnet-ligand complexes. However, in certain cases involving macromolecular complexes, the X-ray crystallographic and NMR results may disagree. For instance, the geometry of the alcohol dehydrogenase active site has been intensively investigated by NMR, X-ray crystallography, and fluorescence spectroscopy (4,93,108). The results obtained from the different methods for a number of ligands bound to Co(II) or Zn(II) alcohol dehydrogenase are qualitatively similar inasmuch as the substrates appear to have the same basic conformation when bound at the active site of the enzyme, regardless of the method used to determine the conformation. However, the results of X-ray crystallographic studies suggest the substrate is directly coordinated with the active site metal ion, whereas NMR studies suggest a second-sphere mode of interaction between metal ion and substrates. Because the crystalline alcohol dehydrogenase is much less active than the enzyme in solution, the discrepancy probably is a result of structural differences between the two states. In this case, then, the NMR method is thought to more accurately describe the geometry of enzyme-bound ligands in the physiological state than does X-ray diffraction.

Measurement of nuclear magnetic relaxation rates of substrate atoms in the presence of enzyme-bound paramagnetic probes has been used to determine ligand conformations for a number of enzyme systems. An excellent comprehensive review by Burton et al. (93) describes the application of the method to twelve different enzyme systems, as well as three nonenzyme systems, and several polynucleotide systems. Of course,
studies of the active site and allostERIC site topography of FBPase by the nuclear magnetic relaxation method are particularly relevant to this literature review, and will be discussed in some detail below.

In 1973, Benkovic and his coworkers (109) investigated both the binary fructose-l-phosphate (F1P)-Mn(II) complex and the ternary F1P-Mn(II)-FBPase complex by using \(^{31}\text{P}\) and \(^{13}\text{C}\) NMR. This group found that F1P is a poor substrate for rabbit liver FBPase, being hydrolyzed at a rate 0.3% that of FBP; in addition, the \(K_m\) for the reaction was 0.62mm, several orders of magnitude higher than the \(K_m\) for FBP. Therefore, it was reasoned that F1P was a better candidate for NMR measurements than FBP, because F1P was less likely to be exchange limited, and less hydrolysis would occur during the course of the experiment when FBPase was present.

In initial experiments, \(T_1\) and \(T_2\) of the \(^{13}\text{C}\) and \(^{31}\text{P}\) resonances of F1P in solution were monitored in the presence and absence of MnCl\(_2\). Both relaxation rates were shortened by the presence of the paramagnetic ion, and the ratio \(T_{1\text{P}}/T_{2\text{p}}\) was greater than 1, indicating \(T_1\) is not limited by chemical exchange. The Stokes-Einstein equation was used to determine that \(r = 5.0 \times 10^{-10}\) seconds for the Mn-F1P complex. By using the Solomon-Bloembergen equation, it was determined that Mn was between 2.1-2.8 Å away from C-1, C-2, C-3, C-4, and C-5 of F1P, whereas the distance between Mn and C-6 could not be determined because \(T_1\) of C-6 was not affected by the presence of Mn. Similarly, the distance between Mn and the phosphate group of F1P was determined to be 2.4 Å at pH = 5.8 and 3.2 Å at pH = 7.5.
In addition, the distance between Mn and the phosphate group of FlP was measured in the presence of FBPase. Again, $1/\tau_{1p}$ was less than $1/\tau_{2p}$, indicating the system is in fast exchange. The correlation time was estimated by using both Stokes' law and the ratio of $T_{1p}/T_{2p}$. The values obtained were $2-5 \times 10^{-8}$ seconds, representing an upper limit for $\tau_C$. The Mn to phosphorus distance obtained for the FlP-Mn-FBPase complex was 2.5 Å at pH = 5.8, and 3.2 Å at pH = 7.5.

The results presented by Benkovic et al. (109) suggest that Mn is directly coordinated to the phosphate group of FlP in both the binary and ternary complexes, because inner coordination complexes typically give distances in the range of 2.8-3.2 Å (93). However, the distances obtained in the presence of FBPase may be spuriously short for two reasons.

Firstly, it is possible that binary complexes of the type FlP-Mn may be contributing to the relaxation rates attributed to the ternary complex, FlP-Mn-FBPase. Because the $K_m$ for FlP is relatively high, it is quite possible that the association constant is quite low, resulting in a significant amount of unbound FlP which may form a binary complex with Mn. Secondly, the value for $\tau_C$ used in the distance measurement calculations was an upper limit because, as previously mentioned, the Stokes-Einstein equation gives only $\tau_T$ and because the $T_{1p}/T_{2p}$ ratio method may include hyperfine contributions to $T_2$ when applied to phosphorus nuclei (102,103).

The $^{31}$P NMR studies of Benkovic et al. (109) on the FlP binding site of rabbit liver FBPase were extended by Cunningham et al., who
determined the Mn-^{31}P distances for enzyme-bound F6P, P_i and AMP, as well as the Mn-^{1}H distances for H-8, H-2, and H-1' of AMP (110). In these studies, \( \tau_c \) was estimated by three independent methods. The ratio of \( T_{1p}/T_{2p} \) (obtained by using results for H-8 of AMP) was used to estimate \( \tau_c \), as was the EPR spectrum of enzyme-bound Mn. In addition, \( T_{1p} \) was determined for P_i and AMP by using \( ^{31}P \) NMR at two frequencies, and the results were used to calculate \( \tau_c \). An average of \( \tau_c \) obtained by the three different methods was \( 1.8 \times 10^{-9} \) seconds, at least an order of magnitude lower than the value presented by Benkovic et al. (109).

Because the ratio of \( T_{1p}/T_{2p} \) obtained by using \( ^{31}P \) NMR was between 100 and 200 for the three ligands used in the study, the criterion that the system be in fast exchange was satisfied; hence, the Solomon-Bloembergen equation could be applied to obtain Mn-nuclei distances. The Mn-^{31}P distances obtained for F6P and P_i were both 6.3 Å, whereas the Mn-^{31}P distance for AMP was 5.2 Å. The Mn-^{1}H distances for H-8, H-2, and H-1' of AMP were 4.1 Å, 6.8 Å, and 7.3 Å, respectively. It was also noted that the Mn-^{31}P distances for F6P and P_i were lengthened slightly when the ligands were simultaneously bound to the enzyme.

In addition, Cunningham et al. (110) introduced an additional paramagnetic probe by reacting the FBPase with a nitroxide spin label which modified four enzyme sulfhydryl groups per tetramer. However, the spin label had no effect on the relaxation rates of F6P and P_i phosphate groups. By using the EPR method, \( \tau_c \) for the spin-labeled enzyme was obtained, making it possible to calculate that the nitroxide group was greater than 9.6 Å removed from P_i and the phosphate of F6P. Finally,
by monitoring the effect of Mn on the EPR spectrum of spin-labeled FBPase, it was possible to estimate that the distance between enzyme-bound Mn and the spin label is about 16 Å (97,110).

In conclusion, important information on the mechanisms of enzyme catalyzed reactions may be obtained by measuring nuclear magnetic relaxation rates in the presence of paramagnetic probes. However, the proper use of the technique requires that a number of criteria be met, and that the appropriate correlation times be carefully determined. The method has previously been applied to the study of the mechanism of rabbit liver FBPase; studies presented in this dissertation also use paramagnetic relaxation techniques to further elucidate the mechanism of bovine liver FBPase.
PART I. THE EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON
THE REVERSE REACTION KINETICS OF BOVINE LIVER
FRUCTOSE 1,6-BISPHOSPHATASE
INTRODUCTION

FBPase (EC 3.1.3.11) catalyzes the hydrolysis of FBP to form F6P and \( P_i \) in the presence of a required divalent cation. The enzyme is a crucial control site in gluconeogenesis and, as such, is highly regulated by several effectors, including AMP and 2,6FBP. The FBPase reaction has been studied extensively in the forward (physiological) direction (9), but these investigations have been ineffective in elucidating the manner of product release because technical problems for some time precluded the use of product inhibition studies, and in addition, the amount of information obtainable in a Uni system is limited inherently. The exergonic hydrolysis of FBP until recently was considered irreversible (58). However, it was demonstrated that the reaction of FBPase is kinetically reversible (7), and studies of the reverse reaction have substantially narrowed the possible kinetic mechanisms (54).

Using results of product inhibition studies in the forward reaction and initial-velocity studies of the reverse reaction of FBPase, it was possible initially to eliminate from consideration the conventional rapid-equilibrium Ordered Uni Bi mechanism. Further studies on the reverse reaction utilizing substrate analogs indicated that the steady-state Ordered and Ping Pong Uni Bi mechanisms were not consistent with the data. In addition, isotope partitioning experiments indicated that the reverse reaction approximates the rapid-equilibrium assumption.
The results obtained thus far were in accord with a rapid-equilibrium Random Bi Uni mechanism, as well as a unique rapid-equilibrium Ordered Bi Uni mechanism of the type proposed by Frieden (59), with F6P binding to a site other than the active site (54). Several methods have been suggested whereby these two mechanisms may be differentiated, including the use of multisubstrate analogs (60).

Recent reports have indicated that 2,6FBP is a potent competitive inhibitor for FBP in the forward reaction (2,66). Pilkis et al. (3) have presented additional evidence suggesting that 2,6FBP may be an allosteric effector of the enzyme and also may act at the active site. These results and the obvious similarity of 2,6FBP to FBP suggested the use of the former compound as a multisubstrate analog for studying the reverse reaction. This section presents the results of these studies and the conclusion that the most probable mechanism for the reverse reaction is rapid-equilibrium Random Bi Uni. In addition, our data serve to shed additional light on the interaction of 2,6FBP with FBPase.
MATERIALS AND METHODS

FBP, trisodium salt, F6P, disodium salt, NADP, sodium salt, L-histidine, Tris(hydroxymethyl)aminomethane (Tris), phenylmethyl-sulfonylfluoride, phosphoglucoisomerase, aldolase, and rabbit muscle FBPase were purchased from Sigma. Affi-gel Blue, 100-200 mesh, was purchased from Bio-Rad. Glucose-6-P dehydrogenase and glycerolphosphate dehydrogenase were purchased from Boehringer Mannheim. All other reagents were of the highest quality commercially available. Distilled deionized water was used in the preparation of all reagents.

Enzyme Preparation

FBPase was purified from bovine liver, as described (54), with the following modification. After phosphocellulose chromatography, the enzyme was dialyzed against 10 mM Tris-HCl, pH 8.5, and applied to an Affi-gel Blue column (1.5 cm x 30 cm). After washing with five column volumes of buffer, the enzyme was eluted with 25 μM AMP in 10 mM Tris-HCl, pH 8.5 (111). Enzyme prepared in this fashion had a specific activity of 22.0 I.U./mg, and a pH 6.5:9.0 activity ratio of at least 2.0.
Enzyme Assays

The coupled spectrophotometric assay used to measure the reverse FBPase reaction has been described previously (7). The assay couples the production of FBP to the aldolase reaction to produce dihydroxyacetonephosphate and D-glyceraldehyde-3-phosphate. D-Glyceraldehyde-3-phosphate is converted to dihydroxyacetonephosphate by triosephosphate isomerase, and dihydroxyacetonephosphate is reduced to glycerol-3-phosphate in the presence of NADH and glycerolphosphate dehydrogenase. All assays contained 25 mM Tris/25 mM histidine (pH 6.5), 8 mM MgSO\(_4\), 0.125 mM NADH, 4.6 units of aldolase, 29 units of triosephosphate isomerase, and 2.6 units of glycerolphosphate dehydrogenase. All assay components except P\(_i\) were incubated at 28°C for 5 minutes, before initiation of the assay by P\(_i\). Absorbance changes at 340 nm were monitored on a Cary 118 spectrophotometer, using the 0.02 optical density full scale setting. 2,6FBP was added in a small volume of 0.01 N NaOH; uninhibited assays contained the same volume of 0.01 N NaOH alone. The final volume of the assays was 1.0 ml.

The forward FBPase reaction was monitored spectrophotometrically by using a coupled assay based on that originally developed by Pontremoli (7,112). The assay mixture contains 25 mM Tris/25 mM histidine, pH 6.5, 5 mM MgSO\(_4\), 0.2 mM NADP, 6.3 units of phosphoglucoisomerase, and 5 μM FBP. The total assay volume was 1.0 ml, and the temperature was maintained at 28°C.
The kinetic data from the reverse reaction studies were analyzed by using a computer program written in the OMNITAB II language (113).

Preparation of 2,6FBP

Several methods for the preparation of 2,6FBP have been described (65,114,115). In all cases, FBP is reacted with dicyclohexylcarbodiimide to form D-fructose-1,2-cyclic 6-bisphosphate (116), which is then hydrolyzed with base to form a mixture of FBP and 2,6FBP. Van Schaftingen and Hers (115) separated the mixture by first converting FBP to F6P by using FBPase, and then separating the resulting P$_i$, F6P and 2,6FBP on a column of Dowex AG1. Pilkis et al. (65) hydrolyzed FBP to F6P by using base hydrolysis at high temperature, and separated the resulting mixture on a column of DEAE-Sephadex. Uyeda et al. (114) also hydrolyzed FBP by using base hydrolysis, but separated the resulting mixture by using paper chromatography.

All three methods were used to prepare 2,6FBP for use in these studies. The method described by Van Schaftingen and Hers (115) was found to be the most successful for the following reasons: first, FBPase was used to degrade FBP in order to facilitate its separation from 2,6FBP. The other methods (65,114) involve heating the sample to 90°C for 30 minutes in the presence of 0.25 N NaOH, which in my hands generated rearrangement products that appeared as contaminants in the final 2,6FBP preparation. Second, Van Schaftingen and Hers (115) used Dowex AG1 column chromatography to separate the final products of the
I found this column to have a higher capacity and superior flow characteristics when contrasted with the other chromatography techniques (65,114). Therefore, 2,6FBP was prepared essentially as described (115) with minor modification.

FBP (2.5 g, trisodium salt) was dissolved in 10.0 ml of water and passed over a 0.9 cm x 27 cm Dowex 5W (X-8) column in the H\(^+\) form. The carbohydrate-containing fractions were detected by charring, and the pooled fractions were adjusted to pH 7.0 by the addition of approximately 2 ml of pyridine. A mixture containing 7 ml of the FBP solution, 0.5 ml of triethylamine and 20 ml pyridine was assembled, and the cyclization reaction was initiated by the addition of 2.4 g dicyclohexylcarbodiimide in 10 ml of pyridine. The reaction was allowed to proceed for 24 hrs at 25°C and then stopped by the addition of 40 ml of water. The mixture was passed through a sintered glass filter, and then extracted with five 200 ml portions of ether.

The D-fructose-1,2-cyclic 6-bisphosphate contained in the aqueous phase was hydrolyzed to FBP and 2,6FBP by the addition of 0.2 volumes of 2.5 N NaOH (approximately 6.0 ml) followed by incubation at 37°C for 30 minutes. Solid glycine was added to a concentration of 20 mM, and the solution placed on ice, after which 2 N HCl was used to adjust the pH to 9.4.

To facilitate the removal of FBP from 2,6FBP in the mixture, FBP was hydrolyzed to F6P and P\(_1\) by using FBPase. The solution was brought to 90 ml by the addition of water, and MnCl\(_2\) was added to a final concentration of 0.5 mM. Rabbit muscle FBPase was added to give 0.4
units of enzyme per ml of solution, and the mixture was incubated for 3 hr at 30°C.

Following the degradation of FBP, the resulting mixture of F6P, P\textsubscript{i} and 2,6FBP was separated by column chromatography. The mixture was diluted with water to a final volume of 450 ml, and applied to a 0.9 cm x 30 cm column of Dowex AG\textsubscript{i} (X-2) in the chloride form. The column was developed with a linear gradient of NaCl, 100 mM-400 mM, in a total volume of 250 ml. Fractions containing 3.0 ml were collected and assayed for F6P by using the method described for assaying the forward FBPase reaction, omitting FBPase. Fractions were also assayed for 2,6FBP, by monitoring the inhibitory effect of aliquots of the fractions on the forward FBPase reaction.

Column fractions containing 2,6FBP were pooled, and the concentration of 2,6FBP was determined by the assay of acid-revealed F6P (65,114, 115). The final yields of the preparations were typically between 3.2-5.0%, and the preparations contained about 5% contaminating FBP. The structure and purity of the final product was confirmed by using \textsuperscript{31}P NMR spectroscopy, with and without proton decoupling (117).
RESULTS AND DISCUSSION

The results of 2,6FBP inhibition of the FBPase reaction are shown in Figures 1 and 2. In both cases, one substrate was held at a constant concentration near its $K_m$ value while the other substrate was varied in the presence of 0-6 µM inhibitor. Figure 1 shows that 2,6FBP gives a linear competitive plot with respect to F6P. The slope replot shown in Figure 3 is also linear. Similarly, inhibition with respect to $P_i$ is linearly competitive with a linear secondary plot, as shown in Figures 2 and 4, respectively. The values for the inhibition constants were determined to 1.16 ± 0.16 µM for F6P and 0.35 ± 0.06 µM for $P_i$ from Figures 1 and 2, respectively. Though theoretically the inhibition constants should be equivalent, some error may be introduced because they are calculated by using a compilation of other previously determined kinetic constants (54), in addition to the experimental data.

In the presence of saturating Mg$^{2+}$, the reverse reaction of FBPase is Bi Uni. The kinetics of the reverse reaction have been investigated by initial-velocity studies and the use of substrate and product analogs. Fromm and Stone showed the product analog 2,5-anhydromannitol-1,6-bis-phosphate is a competitive inhibitor for both $P_i$ and F6P (54), as well as a competitive inhibitor of FBP (52). These data served to eliminate both steady-state Ordered Bi Uni and Ping Pong Bi Uni mechanisms because the rate equation for both predicted that the analog would be a noncompetitive inhibitor with respect to F6P. However, the data still were consistent with two mechanisms described by the rate equation:
Figure 1. Plot of the reciprocal of initial velocity versus the reciprocal of the molar concentration of F6P in the absence (○) and presence of 1.5 μM (●), 3.0 μM (△), 4.5 μM (□), and 6.0 μM (●) 2,6FBP. Assays were performed as outlined in "Materials and Methods" with a fixed concentration of 25 mM P_\text{\text{\text{\text{I}}}} and 0.60 I.U. of FBPase.
Figure 2. Plot of the reciprocal of initial velocity versus the reciprocal of the molar concentration of $P_i$ in the absence (○) and presence of 1.5 μM (●), 3.0 μM (◇), 4.5 μM (△), and 6.0 μM (□) 2,6FBP. Assays were performed as outlined in "Materials and Methods" with a fixed concentration of 0.61 mM F6P and 0.33 I.U. of FBPase.
Figure 3. The slope replot for 2,6FBP inhibition of FBPase with respect to F6P. The data are from Figure 1.
Figure 4. The slope replot for 2,6FBP inhibition of FBPase with respect to $P_i$. The data are from Figure 2.
where \( K_q, K_p, \) and \( K_{ia} \) are the Michaelis constants for \( P_i \) and F6P, and the dissociation constant for the product analog. In previous work on the forward reaction, F6P was shown to be a noncompetitive inhibitor for FBP. Because product inhibition should be competitive for a rapid-equilibrium random Uni Bi mechanism, it is necessary to propose the formation of an inactive enzyme-F6P-F6P complex for this mechanism to be in harmony with all the data.

An equally likely mechanism of the type proposed by Frieden (59) was illustrated in scheme 3. This rapid-equilibrium Ordered mechanism involves the formation of an inactive enzyme-F6P complex, and is consistent with all the data obtained to this point for the reverse reaction.

The two aforementioned mechanisms are not distinguishable by classical kinetic techniques. It has been pointed out, however, that three criteria will permit a differentiation to be made between the rapid-equilibrium Random mechanism and the unique Ordered mechanism (60). One such procedure involves the use of a multisubstrate analog. For a Random mechanism, the multisubstrate analog would be a competitive inhibitor for both substrates. Because the analog would bind both substrate sites on the enzyme simultaneously, only the enzyme term of the rate equation would be affected. Primary plots for this mechanism would be linearly competitive, and secondary plots would be slope linear.
Alternatively, in the case of Frieden's mechanism, the analog would be expected to bind to a subsite for F6P, as well as to the active site. The binding of more than one molecule of the analog per substrate site will result in several squared terms in the rate equation (111). Consequently, the primary plots for this mechanism would be competitive for P_i and noncompetitive for F6P, and secondary plots would be parabolic.

It is well-documented that 2,6FBP is a potent competitive inhibitor of FBPase in the forward reaction, and the compound seemed an ideal choice as a multisubstrate analog for F6P and P_i. The results of our study on the effect of 2,6FBP indicate that it is a powerful inhibitor of the reverse reaction as well. In light of the previous discussion, it also is possible to select the steady-state Random Uni Bi mechanism in which the rapid-equilibrium assumption is approximated (54) as the best possibility for FBPase in the forward direction. In the reverse direction, FBPase seems to be rapid-equilibrium Bi Uni.

The nature of the actual interaction of 2,6FBP with FBPase is far from clear. At low concentrations (< 10 μM), 2,6FBP seems to be strictly a competitive inhibitor of the forward reaction; however, higher concentrations of 2,6FBP cause the hyperbolic substrate concentration curve to become sigmoidal (3). In addition, even at low concentration, the effector potentiates AMP inhibition. Hence, it has been proposed that the action of 2,6FBP on FBPase involves at least some interaction with an allosteric site (3).
It is possible to conceive of two mechanisms for 2,6FBP interaction with FBPase that are in harmony with the data presented here. If the effector were to interact only with the active site, we would expect competitive plots for both substrates and linear replots as seen in Figures 1 and 2. This would, however, be kinetically indistinguishable from a situation in which 2,6FBP binds exclusively to an allosteric site, causing simultaneous release of product or substrates at the active site.

On the basis of the previous study of the effect of the product analog 2,5-anhydromannitol-1,6-bisphosphate on the reverse reaction, interaction with the active site seems most likely. The product analog, thought to act at the FBP active site, gave kinetic plots of the same pattern seen with 2,6FBP. It also is possible that 2,6FBP binds an allosteric site only at concentrations higher than were used in this study.
CONCLUSIONS

The results of this study indicate that the kinetic mechanism of bovine liver FBPase in the reverse reaction direction is rapid-equilibrium Bi Uni. In addition, the inhibition patterns produced by 2,6FBP with respect to F6P and $P_i$ in the reverse direction FBPase reaction are indicative of an active site mode of action for the inhibitor. However, as mentioned, an allosteric mode of action of 2,6FBP is also a possibility. Clearly though, 2,6FBP acts at only one type of site, because the results present no evidence of multiple binding of the inhibitor to the enzyme. In any case, physical studies on the binding of 2,6FBP to FBPase presented in the following section serve to clarify the nature of the interaction.
PART II. NUCLEAR MAGNETIC RESONANCE STUDIES OF FRUCTOSE 2,6-BISPHOSPHATE AND ADENOSINE-5'-MONOPHOSPHATE INTERACTION WITH BOVINE LIVER FRUCTOSE 1,6-BISPHOSPHATASE
INTRODUCTION

Bovine liver fructose 1,6-bisphosphatase, FBPase (EC 3.1.311), catalyzes the hydrolysis of fructose 1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P) and inorganic phosphate (P$_i$) in the presence of a required divalent metal ion (Mn$^{2+}$, Zn$^{2+}$, Mg$^{2+}$). The enzyme is composed of four identical subunits, each capable of binding metal ions (23). FBPase plays a key role in the futile cycle of FBP synthesis and degradation and is highly regulated by several cellular components, including AMP and fructose 2,6-bisphosphate (2,6FBP) (for reviews see 9-11).

Inhibition of FBPase by 2,6FBP in the direction of FBP degradation has been characterized as being synergistic with AMP and is relieved by high concentrations of the substrate, FBP (3,66). However, the inhibitor, 2,6FBP, has also been reported to change the substrate saturation curve from hyperbolic to sigmoidal (66). As described in Part I, in the reverse direction, the inhibitor gives kinetic results consistent with classical competitive inhibition, indicating that 2,6FBP acts either exclusively at the active site or solely at an allosteric site. Competitive inhibition in the forward direction also has been reported for FBPase from pig kidney (67), spinach chloroplast (67), and rabbit liver (118). In addition, Kitajima and Uyeda (69) have reported that 2,6FBP binds only one site per FBPase monomer and that binding of the inhibitor is competitive with F6P, P$_i$, and FBP. Similarly, rat liver FBPase binds only one equivalent of 2,6FBP per enzyme monomer, and
the substrate analog αMeFBP blocks 2,6FBP binding (68). These results, however, could also be interpreted either as 2,6FBP action at the active site or binding of the inhibitor at an allosteric site, which prevents the binding of substrate or products.

To establish whether 2,6FBP acts at the active site or at an allosteric site of FBPase, we have taken advantage of the Mn-binding property of the enzyme by monitoring the effect of the paramagnetic ion on $^{31}$P NMR relaxation rates. Cunningham et al. (110) have utilized this technique to map the active and allosteric site topography of rabbit liver FBPase for the phosphoryl groups of F6P, $P_i$, and AMP and the protons at carbons 2, $H(2)$, 8, $H(8)$, and 1, $H(1)$ of AMP. In a similar fashion, I have measured enzyme-Mn to phosphorus distances for F6P and $P_i$, products of FBPase that are considered to bind only at the active site, and I have compared these with the distances obtained for the C-2 and C-6 phosphate groups of 2,6FBP and the C-1 and C-6 phosphate groups of α-methyl D-fructofuranoside 1,6-bisphosphate (αMeFBP), a nonhydrolyzable competitive inhibitor of FBPase (51). A strong correlation of these distances would indicate that 2,6FBP also acts at the active site of FBPase. In addition, a second locus for the distance measurements can be introduced by spin-labelling the enzyme with a nitroxide group at an active sulfhydryl site because it has been established (23) that bovine liver FBPase has one very reactive thiol group per enzyme monomer.

The synergism of inhibition observed for AMP and 2,6FBP suggests that the sites for these inhibitors may be closely associated. By
comparing the enzyme-Mn to phosphorus distances of one inhibitor in the presence and absence of the other, it is possible to observe perturbations of the AMP site produced by the presence of 2,6FBP or vice versa. The effect of 2,6FBP on the binding of $^{14}$C-AMP to FBPase is also investigated.

AMP is thought to act at an allosteric site of FBPase, as evidenced by the ability of several chemical modifiers to disrupt AMP sensitivity without inhibiting enzyme activity (9). AMP inhibition of the bovine liver FBPase has been investigated for both the forward and reverse direction reactions by using initial-rate and progress-curve-analysis kinetic techniques (55). The nucleotide was found to exhibit noncompetitive inhibition with respect to the substrates in both directions.

Though the interaction of AMP with FBPase has been thoroughly studied by classical kinetic and binding techniques, additional information about the dynamics of the nucleotide-enzyme complex formation could be gleaned through the use of NMR to monitor the exchange reaction between AMP and enzyme. The use of NMR to study the binding of small molecules to macromolecules has been described in detail (4-6,92-98). In this section, $^1$H NMR was used to obtain information on reaction rate constants and activation parameters for the AMP interaction with FBPase in the presence and absence of 2,6FBP.
MATERIALS AND METHODS

FBPase from fresh bovine liver was prepared as described in Part I. The enzyme was then concentrated by ammonium sulfate precipitation and dialyzed against the appropriate buffer for the experiments as detailed below.

2,6FBP was prepared as described in Part I and assayed by acid hydrolysis for F6P release. The concentration of F6P was determined enzymatically by using phosphoglucoseisomerase and glucose-6-P dehydrogenase and monitoring the absorbance at 340 nm of NADPH produced from NADP+. AMP concentrations were determined by measuring the absorbance at 259 nm, using an extinction coefficient of 15,400 M⁻¹ cm⁻¹. Mg²⁺ and Mn²⁺ stock solutions were standardized by the Analytical Service Division of the Ames Laboratory of the United States Department of Energy, Ames, Iowa. [⁵²⁵⁵Mn] MnCl₂ was obtained from New England Nuclear, and ¹⁴C AMP was from Amersham.

Preparation of αMeFBP

The synthesis of αMeFBP by phosphorylation of α-methyl fructoside was previously described (51). The method involves methylating fructose, and separating the resulting α and β anomers by selective crystallization of the anomeric complexes with brucine alkaloid (51,119). Separation of the methylated anomers of fructose by this method was found to be unsatisfactory in my hands; indeed, Benkovic et al. (51)
also reported difficulty isolating a product, presumed by them to be 
α-methyl fructoside, which gave optical rotation results inconsistent 
with the literature values (119). Therefore, α-methyl fructose was 
separated from β-methyl fructoside by using column chromatography (120) 
before phosphorylation of the α anomer, as described in detail below.

Fructose (5.2 g) was treated with anhydrous, acid methanol (125 ml 
methanol plus 0.45 ml concentrated H₂SO₄) for 48 hours at room 
temperature. The mixture was then neutralized by the addition of 
Amberlite IR-45 (OH⁻ form), which was removed by filtration. The 
solution was evaporated to obtain 5.0 g of syrup. The syrup was 
analyzed by using paper chromatography on Whatman 3MM paper using 
n-propanol: 1 N NH₄OH, 7:2 as the solvent (121). The chromatogram 
revealed 3 spots upon treatment with 0.1 N H₂SO₄ in 90% ethanol followed 
by heating at 70°C for 20 minutes. One spot had the same mobility as 
fructose, whereas the other spots were more mobile.

The syrup obtained in the first step was made 50% in methanol and 
applied to a column (3.5 x 50 cm) of Dowex 1X2, 50-100 mesh, in the OH⁻ 
form (120). It is imperative that the 50-100 mesh be used, as the 
200-400 grade gives much poorer separation. The column was developed 
with water at a flow rate of 1.5 ml/minute, and 6.0 ml fractions were 
collected. The fractions were analyzed for carbohydrate by the 
phenol-sulfuric acid method (122). Three peaks of carbohydrate were 
separated, and the appropriate fractions were pooled and the optical 
rotation determined. The first two peaks eluted from the column gave 
negative optical rotation values, whereas the pool of the third peak
gave a value of \([\alpha]^{22}_D = +83.9^\circ\), in agreement with the literature value of +88-93° for \(\alpha\)-methyl fructoside (119). Therefore, the peaks were, in order of elution, \(\beta\)-methyl fructopyranoside, \(\beta\)-methyl fructofuranoside, and \(\alpha\)-methyl fructofuranoside (120).

The \(\alpha\)-methyl fructofuranoside was concentrated by rotoevaporation to obtain 0.96 g of syrup, a yield of 18%. The syrup was dried for 12 hours over \(\text{P}_2\text{O}_5\) in a vacuum desiccator. This material was phosphorylated by using the method described by Hartman and Barker (123) for the phosphorylation of 2,5-anhydro-D-glucitol. The syrup (360 mg) was taken up in 10 ml of dry pyridine at -20°C, and 0.9 ml diphenylphosphorylchloridate was added. After 30 minutes, the temperature was raised to 0°C, and the mixture was incubated for 12 hours. The reaction was stopped by the addition of a few drops of water, and 50 ml of chloroform were added. The chloroform solution was washed successively with two 200 ml portions of 1 N \(\text{H}_2\text{SO}_4\), followed by similar washes with saturated sodium bicarbonate, and finally, water. The resulting solution was evaporated to a syrup (approximately 1 g, 80% yield).

The syrup was taken up in 50 ml of methanol, and the phenyl groups were removed by catalytic hydrogenation. Platinum oxide (0.2 g) was added to the solution, which had been flushed with nitrogen. \(\text{H}_2\) was bubbled through the solution for 12 hours, after which the catalyst was removed by filtration. The sample was evaporated, and the resulting syrup was taken up in 10 ml water. The solution was adjusted to pH 10.00 with cyclohexylamine and sufficient acetone was added to form a precipitate. The solution was stored overnight to allow the formation
of crystals, after which the crystallization was repeated. Approximately 0.5 g of material was collected, for a final yield of 30%.

Paper chromatography (121) of the final product revealed one spot when the chromatogram was treated to reveal carbohydrate, and two spots when treated to reveal phosphorus-containing material (124). The αMeFBP was further analyzed by $^1$H, $^{13}$C and $^{31}$P NMR. $^{31}$P NMR revealed two major and one minor resonances. The minor resonance was identical to that obtained with commercial methyl phosphate, and composed approximately 10% of the total phosphate in the sample. The two major resonances were assigned to the 1 and 6 phosphate groups of αMeFBP based on comparison to FBP and 2,6FBP (117,125). The structure of the αMeFBP was confirmed by using $^1$H and $^{13}$C NMR, and comparing my results to those published previously (125-127).

The cyclohexylammonium salt of αMeFBP was converted to the sodium form by using ion exchange resin (Dowex 50W-X8). The concentration of the αMeFBP was determined by measuring total phosphate as described by Hartman and Barker (123).

Preparation of Spin-labelled FBPase

Spin-labelled FBPase was prepared in a fashion similar to that described by Cunningham et al. (110). The maleimide spin label (3-[Maleimidomethylene]-proxyl, Sigma) was dissolved in 50 mM PIPES buffer, pH 6.5, to a concentration of 2 mg/ml. FBPase (50 μmoles) was incubated with a ten-fold molar excess of the spin label for 2 hours in the dark
at room temperature. The number of FBPase sulfhydryl groups modified was determined before and after spin-labelling by reaction with Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, DTNB, Sigma) (128). For this reaction, 2 μM FBPase was added to a 20-fold excess of DTNB in 0.1 M Tris, pH 8.0, in a total volume of 1.0 ml, and the change in absorbance at 412 nm was monitored. Before spin-labelling, four sulfhydryl groups per enzyme tetramer reacted with the DTNB over a period of five minutes. This was followed by a very slow reaction of 1-2 additional groups over the next 2 hours. After spin-labelling, only the slow reacting sulfhydryl groups remained. Spin-labelled FBPase retained over 75% of the activity of the native enzyme.

All reagents used were of the highest purity available commercially. All solutions, including enzyme, used in NMR experiments were treated with Chelex before the addition of the appropriate metal ion. 2,6FBP, F6P, P1, αMeFBP, and AMP stock solutions were adjusted to pH 6.5 before their addition to NMR samples. NMR tubes were acid washed and thoroughly rinsed before use. Distilled, deionized water was used throughout.

Methods

Measurements were made with a Bruker HM-300 NMR spectrometer operating at 121.5 MHz for 31P and 300 MHz for 1H. The 300-MHz spectrometer is equipped with a variable temperature controller and an Aspect 2000 computer. All experiments were performed at 10°C unless
otherwise noted. Irradiation of the water resonance was used in the $^1$H NMR exchange broadening experiments. In the $^{31}$P NMR experiments, broadband decoupling, if used, was gated by using an automated pulse sequence to avoid a Nuclear Overhauser Effect.

Longitudinal relaxation rates, $T_1$, were determined by using an $180^\circ-\tau-90^\circ$ inversion-recovery pulse sequence and computer fitted to the equation $Y = A_3 + A_2 \exp(\tau/T_1)$, where $Y$ is the normalized peak intensity for a particular value of $\tau$, $A_2$ is the nucleus flip angle in units of $\pi/2$, and $A_3$ is the normalized intensity of the largest peak. The transverse relaxation rates, $T_2$, were measured by obtaining the line width at half height in hertz, $\Delta$, and applying the equation $1/T_2 = \pi\Delta$.

Samples for the distance measurements typically contained 13.5 mM F6P, aMeFBP, 2,6FBP, P$_i$, or AMP in 50 mM PIPES (pH 6.5) and 20% D$_2$O to serve as a heteronuclear lock. When distance measurements were performed on two compounds in the same sample (i.e., AMP and F6P or AMP and 2,6FBP), both compounds were present at 13.5 mM. Either Mn$^{2+}$ or Mg$^{2+}$ was allowed to incubate with FBPase several hours at 5°C before addition to the sample. The final concentration of FBPase tetramer in the samples was 1.2 $\mu$M, whereas the concentration of metal ion was either 2.4 $\mu$M or 3.6 $\mu$M. When spin-labelled FBPase was used in place of Mn-FBPase in distance measurement experiments, the concentration of enzyme was 20 $\mu$M tetramer. Other conditions were identical to those described for experiments using Mn-FBPase.

Exchange measurements made for the H(2) and H(8) of AMP were performed in 99% D$_2$O containing 25 mM PIPES (pH 6.5), 6 mM Mg$^{2+}$, and
0.1 mM EDTA. Other conditions for these experiments are given in the figure legends.

$T_1$ and $T_2$ measurements for the H(2) and H(8) of AMP were performed on samples containing 99% D$_2$O, 50 mM PIPES (pH 6.5), 13.5 mM AMP, and 1.2 μM FBPase tetramer preincubated with metal as just described.

All $T_1$ and $T_2$ determinations were made at least twice and usually three times. Agreement between $T_1$ determinations was ±20%.

$T_1$ determinations at 36.4 MHz were made on a Bruker HX-90 spectrometer.

Distance Measurements

The distances between enzyme-bound Mn$^{2+}$ or nitroxide spin label and the phosphate groups of the various effectors of FBPase were obtained by monitoring the effect of the paramagnetic centers on the longitudinal relaxation rates of the $^{31}$P nuclei of the ligands. The distances were calculated by a modified form of the Solomon-Bloembergen equation (101):

$$r (A) = C \left[ f \times T_{1p} \left( \frac{3 \tau_c}{1 + \omega_1^2 \tau_c^{-2}} \right) \right]^{1/6}$$

where $f$ is the mole fraction of enzyme-bound ligand, $\tau_c$ is the correlation time for the dipolar interaction, $\omega_1$ is the Larmor precession frequency, and $T_{1p}$ is determined using the equation $1/T_{1p} = 1/T_1(Mn) - 1/T_1(Mg)$. $C$ equals $[(2/15) \gamma_1^2 g^2 S^2(S+1)]^{1/6}$, where $\gamma_1$ is the gyromagnetic ratio, $g$ is the electronic g-factor, $S$ is the electron
spin, and $\beta$ is the Bohr magneton. For $^{31}\text{P}$-Mn interactions, $C$ has a value of 601, and for $^{31}\text{P}$-nitroxide interactions, $C$ has a value of 399 (5).

**Dialysis Experiments**

The concentration of Mn-enzyme and the concentration of Mn$^{2+}$ free in solution were measured by using an equilibrium dialysis cell and $^{54}\text{Mn}$. The dialysis cell consists of two Plexiglas chambers, each capable of containing 1 ml. The chambers can be separated by a small piece of dialysis tubing, and clamped together. (A size 14 cork borer can be used to conveniently cut the dialysis tubing to the proper size.) Solutions may then be added to each chamber through a small port by means of a syringe. The ports are sealed with tape, and small molecules may exchange between the two chambers through the separating membrane.

FBPase was incubated for 1 hour at 5°C with two equivalents of $[^{54}\text{Mn}]\text{MnCl}_2$, 20 μCi/μmole, as for the NMR experiments. To each chamber of the equilibrium dialysis cell was added 0.5 ml of a solution containing 13.5 mM PIPES buffer, pH 6.5, and 20% D$_2$O. To one chamber was added a small aliquot of Mn-enzyme to a final concentration of 2.4 μM. The cells were allowed to equilibrate 10 hours at 5°C on an agitating platform. To ensure the attainment of equilibrium, a control sample was included in which $^{54}\text{Mn}$, but not enzyme, was added to one chamber. Both sides of the cell were monitored until the $^{54}\text{Mn}$ concentration on each side was equivalent. After equilibration, small
aliquots from each side were added to 13 x 100 mm glass culture tubes and counted in a Tracer Analytic 1191 gamma counting system. These experiments allowed the determination of the concentration of Mn sup2+ free in solution as well as the concentration of Mn sup2+ bound to FBPase under the same conditions as those used in the NMR experiments.

The contribution of binary complexes of the type Mn-ligand to T sup1p was also evaluated by a dialysis experiment. In this case, FBPase was incubated with 2 or 3 equivalents of Mn sup2+ or Mg sup2+ as for the NMR experiment. Me sup2+-FBPase was added to a mixture of 13.5 mM ligand, 50 mM PIPES pH 6.5 and 20% D 2O to give a final concentration of 2.4 μM enzyme (twice the concentration used in the NMR experiments) and a final volume of 2.5 ml. The sample was transferred to a piece of 6.4 mm dialysis tubing and placed in a 1 x 15 cm glass tube containing 2.5 ml of the same mixture minus FBPase. The top of the tube was sealed with laboratory film, and the tubes were attached to an agitating platform and allowed to equilibrate for at least 4 hours. The exact time needed for equilibration was determined by including a control sample which was assembled as described, except that AMP was added only to the sample inside the dialysis sack. The outside solution was then monitored by removing small aliquots and determining the AMP concentration by using the absorbance of the sample at 259 nm. A 5 mm acid-washed glass bead placed in the dialysis sack was found to aid in mixing of the sample and hence decreased the time needed for equilibration.

After equilibration, 2.0 ml of the dialysate, containing any ligand-Me sup2+ complexes but not enzyme, was transferred to an NMR tube and
$T_1$ was obtained. For each ligand, both a Mg$^{2+}$ and a Mn$^{2+}$ sample was prepared, so that $T_{1P}^*$, the contribution of binary complexes to $T_{1P}$, could be determined.

**Binding Studies**

Binding experiments were performed at 20°C in duplicate by using the method of Paulus (129). PM-10 Diaflow membranes (Amicon) were cut with a number 5 cork borer as described and immersed 4 hours in water before use. Uniformly labelled $^{14}$C-AMP was from Amersham. A typical experiment contained in a final volume of 200 μl, 50 mM PIPES (pH 6.5), 6 mM Mg$^{2+}$, 0.1 mM EDTA, 2 μM FBPase tetramer and AMP (specific activity 8 μCi/μM) from 1 μM to 50 μM. 2,6EBP or F6P, if present, was at a concentration of 50 μM or 0.5 mM, respectively. The samples were incubated at 28°C for 5 minutes, then transferred to the Paulus apparatus. The Paulus cell was run to dryness under 5-10 lbs/square inch of N$_2$, typically taking 15-30 minutes. The outlet ports were each purged with three 5 ml aliquots of ethylene glycol before removal of the membranes. The membranes were counted in 1 ml of water and 15 ml of Bray's scintillation fluid (130) in a Packard liquid scintillation spectrometer with an efficiency of 65%.
Exchange-broadening Measurements

Information on enzyme-bound ligand can be obtained by monitoring the broadening of the NMR signal of the small molecule, and the Swift-Connick equation can be applied to a system in which a nucleus of a ligand exchanges between two environments (100). The observed spin-spin relaxation rate, $1/T_{2obsd}$, for a nucleus undergoing chemical exchange between the free and enzyme-bound states is given by:

$$1/T_{2obsd} = 1/T_{2free} + f/(T_{2m} + \tau_m),$$

where $f$ is the fraction of ligand bound, $1/T_{2free}$ is the relaxation rate of free ligand, $T_{2m}$ is the spin-spin relaxation time in the bound state, and $\tau_m$ is the lifetime of the bound state ($1/k_{diss}$). The use of this equation requires the chemical shifts of the free and bound ligand to be identical and the fraction of free ligand to be in excess over the bound ligand ($f \leq 0.3$).

Either of two exchange mechanisms may describe the ligand-enzyme interaction. In the fast exchange limit, $T_{2m} \gg \tau_m$, and the weighted average width between bulk and bound inhibitor is observed; however, in the slow exchange limit $\tau_m > T_{2m}$, and the relaxation of the inhibitor is governed by the time spent bound to the enzyme, $\tau_m$. The two exchange mechanisms may be differentiated by monitoring the temperature dependence of the line broadening. The residence time of the ligand on the enzyme, $\tau_m$, decreases with rising temperature, whereas $1/T_{2m}$, which is a monotonically increasing function of the correlation time of the enzyme-AMP complex for a variety of relaxation mechanisms, should decrease with a rise in temperature.
Electron Paramagnetic Resonance (EPR) Measurements

EPR experiments were conducted at 20°C by using a Bruker ER 2000 spectrometer, operating at 9.78 GHz; 25-μl samples of 228 μM FBPase and one equivalent of Mn²⁺ were contained in 1.5-mm glass capillary tubes. For some experiments, an equimolar (to enzyme tetramer) quantity of 2,6FDP was included in the sample. For the nitroxide-labelled FBPase, Mn²⁺ was excluded.
RESULTS

It is possible to measure distances between a paramagnetic probe and magnetic nuclei based on that part of the Solomon-Bloembergen equations (101) that is characterized by dipole-dipole interactions between the electron and nuclear spins provided that certain criteria are fulfilled (4-6,92-99). These include (a) a small outer sphere contribution to $1/T_1$, (b) that $1/T_{1p}$ not be exchange-limited, and (c) that the hyperfine contact contribution to $1/T_{1p}$ be very small.

The restrictions imposed by criterion (a) are minimal because outer-sphere contributions, when present, are usually small. Criterion (b) is fulfilled by the findings for the $T_{1p}/T_{2p}$ ratios illustrated in Table III (5). The last criterion (c) is fulfilled because the electron spin relaxation time for Mn$^{2+}$ is long, so contact contributions to $1/T_1$ by Mn$^{2+}$ are nearly always small, especially at high magnetic fields.

The Effect of Mn$^{2+}$-FBPase on $^{31}$P Relaxation Rates of F6P, 2,6FBP, AMP, oMeFBP, and P$_i$

The values of $fT_{1p}$ and $fT_{2p}$ obtained for the phosphorus nuclei of a number of FBPase ligands in the presence of two or three equivalents of metal ion are shown in Table III, along with the enzyme-Mn to phosphorus distances calculated from the Solomon-Bloembergen equation by using the appropriate $T_{1p}$ values.
Table III. Relaxation Rates at 121.5 MHz of FBPase Ligands in Various Solutions and Mn-P Distances in FBPase-Mn²⁺-Ligand Complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>[Mn²⁺]</th>
<th>f₁₁</th>
<th>f₁₁* b</th>
<th>f₂₂</th>
<th>T₁₁/T₂₂</th>
<th>r (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µM)</td>
<td>(x 10⁻⁵ s)</td>
<td>(x 10⁻⁵ s)</td>
<td>(x 10⁻⁵ s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F6P</td>
<td>2.4</td>
<td>27.5</td>
<td>25.4</td>
<td>8.4</td>
<td>32.7</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>19.0</td>
<td>20.2</td>
<td>9.1</td>
<td>20.8</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Pi</td>
<td>2.4</td>
<td>28.9</td>
<td>27.8</td>
<td>6.1</td>
<td>47.4</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>21.0</td>
<td>22.0</td>
<td>3.7</td>
<td>56.8</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>2,6FBP</td>
<td>2P</td>
<td>2.4</td>
<td>15.4</td>
<td>13.6</td>
<td>9.6</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>11.9</td>
<td>5.8</td>
<td>20.5</td>
<td>4.7</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2P</td>
<td>2.4</td>
<td>14.6</td>
<td>12.8</td>
<td>7.3</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>6P</td>
<td>2.4</td>
<td>10.6</td>
<td>6.4</td>
<td>16.6</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>AMP</td>
<td>2.4</td>
<td>5.8</td>
<td>5.2</td>
<td>2.8</td>
<td>20.7</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>5.4</td>
<td>3.3</td>
<td>16.4</td>
<td>4.1</td>
<td>4.1 ± 0.3</td>
</tr>
</tbody>
</table>

The concentration of each ligand was 13.5 mM, and the concentration of FBPase was 1.2 µM tetramer. Other details are described in the text.

b f₁₁* is the T₁₁ value corrected as necessary for binary complexes (in the case of 3 equivalents of Mn²⁺), and calculated using the values of f determined from the data in Table IV (in the case of 2 equivalents of Mn²⁺).
The dissociation constants for 2,6FBP (Part I), F6P (7), AMP (55), αMeFBP (51), and P_i (7) have been tabulated (131) and, with the exception of P_i, are all < 1 mM, confirming that the enzyme is fully saturated with these ligands under the experimental conditions described. The dissociation constant for P_i is 7.7 mM (7) and the enzyme under the conditions used is 65% saturated with P_i.

It is known that K_d for Mn^{2+} is < 1 μM for the binding of the first 2 metal ions to rabbit liver FBPase (43). The very low Mn^{2+}-enzyme dissociation constant suggests that the concentration of Mn^{2+} free in solution will be quite low. The exact concentration of nonenzyme-bound Mn^{2+} in the presence of the various ligands was determined by using equilibrium dialysis and bovine liver enzyme with 2 equivalents of Mn^{2+} per tetramer as described in the "Methods" section. The results from the experiments are shown in Table IV and indicate that, in all instances, more than 85% of the total Mn^{2+} is bound to FBPase. These values allow the calculation of f, as depicted; in Table IV.

To determine r(λ) in the Solomon-Bloembergen equation, it is necessary to take into account contributions to T_{1P} from binary complexes of the type Mn^{2+}-ligand. With a knowledge of the dissociation constants for the four equilibria: E + Mn^{2+} = E-Mn^{2+}, E-Mn^{2+} + ligand = E-Mn^{2+}-ligand, E + ligand = E-ligand, and Mn^{2+} + ligand = Mn^{2+}-ligand, it is possible to determine the distribution and concentration of all the species in solution at equilibrium. This information allows one to experimentally obtain a complex of Mn^{2+}-ligand of comparable concentration to that which would be expected to be present in the T_1 experiments.
Table IV. Determination of $[\text{Mn}^{2+}]$ Free$^a$ and the Value of $f^b$

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$[\text{Mn}^{2+}]$ free (µM)</th>
<th>$f$ ($\times 10^{-4}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6P</td>
<td>0.18</td>
<td>1.64</td>
</tr>
<tr>
<td>$P_i$</td>
<td>0.13</td>
<td>1.68</td>
</tr>
<tr>
<td>2,6FEP</td>
<td>0.29</td>
<td>1.56</td>
</tr>
<tr>
<td>$\alpha$MeFEP</td>
<td>0.25</td>
<td>1.59</td>
</tr>
<tr>
<td>AMP</td>
<td>0.24</td>
<td>1.09</td>
</tr>
</tbody>
</table>

$^a$ $[\text{Mn}^{2+}]$Free is taken to mean $\text{Mn}^{2+}$ that is not associated with enzyme.

$^b$ FBPase was preincubated with 2 equivalents of $^{54}$Mn$^{2+}$. Other experimental details are described in the text.
and then evaluate its contribution to $T_{1p}$ determined in the presence of enzyme.

The potential effect of binary complexes to Mn-ligand on $fT_{1p}$ was evaluated by two independent experimental methods. First, $T_{1p}$ was evaluated twice for each ligand in the presence of enzyme incubated for 2 or 3 equivalents of metal ion. The results of these experiments are shown in Table III. For all ligands, with the exception of $P_i$, F6P, and 2,6FBP, there is no change in the relaxation rates normalized for the concentration of metal ion, $fT_{1p}$, with 3 equivalents of metal ion as compared with 2 metal ions. The $fT_{1p}$ values obtained with 3 metal ions for $P_i$, F6P, and 2,6FBP differ from the respective values obtained with 2 metal ions by an amount greater than that predicted by experimental error alone, indicating either a small contribution of binary complexes to the $T_{1p}$ values for these ligands, or a conformational change in the enzyme induced by metal.

An additional set of experiments was designed to evaluate the contribution of binary complexes to relaxation rates when two equivalents of metal ion were used, as described in the "Methods" section. Samples containing ligand and enzyme with 2 equivalents of Mn$^{2+}$ or Mg$^{2+}$ were dialyzed against solutions containing ligand alone. Three equivalents of metal ion were used in a separate experiment if indications of contributions to $T_{1p}$ from binary complexes existed from the previously described experiments (Table III). The outer solution was then removed and added to an NMR tube, and a $T_1$ determination performed. The results of these experiments are shown in Table V. In
Table V. Determination of the Effect of Binary Complexes and Nonenzyme-Bound Mn\textsuperscript{2+} on the Relaxation Rates of FBPase Ligands\textsuperscript{a}

<table>
<thead>
<tr>
<th>Ligand</th>
<th>([\text{Mn}^{2+}]/[\text{FBPase}])</th>
<th>(T_{1\text{Mn}}/T_{1\text{Mg}})</th>
<th>(T_{2\text{Mn}}/T_{2\text{Mg}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6P</td>
<td>2</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Pi</td>
<td>2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2,6FBP</td>
<td>2P</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>+AMP</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>6P</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>+AMP</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>cMeFBP</td>
<td>1P</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>6P</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>AMP</td>
<td>2</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>+2,6FBP</td>
<td>2</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The experiments were performed as described in Materials and Methods by using 2 or 3 equivalents of Mn\textsuperscript{2+} per FBPase tetramer.
every instance where 2 equivalents of metal ion were used, the ratio of \( T_1(\text{Mn})/T_1(\text{Mg}) \) or \( T_2(\text{Mn})/T_2(\text{Mg}) \) is equal to or near 1.0, indicating that the contributions of binary complexes or free Mn\(^{2+}\) to relaxation rates are negligible in the presence of 2 equivalents of Mn\(^{2+}\) per enzyme tetramer. In the case of F6P and P\(_i\), the data clearly show contributions from binary complexes or free metal ion, when FBPase is equilibrated with three equivalents of Mn\(^{2+}\) or Mg\(^{2+}\); however, a corrected value of \( T_{1p} \) may be obtained by using the appropriate \( T_{1p} \) values from Table III and the results of the dialysis experiments, along with the following expression: \( 1/fT_{1p}^* = 1/fT_{1pB} - 1/fT_{1pF} \), where \( T_{1p}^* \) is the paramagnetic contribution to the longitudinal relaxation rate corrected for free metal ion and binary complexes, and \( T_{1pB} \) and \( T_{1pF} \) are the uncorrected value and the contribution from nonenzyme-bound species, respectively. Interestingly, the discrepancy between the \( T_{1p} \) values for the 2 phosphorus group of 2,6FBP obtained with 2 or 3 equivalents of metal ion does not seem to arise from the existence of either binary complexes or free metal ion. Possibly, the binding of metal ion to FBPase causes a slight change in the position of the 2 phosphorus group of 2,6FBP relative to the active site; however, this effect is not large enough to cause significant changes in the value of \( r \), as shown in Table III.

Because AMP and 2,6FBP are reported to have synergistic inhibitory effects, it was of interest to determine if their simultaneous binding to FBPase would affect the enzyme-Mn to phosphorus distances of either inhibitor. This experiment was accomplished by performing the relaxation time determinations in the presence of 13.5 mM concentrations
of both AMP and 2,6FBP. As shown in Table VI, there was little change in the enzyme-Mn to phosphorus distances for 2,6FBP regardless of whether AMP was present or absent; however, the $fT_{1p}$ values for AMP showed a large change relative to that obtained in the absence of 2,6FBP, suggesting that the enzyme-Mn-P distance for AMP had changed significantly. Thus, the simultaneous presence of 2,6FBP and AMP on the enzyme causes AMP to shift position relative to the active site Mn$^{2+}$, by a factor of 2 Å. F6P, however, had no effect on the relaxation rates determined for AMP.

To investigate the simultaneous interaction of 2,6FBP and AMP with FBPase, binding studies were performed by using one method of Paulus (129). The results of these binding studies are shown in Figs. 5 and 6. In the absence of 2,6FBP, AMP binding is markedly sigmoidal. This fact had been previously reported (33,64). However, these binding studies indicate that 2,6FBP has a strong positive effect on AMP binding to FBPase, as shown in Fig. 5. Though the total amount of AMP bound seems unaffected, the amount of AMP needed to obtain half-saturation decreases from 21 µM in the absence of 2,6FBP to 7 µM in the presence of the effector. In addition, 2,6FBP relieves the sigmoidicity of the AMP binding curve. F6P had no similar effect, as shown in Fig. 6.

To further investigate the nature of 2,6FBP inhibition of FBPase, samples containing F6P and Mn- or Mg-FBPase were titrated with 2,6FBP, and the line width of the phosphorus nucleus of F6P monitored after each addition. The results, shown in Fig. 7, suggest that F6P is displaced from the enzyme by competition with 2,6FBP. It can be seen from Fig. 7
Table VI. Relaxation Rates at 121.5 MHz of 2,6FBP and AMP, and Mn-P Distances in FBPase-Mn$^{2+}$-Ligand Complexes$^a$

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$T_{1p}$ ($\times 10^{-5}$ s)</th>
<th>$T_{2p}$ ($\times 10^{-6}$ s)</th>
<th>$T_{1p}/T_{2p}$</th>
<th>$r$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>63</td>
<td>40.5</td>
<td>15.6</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>2,6FBP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2P</td>
<td>21.2</td>
<td>18.1</td>
<td>11.7</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>6P</td>
<td>22.3</td>
<td>18.3</td>
<td>12.2</td>
<td>5.2 ± 0.4</td>
</tr>
</tbody>
</table>

$^a$The concentration of both ligands was 13.5 mM. FBPase concentration was 1.2 μM tetramer. Other details are described in the text.
Figure 5. Binding of AMP to FBPase in the absence (●) or presence (○) of 50 μM 2,6FBP at 20°C. Other conditions were as described in Methods.
Figure 6. Binding of AMP to FBPase in the absence (●) or presence (○) of 0.5 mM F6P at 20°C. Other conditions were as described in Methods.
Figure 7. The effect of variable concentrations of 2,6FBP on the reciprocal of the line width (v) of the phosphate group of F6P in the presence of Mn-FBPase (o) or Mg-FBPase (●). The concentration of F6P was 10 mM, and other conditions were as described for the distance measurements.
that the line width of the Mn-FBPase sample approaches that of the
control sample containing Mg-FBPase with increasing concentrations of
2,6FBP. In addition, the linearity of the plot is characteristic of
competition between the two ligands for a site on FBPase, presumably the
active site (132). To further substantiate these results, the $T_1$ of the
F6P phosphorus nucleus was obtained before and after the addition of
2 mM 2,6FBP. The $fT_{1p}$ values obtained before and after 2,6FBP addition
were $2.00 \times 10^{-4}$ s and $20.2 \times 10^{-4}$ s, respectively. This distinct
change in the $fT_{1p}$ values is, again, indicative of displacement of F6P
from the enzyme by 2,6FBP. Thus, the NMR experiments described in this
report give results that are in harmony with kinetic and binding studies
which have shown that 2,6FBP and F6P interact in a mutually exclusive
fashion with FBPase.

Determination of Correlation Times in Mn-FBPase Complexes

To calculate internuclear distances from the $T_{1p}$ values given in
Table III, a value of $\tau_c$, the correlation time for the dipolar effect of
Mn-FBPase on the nuclei of the various ligands, must be determined.
Estimates of the value of $\tau_c$ were obtained by three independent methods.
In the first instance, the correlation time was estimated by using
the $T_{1p}/T_{2p}$ ratio for the H(8) of AMP at 300 MHz and applying the
equation:
\[
\frac{T_{1p}}{T_{2p}} = \frac{4 + 3/(1 + \omega_1^2 \tau_c^2)}{6/(1 + \omega_1^2 \tau_c^2)}
\]

The same method was applied to the \(T_{1p}/T_{2p}\) ratios for all the \(^{31}\)P experiments. The \(T_{1p}/T_{2p}\) ratio average was greater than 26. This value is somewhat larger than those obtained by other methods, suggesting scalar effects on the \(T_2\) value, which increase the magnitude of the \(T_{1p}/T_{2p}\) ratio. The correlation times determined from these data were not used in the distance measurement calculations.

A second estimate of \(\tau_c\) was obtained from the \(T_{1p}\) values for the phosphate of F6P determined at 121.5 MHz and 36.4 MHz, assuming that \(\tau_c\) is frequency dependent. The ratio of \(T_{1p}\) for F6P at the two frequencies was 40, and a lower limit value of \(\tau_c\) calculated from the Bloembergen-Morgan equation (133) as modified by Fung et al. (103), is \(3.0 \times 10^{-9}\) s.

Another estimation for the correlation time was obtained by measuring the peak to peak separation of an EPR transition of enzyme-bound Mn\(^{2+}\) and applying the equation:

\[
\frac{1}{T_{2e}} = \left[ \pi (\text{line width}) \ g \ (3)^{1/2} / (7.1 \times 10^{-7}) \right] \ \text{Gs}
\]

where \(g\) has a value of 2. Assuming that \(T_{2e}\), the electron-spin relaxation time for Mn\(^{2+}\)-FBPase, is equal to \(\tau_c\), the line width of
34.5 G results in a correlation time of $1.88 \times 10^{-9}$ s, a lower limit. When 2,6FBP was included in the sample, the line width was 36.7 G, and the resulting correlation time was $1.78 \times 10^{-9}$ s.

An average correlation time of $2.0 \times 10^{-9}$ s was used in the Solomon-Bloembergen equation to calculate Mn-enzyme to phosphorus nuclei distances.

Nitroxide-\textsuperscript{31}P Distance Estimations

The effect of spin-labelled FBPase on the spin lattice relaxation times of the phosphorus nuclei of 2,6FBP and F6P was negligible, as evidenced by a $<20\%$ difference in the $T_1$ values obtained in the presence of native or spin-labelled FBPase. Therefore, it was not possible to determine the distance between the phosphorus nuclei of either ligand and the nitroxide spin label. However, a minimum distance ($>9.1$ A) can be estimated by using a correlation time of $4 \times 10^{-9}$ s, obtained by using EPR.

Exchange-broadening Measurements for H(2) and H(8) of AMP

Fig. 8 shows the dependence of the line widths of resonances arising for H(2) and H(8) of AMP on $f$, the fraction of AMP bound to FBPase. No chemical shift changes were observed for either proton resonance upon binding to the enzyme. Because the changes in line widths observed for the various values of $f$ were rather small, two
Figure 8. Plot of the change in H(8) (●) and H(2) (○) linewidths (υ) of AMP in the presence of 0.12 mM FBPase tetramer (0.24 mM AMP sites) as a function of $f$, the fraction of AMP bound at 15°C. (▲) Denotes an upfield buffer resonance, as described in the text. The total AMP concentrations varied from 0.8 mM to 4.8 mM. Spectra were obtained in 99% D$_2$O containing 25 mM PIPES (pH 6.5), 6 mM Mg$^{2+}$, and 0.1 mM EDTA.
control experiments were carried out to ascertain if the changes in line
widths are indeed due to specific AMP binding to FBPase rather than to
changes in viscosity caused by the introduction of AMP into the enzyme
solution. First, the line width of an upfield resonance arising from a
buffer component was monitored at each value of f and showed little
change throughout the experiment. Second, a sample made up with a value
of f = 0.3, but containing heat-denatured enzyme, gave a narrow line
width (<3 Hz) for both proton resonances.

Fig. 9 shows the variation in line width of the H(8) and H(2) of
AMP as a function of temperature. Between 5°C and 30°C, these line
widths narrowed, indicating that AMP is in fast exchange on the 1H NMR
time scale between free and bound environments, and, therefore, that
T_{2M} > \tau_M. These results are in harmony with the findings shown in
Table III for AMP, which suggest that AMP is in the fast exchange limit.

From the temperature dependence of the line broadening of the proton
resonances, it is possible to obtain the apparent energy of activation
(E_a) of AMP from an Arrhenius-type plot as shown in Fig. 10. The values
of E_a and of AMP association with FBPase for H(2) and H(8) of AMP
obtained from the slope of the Arrhenius plot are 1.64 Kcal/mole and
1.76 Kcal/mole, respectively.

Because the fast exchange region is indicated by the data
presented, T_{2M} > \tau_M, and it is possible to determine a lower limit for
the dissociation rate constant, k_{diss}, inasmuch as \tau_M = 1/k_{diss}. The
average value of T_{2M} was 5.0 \times 10^{-2} s, and the lower limit of k_{diss} is
therefore 0.2 \times 10^2 \text{ s}^{-1}.
Figure 9. Variation of the linewidths ($\Delta\nu$) of the H(8) (●) and H(2) (○) resonances of AMP as a function of temperature. The sample contained 0.17 mM FBPase tetramer, 1.2 mM AMP, 25 mM PIPES (pH 6.5), 6 mM Mg$^{2+}$, and 0.1 mM EDTA in 99% D$_2$O.
Figure 10. Arrhenius plot of the temperature dependence of specific broadening of the H(8) (●) and H(2) (○) resonances of AMP. The data are from Figure 9.
An additional set of experiments was designed to determine if 2,6FBP would produce measurable effects on the H(2) and H(8) resonances of AMP. Fig. 11 shows the proton resonances in the presence and absence of 2,6FBP. Clearly, the addition of 2,6FBP causes the AMP resonances to sharpen markedly, although no chemical shift was observed. The simplest explanation for this observation would be attributed to a competition for a particular binding site between the two compounds. This was unlikely in the present situation because of the dissimilarity of the two compounds and the accumulated evidence indicating that 2,6FBP promotes the binding of AMP on FBPase (68,69, Fig. 5).

It was possible that the AMP line narrowing observed in the presence of 2,6FBP was due to a change in the exchange mechanism of AMP-enzyme complex formation. To investigate this possibility, a variable-temperature experiment identical to that shown in Fig. 9 was performed, except that 0.5 mM 2,6FBP was included in the sample. Throughout the experiment, the line width of H(2) remained within ± 0.1 Hz of an average value of 1.70 Hz, and the line width of H(8) remained within ± 0.3 Hz of 2.05 Hz. This indicates that either a fast or an intermediate exchange mechanism describes the AMP-enzyme complex formation because no line broadening occurred with elevated temperature.
Figure 11. $^1$H NMR spectrum of H(8) and H(2) of AMP in the absence (A) and presence (B) of 2,6FBP. (A) Spectrum of a sample containing 0.17 mM FBPase tetramer, 1.2 mM AMP, 25 mM PIPES (pH 6.5), and 0.1 mM in 99% D$_2$O.
DISCUSSION

The objective of this study was to investigate the enzyme-ligand interaction of two potent inhibitors of FBPase, 2,6FBP, and AMP. Of particular importance was the nature of the site occupied by 2,6FBP and how this site interacts, if at all, with the allosteric site of AMP.

Although Cunningham et al. (110) have made distance measurements using the technique of paramagnetic ion enhancement of relaxation rates of magnetic nuclei with rabbit liver FBPase, it was necessary for us to duplicate some of their experiments with the bovine liver enzyme for at least two reasons. First, the two enzymes do differ in both chemical and kinetic properties. For example, the two enzymes are immunologically different (22), the bovine enzyme contains tryptophan, whereas the rabbit enzyme lacks this amino acid (23), and finally, rabbit liver FBPase is believed to be kinetically irreversible (58), whereas the reverse reaction kinetics of the bovine enzyme have been studied in detail (7). Thus, it could not be assumed, a priorí, that the results of (110) are applicable to bovine liver FBPase. Second, it was necessary to establish the distances from Mn^{2+} on bovine liver FBPase to the active and AMP binding sites as a point of reference for the 2,6FBP binding locus, which is the basis for this report. It will be shown that, although the topography of the two FBPases is similar relative to their substrate and AMP binding sites in some respects, they do differ in other important characteristics.
Despite numerous studies on the interaction of 2,6FBP with FBPase since the discovery of the inhibitor in 1981, there still is controversy over whether the inhibitor acts at an allosteric site or the active site of the enzyme. Inhibition of the rat liver FBPase by 2,6FBP was reported by Pilkis et al. (2) and Van Schaftingen and Hers (1). Both groups describe the inhibition as being synergistic with AMP; however, Pilkis et al. (3) reported purely competitive inhibition, whereas Van Schaftingen and Hers (66) indicated that the inhibitor changes the substrate-saturation curve from hyperbolic to sigmoidal, suggestive of an allosteric mode of competition. Pilkis et al. (3) were later able to confirm the latter result with the rat liver FBPase. However, Gottschalk et al. (67) have since reported exclusively competitive inhibition for pig kidney and spinach chloroplast FBPases, and Pontremoli et al. (134) have reported competitive inhibition of rabbit liver FBPase by 2,6FBP.

The reverse reaction kinetics of the inhibition of bovine liver FBPase gave results indicative of classical linear competitive inhibition with respect to F6P and $P_i$ by 2,6FBP (Part I). These findings are consistent with the action of 2,6FBP at either the active site or an allosteric site of FBPase, but not both. If 2,6FBP does in fact bind to an allosteric inhibitory site, then binding of substrate does not occur at the active site; i.e., binding of the ligands is mutually exclusive.

Kitajima and Uyeda (69) have investigated the binding of 2,6FBP to rabbit liver FBPase by using a column centrifugation technique. Their results indicate the inhibitor binds to a single site on each enzyme.
monomer and that binding of 2,6FBP can be prevented by F6P, P\textsubscript{i}, and FBP. 2,6FBP (69) and FBP (43) both produce nonlinear Scatchard plots at higher ligand concentrations; however, the substrate and inhibitor may differ in the degree of cooperativity expressed (69).

The binding of 2,6FBP to rat liver FBPase also has been investigated (68). In this study, 4 moles of 2,6FBP were found to bind per enzyme tetramer, and 2,6FBP enhanced the binding of AMP to the enzyme. In addition, the substrate analogs oMeFBP and sMeFBP prevented the binding of 2,6FBP to FBPase, indicating an active site mode of binding of 2,6FBP.

2,6FBP protection of the catalytic activity of pig kidney (67) and rabbit liver (134) FBPases from inactivation by pyridoxal 5'-phosphate has been demonstrated, indicative of an active site mode of binding for the inhibitor. On the other hand, 2,6FBP protects both the catalytic activity and AMP allosteric site of the rat liver FBPase from acetylation, whereas FBP protects only the catalytic activity (135). In addition, 2,6FBP, but not FBP, confers protection from proteolysis on the rabbit liver enzyme (134). Also, protection of the catalytic activity of rat liver FBPase is given by low concentrations of 2,6FBP, or high concentrations of the substrate (136). Furthermore, Gottschalk et al. (67) have reported the catalytic activity and sensitivity to 2,6FBP inhibition of pig kidney FBPase to have similar pH profiles, whereas Francois et al. have found that the inhibitory action of 2,6FBP decreases with the increasing pH, though the affinity for substrate is not affected by the same conditions (137). One recent report (138) has
even suggested that 2,6FBP is an activator of rat liver FBPase, as well as an inhibitor. Thus, the accumulated evidence presented in reports on 2,6FBP interaction with several FBPases does not clearly point to a particular mode of binding of the inhibitor.

From experiments presented in this study, estimates of the distances from the Mn-binding site on bovine liver FBPase subunits to the phosphorus atoms of F6P, \( P_i \), AMP, \( \alpha \text{MeFBP} \), and 2,6FBP have been obtained. A direct comparison of the enzyme-Mn to phosphorus distances obtained for the C-6 phosphorus atoms of F6P, \( \alpha \text{MeFBP} \), and 2,6FBP gives an indication of the identity of the site(s) occupied by the compounds. Had the distances been significantly different, an allosteric site for 2,6FBP would have been strongly indicated. Because the three distances are within experimental error, identical, the experiments described herein present circumstantial evidence that 2,6FBP occupies the active site of FBPase, as do F6P and \( \alpha \text{MeFBP} \). Of course, the possibility remains that F6P, \( P_i \), \( \alpha \text{MeFBP} \), and 2,6FBP occupy separate sites, which coincidentally have the same Mn-phosphate distances. However, the distance between enzyme-bound Mn\(^{2+} \) and the C-1 phosphorus atom of \( \alpha \text{MeFBP} \), the C-2 phosphorus atom of 2,6FBP, and \( P_i \), are also identical. These results add credence to the hypothesis of an active site mode of action for 2,6FBP.

The results suggest that the FBPase site for the phosphorus groups of various sugars, as well as \( P_i \), is quite static, as evidenced by the similarity of the enzyme-Mn to phosphorus distances observed for F6P, \( P_i \), \( \alpha \text{MeFBP} \), and 2,6FBP. The relative positions of 2,6FBP, F6P, \( \alpha \text{MeFBP} \), and \( P_i \) to Mn\(^{2+} \) at the active site of FBPase are diagrammed in Figure 12.
Figure 12. Relative spatial arrangements between F6P, αMeFBP, 2,6FBP, Pᵢ, and the metal ion site of FBPase. Distances are in angstroms, and not necessarily drawn to scale. F6P and Pᵢ distances were determined separately, and are shown together only for reference.
Mn$^{2+}$

$\text{2,6FBP} \quad \text{POH}_2\text{C} \quad \text{CH}_2\text{OH}$

$\text{Mn}^{2+}$

$\text{F6Pand Pi}$

$\text{5.3}$

$\text{CH}_2\text{OP}$

$\text{OCH}_3$

$\text{5.0}$

$\text{5.3}$

$\text{CH}_2\text{OH}$

$\text{4.8}$

$\text{4.9}$

$\text{POH}_2\text{C}$
It has been previously noted (110) that Mn\(^{2+}\) at the active site of rabbit liver FBPase is equidistant from the phosphorus groups of F6P and P\(_i\). This result has been substantiated herein for the bovine liver enzyme. Interestingly, Mn\(^{2+}\) is also equidistant from the C-1 and C-6 phosphoryl groups of the product analog, \(\alpha\)MeFBP, and from the C-2 and C-6 phosphoryl groups of 2,6FBP. This again indicates that the inhibitor, like \(\alpha\)MeFBP, F6P, and P\(_i\), occupies the active site of FBPase.

The Mn\(^{2+}\) to phosphorus distances for F6P, P\(_i\), and AMP presented in this report are comparable to the values tabulated previously for rabbit liver FBPase (110). For each ligand, the distance calculated for the bovine liver FBPase is approximately 1 Å shorter than that obtained with the rabbit liver enzyme, perhaps due to species differences. However, the relative positions of the three ligands is quite similar; for both enzymes, AMP is approximately 1 Å closer to the active site Mn\(^{2+}\) than either F6P or P\(_i\), and the latter 2 ligands are in both instances equidistant from Mn\(^{2+}\), as previously noted.

In an attempt to gain an additional locus for mapping the sites occupied by 2,6FBP and F6P, FBPase was reacted with a nitroxide spin label. Bovine liver FBPase has been reported to possess one very reactive sulfhydryl group (33). Blocking this group with chemical modifiers relieves substrate inhibition, but does not otherwise affect the enzyme activity. Spin-labelled FBPase, however, had little (<20%) effect on the spin-lattice relaxation rates of the \(^{31}\)P nuclei of either F6P or 2,6FBP. Calculations indicate that both these compounds are at least 9.1 Å removed from the active sulfhydryl site.
AMP inhibition of bovine liver FBPase has been well-characterized through initial-rate kinetic studies of the forward (57) and reverse (54) reactions, as well as progress-curve analysis of the forward reaction (54). In addition, binding of AMP to the bovine liver FBPase allosteric site has been investigated in detail (57,64). The enzyme is reported to bind 2 (57) or 4 (64) moles of AMP per mole of tetramer. Hill, Scatchard, and double reciprocal plots of the binding data display complex curvature and have been analyzed by assuming mixed cooperativity (64).

Several lines of evidence indicate synergism between AMP and 2,6FBP inhibition of FBPase, and this, in turn, suggests an interaction of the binding sites of the two inhibitors. As previously mentioned, 2,6FBP changes the substrate saturation curve of FBPase from hyperbolic to sigmoidal, and this effect is potentiated by AMP (66). In addition, 2,6FBP protects the AMP allosteric site from inactivation by acetylation, and AMP and 2,6FBP induce similar ultraviolet difference spectra in the presence of enzyme (3). 2,6FBP is also reported to enhance the binding of AMP to FBPase (68); however, AMP is reported not to have an effect on 2,6FBP binding to FBPase (69).

Several experiments were performed to gain further insight into the interaction of AMP with FBPase, especially with regard to the apparent synergism with 2,6FBP. By measuring the temperature dependence of NMR line broadening of the H(2) and H(8) resonances of AMP, it was determined that the nucleotide is in fast exchange with FBPase. These results are supported by the $T_{1p}/T_{2p}$ for the $^{31}$P nucleus of AMP illustrated in Table III. By graphing the line broadening as a function
of temperature in an Arrhenius-type plot, it was possible to obtain estimates of $E_a$ for AMP interacting with FBPase. In addition, the lower limit of $k_{diss}$ at 20°C was estimated from the line broadening.

A plot of the change in the line width of $H(2)$ and $H(8)$ resonances of AMP as FBPase is titrated with nucleotide (Fig. 8) is markedly nonlinear. Because control experiments indicated that the change in line width is indeed due to AMP binding to FBPase, we have interpreted the nonlinearity of the titration curve to be an expression of the cooperative nature of AMP binding to the enzyme.

When 2,6FBP was added to a sample of AMP and FBPase, the AMP resonance sharpened markedly. This rather unexpected result could possibly be attributed to a competition between the two compounds. This explanation was given by Lanier and Navon (132) for a similar effect of p-toluenesulfonamide on the proton resonances of sulfonilamide in a solution containing carbonic anhydrase, and by Neurohr et al. (139) for the effect of $\alpha$-methyl D-galactopyranoside on the $\beta$ anomer of the same sugar in a sample containing peanut agglutin. In light of the accumulated evidence indicating synergism between 2,6FBP and AMP, a competition between the two seemed highly unlikely. Exclusive binding of AMP or 2,6FBP to FBPase can be conclusively ruled out by the binding depicted in Figure 5 which shows that 2,6FBP actually enhances the binding of AMP to FBPase. These results have been substantiated by others (68,69).

A second explanation for the NMR data would be a change in the exchange mechanism of AMP and FBPase. This possibility was tested by
performing the variable-temperature experiment in the presence of 2,6FBP. Because the line width of H(2) and H(8) of AMP remained essentially unchanged throughout the experiment, the slow exchange mechanism, which would have resulted in line broadening, could be discounted. However, because the line widths were quite narrow, even at 5°C, a further narrowing of the line resonances with raising temperature indicative of fast exchange, would not have been detectable within experimental error; therefore, it was not possible to execute an Arrhenius-type plot as shown in Fig. 10 for these data. However, the data do suggest a perturbation of the kinetics of AMP and enzyme interaction.

Additional information on the way 2,6FBP affects the interaction of AMP and FBPase was obtained by measuring the enzyme-Mn to phosphorus distances for each ligand in the presence and absence of the other inhibitor. Though AMP had no effect on the distances obtained for 2,6FBP, the sugar brought about a significant perturbation of the nucleotide, resulting in an increase of the distance between enzyme-bound Mn$^{2+}$ and the phosphate group of AMP. This effect was reproducible even at lower (1 mM) concentrations of 2,6FBP. F6P, however, had no effect on the distance obtained for AMP, nor did AMP affect the F6P distance.

Results presented in this report serve to support the concept of an interaction between the FBPase binding sites for 2,6FBP and AMP. First, 2,6FBP seems to cause a change in the kinetics of AMP interaction with FBPase. Second, 2,6FBP lengthens the distance between enzyme-bound Mn$^{2+}$ and the phosphorus group of AMP. These results, and those of other investigators, strongly suggest that the allosteric site for AMP and the
binding site for 2,6FBP are in proximity on the FBPase molecule. This idea is further substantiated by the distance measurements presented in this study, which indicate that the phosphorus nuclei of both the 2,6FBP and AMP binding sites are within 6.5 Å from the enzyme-bound Mn\(^{2+}\).

The distance between bound Mn\(^{2+}\) and the C-6 phosphorus atoms of F6P and 2,6FBP was essentially identical for bovine liver FBPase. Likewise, the C-2 phosphorus of 2,6FBP and P\(_i\) gave similar Mn-P distances. Also, both F6P and 2,6FBP are at least 9.1 Å removed from the reactive sulfhydryl residue of FBPase. These results strongly suggest that 2,6FBP acts at the active site of bovine liver FBPase.

2,6FBP and the substrate, FBP, differ somewhat in the properties that they induce in FBPase; for instance, the two differ in the ability to protect FBPase from chemical modifiers (135) or proteolysis (134). This seeming anomaly could be ascribed to the different spatial arrangement of the phosphate groups on C-1 and C-2 in FBP or 2,6FBP, respectively; other studies have indicated that the substrate of FBPase is the α-anomer of FBP (48), whereas 2,6FBP occurs as the β-anomer (117). Alternatively, 2,6FBP may act at both the active site and some distant allosteric site; however, this seems unlikely in light of the recent results of Kitajima and Uyeda (69), which do not indicate multiple binding of the inhibitor. Therefore, the most likely action of the inhibitor is a competition with the substrate for the active site of FBPase.
CONCLUSIONS

$^1\text{H}$ and $^{31}\text{P}$ nuclear magnetic resonance were used to investigate the interaction of AMP and 2,6FBP with bovine liver FBPase. Mn$^{2+}$ bound to FBPase was used as a paramagnetic probe to map the active and AMP allosteric sites of FBPase. Distances between enzyme-bound Mn$^{2+}$ and the phosphorus atoms at the C-6 positions of F6P and αMeFBP were identical, and the enzyme-Mn to phosphorus distance determined for the C-6 phosphorus atom of 2,6FBP was very similar to these values. Likewise, the enzyme-Mn to phosphorus distances for $P_\text{i}$, the C-1 phosphorus atom of αMeFBP, and the C-2 phosphorus atom of 2,6FBP agreed within 0.5 Å. The distance between enzyme-bound Mn$^{2+}$ and the phosphorus atom of AMP was significantly shorter than the distances obtained for any of the aforementioned ligands, but the presence of 2,6FBP caused the enzyme-Mn to phosphorus distance for AMP to lengthen markedly. NMR line broadening of AMP protons was studied at various temperatures. The dissociation rate constant was found to be greater than 20 sec$^{-1}$. The presence of 2,6FBP caused the proton resonances to narrow markedly, indicating that 2,6FBP perturbs the exchange between AMP and FBPase. Binding experiments indicated that 2,6FBP changes the AMP binding curve from sigmoidal to hyperbolic and has a positive effect on AMP binding to FBPase without affecting the total amount of AMP bound. It was concluded that 2,6FBP strongly affects the interaction of AMP with FBPase and that the sugar most likely acts at the active site of the enzyme.
GENERAL SUMMARY

This dissertation describes studies undertaken in order to clarify the nature of the interaction of 2,6F6P and AMP with bovine liver FBPase. Of special interest were the nature of the enzyme site occupied by 2,6F6P and the mechanism of the synergism of inhibition exhibited in the presence of both AMP and 2,6F6P.

In Part I, kinetic studies of the inhibition of the reverse FBPase reaction showed classical competitive inhibition for both F6P and P\textsubscript{i} by 2,6F6P, indicative of an active site mode of action for the inhibitor. An allosteric site mode of action might also explain the results obtained in Part I; however, the results clearly showed that the inhibitor acts only at one site on the enzyme, and not at both an allosteric and the active site.

To gain further insight into the site of action of 2,6F6P, the distance between Mn\textsuperscript{2+} bound at the active site of FBPase and the phosphorus groups of several FBPase ligands was determined. The distances obtained for F6P, P\textsubscript{i} and αMeF6P were very similar to the analogous values given by 2,6F6P. Because F6P, P\textsubscript{i}, and αMeF6P all interact with the active site of FBPase, the results present circumstantial evidence that 2,6F6P also binds to the active site of FBPase. More conclusive evidence would have been gained if another locus from which to perform the distance measurements was available; however, the spin label which was introduced proved to be too far removed from either 2,6F6P or F6P to provide significant relaxation for the ligands.
Nonetheless, this observation also indicates that F6P and 2,6FBP occupy similar, probably identical, sites in proximity to the enzyme-bound Mn\(^{2+}\). Furthermore, titration of F6P-Mn-enzyme with 2,6FBP caused a narrowing of the phosphorus resonance of F6P, indicating competition between the two ligands, and therefore an active site mode of action for 2,6FBP.

The interaction of AMP with FBPase was investigated by monitoring the NMR line broadening of AMP protons at various temperatures. This allowed the calculation of a lower limit for the dissociation rate constant, as well as the energy of activation for the interaction of AMP with FBPase.

The distance between the phosphorus group of AMP and Mn\(^{2+}\) bound at the active site of FBPase was obtained, and found to be significantly shorter than the Mn\(^{2+}\)-phosphorus distances for F6P, P\(_i\), αMeFBP, and 2,6FBP. The difference is not unexpected, because AMP is known to interact with an allosteric site on FBPase, whereas the other ligands appear to bind only at the active site of the enzyme.

Interestingly, the phosphorus group of AMP was found to move away from the enzyme-bound Mn\(^{2+}\) when 2,6FBP was present. This suggests that 2,6FBP causes a conformational change in the enzyme which displaces the phosphorus group of AMP relative to the active site Mn\(^{2+}\). Additional evidence that 2,6FBP changes the interaction of AMP with FBPase was gained from binding studies, which showed that 2,6FBP enhances the binding of AMP to FBPase. Apparently, 2,6FBP induces a conformational change in the enzyme about the AMP allosteric site in a fashion which
results in tighter binding of AMP to FBPase.

In conclusion, kinetic studies of the inhibition of the reverse reaction of FBPase by 2,6FBP indicated that the inhibitor interacts only with one class of sites on the enzyme, and also provided inhibition constants with respect to the substrates, F6P and P_i. The Mn-phosphorus distances for a number of FBPase ligands were determined by using \(^{31}\)P NMR. Because of the similarity between the values obtained for 2,6FBP and a number of active site ligands, it was concluded that 2,6FBP also acts at the active site of FBPase. This conclusion is substantiated by \(^{31}\)P NMR studies which monitored a competition between 2,6FBP and F6P. Lastly, the interaction of AMP with FBPase was monitored by using NMR and binding studies. Proton NMR studies gave kinetic and thermodynamic information about the interaction of AMP with FBPase. \(^{31}\)P NMR studies allowed the calculation of the distance between enzyme-bound Mn\(^{2+}\) and the phosphorus group of AMP. The distance lengthened markedly in the presence of 2,6FBP, indicative of an enzyme conformational change. In addition, binding studies showed that the interaction of AMP with FBPase is enhanced by the presence of 2,6FBP.


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