Determinants of substrate recognition and mechanisms of subunit exchange

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Determinants of substrate recognition and mechanisms of subunit exchange

By

Yang Zhou

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Major: Biophysics

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Iowa State University
Ames, Iowa
2007
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ABSTRACT

In biological systems, bonded and non-bonded interactions determine the properties and the behavior of macromolecules. Bonded interactions, such as ionic and covalent bonds, are the basis of chemical properties of biological molecules. Enzymatic reactions, immunological recognition, and ion channel transformations involve bonded interactions. On the other hand, the non-bonded interactions, such as Van der Waals and weak electrostatic interactions, also determine some important properties of macromolecules, such as molecular conformation, the non-covalent binding interactions. Changes in non-bonded interactions can significantly alter the structure and properties of a macromolecule, and thus influence its biological properties.

Adenylosuccinate synthetase (ADSS) offers a good opportunity to measure the effect of non-bonded interactions. Both IMP and 2′-deoxy-IMP are good substrates supporting identical binding affinities and maximal velocities; however, L-aspartate exhibits significant differences in binding affinities depending on which nucleotide is used as a substrate. Crystal structures of both complexes were identical except for the absence of the 2′-OH group in the deoxy-nucleotide complex. The decrease in energy (~2 kcal/mole) in non-bonded interactions due to the loss of one atom explains the nearly 40-fold difference in the binding affinity of L-aspartate and an analog of L-aspartate called hadacidin. Single-atom cavities can profoundly influence the binding affinity of a ligand to a protein or the binding
of two proteins to each other.

Sequence position 273 in *Escherichia coli* adenylosuccinate synthetase has a significant influence on the recognition of one of its substrates L-aspartate. The mutation of Valine\textsuperscript{273} to threonine, alanine, or asparagine causes 15-40 fold changes in the $K_m$ for L-aspartate. Crystallizing these mutant proteins with and without ligands, and subsequent crystallographic investigations, revealed the basis for differences in $K_m$ values. Although the Val273 in the active site doesn’t interact through hydrogen bonds with substrates or any other protein residues, it evidently has effect on the organization of the active site. $K_m$ values are influenced by three mechanisms. The replacement of valine by the threonine, introduces a polar side chain that closes the active site and prevents the binding of L-aspartate. The mutation of position 273 to alanine, results in unfilled space in the active site which allows Arg305 to move into and occupy the binding site of L-aspartate. Asparagine at position 273 occupies too much space (sterically blocking the productive binding mode of L-aspartate and also interacting with surrounding amino acids. All of these factors establish the requirement for valine at position 273, which is the reason Val273 is invariant over all known sequence of adenylosuccinate synthetase.

FBPase catalyzes the formation of fructose 1,6-bisphosphate to fructose 1,6-bisphosphate and inorganic phosphate. It exists in at least two quaternary states called R and T. FBPase is active in its R-state, with its 4 subunits residing in a plane. In the inactive T-state, stabilized by the binding of the allosteric inhibitor AMP, causing the upper
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subunit pair to twist 15 degrees relative to the bottom pair. Different tetramers when combined exchange subunits over time. Active site ligands such as Fru-6-P, Fru-1,6-P₂, and Fru-2,6-P₂ can limit certain subunit exchange reactions, and AMP can abolish exchange altogether. In total, a mixture of two distinguishable FBPases leads to seven different tetramers. Hybrid tetramers have been key in determining the origin of cooperativity in the binding of ligands to FBPase, but the models for cooperative interactions rely on the proper assignment of structural type to each of the seven tetramers. The assignments stem from reasonable assumptions that have gone unchallenged and without direct experimental verification. The model for subunit exchange has the tetramer separating into “top” and “bottom” dimers, and then subsequently each dimer separates into monomers. Verification of the model employed fluorescent probes and monitored the fluorescence change during subunit exchange. By locating the probes at specific positions, the separation of tetramer into dimers, and dimers into monomer, could be monitored independently. Furthermore, by quantifying the time-dependent change in fluorescence the elementary rate constants for forward and reverse reactions in tetramer/dimer and dimer/monomer equilibriums could be determined. These constants allowed the simulation of subunit exchange reactions in agreement with observed fluorescence changes. The approach here is innovative, broadly applicable to any multisubunit system and easier to accomplish than comparable fluorescence resonance energy transfer experiments.
CHAPTER I. GENERAL INTRODUCTION

The role of cavities in ligand selection

The size, shape, and plasticity of binding sites are determinants of ligand binding affinity. In those systems for which electrostatics and/or hydrogen bonds are relatively unimportant, size and shape become the major factors in the docking of substrates and inhibitors to binding sites, and the rational design of drugs and ligand discrimination. Plasticity associated with proteins in induced-fit models is merely a mechanism to couple conformational change to ligand binding interactions. In the final bound state, the binding pocket generally closes over and hugs the ligand. If a molecule is a misfit to the final shape of the binding site, induced conformational changes may not happen. Steric hindrance is a well recognized factor in mechanisms of ligand selection; however, the role of small cavities introduced by a less than optimal fit of a ligand to its binding site are not as broadly appreciated by investigators. Energy penalties due to a cavity equal in volume to a single atom range from 0.5–3 kcal/mol, leading to differences in dissociation constants of 2- to 100-fold.

Studies of T4 lysozyme provided some of the first data on the effects of cavities. L99A mutant of T4 lysozyme introduces a cavity in the core of the enzyme. The core is mostly non-polar and shielded from bulk solvent, resulting in a hydrophobic hole without hydrogen bonding potential. The crystal structure of the L99A complex revealed a sphere of electron density within the constructed cavity. The binding energies and $K_a$ values were determined
for 16 ligands (1,2). Those ligands with an extra ethyl or butyl group that filled the cavity had the highest affinity (2) (Fig 1, Table1). Although the protein undergoes a movement of its backbone which may be partially responsible for the observed variation in affinities, ligands which most effectively occupy the cavity have the highest binding affinities.

Cavities also play an important role in ligand binding to aminoacyl-tRNA synthetases (aaRSs). Aminoacyl-tRNA synthetases catalyze the esterification of an amino acid to its cognate tRNA. In order to maintain the fidelity of mRNA translation, each aaRS must discriminate between its cognate amino acid and other amino acids (3). Fersht proposed the “double-sieve” (two-step substrate selection) model to explain the mechanism of the amino-acid selection. Amino acids larger than the cognate amino acid are excluded by the aminoacylation site, serving as the “first, coarse sieve” (4). Valyl-tRNA synthetase (ValRS) strictly discriminates the larger L-isoleucine at the aminoacylation site: Pro41 accommodates the side chains of L-valine and L-threonine, but has no room for the additional methyl group of L-isoleucine (5). Amino acids with small side chains, however, present no steric barriers, but still have much lower affinities to enzyme that the cognate amino acid. ValRS also activates and edits L-cysteine (6,7) and a noncanonical amino acid, L-α -aminobutyrate (6,8,9), which are one methyl group smaller than L-valine. However, these non-cognate amino acids bind poorly to ValRS. Glutaminyl-tRNA synthetase (GlnRS) binds weakly to glutamate. Moreover, when the noncognate amino acid glutamate is bound to glutaminyl-tRNA synthetase tRNA\textsubscript{Gln} binds some 60-fold more weakly than in the presence
of the cognate amino acid glutamine (10). Unlike most tRNA synthetases, GlnRS does not
catalyze the formation of the aminoacyl-adenylate intermediate without bound tRNA.
Glutamate and glutamine bind to the amino acid pocket of the synthetase, but the negative
charge of glutamate results in a strong electrostatic interaction with an arginyl side chain.
That interaction draws the \( \alpha \)-carboxyl group of glutamate away from the 3'-hydroxyl group of
tRNA\textsubscript{Gln}, causing non-optimal packing between tRNA\textsubscript{Gln}, ATP and glutamate in the active
site. The effect is observed kinetically on the \( K_m \) for the amino acid and the kinetic on
constant (\( k_{on} \)) of tRNA\textsubscript{Gln}.

Cavities have significant implications for drug design. An important criterion to
determine the medical value of a drug is its specificity toward the target protein in order to
minimize undesirable side-effects. A well designed drug with good specificity can
efficiently stimulate or inhibit the target, and have a suitable affinity to its binding site. The
binding cavity can identify the proper candidates: large molecules are excluded by steric
hindrance and small ones, usually can’t bind efficiently in part due to the formation of
cavities. A century ago, Fischer and Ehrlich already used the "lock-and-key" analogy to
describe such specificity. During the last several years, we have witnessed huge progress in
computational biology, which has improved the likelihood of success in rational drug design.
Hundreds of thousands of compounds can be screened to find a promising new drug;
however, qualified candidates are still hard to come by. To improve the process of drug
design, we need to better evaluate the negative impact of cavity formation. Proper
consideration of the impact of cavity formation can eliminate false leads or indicate changes in a lead compound that enhance affinity.

**Recognition of L-aspartate and L-aspartate analogs by adenylosuccinate synthetase**

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP-forming; ADSS); EC 6.3.4.4, converts IMP and L-aspartate to adenylosuccinate, using GTP as an energy source. The synthetase participates in *de novo* purine nucleotide biosynthesis, the purine nucleotide cycle, and/or salvage pathways for nucleotides (11,12). Evidence strongly implicates a two-step reaction mechanism for adenylosuccinate synthetases. First, the γ-phosphoryl group of GTP is transferred to atom O-6 of IMP, forming 6-phosphoryl-IMP (6-PIMP). Second, the α-amino group of L-aspartate displaces the 6-phosphoryl group of 6-PIMP to form adenylosuccinate (11-16) (Fig 2). The enzyme adopts a random sequential kinetic mechanism with a strong bias in favor of the association of L-aspartate after the formation of an enzyme•IMP•GTP complex (17) (Fig 3). Mg$^{2+}$ is generally required for the reaction; however, Ca$^{2+}$ or Mn$^{2+}$ could be alternative cofactors supporting catalysis at rates 5-fold less than Mg$^{2+}$.

Potent inhibition of adenylosuccinate synthetase generally terminates cell growth. Hadacidin (N-formyl-N-hydroxyglycine) inhibits only adenylosuccinate synthetase, being a competitive inhibitor with respect to L-aspartate ($K_i$ $\sim$10$^{-6}$ M) (18) and a tight-binding analogue of L-aspartate in structural investigations of the synthetase (14,15).

Vertebrates have two forms of adenylosuccinate synthetase: a basic isozyme, found
exclusively in muscle (17), is a component of the purine nucleotide cycle and the de novo pathway for the biosynthesis of AMP, and an acidic isozyme, which functions exclusively in de novo AMP biosynthesis (19-21). The two isoforms in mouse are 75% identical in their amino acid sequences, and 40% identical to the enzyme from E. coli. AMP is a weak inhibitor of the mouse basic isozyme, but a potent inhibitor of the mouse acidic isozyme and the E. coli enzyme (22). On the other hand, in the presence of saturating levels of IMP, fructose-1,6-bisphosphate is a potent inhibitor of the basic isozyme, but only a weak inhibitor of the acidic isozyme (23). Hence, in heavily exercised muscle, elevated levels of fructose-1,6-bisphosphate could inhibit the basic isozyme, but in others tissues and in bacteria, AMP is the likely feedback regulator of the synthetase.

AMP and fructose-1,6-bisphosphate bind to the IMP site of the synthetase (23,24). Arguably then, the “regulatory” site of adenylosuccinate synthetase is contained within its active site. Indeed, IMP stabilizes active synthetase dimers by interacting with residues from two subunits (25-27). Moreover, the 5′-phosphoryl group of IMP, by hydrogen bonding with the side chain of Asparagine, organizes residues of the GTP pocket some 30 Å away (27). The mutation of Asparagine to alanine lowers $k_{cat}$ 100-fold, but has no effect on the $K_m$ for any of the substrates (28). An active site organized by IMP avoids the unproductive hydrolysis of GTP that would occur if the nucleoside triphosphate were to bind productively in the absence of IMP. IMP is sufficient to organize the active site of the Escherichia coli synthetase alone, because it can bind to loops that define the active site of...
the synthetase: Asparagine$^{38}$ of the switch loop, Arginine$^{303}$ of the aspartate loop, and several residues of the IMP loop, residues 120–130 (27).

The comparison of ligand-free and ligated complexes of *Escherichia coli* adenylosuccinate synthetase reveals remarkable structural change in several loops (27). Loops 38–53 and 419–421 are ordered structures in the ligand-free state and move significantly when ligands bind. Mg$^{2+}$-GDP is an important factor in the conformational change of loop 38–53, making six hydrogen and metal-coordination bonds to this loop in ligated complexes. Some backbone elements of loop 38–53 move up to 9 Å (14). Loop 120–130 and 299–304, close to IMP and L-aspartate respectively and disordered without ligands, undergo change to well-defined conformations (14,15).

Threonine$^{128}$, Threonine$^{129}$, Valine$^{273}$, Threonine$^{300}$, and Threonine$^{301}$ are conserved in amino acid sequences of adenylosuccinate synthetases. Threonine$^{128}$ and Threonine$^{129}$ interact with the 5′-phosphoryl group of IMP and Threonine$^{301}$ hydrogen bonds directly to the carboxyl group of hadacidin (N-formyl N-hydroxyglycine; an analogue of L-aspartate). They undergo significant conformational change in response to the binding of ligands. These interactions are observed in ligated crystal structures of the synthetase from *E. coli* and mouse muscle (14,16). Most surprising, however, is the significance of Valine$^{273}$ to the recognition of L-aspartate. Its mutation to threonine and alanine cause 15- and 30-fold increases in the $K_m$ for L-aspartate, and the mutation to asparagine causes a 40-fold increase (Table 2). These mutations also reduce $K_i$ values for analogues of L-aspartate by up to
40-fold (29). Obviously, Valine$^{273}$ is essential in binding L-aspartate, although crystal structures reveal no direct hydrogen bonds between Valine$^{273}$ and L-aspartate.

**FBPase subunit exchange**

Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.13.11;FBPase), a key regulatory enzyme of the gluconeogenic pathway, catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P$_2$) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (for a review see (30-32)). It is not only subject to the indirect regulation by glucagon and insulin, but also inhibited directly by AMP and fructose 2,6-bisphosphate (Fru-2,6-P$_2$). AMP binds to an allosteric and Fru-2,6-P$_2$ to the active site of FBPase, with Hill coefficients of 2 and 1, respectively (30-34). Although allosteric and active sites are at least 28 Å apart, the presence of Fru-2,6-P$_2$ will increase the affinity of AMP to FBPase by at least 10-fold. This property is called Fru-2,6-P$_2$/AMP synergism. *In vivo*, fructose-6-phosphate 1-kinase, which catalyzes the formation of Fru-1,6-P$_2$ from ATP and Fru-6-P, combines with FBPase to define a futile cycle, and hence both enzymes are subject to coordinate regulation (35,36).

Porcine liver FBPase is a tetramer with identical subunits (M$_r$=37,000). Each subunit has a substrate binding site for Fru-1,6-P$_2$, (or the inhibitor Fru-2,6-P$_2$) and metal ions, and a distinct allosteric binding site for AMP (37-39). The four subunits of the tetramer occupy each corner of a rectangle, labeled clockwise C1 through C4 (Fig 4). Crystallographic structure determinations reveal FBPase in at least two distinct quaternary conformations,
called R and T. R-state support catalysis and has the four subunits in a plane. The binding of AMP induces a transition from the R-state to the inactive T-state, in which the C1-C2 subunit pair rotates 15° with respect to the C3-C4 subunit pair about one of three intersecting twofold axes (39,40).

A proposed allosteric mechanism of AMP inhibition involves the conformation states of loop 52–72. In the absence of AMP, the loop forms an engaged loop conformation, interacting with the active site (41,42). Metals with products help the stabilization of this conformation. The binding of AMP alone or with F26P₂, putatively removes the loop from the active site, and stabilizes the disengaged loop conformation. The engaged conformation of loop 52–72 only appears in R-state crystal structures and the disengaged conformer only in T-state structures. However, disordered loop conformations have appeared in both the R-state in the absence of metals and/or active site ligands and the T-states of specific mutant FBPases (39,43-45). Mutations of conserved residues in this loop 52–72 cause significant effects on catalysis and allosteric inhibition of catalysis by AMP (42,46).

Distinct homotetramers of FBPase (engineered homotetramers having different subunit pIs) undergo spontaneous subunit exchange to form hybrid tetramers. Anion-exchange chromatography can separate these tetramers by hybrid ratio: 4:0, 3:1, 2:2, 1:3, and 0:4 (47,48). (There are three different 2:2 hybrids that resolve into two peaks. 2:2 Hybrids with subunit C1 identical to subunit C2, but different from C3 and C4 separates by DEAE chromatography from 2:2 hybrids in which C1 and C2 are different.) Active site ligands,
such as Fru-6-P, Fru-1,6-P$_2$, and Fru-2,6-P$_2$, selectively block subunit exchange at the level of individual subunits. Only one kind of 2:2 hybrid is observed in the presence of such ligands (48). As the 6-phosphoryl groups of these ligands bind to residues from both the C1 and C2 subunits (C3 and C4 as well), preventing the dissociation of the dimer into monomers, the 2:2 hybrid that forms is of the type in which subunits C1 and C2 are identical. AMP, on the other hand, abolishes all subunit exchange. This inhibition of subunit exchange must differ from that due to active site ligands as AMP binds to one subunit exclusively (48). One possible model for the effect of AMP assumes an ordered mechanism for subunit exchange (Fig. 5): the tetramer dissociates initially to C1-C2 dimers by loss of the C1-C4 (top-bottom) interface. Then C1-C2 dimers dissociate into monomers. If AMP blocks the formation of C1-C2 dimers, then all exchange reactions stop. In non-denaturing polyacrylamide gels taken at progressive time points, 2:2 hybrids appear first, consistent with the ordered model (47,48).

In order to prove our hypothesis, it is necessary to find a way to monitor this dynamic process. Fluorescence is a good candidate due to its sensitivity. However, the most common fluorescence technique, FRET, is not an appropriate method for our model. A FRET system requires two different probes (donor and acceptor). For each of them, the same four probes in one FBPase tetramer will interact with each other due to their close distance (~30-50 Å). This interaction is called self-quenching (49-51). For such circumstances, FRET signals will be obscured by changes in the level of self-quenching.
Fortunately, the fluorescence signal from time-dependent changes in self-quenching provides information comparable to a FRET experiment, and is simpler to implement, as it requires only a single type of fluorescent probe.

Fluorescence self-quenching has been known since 1888. It occurs due to the existence of the same dye molecules in proximity, but the exact mechanism is not yet clear (49-51). Fluorescence from the dye is reduced when multiple dye molecules are bound to the same substrate. The distance increase between dye molecules results in increasing fluorescence. Self-quenching has been used in studies of membrane fusion, ensembles of proteases and nucleases, of protein-dimer formation, and single molecule protein folding (52-55). By modifying the FBPase enzyme, we can attach probes to special places of each subunit of the tetramer. The dissociation of the FBPase into dimers and monomers increases the distance between probe molecules and leads to an increase in fluorescence. By observing the time dependence of fluorescence change for distinct probe locations, we confirmed an ordered pathway of dissociation from tetramers to C1-C2 dimers, and then to monomers.

REFERENCES
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Table 1: The affinities of different ligands in T4 lysozyme L99A

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<th>Ligand</th>
<th>$K_a \times 10^{-3}$(M$^{-1}$)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$-RT\ln K_a$ (kcal/mol)</th>
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<td>benzene</td>
<td>5.7 ± 1.7</td>
<td>6.32 ± 0.37</td>
<td>-5.19 ± 0.16</td>
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<td>toluene</td>
<td>9.8 ± 0.6</td>
<td>6.53 ± 0.73</td>
<td>-5.52 ± 0.04</td>
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<td>ethylbenzene</td>
<td>14.8 ± 1.7</td>
<td>6.76 ± 0.87</td>
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<td>propylbenzene</td>
<td>55.2 ± 2.0</td>
<td>9.97 ± 0.05</td>
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<td>n-butylbenzene</td>
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<td>isobutylbenzene</td>
<td>51.0 ± 44.9</td>
<td>7.09 ± 0.35</td>
<td>-6.51 ± 0.06</td>
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Table 2: The kinetics assay of *E.coli* ADSS and its mutations in position 273

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<th>Enzyme</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$K_m^{GTP}$ (µM)</th>
<th>$K_m^{IMP}$ (µM)</th>
<th>$K_m^{L-aspartate}$ (mM)</th>
<th>$K_i^{badacidin}$ (µM)</th>
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<td>Wild Type</td>
<td>1.00 ± 0.05</td>
<td>26 ± 2</td>
<td>28 ± 1</td>
<td>0.23 ± 0.04</td>
<td>0.49 ± 0.08</td>
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<tr>
<td>Threonine$^{273}$</td>
<td>1.99 ± 0.02</td>
<td>45 ± 7</td>
<td>43 ± 4</td>
<td>3.4 ± 0.2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Alanine$^{273}$</td>
<td>1.00 ± 0.1</td>
<td>31 ± 3</td>
<td>17 ± 2</td>
<td>7 ± 1</td>
<td>47 ± 2</td>
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<tr>
<td>Asparagine$^{273}$</td>
<td>0.050 ± 0.001</td>
<td>21 ± 2</td>
<td>24 ± 2</td>
<td>9 ± 1</td>
<td>N/A</td>
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</table>
Fig 1. Ligands used to test the difference of affinities in T4 lysozyme L99A.
Fig 2. A proposed two-step reaction mechanism for adenylosuccinate synthetase.
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Fig 5. A proposed mechanism of ordered dissociation pathway for FBPase tetramer.
CHAPTER II

Cavitation as a Mechanism of Substrate Discrimination by Adenylosuccinate Synthetases†

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Coordinates and structures factors for structures reported here (2GCQ and 2DGN) have been deposited with the Research Collaboratory for Structural Bioinformatics, http://www.rcsb.org/pdb/.  
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ABSTRACT

Adenylosuccinate synthetase catalyzes the first committed step in the de novo biosynthesis of AMP, coupling L-aspartate and IMP to form adenylosuccinate. $K_m$ values of IMP and 2'-deoxy-IMP are nearly identical with each substrate supporting comparable maximal velocities. Nonetheless, the $K_m$ value for L-aspartate and the $K_i$ value for hadacidin (a competitive inhibitor with respect to L-aspartate) are 29- to 57-fold lower in the presence of IMP than 2'-deoxy-IMP. Crystal structures of the synthetase ligated with hadacidin, GDP and either 6-phosphoryl-IMP or 2'-deoxy-6-phosphoryl-IMP are identical except for the presence of a cavity normally occupied by the 2'-hydroxyl group of IMP. In the presence of 6-phosphoryl-IMP and GDP (hadacidin absent), the L-aspartate pocket can retain its fully ligated conformation, forming hydrogen bonds between the 2'-hydroxyl group of IMP and sequence-invariant residues. In the presence of 2'-deoxy-6-phosphoryl-IMP and GDP, however, the L-aspartate pocket is poorly ordered. Evidently, interactions involving the 2'-hydroxyl group of IMP maintain a protein conformation favorable to the binding of L-aspartate; however, the absence of the 2'-hydroxyl group in the fully ligated enzyme introduces a cavity with an approximate energy cost of 2.2 kcal/mol. The unfavorable thermodynamics of cavity formation may be the major factor in destabilizing ligands at the L-aspartate pocket.

ABBREVIATIONS

The abbreviations used are: 6-PIMP, 6-phosphoryl-IMP; 2'-deoxy-6-PIMP, 2'-deoxy-6-phosphoryl-IMP.
INTRODUCTION

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP-forming); EC 6.3.4.4), converts IMP and L-aspartate to adenylosuccinate, using GTP as an energy source. The synthetase participates in de novo purine nucleotide biosynthesis, the purine nucleotide cycle, and salvage pathways for nucleotides (1-3). Evidence supports a two-step reaction mechanism for adenylosuccinate synthetases. First, the γ-phosphoryl group of GTP is transferred to atom O-6 of IMP, forming 6-phosphoryl-IMP (6-PIMP). Second, the α-amino group of L-aspartate displaces the 6-phosphoryl group of 6-PIMP to form adenylosuccinate (1-7). The enzyme adopts a random sequential kinetic mechanism with a strong bias in favor of the association of L-aspartate after the formation of an enzyme•IMP•GTP complex (8, 9).

Several potent inhibitors of adenylosuccinate synthetase terminate cell growth: Hydantocidin, a proherbicide activated specifically in plants, binds to the active site in combination with phosphate and GDP (10, 11, 12), the de novo purine nucleotide pathway converts L-alanosine into an analog of adenylosuccinate (13, 14), and hadacidin (N-formyl-N-hydroxyglycine) is a competitive inhibitor with respect to L-aspartate (15, 16). The synthetase also recognizes a limited number of substrate alternatives: 2'-deoxy-IMP and β-D-arabinosyl-IMP for IMP; 2'-deoxy-GTP and GTPγS for GTP; and DL-threo-ß-fluoroaspartate, L-alanine-3-nitronate, L-cysteine sulfinate, L-alanosine, and hydroxylamine for L-aspartate (1, 3, 17). The synthetase requires Mg²⁺ as a cofactor, albeit
Ca\(^{2+}\) or Mn\(^{2+}\) support catalysis at rates 5-fold less than Mg\(^{2+}\) (1, 3).

Vertebrates have two forms of adenylosuccinate synthetase: a basic isozyme, found exclusively in muscle, is a component of the purine nucleotide cycle and the \textit{de novo} pathway for the biosynthesis of AMP, and an acidic isozyme, which functions exclusively in \textit{de novo} AMP biosynthesis (18-23). The two isoforms in mouse are 75\% identical in their amino acid sequences, and 40\% identical to the enzyme from \textit{E. coli}. AMP is a weak inhibitor of the mouse basic isozyme, but a potent inhibitor of the mouse acidic isozyme and the \textit{E. coli} enzyme (23). On the other hand, in the presence of saturating levels of IMP, fructose 1,6-bisphosphate is a potent inhibitor of the basic (muscle) isozyme, but only a weak inhibitor of the acidic isozyme (23). Hence, in heavily exercised muscle, elevated levels of fructose 1,6-bisphosphate could inhibit the basic isozyme, but in other tissues and in bacteria, AMP is the likely regulator of the synthetase.

AMP and fructose 1,6-bisphosphate bind to the IMP site of the synthetase (23, 24). Arguably then, the “regulatory” site of adenylosuccinate synthetase is within its active site. Indeed, IMP stabilizes active synthetase dimers by interacting with residues from two subunits (5, 23, 25). Moreover, the 5'-phosphoryl group of IMP, by hydrogen bonding with the side chain of Asn38, organizes residues of the GTP pocket some 30 Å away (26). The mutation of Asn38 to alanine lowers \(k_{\text{cat}}\) 100-fold, but has no effect on the \(K_m\) for any of the substrates (27). An active site organized by IMP avoids the unproductive hydrolysis of GTP that would occur if the nucleoside triphosphate were to bind productively in the absence
of IMP.

Here we report a role for IMP in the recognition of L-aspartate. Recombinant synthetases from *E. coli* and mouse muscle have similar $K_m$ values for 2'-deoxy-IMP and IMP. Both enzymes, however, exhibit 29- to 57-fold increases in their $K_m$ values for L-aspartate ($K_m^{L\text{-aspartate}}$) and $K_i$ values for hadacidin ($K_i^{\text{hadacidin}}$) when 2'-deoxy-IMP replaces IMP. Crystal structures of the *E. coli* synthetase, having bound GDP, hadacidin and either 6-PIMP or 2'-deoxy-6-PIMP, reveal no conformational differences within experimental uncertainty; the absence of the 2'-hydroxyl group introduces only a cavity. On the other hand, crystalline complexes of the mouse muscle synthetase without hadacidin, but having bound GDP and either 6-PIMP or 2'-deoxy-6-PIMP, differ significantly. Disorder in the L-aspartate pocket is correlated with the loss of hydrogen bonds normally associated with the 2'-hydroxyl group of IMP. Although disorder in the L-aspartate pocket may contribute to increases in $K_i^{\text{hadacidin}}$ and $K_m^{L\text{-aspartate}}$, cavity formation in the fully ligated enzyme by itself can account for the observed increases in kinetic parameters.

**EXPERIMENTAL**

_Materials—_ All reagents, including GTP, IMP, L-aspartate, bovine serum albumin, and DEAE-Sepharose were from Sigma unless noted otherwise. Plasmids for the expression of enzyme are those employed in previous investigations (23, 27).

_Expression and Purification of Mouse Muscle and E. coli Adenylosuccinate Synthetase—_ The recombinant *E. coli* and mouse basic isozyme were prepared as described
previously (27, 28). Enzyme purity was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Investigations here employed the mouse muscle isozyme with its N-terminal polyhistidyl tag in place. The presence of the polyhistidyl tag has no effect on the kinetics of the mouse basic isozyme (28); however, only the tagged form of protein has been crystallized (24, 28, 29). Recombinant *E. coli* adenylosuccinate synthetase used here does not have an affinity tag (27).

**Enzyme Assays**— Protein concentrations were determined by the method of Bradford (30), using bovine serum albumin as a standard. Enzyme activity was determined by the change in absorbance at 290 nm at 22 °C as described previously (8). Using up to 1 µg/mL enzyme, the reaction was linear for at least 1 min. *K*<sub>m</sub> and *V*<sub>max</sub> values for each substrate were obtained by holding the other two substrates at saturating levels (100 µM GTP, 300 µM IMP or 2'-deoxy-IMP, and 2 mM L-aspartate with IMP or 200 mM L-aspartate with 2'-deoxy-IMP) and varying the concentration of the third substrate. In determining *K*<sub>m</sub> values, initial concentrations of GTP varied systematically from 5–100 µM, those of IMP (or 2'-deoxy-IMP) from 25–300 µM, and those of L-aspartate from 0.1–2 mM (for IMP) or 1–96 mM (for 2'-deoxy-IMP). In determining the *K*<sub>i</sub> values of hadacidin, concentrations of GTP and IMP were maintained at saturating levels (100 and 300 µM, respectively) as initial concentrations of L-aspartate and hadacidin varied from 0.125–2 mM and 0.25–16 µM, respectively. *K*<sub>i</sub> values of hadacidin in the presence of 2'-deoxy-IMP employed fixed initial concentrations of GTP and 2'-deoxy-IMP of 100 and 300 µM, respectively, as initial
concentrations of L-aspartate and hadacidin varied from 4–64 mM and 10–160 µM, respectively. For the *E. coli* enzyme, the assay buffer contained 20 mM Hepes, pH 7.7, and 6 mM magnesium chloride, and for the mouse enzyme 20 mM Hepes, pH 7.2, and 8 mM magnesium acetate. All data were analyzed with the computer program GraFit (31). Models for competitive, noncompetitive, uncompetitive and mixed inhibition were fit to hadacidin inhibition data.

**Crystallization**— Crystals were grown by the method of hanging drops. The mouse muscle enzyme crystallized from equal parts of a protein solution (10 mg/ml protein in 50 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 10 mM GTP and 10 mM 2'-deoxy-IMP) and a precipitant solution (100 mM Hepes, pH 7.0, 200 mM magnesium acetate, 12–14% (w/v) polyethylene glycol 8000 or 12–17% (w/v) polyethylene glycol 3350). Crystals of the *E. coli* enzyme grew from equal parts of a protein solution (10 mg/ml protein in 50 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 5 mM GTP, 5 mM 2'-deoxy-IMP, 2 mM hadacidin, and 10 mM magnesium acetate) and a precipitant solution (100 mM Hepes, pH 7.0, 200 mM magnesium acetate, 12–16% (w/v) polyethylene glycol 8000). In all experiments, droplet volumes were 6 µL, and wells contained 500 µL of the precipitant solution. Equal dimensional prisms of 100 microns appeared within 3 days at 22 °C. Crystals were transferred to a solution of 50 mM Hepes, pH 7.0, 100 mM magnesium acetate, 27% (w/v) polyethylene glycol 8000, and 21% (v/v) glycerol and then frozen in liquid nitrogen.
Data Collection, Model Building, and Refinement— Data for the mouse muscle complex was collected at Beamline 9-2 of the Stanford Synchrotron Radiation Laboratory, using a ADSC Quantum 4 CCD detector. The wavelength of radiation was 0.979 Å, and the temperature of data collection was 120 K. Data reduction employed Denzo/Scalepack (32). Data for the *E. coli* complex were collected at Iowa State University on a Rigaku R-AXIS IV++ image plate detector using an Osmic confocal mirror system (CuKa radiation) and a sample temperature of 100 K. Data reduction employed the software package CrystalClear (33). Intensities were converted to structure factors using the CCP4 program TRUNCATE (34). Structures were solved by molecular replacement, using the program AmoRe (35) and models for the fully ligated mouse muscle enzyme (Protein Data Bank identifier 1LON, (29)) and *E. coli* enzyme (Protein Data Bank identifier 1CGO, (7)). Model building and refinement employed the programs XTALVIEW (36) and CNS (37), respectively. Force constants and parameters of stereochemistry came from Engh and Huber (38). Criteria for the addition of water molecules were identical to those of previous studies (5-7). Estimates of coordinate error used the method of Luzzati (39). Evaluation of stereochemistry of the refined model employed PROCHECK (40). Superposition of structures employed software from the CCP4 package (41) or XTALVIEW (36).

Estimates of Cavitation Energies— The contribution to the total potential energy of the 2'-hydroxyl group of 6-PIMP in the fully ligated complexes of the *E. coli* and mouse muscle synthetases was calculated by using AutoDock 3.0 (42). Van der Waals’ interactions were
determined between the 2'-O atom and atoms within a sphere of radius 10 Å. Lennard-Jones parameters in AutoDock 3.0 are not available for interactions involving Mg$^{2+}$; however, as Mg$^{2+}$ is approximately 9.8 Å from the 2'-hydroxyl group of 6-PIMP in synthetase structures, the contribution of a single Mg$^{2+}$-O contact to the total energy is negligible.

**RESULTS**

*Enzyme Purity and Kinetics*— Proteins used in assays and crystallization were at least 95% pure on the basis of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 2'-Deoxy-IMP is a substrate of the synthetase (17), but prior to the present study no information was available regarding the kinetics of an adenylosuccinate synthetase reaction supported by 2'-deoxy-IMP. $K_m$ values for 2'-deoxy-IMP and IMP (Table 1) for both the *E. coli* and mouse muscle enzyme are equal within experimental uncertainty. All other parameters are equivalent for the two reactions except those associated with L-aspartate and hadacidin. $K_m^{\text{L-aspartate}}$ increases 57- and 29-fold, and $K_i^{\text{hadacidin}}$ increases 35- and 44-fold, for *E. coli* and mouse muscle enzymes, respectively, in reactions that use 2'-deoxy-IMP instead of IMP. Evidently, the *E. coli* and mouse muscle adenylosuccinate synthetases, broadly representative of microbial and vertebrate systems, have a mechanism to distinguish ribonucleotide and 2'-deoxy-ribonucleotide substrates. That mechanism is indirect; the recognition of the nucleotide itself is equally robust, but in some way the 2'-hydroxyl group of the ribonucleotide is an essential determinant in the recognition of L-aspartate and for
high-affinity binding of hadacidin.

**Existing Crystal Structures of Ribonucleotide Complexes of E. coli and Mouse Muscle Synthetases**— Crystal structures of synthetases from *E. coli* and mouse muscle exist in ligand-free and fully ligated complexes (7, 24, 29). For the latter, GDP, 6-PIMP, hadacidin (as an analog of L-aspartate) and Mg$^{2+}$ are in the active site. In addition, a structure is available for the 6-PIMP•GDP complex for the mouse muscle enzyme (29) and for the Arg303→Leu *E. coli* enzyme, which has low affinity for hadacidin and a high $K_m$ for L-aspartate (7, 43). In fully ligated complexes, the 2'-hydroxyl group of IMP hydrogen bonds with a backbone carbonyl group of a peptide link between sequence invariant residues (Val273 and Gly274 in the *E. coli* synthetase and Val305 and Gly306 in the mouse muscle enzyme). The 2'-hydroxyl group also hydrogen bonds with an arginyl side chain (Arg303 and Arg335 in the *E. coli* and mouse muscle enzymes, respectively). Arg303 (and probably the corresponding position in the mouse muscle isozyme Arg335) is essential for the recognition of L-aspartate, as its mutation to leucine causes a 200-fold increase in the $K_m$ for L-aspartate (43). Hadacidin makes only nonbonded contacts with 6-PIMP in fully ligated complexes.

**Fully Ligated 2'-Deoxy-ribonucleotide Complex of the E. coli Synthetase (Protein Data Bank identifier 2GCQ)**— Statistics of data collection and refinement for crystals of the *E. coli* synthetase grown in 2'-deoxy-IMP, GTP, hadacidin and Mg$^{2+}$ are in Table 2. The active site contains a single Mg$^{2+}$ to which are coordinated 2'-deoxy-6-PIMP, GDP, and
hadacidin (Figure 1). Electron density associated with 2'-deoxy-6-PIMP is strong, but no observable density is associated with what would be the position of a 2'-hydroxyl group (Figure 1). Interactions between active site ligands and the protein are identical to those of the 6-PIMP, GDP, hadacidin and Mg\(^{2+}\) complex (PDB identifier 1CGO) (Figure 2). Specifically, as the focus here is on the binding of hadacidin, the oxygen atom of its N-formyl group coordinates Mg\(^{2+}\) (2.27 Å), and its β-carboxyl group hydrogen bonds with Thr301 OG1 (2.68 Å), Arg303 HN2 (2.83 Å), and backbone amide groups of Thr300 (3.03 Å) and Thr301 (2.66 Å). The N-hydroxyl group of hadacidin is 3.45 Å and 2.52 Å from the side chains of Arg305 and Asp13, respectively. The aforementioned distances in the 6-PIMP and 2'-deoxy-6-PIMP complexes exhibit a root-mean-squared deviation of 0.22 Å, well within the estimated uncertainty in coordinates of 0.25 Å, and no distance between corresponding atoms in the two complexes exceeds 0.4 Å. In fact, the principal difference in the fully ligated E. coli structures is the void left by the absence of the 2'-hydroxyl group in the 2'-deoxy-IMP complex.

*Partially Ligated 2'-Deoxy-ribonucleotide Complex of the Mouse Muscle Synthetase (Protein Data Bank identifier 2DGN)—* Statistics of data collection and refinement appear in Table 2 for the mouse basic enzyme crystallized with 2'-deoxy-IMP, GTP and Mg\(^{2+}\). The active site contains a single Mg\(^{2+}\) that coordinates 2'-deoxy-6-PIMP and GDP. Electron density associated with the ligands is strong, but none is present at what would be the position of the 2'-hydroxyl group.
Unlike the fully ligated structures of the *E. coli* and mouse muscle enzymes, the partially ligated complexes of the mouse basic isozyme exhibit significant conformational differences in their L-aspartate binding pockets (Figure 3). The mouse muscle enzyme with 6-PIMP, GDP and Mg$^{2+}$ (Protein Database Base identifier 1LNY) has a dimer in the asymmetric unit. Backbone carbonyl groups of residues 332–334 of chain B hydrogen bond in a lattice contact that stabilizes the aspartate binding loop (residues 330–336) some 5 Å away from its position in fully ligated structures of the mouse muscle and *E. coli* enzymes (7, 29). Distances from Thr332 OG1 to Asn68 O and Gly70 N are 9.46 and 9.95 Å, respectively, about 6.2 Å longer than corresponding distances in the fully ligated mouse muscle enzyme. Arg335 (equivalent to Arg303 in the *E. coli* synthetase) and the backbone carbonyl group of Val305 (corresponding to Val273 of the *E. coli* enzyme) do not hydrogen bond with the 2'-hydroxyl group of the ribonucleotide in chain B. The aspartate loop of chain A participates in a different set of lattice contacts involving backbone carbonyl groups of residues 333 and 334, as well as a side chain stacking interaction involving Trp329. Nonetheless, the conformation of the aspartate loop in chain A is similar to that of the fully ligated structure. Specifically, Arg335 and the backbone carbonyl of Val305 hydrogen bond with the 2'-hydroxyl group of IMP, as observed in fully ligated systems. Distances in chain A from Thr332 OG1 to Asn68 O and Gly70 N are 6.81 and 6.77 Å, respectively, about 3.5 Å longer than those in the fully ligated mouse muscle enzyme.

In contrast to the 6-PIMP•GDP•Mg$^{2+}$ complex, the asymmetric unit of the
2'-deoxy-6-PIMP•GDP•Mg^{2+} mouse muscle complex has but a single subunit. The enzyme is still a dimer, however, its axis of molecular symmetry coincides with a crystallographic axis of twofold symmetry. The 6-PIMP•GDP•Mg^{2+} and 2'-deoxy-6-PIMP•GDP•Mg^{2+} complexes have nearly equivalent structures (root mean square deviation for superimposed C\alpha carbons of 0.4 Å) except for their aspartate loops. Interactions involving Arg335 and the backbone carbonyl of Val305 are absent as expected. Moreover, the aspartate loop is withdrawn from the active site: distances from Thr332 OG1 to Asn68 O and Gly70 N are 9.46 and 9.85 Å, respectively. The conformation of the aspartate loop in the 2'-deoxy-6-PIMP•GDP•Mg^{2+} is much like that of the aspartate loop in chain B of the 6-PIMP•GDP•Mg^{2+} structure, but the outwardly displaced loop cannot be attributed to a lattice contact.

**Energy differences in 6-PIMP and 2'-deoxy-6-PIMP complexes**— The crystal structures of the fully ligated complexes 6-PIMP and 2'-deoxy-6-PIMP of the *E. coli* synthetase are identical to within experimental error. Hence, the energy of interaction involving the 2'-hydroxyl group in the fully ligated 6-PIMP complex is an estimate of the energy penalty due to the absence of that group. As fully ligated 6-PIMP complexes are available for the *E. coli* and mouse muscle synthetases (PDB identifiers 1CG0 and 1LON, respectively), the interaction energies can be calculated for both systems. The interaction energies of the 2'-hydroxyl groups in the *E. coli* and mouse muscle systems from AutoDock 3.0 (Table 3) are nearly identical, in harmony with the nearly identical sequences and structures of the two
complexes in the region of consideration (29).

**DISCUSSION**

The kinetic mechanism for the *E. coli* enzyme is rapid-equilibrium random (8, 9). Hence, $K_{m}^{L\text{-aspartate}}$ is a reasonable approximation of the equilibrium constant governing the dissociation of L-aspartate from the enzyme•IMP•GTP•L-aspartate•Mg$^{2+}$ complex. Moreover, changes in $K_{i}^{\text{hadacidin}}$ are consistent with those of $K_{m}^{L\text{-aspartate}}$, further evidence of rapid equilibrium kinetics. L-Aspartate and hadacidin then dissociate more readily from the 2'-deoxy-IMP complex than from the IMP complex, and this is true to the same extent for the *E. coli* and mouse muscle enzymes.

A factor in the relatively weak association of L-aspartate and hadacidin in the presence of 2'-deoxy-IMP is the loss of hydrogen bonds involving the side chain of a conserved arginine (positions 303 and 335 in *E. coli* and mouse muscle enzymes, respectively) and the backbone carbonyl group of a conserved valine (positions 273 and 305 in *E. coli* and mouse muscle enzymes, respectively). Complexes of 6-PIMP, GDP and Mg$^{2+}$ of the mouse muscle enzyme (Protein Data Bank identifier 1LNY, space group C2) indicate a predisposition to form hydrogen bonds involving the 2'-hydroxyl group of 6-PIMP even in the absence of hadacidin. When these hydrogen bonds are intact (as in chain A of 1LNY), the L-aspartate loop assumes a conformation much like that of the fully ligated complexes of the synthetase. Lattice contacts observed in the mouse muscle crystal structure (1LNY), however, introduce an element of uncertainty. We do not know whether hydrogen bonds involving the
2'-hydroxyl of 6-PIMP form in solution in the absence of L-aspartate. If such hydrogen bonds do form, they have no influence on the $K_m$ of IMP, and hence the free energy from such interactions must go toward stabilizing a subset of conformations favorable to the binding of L-aspartate.

A more intriguing contribution to the elevated kinetic parameters for L-aspartate and hadacidin is the cavity that replaces the 2'-hydroxyl group of the fully ligated 2'-deoxy-ribonucleotide complex. Water does not occupy the site left vacant by the absent 2'-hydroxyl group, and the protein structure does not relax to fill the void. Hence, the cost of cavity formation to a first approximation is the loss of all interactions involving the 2'-hydroxyl group in the fully ligated complex. Assuming the entropy is not changed significantly in a thermodynamic transition from a 6-PIMP to a 2'-deoxy-6-PIMP complex (and we see no evidence from the crystal structures to suggest differences in conformational entropy), the calculated difference in potential energy is a fair approximation of the free energy differences in the ribonucleotide and deoxy-ribonucleotide complexes. The energy difference ranges from 2.2 to 2.3 kcal/mol, which would increase dissociation constants for hadacidin by 41- to 49-fold. The calculated increases are close to those of observed kinetic parameters, and suggest that cavity formation is the primary cause of the observed behavior.

Curiously, the absence of the 2'-hydroxyl group only influences kinetic parameters of hadacidin and L-aspartate. One would expect an increase in the $K_m$ of 2'-deoxy-IMP relative to IMP, as the loss in hydrogen bonding potential and nonbonded interactions are due
to the absence of a functional group covalently attached to the nucleotide. An explanation of this phenomenon may rest with the kinetic mechanism of the synthetase. Although that mechanism is random, isotope exchange kinetics indicate that in 90% of synthetase turnovers L-aspartate is the last substrate to bind to the enzyme (9). Evidently, when IMP binds to the active site, its base encroaches upon the L-aspartate pocket (29). Phosphorylation at the 6-position of IMP introduces numerous interactions that “bend” the purine base away from the L-aspartate pocket (24). Hence, the formation of 6-PIMP removes steric barriers to the productive binding of L-aspartate. As the cavity due to the absent 2'-hydroxyl group does not exist until L-aspartate binds (and for the vast majority of turnovers, binds last of the three substrates), the negative impact of cavity formation is felt entirely by the amino acid substrate.

The unfavorable free energy of cavity formation may be a factor in the weak binding of small amino acids (glycine, alanine and serine) to the synthetase (Aaron ver Heul and Richard B. Honzatko, unpublished) and may account for the relatively weak inhibition of adenylosuccinate synthetases by succinate. Succinate has the carboxyl groups of L-aspartate, but lacks the steric encumbrance of an α-amino group, and yet $K_{m}^{L\text{-aspartate}}$ is 4-fold lower than the $K_{i}^{\text{succinate}}$ for the *E. coli* enzyme (44). If, however, the succinate complex of the synthetase leaves a void where an α-amino group should be, its $K_{i}$ would be high relative to the $K_{m}$ of L-aspartate.

Cavity formation as a means of substrate discrimination is not unprecedented. In fact,
the selection of the correct amino acid by aminoacyl-tRNA synthetases is due at least in part to the penalty in placing a hydrogen atom in a binding pocket designed for a larger functional group. L-Valine suffers approximately a 3.4 kcal/mol penalty relative to L-isoleucine in binding to the recognition pocket of isoleucyl-tRNA synthetase, and a penalty of similar magnitude occurs in the binding of L-α-aminobutyric acid to valyl-tRNA synthetase (45-47). Energy shortfalls for the association of L-alanine to cysteinyl-tRNA synthetase and L-phenylalanine to tyrosyl-tRNA synthetase are even greater (9.1 and 7.0 kcal/mol, respectively), and result in effective substrate discrimination without recourse to editing mechanisms (47, 48). The high energy penalties of tRNA synthetases, attributed to the loss of interactions of a missing carbon, oxygen or sulfur atom, infer a rigid pocket for the amino acid side chain, surrounded by densely packed protein atoms. By comparison, the energy penalty caused by 2'-deoxy-IMP (2.2–2.3 kcal/mol) is relatively modest, and clearly, one observes “empty space” in the vicinity 2'-hydroxy group even for the correct substrate (Figure 4).

Cavity formation is a significant consideration in the design of tight-binding inhibitors. In the case of adenylosuccinate synthetase and perhaps for other multi-substrate systems, it may be possible to design or discover “complementary” inhibitors, molecules that by themselves inhibit weakly, but when combined result in potent inhibition. In fact, hydantocidin owes its potent inhibition ($K_i$~20 nM) of adenylosuccinate synthetase to its tight packing with phosphate and GDP in the active site (12). A screen of adenylosuccinate
synthetase in its complex with 2'-deoxy-6-PIMP and GDP or fructose 1,6-bisphosphate and GDP could lead to tight-binding alternatives to hadacidin.

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Table 1. Kinetic parameters for recombinant adenylosuccinate synthetases from *E. coli* and mouse muscle using IMP and 2'-deoxy-IMP.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th><em>E. coli</em></th>
<th>Mouse muscle</th>
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<tr>
<td><strong>IMP reaction:</strong></td>
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<tr>
<td>$k_{cat}$ (sec$^{-1}$)</td>
<td>1.00±0.05</td>
<td>5.4±0.4$^b$</td>
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<tr>
<td>$K_m$ IMP (µM)</td>
<td>28±1</td>
<td>45±7$^b$</td>
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<tr>
<td>$K_m$ GTP (µM)</td>
<td>26±2</td>
<td>12±2$^b$</td>
</tr>
<tr>
<td>$K_m$ L-aspartate (µM)</td>
<td>230±40</td>
<td>140±20$^b$</td>
</tr>
<tr>
<td>$K_i$ hadacidin (µM)</td>
<td>0.49±0.08</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td><strong>2'-Deoxy-IMP reaction:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (sec$^{-1}$)</td>
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<td>5.5±0.2</td>
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<tr>
<td>$K_m$ 2'-deoxy-IMP (µM)</td>
<td>41±4</td>
<td>58±8</td>
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<tr>
<td>$K_m$ GTP (µM)</td>
<td>21±1</td>
<td>15±2</td>
</tr>
<tr>
<td>$K_m$ L-aspartate (mM)</td>
<td>13±5</td>
<td>4±0.2</td>
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<tr>
<td>$K_i$ hadacidin (µM)</td>
<td>17±3</td>
<td>14±3</td>
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*Footnotes.*

$^a$Conditions of assay are in the experimental section.

$^b$From reference (23).
Table 2. Statistics of data collection and refinement

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<tr>
<th>Complex</th>
<th>( E.\ cb: \text{GDP}^-\text{2'-deoxy-6-PIMP\cdot hadacinin} )</th>
<th>Mouse muscle: ( \text{GDP}^-\text{2'-deoxy-6-PIMP} )</th>
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<tr>
<td>Space group</td>
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<td>P4(_3)2(_1)2</td>
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<td>( a ) (Å)</td>
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<td>70.11</td>
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<tr>
<td>( b ) (Å)</td>
<td>80.59</td>
<td>70.11</td>
</tr>
<tr>
<td>( c ) (Å)</td>
<td>158.5</td>
<td>199.0</td>
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<td>Resolution limits (Å)</td>
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<td>No. of unique reflections</td>
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<td>20,371</td>
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<td>No. of refls. in refinement(^c)</td>
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<td>No. of atoms</td>
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### Table 2. Footnotes.

*Resolution range for the last shell is 2.0–2.07 Å for the *E. coli* complex and 2.4–2.5 Å for mouse muscle complex.

\[ R_{\text{merge}} = \frac{\sum_i \sum_j|I_{ij} - <I_j>|}{\sum_i \sum_j I_{ij}}, \]

where *i* runs over multiple observations of the same intensity and *j* runs over crystallographically unique intensities.

*All data for which \( |F_{\text{obs}}| > 0 \).*

| R-factor = \( \frac{\sum ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum |F_{\text{obs}}|} \), \( |F_{\text{obs}}| > 0 \). |
Table 3. Energy calculations involving the 2'-hydroxyl group of 6-PIMP in fully ligated complexes of *E. coli* and mouse muscle adenylosuccinate synthetase.

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<tr>
<td>Total</td>
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<td>-2.190</td>
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</table>
FIGURE LEGENDS

Figure 1. The 2'-deoxy-6-PIMP•GDP•hadacidin•Mg\textsuperscript{2+} complex. The left panel provides a view of a single subunit of the dimer with bound ligands. The right panel shows electron density from an omit map covering 2'-deoxy-6-PIMP, using a contour level of 1.5-\(\sigma\) and a cutoff radius of 1.5 Å. The labels Had and d-6-PIMP represent hadacidin and 2'-deoxy-6-PIMP, respectively. Parts of this figure were drawn with MOLSCRIPT (49).

Figure 2. Stereoview of superpositions of fully ligated complexes of E. coli adenylsuccinate synthetase. The complex of 6-PIMP, GDP, hadacidin and Mg\textsuperscript{2+} (white with thin lines) nearly overlays that of 2'-deoxy-6-PIMP, GDP, hadacidin and Mg\textsuperscript{2+} (black with bold lines). Labels Had and d-6-PIMP represent hadacidin and 2'-deoxy-6-PIMP, respectively. The viewing orientation is that of Figure 1. This figure was drawn with MOLSCRIPT (49).

Figure 3. Stereoview of superpositions of partially ligated complexes of mouse muscle adenylsuccinate synthetase. Complexes of 6-PIMP, GDP and Mg\textsuperscript{2+} (Chain A, white with thin lines, and Chain B, gray with medium lines) are superimposed on the complex of 2'-deoxy-6-PIMP (black with bold lines). Label d-6-PIMP represents 2'-deoxy-6-PIMP. The viewing orientation is that of Figure 1. This figure was drawn with MOLSCRIPT (49).

Figure 4. Space filling models of the fully ligated complex of the E. coli synthetase. Packing voids in the vicinity of the 2'-hydroxy group of 6-PIMP (left) and in its absence in the 2'-deoxy-6-PIMP complex (right) are indicated by dark outlines of the atoms determining...
to the void. The viewing orientation is that of Figure 1. This figure was drawn with PyMOL (50).
Fig. 1. Iancu, et al.
Fig. 2. Iancu, et al.
Fig 3. Iancu, et al.
Fig. 4. Iancu, et al.
Table of Contents graphic, Iancu et al.
CHAPTER III

The Role of Valine\textsuperscript{273} in the Recognition of L-Aspartate in Adenylosuccinate Synthetase

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Ames, IA 50011

ABSTRACT

Adenylosuccinate synthetase, with the hydrolysis of GTP, catalyzes the first step of \textit{de novo} purine nucleotide biosynthesis in the formation of adenylosuccinate from L-aspartate and IMP. Valine in position 273 of the enzyme from \textit{Escherichia coli} is conserved over all sequences of adenylosuccinate synthetase. Mutation of this site in the synthetase from \textit{E. coli} reveals the significant role of Valine\textsuperscript{273} in the recognition of L-aspartate, even though it cannot hydrogen bond with the substrate. Mutations of Valine\textsuperscript{273} to alanine, threonine, or asparagine increase the \(K_m\) for L-aspartate from 15- to 40-fold, and decrease the \(K_i\) for dicarboxylate analogues of L-aspartate by up to 40-fold. Here we show the size and apolar properties of residue 273 are responsible for proper conformational change of the L-aspartate loop in response to the binding of L-aspartate. Structure-based mechanisms, by which the residue influences substrate recognition, are presented here to explain the conservation of Valine\textsuperscript{273}. 

INTRODUCTION

In the *de novo* biosynthesis of AMP, adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP-forming); EC 6.3.4.4) catalyzes the first reaction: generating adenylosuccinate from IMP and L-aspartate. The liagse reaction is energetically unfavorable and is coupled with the hydrolysis of GTP to favor product formation in a two step reaction mechanism (1-3): The $\gamma$-phosphoryl group of GTP is transferred to the O-6 atom of IMP, forming 6-phosphoryl-IMP (6PIMP). Then, the $\alpha$-amino group of L-aspartate displaces the 6-phosphoryl group of 6PIMP to form adenylosuccinate (SAMP) (1-7). The kinetic mechanism is random sequential with a strong bias in favor of the association of L-aspartate after the formation of an enzyme-IMP-GTP complex (8). Mg$^{2+}$ is an essential cofactor in the reaction (2).

Besides its role in *de novo* biosynthesis of purine nucleotides, adenylosuccinate synthetase participates in salvage pathways for nucleotides (1-3). The position of adenylosuccinate synthetase in the nucleotide metabolism makes it important to the growth of cells: the synthetases are targets in the development of drugs in the treatment of cancer and infectious disease.

The active form of the synthetase from *Escherichia coli* is a homodimer (9). IMP, is an essential factor in the formation and stability of the homodimer. The 5$'$-phosphoryl group of IMP hydrogen bonds with the Arginine$^{143}$ from the symmetry-related subunit of the dimer. Mutation of Arginine$^{143}$ significantly lowers the affinity of IMP for the enzyme and abolishes
ligand-induced dimerization of the synthetase (2). IMP in the form of its phosphorylated intermediate 6-PIMP, binds Mg$^{2+}$ and three dynamic loops of the active site (L-aspartate loop, loop 120-130 and loop 38-53). IMP alone is sufficient to organize the active site of the E. coli synthetase alone (10) through its interaction between the 5$'$-phosphoryl group and Asparagine$^{38}$.

The comparison of ligand-free and ligated complexes of E. coli adenylsuccinate synthetase reveals remarkable conformational change in several loops (3). Loops 38–53 and 419–421, ordered structures in the ligand-free enzyme, exhibit significant movement when ligands bind. Some backbone elements of loop 38-53 move up to 9 Å (5). Mg$^{2+}$-GDP seems an important factor in stabilizing the active conformation of loop 38-53, making six hydrogen and metal coordination bonds to this loop. Loop 120-130 and 299-304, close to the IMP and L-aspartate pockets, respectively, and disordered without ligands, assume well-defined conformations in the presence of IMP and L-aspartate (5,6). Conformational change in the L-aspartate loop 299-304 and the binding of L-aspartate are coupled with the binding of IMP. The hydrogen bond from atom O2' of IMP to Arginine$^{303}$ NH2 can draw the L-aspartate loop into a conformation ready to bind L-aspartate. The loss of this hydrogen bond, by the replacing IMP with 2'$'$-deoxy-IMP, increases the $K_m$ for L-aspartate by 40-fold (11).

Threonine$^{128}$, Threonine$^{129}$, Valine$^{273}$, Threonine$^{300}$, and Threonine$^{301}$ are sequence conserved, indicating they probably have important functions. Threonine$^{128}$ and
Threonine\textsuperscript{129} interact with the 5′-phosphoryl group of IMP and Threonine\textsuperscript{301} hydrogen bonds directly to the carboxyl group of hadacidin (\textit{N}-formyl \textit{N}-hydroxyglycine; an analogue of \textit{L}-aspartate) (5,7). However, the apparent significance of Valine\textsuperscript{273} to the recognition of \textit{L}-aspartate is puzzeling. Threonine and alanine in position 273 cause 15- and 30-fold increases, respectively, in the $K_m$ for \textit{L}-aspartate, and the mutation to asparagine causes a 40-fold increase. At the same time, it reduces $K_i$ values for analogues of \textit{L}-aspartate by up to 40-fold (12). Obviously, Valine\textsuperscript{273} is essential to the recognition of \textit{L}-aspartate although its side chain cannot hydrogen bond.

Hadacidin is a competitive inhibitor with respect to \textit{L}-aspartate (13), and provides the basis in crystal structures for modeling interactions between \textit{L}-aspartate and the active site. Here we report four new crystal structures of mutant \textit{E. coli} adenylosuccinate synthetase in which position 273 is alanine or threonine. In its ligated complexes, the side chain of Threonine\textsuperscript{273} exhibits several interactions that don’t exist for wild type enzyme. Threonine\textsuperscript{273} OG1 hydrogen bonds with Arginine\textsuperscript{305} and thus closes the \textit{L}-aspartate loop onto the binding pocket for \textit{L}-aspartate. The Alanine\textsuperscript{273} mutant has the \textit{L}-aspartate loop conformation of the wild type complex; however, the absence of the two $\gamma$-CH\textsubscript{3} groups leaves excess room in the \textit{L}-aspartate binding pocket, allowing the side chain of Arginine\textsuperscript{305} to fill the space normally occupied by \textit{L}-aspartate. Both mutations force \textit{L}-aspartate to expend more binding energy to organize the active site into the catalytically product state.
EXPERIMENTAL

Materials— Restriction enzymes came from Promega. Pfu DNA polymerase and *E. coli* strain XL-1 blue were obtained from Stratagene. Phenyl-Sepharose CL-4B came from Amersham Biosciences, Inc. All reagents, including GTP, IMP, L-aspartate, bovine serum albumin, and DEAE-Sepharose were from Sigma unless noted otherwise.

Molecular Biology— The construction of the *Escherichia coli* adenylosuccinate synthetase mutants on position 273 is described previously (12).

Purification of Enzyme from *Escherichia coli*— The synthetase was prepared as described previously from a genetically engineered strain of *E. coli*. (15). Enzyme purity exceeded 95% as determined by SDS-polyacrylamide gel electrophoresis (data not shown).

Enzyme Assay— Protein concentration was determined by the Bradford method (16), using bovine serum albumin as the standard. Enzyme activity was determined at an absorbance of 290 nm and 22 °C as described previously (17). For the *E. coli* adenylosuccinate synthetase, the assay buffer contained 20 mM Hepes, pH 7.7, and 6 mM magnesium chloride. Using up to 1 µg/ml enzyme, the reaction was linear for 1 min. $K_m$ and $V_{max}$ values for each substrate were obtained by holding the other two substrates at saturating levels (100 µM for GTP, 300 µM for IMP, and 2000 µM for L-aspartate) and varying the third substrate. GTP was varied from 5 to 100 µM, IMP varied from 25 to 500 µM, L-aspartate varied from 100 to 1000 µM and hadacidin varied from 0.25 to 100 µM. All of the kinetic data were analyzed with the computer program GraFit (18).
Crystallization—Crystals were grown by the method of hanging drops. Equal parts of a protein solution (10 mg/ml of protein in 50 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA) and precipitant solution (200 mM MgCl₂, 100 mM HEPES, pH 7.0, 12-14% polyethylene glycol 8000) were mixed in 6-µl droplets. The wells contained 500 µl of the precipitant solution. Equal dimensional prisms of 100 microns appeared within 3 days at 22 °C. The crystals were transferred in three steps (10-min intervals) to solutions containing 100 mM MgCl₂, 50 mM HEPES, pH 7.0, 27% (w/v) polyethylene glycol 8000, and glycerol at 7, 14, and 21% (v/v) and then frozen in liquid nitrogen.

Data Collection, Model Building, and Refinement—Data were collected at Beamline 9-2 of Stanford Synchrotron Radiation Laboratory, using the CCD detector, ADSC Quantum 4. The wavelength of x-radiation was 0.979 Å, and the temperature of data collection was 120 K. Data reduction was done with Denzo/Scalepack package (19). Other data were collected on a Rigaku R-AXIS IV++ image plate detector using an Osmic confocal mirror system (CuKa radiation) and a sample temperature of 100 K. Data reduction employed CrystalClear software. Structures were solved by molecular replacement with the program AmoRe (20). The initial model was the E. coli synthetase (Protein Data Bank identifier 1CG0) (7). Model building and refinement employed the programs XTALVIEW (21) and CNS (22), respectively. Force constants and parameters of stereochemistry came from Engh and Huber (23). Criteria for the addition of water molecules were identical to those of
previous studies (5-7). Estimates of coordinate error used the method of Luzzati (24). Evaluation of stereochemistry of the refined model employed PROCHECK (25). Superposition of structures employed software from the CCP4 package (26) or XTALVIEW (21).

**RESULTS**

In order to explore the role of the amino acid at position 273 in *E. coli* adenylosuccinate synthetase mutant enzymes Valine$^{273} \rightarrow$Threonine and Valine$^{273} \rightarrow$Alanine were constructed. The mutant enzymes were assayed and crystallized in partial ligation (GDP, 6PIMP, and Mg$^{2+}$) or in full ligation (GDP, 6PIMP, Mg$^{2+}$, and hadacidin). The results of kinetic and structural analyses are in Tables 1&2. $K_m$ values for L-aspartate and $K_i$ values for hadacidin increase significantly, whereas $k_{cat}$, and $K_m$ values for IMP and GTP remain comparable to those of the wild-type enzyme.

*Threonine$^{273}$ Adenylosuccinate Synthetase, Fully Ligated*— This structure is nearly identical to that of the wild type enzyme, fully ligated (Figure 1). The RMSD for these two structures based on Ca atoms is 0.17 Å, the approximate level for the uncertainty in atomic coordinates. The superposition of structures reveals identical backbone and side-chain conformations. Atom O2’ of IMP O2’ is 2.7 Å from the atom NH2 of Arginine$^{303}$, indicative of a hydrogen bond. The $\chi_1$ angle of Threonine$^{273}$ differs from that of Valine$^{273}$ of the wild-type complex (–46° vs. –124° in the wild-type enzyme). Threonine$^{273}$ OG1 forms a hydrogen bond with the OD2 of Aspartate$^{13}$ (3.1 Å), as well as with a water molecule.
in the active site (3.1 Å). There are also two potential hydrogen bonds between Threonine\textsuperscript{273} OG1 and hadacidin atom OB and Arginine\textsuperscript{305} NE, both with distances of 3.6 Å. These extra interactions do not change the conformation of residues (such as Arginine\textsuperscript{305} and Aspartate\textsuperscript{13}) that interact with ligands (hadacidin or water molecules).

\textit{Alanine}\textsuperscript{273} \textit{Adenylosuccinate Synthetase, Fully Ligated}— The RMSD for the C\textalpha{} atoms from the wild type and Alanine\textsuperscript{273} complexes is 0.25 Å. Moreover, the corresponding RMSD for the C\textalpha{} atoms from the Alanine\textsuperscript{273} and Threonine\textsuperscript{273} complexes is 0.24 Å. The absence of two $\gamma$-CH\textsubscript{3} groups in the Alanine\textsuperscript{273} enzyme creates a small cavity around the $\beta$-carbon atom: a hemisphere of ~4 Å radius around the $\beta$-carbon atom is clear of contacts (Figure 1). The loss of two methyl groups without a compensating conformational change in the enzyme to enhance the packing density of atoms could result in energy differences of 5 kcal/mole, more than enough to account for the increase in $K_m$ for L-aspartate.

\textit{Threonine}\textsuperscript{273} \textit{Adenylosuccinate Synthetase, Partially Ligated}— GDP, IMP and Mg\textsuperscript{2+}, have the same conformation as in the fully ligated complex, and Threonine\textsuperscript{273} itself, has the same conformation (Figure 2). Other differences, however, are evident between the partially ligated Threonine\textsuperscript{273} complex and the wild-type complex. In the wild-type system atom O2’ of IMP hydrogen bonds with atom NH2 of Arginine\textsuperscript{303}. In the partially ligated Threonine\textsuperscript{273} complex this structure, atom O2’ of IMP is 4.1 Å from atom NH2 of Arginine\textsuperscript{303}. At the same time, atom OG1 of Threonine\textsuperscript{273} hydrogen bonds with atom NE of Arginine\textsuperscript{305}. This interaction cannot exist in the wild type system. Perhaps as a consequence of the
Threonine$^{273}$-Arginine$^{305}$ interaction, the L-aspartate loop closes over the active site. We see such closed conformations in fully ligated complexes, but this is the first instance of a closed active site in a partially ligated complex.

**Alanine$^{273}$ Adenylosuccinate Synthetase, Partially Ligated**— The partially ligated Alanine$^{273}$ structure is like the wild-type enzyme with respect to the conformation of the L-aspartate loop (Figure 3). Atom O2’ of IMP is 3.0 Å from atom NH2 of Arginine$^{303}$, and L-aspartate loop stands off from the active site; however, specific side chains exhibit differences. Arginine$^{305}$ swings almost 30 degrees toward to Alanine$^{273}$ in the binding site. Atom NE of Arginine$^{305}$ binds (2.9 Å) to the backbone carbonyl of Arginine$^{303}$, and atom NH2 of Arginine$^{305}$ forms hydrogen bonds with atom OD2 of Aspartate$^{13}$ (2.7 Å) and a water molecule (3.4 Å). Atom NH1 of Arginine$^{305}$ also hydrogen bonds with atom OD2 of Aspartate$^{13}$ (2.6 Å). Of these interactions, only the last one exists in partially ligated complexes of the wild-type enzyme.

**DISCUSSION**

There are two important differences between partially ligated complexes of the Threonine$^{273}$ mutant and wild-type enzymes (Figure 2). First, the Threonine$^{273}$ complex lacks the IMP O2’-Arginine$^{303}$ NH2 hydrogen bond. This hydrogen bond in the wild-type enzyme positions Arginine$^{303}$ favorably for the binding of the β-carboxyl group of L-aspartate. Second, the L-aspartate loop is like the L-aspartate loop in the fully ligated complex, that is, in its closed conformation. Stabilization of the closed conformation in the absence of
L-aspartate is due evidently to the interaction between atom OG1 of Threonine\textsuperscript{273} and the side chain of Arginine\textsuperscript{305}, the latter being critical to the binding of the \(\alpha\)-carboxyl group of L-aspartate. Both of these conditions are unfavorable to the binding of L-aspartate, and not surprisely, the \(K_m\) for L-aspartate for the Threonine\textsuperscript{273} enzyme is high relative to that of the wild-type enzyme. In contrast, the fully ligated complexes of the wild-type and Threonine\textsuperscript{273} enzymes are almost identical. As the fully ligated complexes are the closest structural approximations of the substrate-bound complexes, their similarity suggests comparable energies for transition states. The structures then are consistent with comparable \(k_{cat}\) values determined from activity assays in the presence of saturating substrates.

Destabilization of the Alanine\textsuperscript{273}-L-aspartate complex relative to the wild-type complex differs appreciable from that of the Threonine\textsuperscript{273} complex. The partially ligated structure of the Alanine\textsuperscript{273} complex is very similar to that of the wild-type enzyme. Some organizational differences exist, particularly with respect to the conformation of Arginine\textsuperscript{305}, but for the most part L-aspartate has free access to the active site (Figure 3). In the fully ligated complex, however, the Alanine\textsuperscript{273} enzyme has a less densely packed active site than that of the wild-type enzyme because of the absence of two methyl groups from the side chain at position 273. Hence, a cavity exists in the active site in the fully ligated complex of the Alanine\textsuperscript{273} enzyme that does not exist in the fully ligated complex of the wild-type enzyme. The loss of non-bonded contacts due to a less tightly packed active site raises the
$K_m$ for L-aspartate. Nonetheless, the conformation of substrates in the fully-ligated complex of Alanine$^{273}$ and the wild-type enzymes are virtually identical. Hence, the $k_{cat}$ values are similar for the two systems.

The basis for sequence conservation in position 273 ultimately rests with the optimization of packing density in the active site because of a complementary fit of the isopropyl group of value, which can be achieved on only the isoteric side chain of threonine. In the presence of side chains smaller than that of valine, the active site has destabilizing internal voids in its fully ligated state. In the presence of threonine, however, the active site closes in the absence of L-aspartate. Hence, more binding energy of the L-aspartate interaction must go toward “lifting” the partially-ligated ground state to the fully-ligated transition state.

REFERENCES

63


Table 1: Statistics of data collection and refinement.

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Table 2: Kinetic parameters for recombinant adenylosuccinate synthetases from *E. coli*.

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<td>1.00 ± 0.1</td>
<td>31 ± 3</td>
<td>17 ± 2</td>
<td>71</td>
<td>47 ± 2</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Stereoview of superpositions of fully ligated complexes of wild type, Thr273, Ala273 *E. coli* adenylosuccinate synthetases. Active sites of Thr273 and Ala273 complexes (Thr273 complex, white with thin lines, and Ala273 complex, gray with medium lines) are superimposed on the active site of wild type complex (1CG0, black with bold lines). This figure was drawn with MOLSCRIPT.

Figure 2. Stereoview of superpositions of fully ligated and partially ligated complexes of Thr273 *E. coli* adenylosuccinate synthetase. Active site of fully ligated Thr273 complexes (white with thin lines) is superimposed on the active site of partially ligated Thr273 complex (black with bold lines). The viewing orientation is that of Figure 1. This figure was drawn with MOLSCRIPT.

Figure 3. Stereoview of superpositions of fully ligated and partially ligated complexes of Ala273 *E. coli* adenylosuccinate synthetase. Active site of fully ligated Ala273 complexes (white with thin lines) is superimposed on the active site of partially ligated Ala273 complex (black with bold lines). The viewing orientation is that of Figure 1. This figure was drawn with MOLSCRIPT.
Fig. 1. Zhou, *et al.*
Fig. 2. Zhou, et al.
Fig. 3. Zhou, et al.
CHAPTER IV

Time-dependent Fluorescence Change as a Probe of Subunit Exchange Kinetics in Porcine Fructose-1,6-Bisphosphatase

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ABSTRACT

Fructose-1,6-bisphosphatase (FBPase) governs a control point in gluconeogenesis, the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P₂) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (Pᵢ). Mammalian FBPases are stable homotetramers in solution, and yet subunits exchange spontaneously between distinct homotetramers to produce hybrid heterotetramers. Fluorescent probes (eosin-5-iodoacetamide), attached specifically to subunits, exhibit self-quenching if brought into proximity by the subunit arrangement of the FBPase tetramer. Elimination of self-quenching occurs when the tetramer dissociates into subunits upon dilution or if unlabeled subunits exchange with probe-labeled subunits. By monitoring the time-dependent fluorescent change, disruption of the C1-C4 interface of the tetramer occurs without a time lag; however, disruption of the C1-C2 interface occurs after a lag of approximately 30 minutes. Forward/reverse rate constants for subunit dissociation/association reactions account for time-dependent fluorescent changes due to the
dilution of labeled tetramer and also to subunit exchange between modified and wild-type tetramers. The exchange pathway begins with the formation of C1-C2 dimers from tetramers, followed by the formation of single subunits from C1-C2 dimers. The pathway observed here is consistent with subunit arrangements previously assigned to hybrid heterotetramers of FBPase. Moreover, the most kinetically liable interface of the mammalian tetramer (C1-C4 interface) corresponds to the interface of FBPase from Escherichia coli subject to stabilization by allosteric activators.

Key Words: Protein-protein interactions, Kinetic mechanisms, Protein self-assembly, Subunit exchange, Protein engineering, Macromolecular dynamics

INTRODUCTION

Fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) is a key regulatory enzyme in the gluconeogenesis, catalyzing the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P₂) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (Pᵢ) (1-3). FBPase, together with fructose-6-phosphate 1-kinase, composes a futile cycle in the gluconeogenic/glycolytic pathways (4, 5). AMP and fructose 2,6-bisphosphate (Fru-2,6-P₂) inhibit the enzyme by binding to the allosteric and active sites respectively. Inhibition of FBPase by AMP is nonlinear (Hill coefficient of ~2) and noncompetitive with respect to Fru-1,6-P₂, whereas inhibition by Fru-2,6-P₂ is linear and competitive with respect to substrate (1-3, 6, 7). Fru-2,6-P₂ increases the binding affinity of AMP to FBPase by as much as 10-fold (8). AMP/Fru-2,6-P₂ synergism stems from distinct
mechanisms of conformational change that lead to similar inhibited end states of the enzyme (8-10).

FBPase is a tetramer of identical subunits \((M_r=37,000)\). Each subunit has binding sites for Fru-1,6-P\(_2\) (or Fru-2,6-P\(_2\)) and metal ions, defining the active site, and a distinct AMP binding site, which is at least 28 Å from the nearest active site (11-13). The four subunits of the tetramer adopt an approximate square-planar arrangement. Subunit C1 occupies the upper-left position, with subunits C2 through C4 following clockwise (Figure 1). FBPase exists in at three quaternary states called R, I and T, distinguished by the rotation of subunit pair C1-C2 relative to C3-C4 about the molecular twofold axis that lies between subunits C1 and C2 as well as C3 and C4. With the R-state defining a subunit pair rotation of 0°, the I-state (observed in complexes of porcine FBPase with Fru-2,6-P\(_2\)) and the T-state (observed in complexes with AMP or AMP and Fru-2,6-P\(_2\) combined) have subunit-pair rotation angles of 13 and 15°, respectively. (14-16)

Loop 52–72 plays an important role in the allosteric mechanism of AMP inhibition. In the absence of AMP, the loop forms an engaged loop conformation, interacting with the active site (17, 18). The binding of AMP to its allosteric pocket putatively removes the loop from the active site, and stabilizes the disengaged loop conformation. Mutations of conserved residues in Loop 52–72 cause significant effects on catalysis and allosteric inhibition of catalysis by AMP (18, 19).

Previous work reveals different homotetramers of FBPase when mixed undergo
spontaneous subunit exchange. Initially, a hybrid tetramer forms with two subunits from each of two homotetramers, followed later by the formation of hybrid tetramers that combine one subunit of tagged forming hybrid tetramers to a large extent separable by anion-exchange chromatography (20, 21). AMP eliminates subunit exchange, through presumably the formation of the T-quaternary state. The disengaged loops in T-state crystal structures participate in hydrophobic interactions that involve residues from three of four subunits of the FBPase tetramer. Active site ligands, such as Fru-6-P, Fru-1,6-P₂, and Fru-2,6-P₂, block the exchange at the level of individual subunits, but allow the exchange of subunit dimers (21). The 6-phosphoryl groups of these ligands bind to residues from both the C1 and C2 subunits (C3 and C4 as well), and hence, the assignment of hybrid tetramers of FBPase formed in the presence of these 6-phosphoryl ligands has assumed the immutability of the C1-C2 (and C3-C4) subunit pair. Significant conclusions regarding the function of FBPase rests on the assumption of a stable C1-C2 dimer in exchange processes in the presence of 6-phosphoryl ligands (21). In fact, current models for the AMP-induced conformational change in FBPase (21) would be invalid if the C1-C4 subunit pair were the stable dimer. Hence, direct verification of the pathway of subunit places current models of allosteric action in mammalian FBPases on a firm footing.

Self-quenching is the decrease in the change of fluorescence intensity as fluorophore concentrations increase. Self-quenching occurs under conditions (such as high concentration) where fluorophore molecules interact. Although the physical mechanism is
unclear, quenching may result from transient excited-state interactions (collisional quenching) or from formation of nonfluorescent ground-state species (22). The self-quenching of carboxyfluorescein involves energy transfer to nonfluorescent dimmers (23). Investigators has used fluorescence self-quenching to probe ensemble protease and nuclease activities (24), membrane fusion (25), protein-dimer formation (26), and single molecule folding (27). Here, the fluorescence self-quenching arising from a specific placement of probes in the FBPase tetramer, reveals the pathway of subunit exchange in mammalian FBPase. Probes on individual subunits are in proximity near a specific subunit interface. Rapid dilution of such labeled tetramers of FBPase results in a time-dependent rise in fluorescence due to the dissociation of the tetramer into dimers or single subunits. Alternatively, the rapid mixing of unlabeled and labeled tetramer also results in a time-dependent increase in fluorescence as labeled subunits recombine with unlabeled subunits to form hybrid tetramers of FBPase. As self-quenching requires conjugation of only one type of fluorophore with the macromolecule, the technique is relatively easy to implement in comparison to FRET which requires labeling with appropriately matched fluorophores.

Time-dependent change in fluorescence self-quenching of strategically labeled subunits of a mutant FBPase directly confirms the first kinetic step in the dissociation of the tetramer into C1-C2 and C3-C4 subunit pairs. After a substantial time lag, subunit exchange processes involving the disruption of the C1-C2 and C3-C4 interfaces becomes measurable. Forward and reverse rate constants for the dissociation of tetramers into C1-C2 and C3-C4
dimers and the dissociation of C1-C2/C3-C4 subunit pairs into monomers reproduce time
dependent fluorescence change. The kinetic mechanism of subunit exchange is consistent
with a initial rapid dissociation of the tetramer into C1-C2 dimers, followed by a much
slower dissociation of C1-C2 dimers into monomeric subunits.

**EXPERIMENTAL**

*Materials*— Fru-1,6-P₂, Fru-2,6-P₂, NADP⁺, DEAE-Sepharose and Cibacron
Blue-Sepharose came from Sigma. Glucose-6-phosphate dehydrogenase and
phosphoglucose isomerase were from Roche Applied Sciences. Kanamycin sulfate from
Gibco, *E. coli* strains BL21(DE3) and chemically competent DH5α from Invitrogen,
isopropyl-β-d-thiogalactopyranoside (IPTG) from Anatrace, 2′-deoxyribonucleoside
triphosphates and restriction endonuclease DpnI from New England Biolabs, *PfuTurbo* DNA
polymerase from Stratagene, QIAprep Spin Miniprep kit for plasmid isolation from Qiagen,
oligonucleotides from IDT, and eosin-5-iodoacetamide from Molecular Probes. The pET 24
vector containing the insert coding for porcine FBPase came from previous work (20). All
other chemicals were of reagent grade.

*Construction of mutant FBPases*— Directed mutagenesis employed protocols from
Stratagene using the Polymerase Chain Reaction (PCR), *PfuTurbo* DNA polymerase,
2′-deoxyribonucleoside triphosphates, template DNA (pET 24 vector containing the porcine
FBPase insert), and the following primers:

5′-CAGCTGCTCAACTCGTG GCC ACCGC -3′,
5′-GGTGAATGGAGTCAATTCCCTCAT\textbf{GCT}GGACCCG-3′,
5′-GGGTAATACGTTGGTC\textbf{GCT}TTTGATCCCCTCG-3′,
5′-GGATCGTGAACATCGAC\textbf{GCC}CTGTGTGC-3′,
5′-GGTGAATGGAGTCAAT\textbf{GCC}TTCATGC-3′, and
5′-GACTGCTATACGAA\textbf{GCT}AACCGATGGC-3′.

The primers insert codons for alanine (bold typeface) in place of those for Cys38, 92, 116, 128, 183 and 281, respectively. Additional primers, as follow respectively, changed Ala183 to serine, and Thr144, Asn236 and Lys337 to cysteines (altered codons in bold typeface):

5′-GGTGAATGGAGTCAAT\textbf{TCC}TCATGCTGGACCCG-3′,
5′-CAGAAAGAATTCA\textbf{TGC}GATGAACCTTC-3′,
5′-GTTTCCCCCAGAC\textbf{TGC}TCAGCCCCCTAC-3′, and
5′-CAGAAGCAGCGAGCC\textbf{TGC}GAAAAACCTCAGCAGTTTATG-3′.

A programmable theromocycler (BioRad iCycler) amplified mutant plasmids in a reaction volume of 50 µL. Incubation of the reaction mixture with 20 U of DpnI for 2 hrs. at 37 °C removed the starting template, leaving mutated and nicked circular DNA. 5 µL of the incubation mixture was combined with 30 µL of chemically competent \textit{E. coli} strain DH5α cells, left on ice for 30 min., heated for 45 sec. at 42 °C, and then cooled on ice for 2 min. Incubation of the resulting 35 µL culture with 1 mL of Luria broth for 2 hrs. at 37 °C completed the transformation. Transformed cells were selected on Luria broth agar plates
with kanamycin (30 µg/mL). Small volumes of Luria broth with kanamycin (30 µg/mL) were inoculated with single colonies and the cultures grown overnight. The isolation of plasmids from over-night cultures employed the QIAprep Spin Miniprep kit. Complete mutant vectors were then transferred to FBPase-deficient *E. coli* (DF657) for the expression of protein. Mutations, and the integrity of the resulting gene, were confirmed by sequencing the promoter region and the entire open reading frame. The Iowa State University sequencing facility performed all DNA sequencing, using the fluorescent dye dideoxy terminator method.

*Purification of the protein*— Protocols for protein expression and purification are in the literature (19). Recombinant FBPase was expressed in FBPase-deficient *E. coli* (DF657). FBPase, eluted from Cibacron Blue-Sepharose chromatography, was adjusted to pH 7.5–8.3 before loading onto a DEAE-Sepharose column (equilibrated with 5 mM MgCl$_2$ and 20 mM Tris-HCl, pH 7.5). FBPase eluted from DEAE-Sepharose was dialyzed against 20 mM Tris-HCl, pH 7.5, and used immediately in experiments without freezing or storage. Protein purity was monitored by SDS-PAGE (28) and protein concentrations determined by the method of Bradford (21).

*FBPase activity assay*— Activity assays employed the coupling enzymes, phosphoglucone isomerase (10 µg/mL, 5 units) and glucose-6-phosphate dehydrogenase (5 µg/mL, 2 units) (1). All kinetic assays were performed at 22° C. 5 µg of FBPase construct was used in each assay of total volume 1 mL. For assays that employed saturating levels of
Fru-1,6-P$_2$, the reduction of NADP$^+$ to NADPH was monitored by absorbance change at 340 nm, using an extinction coefficient of 6.022$x10^3$. For assays with non-saturating substrate, NADPH production was monitored by fluorescence emission at 470 nm using an excitation wavelength of 340 nm. Initial velocity determinations at pH 7.5 for all mutant constructs employed 100 µM Fru-1,6-P$_2$, 5 mM MgCl$_2$, 150 mM KCl, 0.1 mM EDTA, 0.15 mM NADP+, and 50 mM HEPES, and such determinations at pH 9.5 used the same concentrations of reagents in 50 mM CAPS. Maximal velocity scaled linearly with the amount of FBPase construct, indicating a sufficient excess of coupling enzyme under all conditions of assay.

Determination of the $K_a$ for Mg$^{2+}$, the $K_m$ for Fru-1,6-P$_2$, and the Hill coefficient for Mg$^{2+}$ employed a 6x6 array of conditions that systematically varied the total concentration of Mg$^{2+}$ from 1–50 mM and total concentration of Fru-1,6-P$_2$ from 5–100 µM. The concentration of free Mg$^{2+}$ in assays is taken as its total concentration less the concentration of EDTA (0.1 mM). Loss of free Mg$^{2+}$ due to complexation with substrate was negligible. Determination of the concentration of free Fru-1,6-P$_2$ employed a stability constant of 350 for the binary complex of Mg$^{2+}$ with substrate (29). The determination of $I_{0.5}$ for AMP (concentration of AMP that causes 50% inhibition) and the Hill coefficient for AMP employed 50 mM HEPES, pH 7.5, 50 µM Fru-1,6-P$_2$, 5 mM MgCl$_2$, 150 mM KCl, 0.1 mM EDTA, 0.15 mM NADP+, and AMP from 0.5–200 µM. Determination of the concentrations of free AMP used a stability constant of 100 for the binary complex of Mg$^{2+}$.
with nucleoside monophosphate.

Data were fit to the following equation:

\[ V = \frac{V_{\text{max}}}{(I/I_{0.5})^n + 1}, \quad \text{Equation 1} \]

where \( V \) and \( V_{\text{max}} \) represent velocity and maximal velocity, \( I \) the concentration of AMP, \( I_{0.5} \) the concentration of AMP that causes 50% inhibition, and \( n \) the Hill coefficient for AMP. A similar expression is used to determine the Hill coefficient \( (n) \) for Mg\(^{2+}\), in which \( I \) represents the concentration of Mg\(^{2+}\) and \( I_{0.5} \) is the concentration of Mg\(^{2+}\) that causes 50% activation. Values for \( k_{\text{cat}} \), \( K_a \) for Mg\(^{2+}\) and \( K_m \) for Fru-1,6-P\(_2\) come from fits using the following equation:

\[ V = \frac{(V_{\text{max}} \cdot [M]^2 \cdot [S])}{([M]_0^2 \cdot [S] + K_a \cdot [S] + K_b \cdot [M]^2 + K_{ia} \cdot K_{ib})} \quad \text{Equation 2} \]

where \( V \) represents velocity, \( V_{\text{max}} \) maximum velocity, \([S]\) the concentration of Fru-1,6-P\(_2\), \([M]\) the concentration of Mg\(^{2+}\), \([E_o]\) the total enzyme concentration, and \( k_{\text{cat}} \) the catalytic rate constant. Equation 2 comes from a rapid-equilibrium Random model used in previous work (30). All fits employed the program Grafit (31).

**Labeling of FBPase with fluorescent probe**— Eosin-5-iodoacetamide (C\(_{22}\)H\(_{10}\)Br\(_4\)INO\(_6\), \( M_r=830.84 \)) was chosen as the fluorescent probe because of its high extinction coefficient \((1 \times 10^5 \text{ cm}^{-1}\text{M}^{-1}, \text{pH} 7.5) \) at \( \lambda_{\text{max}}=519 \text{ nm} \) (32). Fluorescence was monitored at the wavelength of maximum emission, 540 nm. Solid eosin-5-iodoacetamide was desolved in dimethylformamide (DMF) to a concentration of 10 mM. 0.1 \( \mu \)mole of the desired FBPase mutant was incubated with 0.1 \( \mu \)mole of dithiothreitol (DTT) in 5 mL of Tris-HCl (20 mM),
pH 7.5 for 1 hour to fully activate the thiol groups. 1 µmole eosin-5-iodoacetamide (100 µL of 10 mM stock solution) was added, and the solution kept in darkness at 4° C for 12 hrs, with gentle rocking. Passage the reaction mixture through a G25 Sepharose column (equilibrated and run with Tris-HCl (20 mM), pH 7.5 at rate ~1ml/min) separated modified protein (first UV-absorbing peak) from unreacted probe. Bradford method (absorbance corrected for the attached probe) was used to check the protein concentration, and the quantity of attached probe was determined by its absorbance at 519 nm and known coefficient extinction.

*Time-dependent Fluorescence Assays*— Fluorescence data were collected using a SLM 8100C fluorimeter from Spectronic Instruments, using an excitation wavelength 519 nm was and an emission wavelength at 540 nm. Each datum was recorded for a time span of 1 minute. All measurements came from freshly prepared enzyme; enzyme with or without probes was never frozen. (The reason for this is discussed in the results section).

Two kinds of experiments were performed: the dilution of labeled enzyme and subunit exchange between labeled and unlabeled enzyme. In the former experiment, labeled FBPase at a concentration of 5.0 µM in Tris-HCl (20 mM), pH 7.5, was mixed (10 seconds of manual mixing in a 2 mL cuvette) with Tris-HCl (20 mM), pH 7.5 to give final protein concentrations from 0.1 to 0.6 µM. Fluorescence changes with time were recorded over a period of 24 hrs. Specific activities of the enzyme were determined at the beginning and at the end of each experiment. Data from the dilution experiments determined $K_{eq}$ governing
the equilibrium between dimers and tetramers (or monomers and dimers), as well as response constants for the difference in fluorescence emission between the self-quenched construct and the construct without self-quenching. Time-dependent fluorescence changes due to subunit exchange employed probe-labeled FBPase, pre-equilibrated with Tris-HCl (20 mM), pH 7.5, at a final concentration of either 0.125 or 0.25 µM. 1.95 mL of the equilibrated probe-labeled FBPase was combined with 50 µL of wild-type FBPase. Ratios of probe-labeled FBPase to wild-type FBPase were 1:1, 1:4 and 1:8. Time-dependent fluorescence exchange experiments were done in triplicate. Ligands such as AMP, Fru-6-P or Mg$^{2+}$, were added to probe-labeled FBPase at the onset of pre-equilibration with Tris-HCl (20 mM), pH 7.5.

**Analysis of Time-dependent Fluorescence Data**— Programs Grafit (31) and Dynafit (33) were used for fitting equilibrium and kinetics models, respectively. Details of the analysis are provided in the results section.

**RESULTS**

**Rationale for directed mutations**— Six cysteines are in wild-type FBPase (Figure 1). Over an incubation of 24 hrs., four mole equivalents of cysteine react with DTNB in the absence of ligands and three mole equivalents react with DTNB in the presence of AMP and Fru-2,6-P$_2$. AMP binds to an allosteric site, stabilizes the quaternary T-state of FBPase, and arrest subunit exchange (14, 20, 21), and Fru-2,6-P$_2$ binds synergistically with AMP at the active site favoring a T-like quaternary state (16, 34). Evidently, a subset of cysteines in
wild-type FBPase could react with the fluorescent probe. Sequence alignments of FBPase sequences revealed alanine, serine, and in some instances lysine at positions corresponding to Cys38, 92, 116, 128, 183, and 281. Substitution of the aforementioned cysteines with alanine would have a reasonable prospect of producing a tetramer with functional properties similar to that of the wild-type enzyme. Ala(38,92,116,128,183,281) FBPase, however, could not be isolated. Constructs with partial substitutions of alanine for cysteine were stable and active, and from these the mutation of Cys183 to alanine seemed the source of the problem. The mutation of Cys183 to alanine would create a void in the tightly packed hydrophobic interior of the subunit. Such voids can destabilize a structure by as much as 7 kcal/mole (35). Position 183 was changed to serine, and the resulting mutant FBPase exhibited substantial activity. Hereafter, the construct Cys38, 92, 116, 128, 281→Ala and Cys183→Ser is called Cys° FBPase.

Cysteine residues engineered into Cys° FBPase serve as specific sites for the covalent attachment of the fluorescent probe. Asn236 and Thr144 are surface side chains near the C1-C2 and C1-C4 interfaces, respectively, whereas Lys337 is at the C-terminus and remote from any interface (Figure 1). Distances between the $\gamma$ atoms of cysteines at positions 144, 236, and 337 are in Table 1. Probes attached to Cys144 should be sensitive to disruption of the C1-C4 interface, but insensitive to changes at the C1-C2 interface. Conversely, probes attached to Cys236 should be sensitive to changes at the C1-C2 interface, but not the C1-C4 interface. Modification of Cys337 puts fluorescent probes at large separations. Hence,
probe-modified Cys337 should be insensitive to changes at either the C1-C2 or C1-C4 interfaces. Positions 144, 236 and 337 sites are not part of any known ligand bind site of porcine FBPase, although position 144 is adjacent to the AMP binding pocket.

Properties of FBPase Constructs— Wild-type and mutant proteins behaved identically throughout purification and were at least 95% pure by sodium dodecylsulfate-polyacrylamide gel electrophoresis (data not shown). The ratio of catalytic rates at pH 7.5/9.5 (Table 1) is a sensitive indicator of proteolysis of mammalian FBPases. A value of 3 or above indicates an intact enzyme, whereas a value of 1.6 or less indicates at least one subunit of the tetramer has undergone proteolysis. Mutations that impair the function of the critical catalytic loop of FBPase also exhibit low activity ratios (18, 20).

The $k_{cat}$ parameters of all constructs are 20–30% of that of the wild-type enzyme. The activity loss is linked to Cys° FBPase, and may be due to the mutation of Cys128 which is near the active site and the C1-C2 interface (Figure 1). Reduced activity may result from the 10-fold increase in the $K_a$ for Mg$^{2+}$ in Cys° FBPase. Modifications have weakened metal association with the active site, but the Hill coefficient for Mg$^{2+}$ remains close to that of the wild-type enzyme. Work of Nelson et al. (30) attributed cooperativity in Mg$^{2+}$-activation of FBPase to the binding of metal ions to the same active site within a subunit. Hill coefficients near 1.9 are consistent with the binding of metal ions to sites 2 and 3, with site 1 fully occupied at concentrations of free Mg$^{2+}$ much less than the minimum concentration used in assays (36, 37). The weaker interaction with the active site, however,
necessitated elevated concentration of Mg$^{2+}$ in assays which lead to significant formation of Mg$^{2+}$•Fru-1,6-P$_2$ complexes. The $K_m$ for Fru-1,6-P$_2$ based on the total concentration of substrate increased systematically as the fixed concentration of Mg$^{2+}$ increased. Subtracting amount of total substrate in the form of binary complexes of Mg$^{2+}$•Fru-1,6-P$_2$, and using the free concentration of Fru-1,6-P$_2$ provided consistent values for the $K_m$ of Fru-1,6-P$_2$ listed in Table 2.

The Hill coefficient for inhibition by AMP indicates modest perturbation due to the mutation of The144→Cys, and still more significant perturbation as a consequence of modifying the enzyme with the fluorescent probe. Sufficient concentrations of AMP, however, still cause complete inhibition consistent with the behavior of the wild-type enzyme (14).

The extent of labeling of FBPase constructs was taken as the ratio of the concentration of covalently attached probe (as measured by absorbance at 519 nm) to the concentration of protein given by the Bradford assay. All constructs exhibited labeling at their theoretical maximum levels of 100±6%.

*Subunit Dissociation by Dilution*— Dilution of labeled FBPase will change the ratio of tetramers, dimers and monomers in the solution. Time-dependent fluorescence change results from the slow approach to equilibrium. In order to monitor whether dissociation occurs by way of a series pathway (for instance, loss of the C1-C4 interface, followed by loss of the C1-C2 interface) or by a parallel pathway (loss of the C1-C4 and C1-C2 interfaces by
comparable time-courses), the attached probes must provide information about the integrity of one interface only. Probe molecules (we hypothesize) at a distance of separation of approximately 40 Å interact, whereas at a distance of 100 Å they do not. Dilution of probe-modified Cys°337 FBPase is a negative control, and should be insensitive to changes at the C1-C4 and C1-C3 interfaces, and perhaps insensitive to changes in the C1-C2 interface (Table 1). Indeed, no time-dependent change in fluorescence is evident upon dilution of Cys°337 FBPase (Figure 2a).

Dilution of probe-modified Cys°144 FBPase should be sensitive to changes at the C1-C4 interface and no other (Table 1). In the absence of ligands or in the presence of Fru-6-P, an immediate and virtually identical time-dependent increase in fluorescence is evident in Figure 2b. On the other hand, no increase in fluorescence occurs with time in the presence of AMP. These findings are consistent with an arrest of subunit exchange by AMP, and that Fru-6-P has no effect on the stability of the C1-C4 interface (21).

Dilution of probe-modified Cys°236 FBPase should be sensitive to changes at the C1-C2 interface alone (Table 1). In the absence of ligands, a time lag of approximately 30 minutes occurs before time-dependent fluorescence changes begin (Figure 2c). Moreover, AMP or Fru-6-P abolishes time-dependent fluorescence change. Hence AMP and Fru-6-P arrest disruption or stabilize the C1-C2 interface. The observation of a time-lag before the onset of fluorescence change is consistent with a serial pathway in which the loss of components with an intact C1-C2 interface depends on a preceding event. That event presumably is the
dissociation of the tetramer into C1-C2 dimers (the loss of components with an intact C1-C4 interface). The proposed mechanism of subunit exchange and subunit dissociation is in Scheme I.

**Determination of Rate Constants for the Tetramer to Dimer Dissociation**— The qualitative findings above are consistent with an equilibrium between tetramers and C1-C2 dimers and a second equilibrium between C1-C2 dimers and monomers. An equilibrium between tetramers and trimers/monomers would not account for the time-lag in the loss of components with C1-C2 interfaces. At elevated concentrations (200 µg/mL), FBPase is overwhelmingly a tetramer. Hence, we assume the predominance of the tetramer, with the formation of some C1-C2 dimer. Loss of the C1-C2 dimer due to further dissociation into monomers is assumed negligible. At low concentrations of fluorophore, fluorescence is linearly proportional to the concentration; however, because of self-quenching the proportionality constant will differ for the various components. For instance, for probe-modified Cys°144 FBPase, self-quenching occurs in the tetramer, but not in the C1-C2 dimer or the monomer.

The fluorescence at low concentrations of total protein then scales linearly with the concentration of tetramer and dimer:

\[
F = aT + bD
\]

Equation 2

where \(a\) and \(b\) are proportionality constants and \(T\) and \(D\) are concentrations of tetramer and dimer. As the stock solution of probe-modified Cys°144 FBPase is primarily tetramer,
fluorescence emission from various dilutions of the stock solution taken at time zero (before equilibration and further dissociation of the tetramer) should vary linearly with total protein concentration. Indeed this is the case (Figure 3) where the slope of an $F$ vs. $T$ plot represents an accurate measure of the proportionality constant $a$. No method is available, however, for the direct measurement of the constant $b$, as dimers in the absence of tetramers occurs only at infinite dilution.

The time-dependent change in fluorescence of a rapidly diluted stock solution of the tetramer can provide sufficient data for a reasonable estimate of $a$, $b$ and rate constants $k_1$ for the dissociation of tetramer to dimer and $k_2$ for the association of dimer to tetramer. The time-dependent fluorescence change associated with six different dilutions of probe-modified Cys°144 FBPase (Figure 3) can be modeled in Dynafit. As is evident from Table 3, parameter $a$ is close to the value determined from a plot of initial fluorescence vs. the total concentration of protein as tetramer. Parameters $b$, $k_1$, and $k_2$ have appreciably higher levels of uncertainty due in part to the correlation between parameter $b$ and the $k_1/k_2$ (that is, $K_{eq}$).

Irrespective of the uncertainty in parameters $b$, $k_1$ and $k_2$, they provide accurate predictions of the outcome of two distinct experiments. The measurement of fluorescence emission from different concentrations of probe-modified Cys°144 FBPase at time zero (immediately upon dilution) and after equilibration (24 hrs.) conforms to the following:

$$\Delta F = a\Delta T + b\Delta D \quad \text{Equation 2}$$

Where $\Delta T$ and $\Delta D$ represent the change in concentrations of tetramer and C1-C2 dimer,
respectively, \(a\) and \(b\) the corresponding proportionality constants, and \(\Delta F\) the change in fluorescence. The final concentrations of tetramer (\(T_f\)) and dimer (\(D_f\)) are given by a simple equilibrium expression:

\[
T_f = \frac{D_f^2}{K_{eq}} \tag{Equation 3}
\]

Where \(K_{eq}\) is a dissociation constant for the tetramer into dimers. Similarly, the stock solution of protein prior to dilution is at equilibrium:

\[
T_{stock} = \frac{(D_{stock})^2}{K_{eq}} \tag{Equation 4}
\]

The initial concentrations of tetramer and dimer immediately upon dilution (\(T_i\) and \(D_i\), respectively) equal \(d\cdot T_{stock}\) and \(d\cdot D_{stock}\), where \(d\) is a dilution factor. Expressing \(\Delta T\) as \(T_f - d\cdot T_{stock}\) and \(\Delta D\) as \(D_f - d\cdot D_{stock}\), substitution of Equations 3&4 in Equation 2 gives

\[
\Delta F = \left(\frac{a}{K_{eq}}\right) \left(\frac{D_f^2}{d^2} - d^2 \cdot \frac{D_{stock}^2}{d^2}\right) + b\cdot \left(D_f - d\cdot D_{stock}\right) \tag{Equation 5}
\]

The concentrations \(D_f\) and \(D_i\) are not known, but can be related to the total concentration of protein as tetramer in the final solution (\(T_0\)) and in the stock (\(T_{0stock}\)): \(T_{0stock} = T_{stock} + \frac{1}{2}D_{stock}\) and \(T_0 = T_f + \frac{1}{2}D_f\). Substitution of Equations 3&4 for \(T_f\) and \(T_{stock}\) results in quadratic equations, one in terms of \(D_f\) and the other in terms of \(D_{stock}\). Solutions to these quadratic equations are given by the quadratic formula:

\[
D_f = -\frac{K_{eq}}{4} + \left(\frac{K_{eq}^2}{16} + K_{eq} \cdot T_0\right)^{1/2} \quad \text{and} \quad D_{stock} = -\frac{K_{eq}}{4} + \left(\frac{K_{eq}^2}{16} + K_{eq} \cdot T_{0stock}\right)^{1/2}
\]

Substitution of these into Equation 5 directly relates an observed fluorescence change (\(\Delta F\)) to the known total concentration of protein as tetramer (\(T_0\)):

\[
\Delta F = \left(\frac{a}{K_{eq}}\right) \left[\left(-\frac{K_{eq}}{4} + \left(\frac{K_{eq}^2}{16} + K_{eq} \cdot T_0\right)^{1/2}\right)^2 - d^2\left(-\frac{K_{eq}}{4} + \left(\frac{K_{eq}^2}{16} + K_{eq} \cdot T_{0stock}\right)^{1/2}\right)^2\right]
\]
In Equation 6, proportionality constants, $a$ and $b$ are the equilibrium constant $K_{eq} = k_1/k_2$ are available from Dynafit. The dilution factor $d$ and total concentration of the protein as tetramer in the stock solution $T_{0stock}$ are known. Hence Equation 6 determines an expected $\Delta F$ which can be compared to observed values (Figure 4).

A second test of parameters in Table 3 is their prediction of the time-dependent fluorescent change in subunit exchange experiments. In these experiments, total protein is constant, and it is the exchange of dimers between tetramers that diminishes self-quenching of the fully-labeled tetramer. The observed and modeled time courses (using Dynafit with fixed values for the four parameters) are again in agreement (Figure 5).

**Determination of Rate Constants for the Dimer to Monomer Dissociation**— In Scheme I, dimer dissociation to monomer follows the tetramer. Only the dimer-monomer dissociation/association influences fluorescence of probe-modified Cys°236 FBPase. Dissociation of the tetramer to dimer by disruption of the C1-C4 interface does not disrupt the self-quenching relationship of probe molecules juxtaposed with respect to the C1-C2 interface (Figure 1). Hence, fluorescence is related to concentrations of tetramer $T$, dimer $D$, and monomer $M$ through proportionality constants $a$, $b$, and $c$:

$$F = a*T + b*D + c*M.$$  \textit{Equation 7}
As before, an estimate of $a$ comes from a plot of fluorescence vs. total protein concentration as tetramer. Two dimer form from one tetramer, hence into to have no in fluorescence due to the conversion of tetramer into dimer, $b = \frac{1}{2}a$. Using values for $k_1$ and $k_2$ determined from probe-modified Cys°144 FBPase, a fit of the time-dependent change in fluorescence (Figure 6) using $a$, $c$, $k_3$ and $k_4$ as adjustable parameters results in the values of Table 4. Using Dynafit, $k_1$ and $k_2$ from Table 3 and $a$, $c$, $k_3$, and $k_4$ from Table 4, simulation of time-dependent fluorescence change in the subunit-exchange reaction between probe-modified Cys°236 FBPase and wild-type enzyme closely follows the observed change in fluorescence (Figure 7).

**DISCUSSION**

The application of self-quenching has a drawback common to all techniques that introduce a non-native probe: the probe must be a reporter group, providing information without influencing function. Detailed information regarding ligand binding sites and the structure of FBPase facilitated the design of experiments, determining the optimal placement of probes so as to enhance self-quenching without significantly perturbing properties of kinetics (Table 2). A distinct advantage of self-quenching lies in its simplicity: it avoids complications due to the overlap of fluorescence emissions from donor and acceptor, a concern in fluorescence resonance energy transfer (FRET) experiments. Moreover, the self-quenching approach requires the covalent coupling of only one type of fluorophore, whereas FRET requires two fluorophores with properly paired absorbance and emission.
properties. A drawback lies in the absence of a quantitative relationship between probe separation and the extent of self-quenching, which is circumvented here by the use of a negative control (Figure 2) that clearly demonstrates the absence of self-quenching at probe separations in excess of 60 Å.

Other applications of self-quenching are in the literature. A decrease in fluorescence emission intensity attends the dimerization of leucine zipper constructs, to which fluorescein is attached (26). Conversion of the dimer to the monomer increased fluorescence. The determination of forward and reverse rate constants in the dimer/monomer equilibrium were determined from fluorescence changes that occurred subsequent to rapid dilution and exchange reactions between labeled and nonlabeled constructs. Chu et al. (27) used self-quenching to monitor the refolding of a single molecule of titan, a protein of ~30,000 amino acids. Fluorescence emission decreases as labeled and denatured titan refolds to its native state.

The formation of tetramer hybrids of FBPase through subunit exchange have provided new tools in the exploration of cooperativity in AMP inhibition (38) and Mg\(^{2+}\) activation (30) of the wild-type tetramer. The process of subunit exchange between a polyglutamyl-tagged homotetramer of FBPase and an untagged tetramer produces seven species (20). DEAE chromatography readily separates tetramers with no tagged subunits, one tagged subunit, three-tagged subunits and four tagged subunits; however, three distinct hybrid tetramers with two tagged subunits separates into a single peak and an unresolved doublet. Assignment of
the correct two-tagged tetramer to the single peak is critical to the valid interpretation of experimental results, particularly those dealing with the mechanism of AMP cooperativity (20, 38, 39)

In the presence of Fru-6-P, Fru-1,6-P2 or Fru-2,6-P2, Nelson et al. (20, 21) observed only three species: tetramers with no tagged subunits, tetramers with four tagged subunits, and a peak matching the retention time of the resolved tetramer with two tagged subunits. As the 6-phosphoryl binding pocket involves residues from subunits C1 and C2, Nelson et al. accounted for the absence of tetramers with one tagged and three tagged subunits by a mechanism of C1-C2 dimer exchange in the presence of active site ligands. By this reasoning, the tagged subunits in the resolved two tagged tetramer are in the same C1-C2 dimer. Recent work of Slebe and co-workers (40), however, suggests that in the presence of high concentrations of guanidinium chloride, the C1-C2 interface becomes solvent exposed before the C1-C4 interface (40). The uncertainty over the relative stability of subunit interfaces in FBPase in the absence of a denaturant, in part motivated the present investigation.

Studies here show unequivocally the stabilization of the C1-C2 interface by Fru-6-P, and the stabilization of the C1-C2 and C1-C4 interfaces by AMP. The effect of AMP is consistent with an ordered pathway of subunit dissociation. If loss of the C1-C4 interface must precede the loss of the C1-C2 interface, then ligand stabilization of the C1-C4 interface must also stabilize the C1-C2 interface. Conversely in an ordered pathway, the stabilization
of the C1-C2 interface by Fru-6-P should have no effect on the stability of the C1-C4 interface, as clearly indicated from the data from Figure 2B showing equivalent time-dependent increases of fluorescence in the presence and absence of Fru-6-P. As mentioned in the results section, the time lag before the appearance of fluorescence change in Figure 2C is consistent with the breakdown of tetramers into C1-C2 dimers prior to the dissociation of C1-C2 dimers into monomers. No evidence here suggests the loss of the C1-C2 interface directly from the tetramer. Such a pathway would generate an immediate rise in fluorescence in Figure 2C upon dilution of probe-modified Cys°236 FBPase, which is not evident from the data. Finally, the rate and proportionality constants determined in the results section also adequately account for the time-dependent fluorescence change due to the exchange of subunits between probe-modified constructs and wild-type FBPase. Of note is the assumption of the same set of rate constants for all constructs in Scheme I, which appears valid in simulations of exchange phenomenon.

The mechanism of ligand stabilization of the C1-C2 interface is clear from structures of FBPase, which reveal hydrogen bonds between the 6-phosphoryl group of Fru-6-P and residues from subunits C1 and C2. But how does AMP abolish exchange processes altogether? AMP transforms FBPase into the T-state, whereas Fru-6-P and Fru-6,2-P₂ stabilize porcine FBPase in the R-state and a T-like state (called I₁₁ (16)), respectively. Fru-6-P and Fru-2,6-P₂ do not abolish dimer exchange via the loss of the C1-C4 interface even though each active site ligand stabilizes a different quaternary of the tetramer. The
disassembly of the tetramer into C1-C2 dimers may occur from a conformational state of FBPase readily accessible to the R- and I_T-states, but not to the T-state AMP complex.

The binding of AMP causes a shear between helices H1 and H2 within the FBPase subunit (15), and may result in a better fit of helices H1 and H2 with their symmetry related mates across the C1-C4 interface. In addition, AMP through its hydrogen bonding interactions may limit the dynamic motion of helices H1 and H2, increasing the depth and steepness of the potential energy well of the T-state relative to the I_T-state (Figure 8). Perhaps in the absence of AMP, FBPase is in rapid exchange between its R- and I_T-states. In other words, a low energy barrier divides the two states, but as the tetramer crosses this barrier, interactions at the C1-C4 interface become weak. Glu192, critical to hydrogen bonds across the C1-C4 interface, undergoes a conformational change between the R- and T-states which may result in the transitory loss of hydrogen bonds (15). The forward and reverse rates in the tetramer/dimer equilibrium would be high relative to the wild-type enzyme in mutant FBPases that favor the intermediate states of the tetramer.

An ordered pathway of subunit exchange is also consistent with the properties of the Type I FBPase from *Escherichia coli* (16, 34, 41, 42). *E. coli* FBPase has an allostERIC activation site at the C1-C4 interface of the tetramer. The activation site favors anions in general, and phosphoenolpyruvate or citrate specifically (34, 42). In the absence of anions, however, the *E. coli* enzyme transforms into a species with a mass comparable to that of a dimer (42). These observations regarding the Type I FBPase from *E. coli* are consistent
with a tetramer/dimer equilibrium in which C1-C2 dimers form due to a weakened C1-C4 interface in the absence of anions. The possibility of a common pathway of dissociation for enzymes as evolutionary distinct as those from pig and E. coli suggest that the labile characteristics of the C1-C4 interface may be a general property of FBPases regardless of source.

REFERENCES

the neutral form of fructose-1,6-bisphosphatase complexed with the product fructose 6-phosphate at 2.1-A resolution, *Proc Natl Acad Sci U S A* 88, 2989-2993.


Table 1. Characteristic distances (in Å) between probe molecules. The $\chi_1$ angle of the cysteine residue adopts a favored minimum energy conformer that allows the fluorescent probe to extend away from the surface of the enzyme. Distances are taken in the R-state structure between the S$\gamma$ atom of the cysteine from subunit C1 to corresponding S$\gamma$ atoms of subunits C2, C3 and C4.

<table>
<thead>
<tr>
<th>FBPase Construct</th>
<th>Distance C1-C2</th>
<th>Distance C1-C3</th>
<th>Distance C1-C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys°144</td>
<td>89</td>
<td>91</td>
<td>39</td>
</tr>
<tr>
<td>Cys°236</td>
<td>37</td>
<td>104</td>
<td>97</td>
</tr>
<tr>
<td>Cys°337</td>
<td>59</td>
<td>127</td>
<td>116</td>
</tr>
</tbody>
</table>
Table 2. Kinetics of FBPase mutants. Cys° FBPase replaces Cys38, 92, 116, 128, and 281 with alanine, and Cys183 with serine. Cys°144 FBPase, Cys°236 FBPase, and Cys°337 FBPase are single mutations of Cys° FBPase in which cysteine replaces Thr144, Asn236 and Lys337, respectively. Assay conditions and definitions of kinetic parameters are in the experimental section.

<table>
<thead>
<tr>
<th>Enzyme type</th>
<th>Wild-type FBPase</th>
<th>Cys° FBPase</th>
<th>Cys°144 FBPase</th>
<th>Cys°236 FBPase</th>
<th>Cys°337 FBPase</th>
<th>Cys°144 FBPase Probe modified</th>
<th>Cys°236 FBPase Probe modified</th>
<th>Cys°337 FBPase Probe modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (sec⁻¹)</td>
<td>22±1</td>
<td>6.7±0.49</td>
<td>7.1±0.54</td>
<td>3.8±0.14</td>
<td>4.9±0.41</td>
<td>6.5±0.31</td>
<td>4.1±0.17</td>
<td>3.7±0.14</td>
</tr>
<tr>
<td>Activity ratio, pH 7.5:9.5</td>
<td>3.3</td>
<td>3.1</td>
<td>3.31</td>
<td>3.0</td>
<td>3.37</td>
<td>3.1</td>
<td>3.12</td>
<td>3.2</td>
</tr>
<tr>
<td>$K_a$ – Mg²⁺ (mM)</td>
<td>0.67±0.04</td>
<td>8±1</td>
<td>8±1</td>
<td>4.1±0.5</td>
<td>8±1</td>
<td>3.6±0.4</td>
<td>3.9±0.4</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>Hill Coefficient, Mg²⁺</td>
<td>1.9±0.1</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.6±0.1</td>
<td>1.4±0.1</td>
<td>1.7±0.2</td>
<td>1.6±0.09</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>$K_m$ – Fru-1,6-P₂ (µM)</td>
<td>1.8±0.1</td>
<td>6±1</td>
<td>7±1</td>
<td>3.0±0.4</td>
<td>8±1</td>
<td>3.6±0.4</td>
<td>2.9±0.4</td>
<td>4.4±0.3</td>
</tr>
<tr>
<td>$I_{0.5}$ – AMP (µM)</td>
<td>1.61±0.05</td>
<td>6.4±0.6</td>
<td>3.4±0.3</td>
<td>4.7±0.2</td>
<td>3.4±0.2</td>
<td>33±5</td>
<td>4.7±0.3</td>
<td>9±1</td>
</tr>
<tr>
<td>Hill Coefficient, AMP</td>
<td>2.2±0.1</td>
<td>2.0±0.3</td>
<td>1.3±0.2</td>
<td>1.8±0.1</td>
<td>1.45±0.09</td>
<td>1.2±0.3</td>
<td>1.8±0.2</td>
<td>1.4±0.2</td>
</tr>
</tbody>
</table>

Table 2. Footnotes.

aData from reference (20).
Table 3: Parameters from the time-dependent change in fluorescence due to the dilution of probe-modified Cys°144 FBPase. Data from Figure 3 are fit by differential equations \( \frac{dF}{dt} = a\frac{dT}{dt} + b\frac{dD}{dt} \), where \( F \) is the observed fluorescence, and \( T \) and \( D \) are the concentrations of tetramer and dimer, respectively. \( \frac{dT}{dt} = \left( \frac{1}{2} \right)\frac{dD}{dt} = -k_1 T + k_2 D^2 \), where parameters \( a, b, k_1 \) and \( k_2 \) are defined in the results section.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fitted value</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1 ) (min(^{-1}))</td>
<td>0.0029±0.0006</td>
<td>21.0</td>
</tr>
<tr>
<td>( k_2 ) (min(^{-1})M(^{-1}))</td>
<td>0.06±0.01</td>
<td>21.5</td>
</tr>
<tr>
<td>( a ) (Arbitrary fluorescence units*min(^{-1}))</td>
<td>2.13±0.004</td>
<td>0.2</td>
</tr>
<tr>
<td>( b ) (Arbitrary fluorescence units*min(^{-1}))</td>
<td>3.2±0.4</td>
<td>12.8</td>
</tr>
</tbody>
</table>
Table 4: Parameters from the time-dependent change in fluorescence due to the dilution of probe-modified Cys°236 FBPase. Data from Figure 6 are fit by differential equations dF/dt = a*dT/dt + b*dD/dt + c*dM/dt, where F is the observed fluorescence, and T, D, and M are the concentrations of tetramer, dimer, and monomer respectively. dT/dt = (½)dD/dt = –k_1*T + k_2*D^2, where parameters k_1 and k_2 are those from Table 3. dD/dt = (½)dM/dt = –k_3*D + k_4*M^2, where parameters k_3 and k_4 are defined in the results section. Parameters a and b do not assume the values of Table 3, but for probe-modified Cys°236 FBPase, b = (½)a, where a is approximated by the slope of a linear plot of F vs. T at time zero.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fitted value</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_3 (min⁻¹)</td>
<td>0.020±0.003</td>
<td>17.1</td>
</tr>
<tr>
<td>k_4 (min⁻¹M⁻¹)</td>
<td>0.49±0.06</td>
<td>12.8</td>
</tr>
<tr>
<td>a (Arbitrary fluorescence units*min⁻¹)</td>
<td>2.205±0.04</td>
<td>0.2</td>
</tr>
<tr>
<td>c (Arbitrary fluorescence units*min⁻¹)</td>
<td>1.7±0.1</td>
<td>8.3</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Overview of the FBPase tetramer. Filled circles indicate positions of the Cα atoms of cysteine residues, Thr144, Asn236 and Lys337. Wireframe models represent bound AMP and Fru-6-P at the allosteric inhibition and active sites, respectively. The symmetry unique interfaces of FBPase (C1-C2, C1-C3, and C1-C4) are between subunits bearing the same labels.

Figure 2. Time-dependent changes in fluorescence subsequent to the dilution of probe-labeled FBPase. 50 µL of a stock solution of 20 µM probe-modified FBPase in Tris-HCl, pH 7.5, was diluted at 25 °C into 1950 µL of the same buffer without protein and with and without AMP and/or Fru-6-P. Fluorescence was monitored continuously for 2 hrs. A. Probe-modified Cys°337 FBPase with 1 mM AMP (■), with 1 mM Fru-6-P (◇) and without ligands (●). B. Probe-modified Cys°144 FBPase with 1 mM AMP (■), with 1 mM Fru-6-P (◇) and without ligands (●). C. Probe-modified Cys°236 FBPase with 1 mM AMP (■), with 1 mM Fru-6-P (◇) and without ligands (●).

Figure 3. Time-dependent changes in fluorescence subsequent to varying dilutions of probe-labeled Cys°144 FBPase. Variable (from 40 to 240 uL) stock solution of 20 µM probe-modified FBPase in Tris-HCl, pH 7.5, was diluted at 25 °C into the same buffer to a total volume 2 mL without protein and with and without AMP and/or Fru-6-P. Fluorescence was monitored continuously for 2 hrs. The lines represent calculated fluorescence values based on the parameters of Table 3. Points represent data selected arbitrarily at
three-minute intervals. Inset: Plot of fluorescence $F$ versus the total concentration of tetramer $T_0$ at time zero. Slope of the plot approximates $a$ to the extent that all protein is tetramer.

**Figure 4. Total fluorescence changes due to varying dilutions of probe-modified Cys$^{144}$ FBPase.** Variable (from 25 to 200 uL) stock solution of 20 µM probe-modified FBPase in Tris-HCl, pH 7.5, was diluted at 25 °C into the same buffer to a total volume 2 mL without protein and with and without AMP and/or Fru-6-P. Fluorescence was measured right after dilution and after equilibrium (24hrs). The line represents calculated fluorescence change based on the parameters of Table 3 and Equation 6.

**Figure 5. Fluorescence change due to subunit exchange between probe-modified Cys$^{144}$ FBPase and wild-type FBPase.** 1.95 mL of a stock solution of 0.5 µM probe-modified FBPase in Tris-HCl, pH 7.5, 1 mM Fru-1,6-P$_2$ was mixed with 50µL of stock solution of 20 µM wild-type FBPase in same buffer at 25 °C. Fluorescence was monitored continuously for 2 hrs. The solid line represents the calculated fluorescence based on the parameters of Table 3. Points represent data selected arbitrarily at three-minute intervals.

**Figure 6. Time-dependent changes in fluorescence subsequent to varying dilutions of probe-labeled Cys$^{236}$ FBPase.** Variable (from 12.5 to 75 uL) stock solution of 20 µM probe-modified FBPase in Tris-HCl, pH 7.5, was diluted at 25 °C into the same buffer to a total volume 2 mL without protein and with and without AMP and/or Fru-6-P. Fluorescence was monitored continuously for 2 hrs. The lines represent calculated fluorescence values
based on the parameters of Tables 3&4. Points represent data selected arbitrarily at three-minute intervals.

**Figure 7. Fluorescence change due to subunit exchange between probe-modified Cys²³⁶ FBPase and wild-type FBPase.** 1.95 mL of a stock solution of 0.65 µM probe-modified FBPase in Tris-HCl, pH 7.5, was mixed with 50 µL of stock solution of 208 µM wild-type FBPase in same buffer at 25 °C. Fluorescence was monitored continuously for 2 hrs. The solid line represents the calculated fluorescence based on the parameters of Tables 3&4. Points represent data selected arbitrarily at three-minute intervals.

**Figure 8. Energy relationships of the known quaternary states of FBPase.** The ordinate represents the rotation of subunit pair C1-C2 relative to C3-C4 in the quaternary state, with the R-, I⁻, and T-states having 0, 13, and 15° subunit pair rotations. Dissociation of C1-C2 dimers from the tetramer may occur at the height of the energy barrier between R and I⁻ states.
Fig. 1. Zhou, et al.
Fig. 2. Zhou, et al.
Fig. 3. Zhou, et al.

Fig. 4. Zhou, et al.
Fig. 5. Zhou, et al.

Fig. 6. Zhou, et al.
Fig. 7. Zhou, et al.

Fig. 8. Zhou, et al.
CHAPTER V. GENERAL CONCLUSION

The affinity of ligands to protein is determined by many factors such as size, shape and plasticity; however, the effect of cavities on ligand recognition is often overlooked. Using adenylosuccinate synthetase (ADSS) as a model, we test the effect of an induced-fit cavity on ligand affinity. Both IMP and 2′-deoxy-IMP serve as good substrates in ADSS and forming expected intermediates and products. Crystal structures of the intermediate complexes are identical except for the presence/absence of the 2′-hydroxyl group. However, ADSS shows significantly different affinities for L-aspartate in the presence of IMP and 2′-deoxy-IMP. Using Lennard-Jones 6-12 model, we get a 2 kcal/mole decrease in energy in the absence of the hydroxyl group. According to thermodynamics formula \( \Delta G = -RT\ln K \), we estimate a ~40 fold affinity difference, which is consistent with our experiment data. Such a model illustrates that an induced cavity can contribute significantly to the recognition of ligands.

The recognition of L-aspartate by Valine\(^{273}\) in ADSS is very interesting since it is a non-polar amino acid and not capable of forming hydrogen bonds or coordinate bonds with metal cations. However, the mutations of Valine\(^{273}\) to threonine, alanine and asparagine increase the \( K_m \) for L-aspartate from 15-40 folds. Sequence alignments reveal Valine\(^{273}\) as an invariant residue, not altered through evolution. Crystal structures of mutant Threonine\(^{273}\) and Alanine\(^{273}\) ADSS with and without ligands reveal different contributions in the poor recognition of L-aspartate. Threonine\(^{273}\), which introduces a polar side chain into the active site, induces a confirmation change of L-aspartate loop (299-304). The loop
closes on the active site making access to the active site difficult for L-aspartate. The change of Valine$^{273}$ to alanine, on the other hand, creates empty space in the active site, allowing Arginine$^{305}$ to drifts into the active site again sterically blocking L-aspartate. Asparagine$^{273}$ occupies extra space in the active site and interacts with neighboring amino acids. All the factors above determine Valine$^{273}$ is the only residue that maintains the correct structure of the active site without causing steric restrictions in the binding of L-aspartate.

Determining the pathway of subunit exchange in FBPase confirms the assignment of hybrid constructs of FBPase, which form the basis for proposed mechanisms of cooperativity in AMP association. By attaching probes at specific positions and monitoring time-dependent fluorescence change due to changes in self-quenching in dilution and subunit exchange experiments, we are able to elucidate the pathway of dissociation of the FBPase tetramer. FBPase dissociates by way of an ordered pathway with the formations of C1-C2 dimer, followed by the the breakdown of C1-C2 dimers into omonomers. Furthermore, fitting the time dependence of fluorescence to the ordered pathway of dissociation determined numerical values for rate constants that allow accurate simulations of data gotten from subunit exchange experiments. The results firmly establish the mechanism of subunit exchange dynamics of FBPase in vitro and may provide a valuable tool in probing rates of quaternary transitions in FBPase. The approach here may in fact be broadly applicable to the study of subunit dissociation of other complex biological systems for which adequate
structural information is available.