Chemistry of the AMP binding site in glycogen phosphorylase

Richard Allen Anderson
Iowa State University

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CHEMISTRY OF THE AMP BINDING SITE IN
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Iowa State University, Ph.D., 1973
Biochemistry

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Chemistry of the AMP binding site in glycogen phosphorylase

by

Richard Allen Anderson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work
Signature was redacted for privacy.
For the Major Department
Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa

1973
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURE</td>
<td>6</td>
</tr>
<tr>
<td>RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>65</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>75</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>77</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>81</td>
</tr>
</tbody>
</table>
DEDICATION

To My Wife, Sylvia,  
and Children,  
Brent and Deborah
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>glucose-6-phosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>glycerol-P</td>
<td>β-glycerophosphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
</tbody>
</table>
INTRODUCTION

Phosphorylase catalyzes an important control step in carbohydrate metabolism which involves the breakdown of glycogen to ultimately yield energy in the form of high energy phosphate compounds. The reaction catalyzed by phosphorylase is shown in equation 1:

$$\text{G}_n + \text{P}_i \leftrightarrow \text{G}_{n-1} + \text{glucose-1-PO}_4$$

where \( \text{G}_n \) and \( \text{G}_{n-1} \) represent glucose polymers linked \( \sim 1 \rightarrow 4 \) containing \( n \) and \( n-1 \) residues. In skeletal muscle there are two interconvertible forms of phosphorylase, phosphorylase \( b \), which requires AMP for enzymatic activity, and phosphorylase \( a \), which is active in the absence of adenosine monophosphate. Muscle phosphorylase \( b \) has specific requirement for AMP, but no evidence has been obtained to demonstrate that a nucleotide participates in the catalytic reaction (Cohn and Cori, 1948). In contrast to the enzyme from skeletal muscle, inactive phosphorylase from liver is not activated by AMP unless a salt e.g. \( \text{Na}_2\text{SO}_4 \) is added (Appleman et al., 1966). Similar results have been observed with corpus luteum phosphorylase (Yunis and Arimura, 1964). Varying degrees of activation of phosphorylase by AMP have been reported from kidney (Villar-Palasi and Gasques-Martinez, 1968) and leucocytes (Yunis and Arimura, 1964; 1968). Plant phosphorylases are not activated by AMP (Tsai and Nelson, 1968; Lee, 1960) which suggests different
methods of control in plants than in animals.

Phosphorylase b can be converted to phosphorylase a by phosphorylase kinase and the reverse reaction is catalyzed by phosphorylase phosphatase as shown in equation 2:

\[ \text{Ca}^{++}, \text{Mg}^{++}, 4 \text{ATP} \rightarrow \text{phosphorylase kinase} \]

\[ 2 \cdot 2 \text{phosphorylase } b \rightarrow \text{phosphorylase } a + 4 \text{ADP} \]

Adenosine monophosphate has been shown to affect the phosphorylase phosphatase reaction by binding to phosphorylase (Nolan et al., 1964) but AMP has little or no affect on the phosphorylase kinase reaction (Krebs et al., 1964).

AMP exerts its effect by inducing changes in enzyme conformation (Ulman et al., 1964; Hedrick, 1966), subunit interactions (Kastenschmidt et al., 1968b) and the binding of substrates. Helmreich and Cori (1964) have shown that the Km of phosphorylase b for inorganic phosphate and for glycogen decreased progressively as the concentration of AMP was increased. Conversely, the addition of either substrate in increasing concentration caused a decrease in the Km for adenosine monophosphate. No interaction between the two substrate binding sites could be demonstrated when the concentration of nucleotide was held constant and the concentration of the substrates were varied.
monophosphate is thought to affect enzymatic activity by inducing changes in enzyme conformation. These effects have been explained through models that describe the binding of adenosine monophosphate as an allosteric effector (Helmreich et al., 1964; Buc, 1967).

Previous workers (Madsen and Cori, 1957) have shown that there are 2 binding sites for AMP per dimer of phosphorylase b but more recent work by Wang et al. (1970b) suggests there may be 2 AMP binding sites per monomer. One binding site with a $K_D$ of $2.1 \times 10^{-4}$ M and a second site with a $K_D$ of 3mM. Adenosine triphosphate also binds at the AMP binding site of phosphorylase and is a competitive inhibitor with respect to AMP; ATP inhibition can also be reversed by glucose-1-PO$_4^-$, but ATP inhibition is not competitive with respect to glucose-1-PO$_4^-$. A plot of velocity versus substrate (glucose-1-PO$_4^-$) concentration shows a sigmoidal shape typical of other allosteric proteins.

The binding of AMP to phosphorylase is dependent upon a number of factors. Polyvalent organic cations, protamine, polylysine, and polyamines (Krebs, 1954; Wang et al., 1968; Mott and Bieber, 1970) and divalent metal ions Mg$^{++}$, Mn$^{++}$, Sr$^{++}$, and Ca$^{++}$ (Kastenschmidt et al., 1968a; Madsen, 1965), as well as substrate anions, phosphate and glucose-1-PO$_4^-$ (Kastenschmidt et al., 1968b; Helmreich and Cori, 1964), have all been shown to enhance the binding of AMP.
While phosphorylase α is active in the absence of AMP, it is stimulated by this nucleotide. Adenosine monophosphate activation of phosphorylase α is due to an increase in the affinity of the enzyme for substrates and is therefore more pronounced at low substrate levels (Lowry et al., 1964).

These facts and the effects of hormones on animal phosphorylase clearly point out the regulatory significance of the interconversion reactions in the control of glycogen metabolism.

Several reports have appeared concerning the nucleotide specificity of glycogen phosphorylase (Mott and Bieber, 1968; 1970; Okazaki et al., 1968; Black, 1969; Black and Wang, 1968; Steiner, 1972) which have shown that a large number of nucleotides and related compounds can bind at the AMP binding site of phosphorylase. From these studies it was concluded that no direct parallel exists between nucleotide specificity for binding and their structural requirement for activation. It was concluded, however, that both the 5'-phosphate group and the purine ring of adenosine monophosphate are involved in binding.

Irreversible modification of the AMP binding site of phosphorylase has been performed by Hulla and Fasold, (1972) using 6-(purine-5'-ribonucleotide)-5-(2-nitrobenzoic acid)-thioether which binds covalently to glycogen phosphorylase after elimination of 2-nitro-4-mercaptobenzoic acid.
The enzyme:AMP derivative complex was active in the absence of added nucleotide but modification could not be carried further than 35% due to precipitation of the enzyme. Danchin and Buc, (1973) have also irreversibly modified the nucleotide binding site using adenosine-5'-monophosphate-cobalt (III) complex which results in an inactive enzymatic form. Modification using this AMP:cobalt (III) complex must be carried out in the cold at saturating levels of AMP to prevent precipitation of the modified enzyme.

It is the object of this work to determine the chemistry of the nucleotide binding site of phosphorylase and to affinity label this site using sulfonyl fluoride derivatives of adenine. Sulfonyl fluoride derivatives were chosen due to their low reactivity and highly specific mode of binding.
EXPERIMENTAL PROCEDURE

Materials and Methods

Phosphorylase b was isolated from rabbit skeletal muscle by the procedure of Fischer and Krebs (1962). The enzyme was recrystallized at least three times at 0 degrees from pH 6.8 buffer consisting of 0.05 M glycerol-P, 0.05 M 2-mercaptoethanol, 0.001 M AMP, and 0.01 M Mg++. No additional AMP or Mg++ was added after the first recrystallization. Residual AMP was removed by acid washed Norit A. A ratio of $A_{260}/A_{280}$ of 0.53 or less was considered satisfactory.

Phosphorylase was assayed in the direction of glycogen synthesis (Illingworth and Cori, 1953) except in the determination of the $K_m$ for glycogen, which was determined by using the coupled assay method of Helmreich and Cori (1964). The reaction mixture (total 1.4 ml) contained 0.0006 M NADP, 0.3 ml of varying levels of glycogen, 0.015 M inorganic phosphate, 0.005 M MgCl$_2$, 0.001 M EDTA, 0.001 M 2-mercaptoethanol, glucose-6-phosphate dehydrogenase, phosphoglucomutase and phosphorylase, pH 7.5. Glucose-6-phosphate dehydrogenase, phosphoglucomutase, and NADP were obtained from Calbiochem.
Enzyme concentration was determined spectrophotometrically at 280 nm. An absorbancy index (1% x cm⁻¹) of 13.2 was used (Kastenschmidt et al., 1968a). A molecular weight of 100,000 for monomer phosphorylase b was used in calculations involving molecular weight (Cohen et al., 1971).

Ultracentrifugation experiments were performed with a Spinco model E analytical ultracentrifuge at a rotor speed of 52,000 rpm at a temperature of 12.8 ± 1 degree. Sedimentation coefficients were determined with a Nikon model 6C microcomparator and were corrected for viscosity of the buffer to water at 20 degrees.

Inhibitor constants for the adenine analogues were calculated by the procedure outlined by Okazaki et al. (1968), except that the reaction was conducted in the direction of glycogen synthesis. No cooperativity was observed under the assay conditions employed. The assay consisted of 0.075 M glucose-1-phosphate, 0.5% glycogen, 18% dimethylsulfoxide to solubilize the inhibitor, AMP (5.3 x 10⁻⁵ M - 3.7 x 10⁻⁴ M), and adenine analogues (7.5 x 10⁻⁴ M - 7.5 x 10⁻⁵ M). The adenine analogues were first dissolved in 100% dimethylsulfoxide and then diluted into the assay mixture. Twenty-five-percent dimethylsulfoxide had no affect on phosphorylase maximal activity or affinity for AMP.
The amount of irreversible inhibition was determined by incubating phosphorylase b at 2-2.3 x 10^{-5} M in 0.05 M glycerophosphate - 0.002 M (ethylenedinitrilo)tetraacetic acid (pH 7.8) with irreversible inhibitor. At prescribed times, aliquots were diluted fiftyfold in 0.05 M glycerophosphate - 0.05 M 2-mercaptoethanol (pH 6.8) and assayed in the presence and absence of AMP by the method of Illingworth and Cori (1953).

Sodium glycerophosphate, cysteine hydrochloride, EDTA, adenosine monophosphate, citric acid, and shellfish glycogen were obtained from Sigma Chemical Company. Glucose-1-phosphate was purchased from Calbiochem. Imidazole and N-1-naphthylethylenediamine dihydrochloride were obtained from Eastman Organic.

Glycogen was purified by precipitating a 10% solution of glycogen in water with 100% trichloroacetic acid. TCA (100%) was added to a final concentration of 15% by volume. After filtration, the pellet was redissolved in water to a final concentration of 5%. The glycogen solution was then passed through a column consisting of 2 parts acid-washed Norit A and 1 part, by weight of Celite 560. The glycogen solution was passed at that time through a Dowex 1 column in the hydrogen ion form. The glycogen solution
was precipitated with 95% ethanol, redissolved in water, lyophilized, and stored frozen.

**Determination of the amount of 8-[^m(m-\text{fluorosulfonyl}-benzamido)benzylthio] adenine bound**

The amount of adenine derivative bound per mole of enzyme was determined by a modification of the procedure of Daniel (1961) for aromatic amines. An aromatic amine could be generated by hydrolysis of the amide linkage of 8-[^m(m-\text{fluorosulfonylbenzamido})benzylthio] adenine. Phosphorylase b, which had been reacted with the adenine derivative, was recrystallized twice, dialyzed against water for 8 hours, and lyophilized. Constant-boiling hydrochloric acid (5.7 N) was added, and the flask was sealed under vacuum. The protein solution was then hydrolyzed for 36 hours at 110 degrees. After hydrolysis, 1.25 ml of H₂O and 0.25 ml of 0.25% sodium nitrite were added to 0.25 ml of the hydrolyzed protein solution in 5.7 N HCl. After 15 minutes, 0.25 ml of 2.5% ammonium sulfamate and 0.25 ml of N-1-naphthylethylene-diamine were added. After 15 minutes, the optical density was determined at 549 nm with the aid of a Cary 15 spectrophotometer. A standard curve was prepared by adding a known amount of analogue to native protein before acid hydrolysis. The blank consisted of native hydrolyzed protein, plus the
usual reaction mixture. This assay will detect $10^{-8}$ moles of aromatic amine in the assay mixture.

The adenine derivatives used in this investigation were a generous gift of the late Dr. B. R. Baker of the University of California, Santa Barbara, California.

Radioactivity of fractions eluted from Dowex 50 was measured in planchets by using a gas-flow model 1042B of Nuclear-Chicago Corp. The amount of incorporation of $^{14}C$-[m-fluorosulfonylbenzamido] benzylthio adenine into phosphorylase $b$ was determined by a slight modification of the procedure described by Reiman et al. (1971).

The time course of the heat inactivation of native and modified phosphorylase $b$ was initiated by dilution (final concentration 100–150 μg/ml) of the respective enzyme into buffer previously equilibrated at 52 degrees. At designated intervals, aliquots were removed and placed in ice-cold test tubes. The tubes were left on ice until all samples were taken and then assayed at 30 degrees. A zero time point was obtained by making a separate dilution into buffer at 30 degrees.

Phosphorylase kinase was prepared by the method of Brostrom et al. (1971). The conversion of phosphorylase $b$ to phosphorylase $a$ was followed by measuring
the incorporation of $^{32}\text{P}$ into phosphorylase a (Reiman et al., 1971). The increase in specific activity upon conversion to phosphorylase a also was followed by using the method of Brostrom et al. (1971). Phosphorylase phosphatase was prepared by the method of Hurd (1967). The phosphorylase phosphatase reaction was followed by release of $^{32}\text{P}$ (Hurd, 1967).

Amino acid analysis of the peptide was performed on a Beckman 120C analyzer. The peptide was hydrolyzed in vacuo in glass-distilled, azeotropic 5.7 N HCl at 110 degrees for 24 hours. Tryptophan analysis was determined by the method of Matsubara and Sasaki (1969).

The sequence of the peptide which 8-[[m(m-fluoro-sulfonylbenzamido)benzylthio] adenine was bound was determined using leucine amino peptidase (Light, 1972) and carboxypeptidase A (Ambler, 1972). Quantitative release of amino acids was determined with the aid of a Beckman 120C analyzer equipped with a high sensitivity card. Leucine amino peptidase and carboxypeptidase A were purchased from Worthington.

High-voltage electrophoresis was performed with Whatman 3 MM paper in pyridine, acetic acid, water buffer
(1:3.4:409), pH 4.0 with a Gilson Model D High-Voltage Electrophorator.

Thin-layer chromatography was performed on pre-coated silica-gel sheets obtained from Eastman Organic.
RESULTS

Seventeen adenine and hypoxanthine derivatives were tested for their ability to inhibit glycogen phosphorylase b (Table I). Both adenine and hypoxanthine showed little inhibition (7 and 3% respectively) under the conditions employed. Substitution of hydrophobic groups at various positions of the purine ring increased the inhibitory capacity of both adenine compounds (III-X) and hypoxanthine compounds (XII-XVI) to act as inhibitors of phosphorylase b. Substitution of a phenyl group at the 8 position of adenine (compound VI) resulted in the largest inhibition (35%).

Since 8-phenyladenine proved to be the best inhibitor tested, further studies were performed on derivatives of this compound. In Table II, it can be seen that compounds with increasing substituent constants, $\pi$, and therefore of increasing hydrophobic character, are more effective inhibitors of glycogen phosphorylase b than compounds less hydrophobic in nature. $\pi$ is defined as:

$$\log P_X - \log P_H,$$

where $P_H$ is the partition coefficient of a parent compound between octanol and water, and $P_X$ is that of the derivative $X$ (Fujita et al., 1964). Therefore, compounds with increasing substituent constants
Table I. Inhibition of phosphorylase b by substituted purines

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>adenine</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>adenosine</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>9-phenyladenine</td>
<td>17</td>
</tr>
<tr>
<td>IV</td>
<td>9-methyladenine</td>
<td>24</td>
</tr>
<tr>
<td>V</td>
<td>8-methyladenine</td>
<td>22</td>
</tr>
<tr>
<td>VI</td>
<td>8-phenyladenine</td>
<td>35</td>
</tr>
<tr>
<td>VII</td>
<td>8-benzyladenine</td>
<td>13</td>
</tr>
<tr>
<td>VIII</td>
<td>8-phenylvinyladenine</td>
<td>12</td>
</tr>
<tr>
<td>IX</td>
<td>8-phenylethyladenine</td>
<td>3</td>
</tr>
<tr>
<td>X</td>
<td>1-benzyladenine</td>
<td>13</td>
</tr>
<tr>
<td>XI</td>
<td>hypoxanthine</td>
<td>3</td>
</tr>
<tr>
<td>XII</td>
<td>9-phenylhypoxanthine</td>
<td>23</td>
</tr>
<tr>
<td>XIII</td>
<td>9-methylhypoxanthine</td>
<td>23</td>
</tr>
<tr>
<td>XIV</td>
<td>1-benzylhypoxanthine</td>
<td>11</td>
</tr>
<tr>
<td>XV</td>
<td>9-benzylhypoxanthine</td>
<td>13</td>
</tr>
<tr>
<td>XVI</td>
<td>3-benzylhypoxanthine</td>
<td>18</td>
</tr>
<tr>
<td>XVII</td>
<td>7-benzylhypoxanthine</td>
<td>12</td>
</tr>
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The assay mixture contained phosphorylase b (0.16 mg/ml) in 0.1 M maleate - 0.045 M mercaptoethanol at pH 6.7 with AMP (4.5 x 10^{-5} M), glucose-1-P (0.016 M), glycogen (1%), and inhibitor (1.8 x 10^{-4} M). Reaction mixtures contained 10% dimethylsulfoxide to solubilize the inhibitors. After 5' of reaction at 30 degrees C, the inorganic phosphate released was determined by method of Illingworth and Cori.
TABLE II. Correlation of Apparent Ki's of Adenine Derivatives with Their Substituent Constants

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Substitution at 8-position of adenine</th>
<th>Ki (M)</th>
<th>π&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (adenine)</td>
<td>H</td>
<td>2.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>XVIII</td>
<td><img src="image" alt="Substitution" /></td>
<td>1.3 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>XIX</td>
<td><img src="image" alt="Substitution" /></td>
<td>3.2 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.03</td>
</tr>
<tr>
<td>XX</td>
<td><img src="image" alt="Substitution" /></td>
<td>2.2 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.02</td>
</tr>
<tr>
<td>XXI</td>
<td><img src="image" alt="Substitution" /></td>
<td>2.1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>0.81</td>
</tr>
<tr>
<td>XXII</td>
<td><img src="image" alt="Substitution" /></td>
<td>4.0 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.68</td>
</tr>
<tr>
<td>XXIII</td>
<td><img src="image" alt="Substitution" /></td>
<td>6.23 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.63</td>
</tr>
<tr>
<td>XXIV</td>
<td><img src="image" alt="Substitution" /></td>
<td>4.9 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.62</td>
</tr>
</tbody>
</table>

<sup>a</sup> The π<sub>a</sub>values in this table are derived from phenylacetic acid (Fujita et al., 1964).

<sup>b</sup> Substituent constant is for a 3 substituted group.
Table II. (continued)

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Substitution at 8-position of adenine</th>
<th>Ki (M)</th>
<th>( \pi^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXV</td>
<td><img src="image" alt="XXV" /></td>
<td>1.2 x 10(^{-3})</td>
<td>0.12</td>
</tr>
<tr>
<td>VII</td>
<td><img src="image" alt="VII" /></td>
<td>6.0 x 10(^{-4})</td>
<td>0</td>
</tr>
<tr>
<td>XXVI</td>
<td><img src="image" alt="XXVI" /></td>
<td>7.2 x 10(^{-4})</td>
<td>-0.04</td>
</tr>
<tr>
<td>XXVII</td>
<td><img src="image" alt="XXVII" /></td>
<td>2.0 x 10(^{-3})</td>
<td>-0.28(^c)</td>
</tr>
<tr>
<td>XXVIII</td>
<td><img src="image" alt="XXVIII" /></td>
<td>2.1 x 10(^{-3})</td>
<td>-0.61</td>
</tr>
<tr>
<td>XXIX</td>
<td><img src="image" alt="XXIX" /></td>
<td>1.6 x 10(^{-3})</td>
<td>-0.77(^d)</td>
</tr>
</tbody>
</table>

\(^c\) \( \pi \) constant is taken from benzene.

\(^d\) \( \pi \) value for NH \(_2\) is taken from phenol. Assay conditions are described in Methods.
exhibit corresponding increases in hydrophobicity. Replacement of the hydrogen at the 8 position of adenine by a phenyl group (compound VI) results in a compound that binds approximately 4 times more tightly to phosphorylase b than adenine. In Table II are shown the apparent inhibition constants, with respect to AMP, of a number of 8-phenyladenine derivatives. Derivatives with increasing hydrophobic character, as measured by substituent constants ($\Phi$), are correspondingly better competitive inhibitors. Compound XXI, was not only the best inhibitor tested, but binds approximately twofold more tightly than the native activator, AMP ($K_i$ compound XXI = $2 \times 10^{-5} \text{ M}$, $K_a_{\text{AMP}} = 5 \times 10^{-5} \text{ M}$).

Irreversible Inhibition of Phosphorylase b

After establishing that hydrophobic substituents on the 8 position of adenine increase the effectiveness of adenine to act as a competitive inhibitor of AMP, a number of 8 substituted adenine derivatives bearing a sulfonyl fluoride group were tested as irreversible inhibitors. No definite relation of structure and degree of inhibition was observed (Table III). Compounds XXXVI, XXXVIII, and XL gave significant inhibition under the conditions
TABLE III: Irreversible Inhibition of Phosphorylase b by 8-substituted Adenine Derivatives

<table>
<thead>
<tr>
<th>Compound #</th>
<th>R</th>
<th>% Irreversible Inhibition</th>
<th>% Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXX</td>
<td>-Ph2-N-C-Ph</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XXXI</td>
<td>-Ph-N-C-Ph</td>
<td>&lt; 5</td>
<td>0</td>
</tr>
<tr>
<td>XXXII</td>
<td>-Ph-N-C-Ph</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XXXIII</td>
<td>-Ph-N-C-CH2CH2Ph</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XXXIV</td>
<td>-Ph-N-C-Ph</td>
<td>&lt; 5</td>
<td>0</td>
</tr>
</tbody>
</table>
Table III. (continued)

<table>
<thead>
<tr>
<th>Compound #</th>
<th>R</th>
<th>% Irreversible Inhibition</th>
<th>% Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXXV</td>
<td></td>
<td>&lt; 5</td>
<td>0</td>
</tr>
<tr>
<td>XXXVI</td>
<td></td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>XXXVII</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XXXVIII</td>
<td></td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>XXXIX</td>
<td></td>
<td>&lt; 5</td>
<td>0</td>
</tr>
</tbody>
</table>
Table III. (continued)

<table>
<thead>
<tr>
<th>Compound #</th>
<th>R</th>
<th>% Irreversible Inhibition</th>
<th>% Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL</td>
<td>-S-CH₂-_VENDOR_MOLECULE_PATTERN-</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

Incubation mixture contained $2.21 \times 10^{-5}$ M phosphorylase b, 0.04 M glycerophosphate, 0.002 M EDTA, 4.0 $\times 10^{-5}$ M inhibitor, 2% dimethylsulfoxide, at pH 7.8. Reaction was allowed to proceed 63 minutes, diluted 1/50 in 0.05 M glycerophosphate 0.05 2-mercaptoethanol (pH 6.8) and then assayed in the presence and absence of AMP.
employed while similar compounds gave either no or less than 5% inhibition. Compound XL was the most effective inhibitor tested but, more importantly, in the absence of AMP, also yields 24% of the activity that would be observed at saturating levels of AMP. Compound XXXIX, which is similar to XL except that the sulfonyl fluoride group is in the para instead of the meta position (Table III), exhibits less than 5% inhibition and does not activate phosphorylase b in the absence of AMP. Since glycogen phosphorylase b is inactive in the absence of AMP, compound XL (8-[m (m-fluorosulfonylbenzamido) benzylthio]-adenine) must be acting in a manner similar to AMP. Interestingly, activation can occur with an adenine derivative that contains no ribose or phosphate group.

Activation and Inhibition of Phosphorylase b by 8-[m (m-fluorosulfonylbenzamido) benzylthio] adenine

A time-dependent inactivation of phosphorylase b activity was observed when 8-[m (m-fluorosulfonylbenzamido)-benzylthio] adenine (compound XL) was incubated with phosphorylase b. This time-dependent inactivation was demonstrated by removing aliquots from the incubation mixture, diluting fiftyfold, and assaying in the presence of 10^-3 M AMP, 0.016 M glucose-1-phosphate, and 1% glycogen at
pH 6.8 (Fig. 1). Inhibition of phosphorylase b activity did not proceed to zero activity, but instead, there remained 24% residual activity. This remaining activity is seemingly due to the ability of the adenine derivative to substitute for AMP. If assays were carried out in the absence of AMP, a time-dependent activation of phosphorylase b could be demonstrated (Fig. 1, lower curve).

Kinetics of Activation and Inhibition of Phosphorylase b by Irreversible Inhibitors

Evidence for the existence of a reversible complex between an irreversible inhibitor and enzyme prior to covalent bond formation has been demonstrated (Kitz and Wilson, 1962; Petra, 1971). The model is shown in equation 1 where E·I is the reversible complex, E−I is the irreversible complex, V_{sat} the maximum rate of inactivation, and k_{2} the first order rate constant (Petra, 1971).

\[ E + I \xrightleftharpoons[k_{-1}]{k_{1}} E·I \xrightarrow{k_{2}} E−I \]  

\[ K_{I} = \frac{[E][I]}{[E·I]} \]  

\[ V_{sat} = k_{2}[E·I] \]  

The irreversibility of the k_{2} step was demonstrated by the inability of phosphorylase b to gain additional
Figure 1. Inhibition and activation of phosphorylase b (2 x 10^{-5} M) by 8-[m-(m-fluorosulfonylbenzamido)benzylthio]adenine (3.45 x 10^{-5} M) in 0.04 M glycerol-P - 0.002 M EDTA (pH 7.8) and 11% dimethylsulfoxide. Enzyme was diluted 50-fold in 0.05 M glycerol-P - 0.05 M 2-mercaptoethanol (pH 6.8) and assayed in 1% glycogen, 0.016 M glucose-1-phosphate, and 1 x 10^{-3} M AMP (upper curve □). Lower curve (△) did not contain AMP in the assay mixture.
% ORIGINAL ACTIVITY VS. TIME (min.)

TIME (min.)

20 40 60 80 100

40 32 24 16 8
activity after dilution nor was the analogue released after dialysis in 0.0015 M hydrochloric acid. Perchloric acid (0.3N) precipitation of the protein was also ineffective in removing the adenine derivative.

\[ E_0 = E^i + E-I \quad E_0 = \text{total enzyme} \quad (4) \]
\[ E^i = E + E-I \quad E^i = \text{concentration of unbound enzyme} \quad (5) \]

Under nonsaturation conditions, the rate equation is as follows:

\[ \frac{-d[E^i]}{dt} = k_2 \left[ \frac{[E]}{K_I} + 1 \right] \quad (6) \]

Integrating:

\[ \frac{\ln E^i}{E_0} = \frac{k_2 t}{K_I} + 1 \quad (7) \]

The rate of inhibition can be obtained by plotting the log of the percent activity vs. time. This can be seen in Figure 2. Compound XXXIX shows only inactivation (see Table III) and should therefore display an inactivation pattern that can be predicted by the kinetic equations previously outlined in this section. The pseudo first order rate constants are obtained from the slope of each
Figure 2. Rate of inactivation of phosphorylase b \((2.10 \times 10^{-5} \text{ M})\) as a function of 8-\[\text{[p-fluorosulfonylbenzamido]benzylthio}\] adenine concentration in 0.04 M glycerol-P - 0.002 M EDTA (pH 7.8), and 11% dimethylsulfoxide at 30 degrees. (□) \(9.09 \times 10^{-4} \text{ M}\) inhibitor, (△) \(7.27 \times 10^{-4} \text{ M}\), (□) \(5.45 \times 10^{-4} \text{ M}\), (○) \(3.64 \times 10^{-4} \text{ M}\), (●) \(1.82 \times 10^{-4} \text{ M}\).
line of the five inhibitor concentrations (Fig. 2).

Therefore, substituting into equation 7 and rearranging (Kitz and Wilson, 1962)

\[
\frac{1}{k_{\text{app}}} = \frac{K_1}{k_{21}} + \frac{1}{k_2}
\]

When the pseudo first order rate constants are plotted according to equation 8, an apparent binding constant, 
\[k_D = 1.25 \times 10^{-3} \pm 0.20,\] and a first order rate constant at saturation, \[k_2 = 0.25 \pm 0.01,\] can be evaluated from Figure 3 using the slope and intercept, respectively.

The previous equations for irreversible inhibitors are not sufficient to evaluate the apparent binding constant and first order rate constant at saturation of 8-[[m(m-fluorosulfonylbenzamido)benzylthio]adenine since these equations do not account for the ability of the meta analogue to substitute for AMP to yield an enzymically active species. In the following equations which allow a kinetic evaluation of 8-[[m(m-fluorosulfonylbenzamido)benzylthio]adenine bound to phosphorylase \(b\), the specific activity of the free enzyme is represented by \(\alpha\), and by \(\beta\) for the specific activity of the modified enzyme. Then where \(\lambda_o\) is equal to the activity of the enzyme not
Figure 3. Reciprocal of the pseudo first order rate constants (evaluated from Figure 2) as a function of the reciprocal 8-[m(p-fluoro-sulfonylbenzamido)benzylthio] adenine concentrations. $k_2$ and $K_D$ were evaluated from the y intercept and slope, respectively.
modified irreversibly, $A_t$ equals total activity at any
time, and $A_{t}'$ is equal to the activity due to incorporation
of analogue measured in the absence of AMP. $E$, $E\cdot I$, and
$E-I$ have the same meaning as in the first part of this
section, namely, free enzyme, enzyme in a reversible
complex with inhibitor, and enzyme in an irreversible
complex, respectively.

\[ E' = E + E \cdot I \]  
(12)

\[ A_{t}' = \alpha E' + A_{t}' \]  
(13)

\[ E' = \frac{A_t - A_{t}'}{\alpha} \]  
(14)

\[ \ln \frac{A_t - A_{t}'}{A_0} = \ln \left( \frac{A_t - A_{t}'}{A_0} \right) = \frac{k_2t}{K_I} \]  
(15)

A plot of $\log \left[ \frac{A_t - A_{t}'}{A_0} \right]$ vs. time can be used to
evaluate $k_{\text{app}}$. This is shown in Figure 4 for 8-[(m-(m-fluorosulfonylbenzamido)benzylthio) adenine binding to phosphorylase b. Linear lines are obtained at all inhibitor concentrations. In Figure 5 the pseudo first order rate constants evaluated from Figure 4 are plotted according to equation 8. Saturation kinetics are observed from which an apparent binding constant $K_D = 1.8 \times 10^{-4} \pm 0.4$ M, and a first order rate constant at saturation, $k_2 = 0.72 \pm 0.11$ min$^{-1}$, can be calculated from the slope and intercept of Figure 4. 8-[(m-(m-fluorosulfonylbenzamido)benzylthio) adenine binds approximately fourteen times more tightly than the para substituted derivative, compound XXXIX. The first order rate constant at saturation is also approximately fourteen times greater for meta derivative (compound XL) than its para substituted counterpart. In terms of half time of inactivation at saturating levels of inhibitor this would be equal to 0.97 minutes for compound XL and 13.86 minutes for compound XXXIX.

Preparation of 8-[(m-(m-fluorosulfonylbenzamido)benzylthio) adenine Modified Phosphorylase b and Determination of the Amount of Bound Adenine Analogue

Reaction of phosphorylase b 2 - 2.3 x 10$^{-5}$ M in 0.04 M glycerol-P - 0.002 EDTA, pH 7.8 was reacted with 1.2-1.4
Figure 4. Rate of inactivation of phosphorylase b (2.10 x 10\(^{-5}\) M) as a function of 8-[m-(m-fluorosulfonylbenzamido)benzylthio] adenine concentration in 0.04 M glycerol-P - 0.002 M EDTA (pH 7.8), and 11% dimethylsulfoxide at 30 degrees (△) 9.0 x 10\(^{-5}\) M inhibitor, (○) 1.22 x 10\(^{-4}\) M, (△) 1.45 x 10\(^{-4}\) M, (○) 1.81 x 10\(^{-4}\) M, (□) 2.43 x 10\(^{-4}\) M.
Figure 5. Reciprocal of the pseudo first order rate constants evaluated from Figure 4 as a function of the reciprocal 8-[m(m-fluorosulfonyl-benzamido)benzylthio]adenine concentration. $k_2$ and $K_D$ were evaluated from the y intercept and slope, respectively.
molar excess of 8-[m(m-4-fluorosulfonylbenzamido)benzylthio]-adenine. The reaction was allowed to proceed for 50 minutes at 30 degrees. An enzymatic check on the amount of modification could be used as an initial check on the amount of modification since the fully modified enzyme has the same activity in the presence or absence of AMP (see Fig. 1). After 50 minutes an equal volume of saturated ammonium sulfate was added. Following centrifugation, the pellet was dissolved in 0.05 M glycerol-P 0.05 M 2-mercaptoethanol (pH 6.8) and dialyzed against the same buffer at 4 degrees to remove excess adenine derivative. The modified enzyme crystallized during dialysis in the absence of AMP or Mg++. The modified enzyme was then recrystallized prior to analysis for the amount of analogue bound. A 1.2 - 1.4 molar excess of analogue to enzyme gives 0.93 ± 0.09 moles bound per mole of enzyme (see Table IV). Reaction of phosphorylase b with a five fold excess of analogue to enzyme for an extended period of time will result in more than one mole of adenine derivative bound per mole of enzyme. When more than one mole of analogue was bound per mole of enzyme there is a loss of enzymic activity to a level lower than the enzymatic activity
Table IV. Binding of 8-[^m(m-fluorosulfonylbenzamido)-benzylthio] adenine to phosphorylase b

<table>
<thead>
<tr>
<th>Molar ratio^a</th>
<th>Amount bound^b</th>
<th>S.A. (μmoles P_i/min/mg)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>0.93 ± 0.1</td>
<td>9.91</td>
</tr>
<tr>
<td>5</td>
<td>1.8 ± 0.1</td>
<td>7.00</td>
</tr>
</tbody>
</table>

^a Molar ratio of 8-[^m(m-fluorosulfonylbenzamido)benzylthio] adenine to phosphorylase b in the incubation mixture.

^b Moles 8-[^m(m-fluorosulfonylbenzamido)benzylthio] adenine bound per mole of enzyme.

^c Assay mixture consisted of 0.016 M glucose-1-phosphate, 1% glycogen, 0.025 M 2-mercaptoethanol - 0.025 M β-glycerophosphate, pH 6.8. Incubation conditions and assay methods are described in text. Aliquots were removed after incubation for one hour at 30 degrees and assayed for enzymic activity.
observed when one mole of analogue is bound per mole of enzyme (Table IV).

Effect of 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine on AMP Binding to Phosphorylase b

AMP was shown to effectively block the inhibition of phosphorylase b by 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine (see Fig. 6). At an inhibitor concentration of $3.6 \times 10^{-4}$ M, in the absence of AMP, 57% of the enzymic activity was lost in six minutes. Under similar conditions, but in the presence of $7.26 \times 10^{-3}$ M AMP, less than 2% loss in activity was observed. This indicates that AMP and the adenine derivative are competing for the same site. More convincing evidence that 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine is binding at the allosteric site is the fact that the adenine derivative binding to phosphorylase b yields an enzymically active enzyme. In general, activation by a covalently bound reagent should give more confidence in a specific reaction than inhibition (Hulla and Fasold, 1972).

Incorporation of $^{14}$C-8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine into Phosphorylase b

Incorporation of $^{14}$C-8-[m(m-fluorosulfonylbenzamido)
Figure 6. Effect of $8-\left[m\left(m\text{-fluorosulfonylbenzamido}\right)\text{benzylthio}\right]$ adenine on AMP binding to phosphorylase b (conditions are the same as Figure 2).

Lower curve ($\square$) contains $3.6 \times 10^{-5}$ M $8-\left[m\left(m\text{-fluorosulfonylbenzamido}\right)\text{benzylthio}\right]$ adenine, all other curves contain this concentration of inhibitor plus varying levels of AMP. ($\triangle$) $3.64 \times 10^{-4}$ M AMP, ($\blacktriangle$) $7.27 \times 10^{-4}$ M AMP, ($\bigcirc$) $3.63 \times 10^{-3}$ M AMP, ($\blacksquare$) $7.26 \times 10^{-3}$ M AMP.
benzylthio)adenine corresponded with the increase in phosphorylase activity when the modified enzyme was assayed in the absence of AMP (Figure 7). Phosphorylase b activity increased to a specific activity of 20.8 μmoles P_i/min/mg of protein (75 mM glucose-1-phosphate in assay). Native enzyme, when assayed under similar conditions, had a specific activity of 62.1 μmoles P_i/min/mg of protein in the presence of saturating level of AMP (1 x 10^{-3} M). The activity of the isolated modified enzyme was not increased by the addition of AMP from 2.5 x 10^{-5} M to 2 x 10^{-3} M, suggesting that the allosteric site in the modified enzyme is fully derivatized.

Wang et al. (1970a) have demonstrated that glucose-6-phosphate is a partial competitive inhibitor of AMP in phosphorylase b. Therefore, when the AMP site in phosphorylase b is irreversibly modified, the inhibition by glucose-6-phosphate should be abolished. In Table V, it can be seen that, although glucose-6-phosphate decreases the activity of the native enzyme by 75%, it has no affect on the modified enzyme.

**Effect of 8-[m(m-fluorosulfonylbenzamido)-benzylthio]adenine on Quaternary Structure of Phosphorylase b**

Since the quaternary structure of phosphorylase b is known to be affected by AMP (Appleman, 1962; Kasten-
Figure 7. Effect of the amount of incorporation of 8-[m(m-fluorosulfonylbenzamido)benzylthio]-adenine on enzymatic activity. Phosphorylase b (9.53 x 10^{-5} M) in 0.04 glycerol-P - 0.002 M EDTA, pH 7.8 in the presence of 1.6 x 10^{-4} M 8-[m(m-fluorosulfonylbenzamido)benzylthio]-adenine. □ Percent maximal incorporation of 14C-8-[m(m-fluorosulfonylbenzamido)benzylthio]-adenine (maximal incorporation was defined as one mole of analogue incorporated per mole of enzyme), Δ percent maximal phosphorylase activity.
Table V. Effect of glucose-6-phosphate on activity of native and 8-([m-[m-fluorosulfonylbenzamido]-benzylthio] adenine modified phosphorylase b

<table>
<thead>
<tr>
<th></th>
<th>10mM G-6-P (S.A.)</th>
<th>No G-6-P (S.A.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native phosphorylase b</td>
<td>4.4</td>
<td>37.9</td>
</tr>
<tr>
<td>Modified phosphorylase b</td>
<td>9.4</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Specific activity (μmoles P<sub>i</sub>/min/mg of protein) was determined in 0.016 M glucose-1-phosphate, 1% glycogen, 1 x 10<sup>-3</sup> M AMP, 0.025 M β-glycerophosphate, 0.025 2-mercaptoethanol, pH 6.8.
Schmidt et al., 1968a), ultracentrifugation experiments were performed to determine whether 8-[(m-fluorosulfonyl-benzamido)benzylthio]adenine and AMP had similar effects on the state of aggregation of phosphorylase b. The adenine derivative modified phosphorylase b, sediments primarily with an $S_{20,w}$ of 12.7 S (Fig. 8A) in the absence of AMP. The quaternary structure of the modified phosphorylase b is similar to native phosphorylase b, which, in the presence of $1 \times 10^{-3}$ M AMP, has an $S_{20,w}$ of 12.8 S (Fig. 8B, lower curve). The upper curve (Fig. 8B) shows the sedimentation pattern of native phosphorylase b in the absence of AMP ($S_{20,w}$, 8.30 S). The modified enzyme shows little or no response to AMP. With $5 \times 10^{-5}$ M AMP, the ultracentrifugal pattern of the modified enzyme remained unchanged, whereas native phosphorylase b showed significant amounts of the tetrameric form. With $1 \times 10^{-3}$ M AMP (Fig. 8A, lower curve), a slight increase in an amount of a slow component was observed. If the allosteric site were not fully modified in the derivatized enzyme, addition of AMP would be expected to cause tetramer formation. The small increase in the amount of the slow component could be due to an additional binding of AMP at a different enzyme site (Wang et al., 1970b). The sedimentation pattern
Figure 8. Ultracentrifugation patterns of native and 8-[m(m-fluorosulfonylbenzamido)benzylthio]-adenine modified phosphorylase b. Enzymes were centrifuged at 52,000 rpm at 12.8 degrees in 0.05 M glycerol-P - 0.05 M 2-mercaptoethanol buffer, pH 6.8. Lower patterns with $1 \times 10^{-3}$ M AMP; upper patterns, no additions. a, modified phosphorylase b (5.59 mg/ml); b, native enzyme (5.55 mg/ml). Direction of sedimentation is from left to right.
of modified phosphorylase b also is temperature dependent; at 23 degrees, the modified enzyme sediments mainly as a dimer, whereas, at lower temperatures, increasing amounts of the tetrameric form are present.

Stability of 8-[m(m-fluorosulfonylbenzamido)-benzylthio]adenine Modified Phosphorylase b

The modified enzyme was more heat stable, and pyridoxal phosphate was more difficult to remove from the covalently modified enzyme, compared with native phosphorylase b. In Figure 9 is a comparison of the heat stability of modified phosphorylase b and native phosphorylase b in the presence (1 x 10^{-3} M) and absence of AMP. Although AMP has a pronounced effect on the heat stability of phosphorylase b, the native enzyme is considerably less stable than the covalently modified phosphorylase. The change in conformation induced by AMP apparently changes phosphorylase b to a more stable form. Phosphorylase is less stable at higher temperatures in L-cysteine than in 2-mercaptoethanol, presumably because L-cysteine can remove the PLP from the holoenzyme, but 2-mercaptoethanol cannot (Shaltiel et al., 1966). In experiments involving heat inactivation using L-cysteine buffer, we observed a similar pattern of inactivation with L-cysteine, but the
Figure 9. Heat stability of native and 8-[m-fluorosulfonylbenzamido]benzylthio adenine modified phosphorylase \( b \). Native phosphorylase \( b \) 11.85 mg/ml (\( \triangle \)) was diluted 1/100 in 0.05 M glycerol-P - 0.05 M 2-mercaptoethanol at 52 degrees; (\( \square \)) same but buffer contained 0.001 M AMP. Modified phosphorylase \( b \) 13.00 mg/ml (\( \square \)) diluted 1/100 in 0.05 M glycerol-P - 0.05 M 2-mercaptoethanol, pH 6.8. Aliquots were removed at designated times and assayed for inorganic phosphate released at 30 degrees. Time on the abscissa refers to time of incubation at 52 degrees.
rate of inactivation was greater in L-cysteine than in 2-mercaptoethanol. The covalently modified enzyme was stable in both 2-mercaptoethanol and L-cysteine.

The resolution of phosphorylase by L-cysteine is inhibited by AMP (Shaltiel et al., 1966). Therefore, if $8-\left[m(m\text{-fluorosulfonylbenzamido)}\text{benzylthio}\right]$ adenine modified phosphorylase b has a conformation similar to the native enzyme in the presence of AMP, the modified enzyme should be difficult to resolve. When we tried to resolve the modified enzyme by use of the procedure of Shaltiel et al. (1966), we were unable to detect any loss of PLP.

Since AMP seemingly induces a more stable conformation, which is manifested in the binding of $8-\left[m(m\text{-fluorosulfonylbenzamido)}\text{benzylthio}\right]$ adenine binding to phosphorylase, we tested the effects of substrates on the stability of the modified enzyme to determine if the binding of the substrates would induce the formation of a less stable complex. Neither glycogen (0.2%) nor glucose-1-phosphate (0.016 M) had any affect on the heat stability of modified phosphorylase b. Interestingly, although the enzyme was stable at 52 degrees, it had no activity when assayed at 52 degrees. In this experiment,
we incubated modified enzyme and substrate (0.032 M glucose-1-phosphate, 2% glycogen, pH 6.8) separately at 52 degrees and then mixed together at zero time. Under these conditions, the modified enzyme was inactive, but, if the enzyme substrate mixture was cooled to 30 degrees, there was a return of enzymic activity to a level essentially that of the control. In contrast to modified phosphorylase b, native phosphorylase b in the presence of AMP was active at 52 degrees.

Interconversion of Modified Phosphorylase b

The conversion of phosphorylase b to phosphorylase a is relatively insensitive to adenosine monophosphate; AMP at $2 \times 10^{-4}$ M had no significant effect on the phosphorylase b kinase reaction (Krebs et al., 1964). Since there is little effect of AMP on the conversion of phosphorylase b to phosphorylase a, we expected a similar rate of conversion of 8-[(m-fluorosulfonylbenzamido)-benzylthio]adenine modified and native phosphorylase b. This is demonstrated in Figure 10, where the conversion of modified phosphorylase b is compared with native phosphorylase b. The specific activity of the modified enzyme increased from 9.2 μmoles P$_i$/min/mg of protein in the nonphosphorylated form to 29.6 μmoles P$_i$/min/mg
Figure 10. Rate of phosphorylation by phosphorylase \( b \) kinase of native and 8-\([m(m\text{-fluorosulfonylbenzamido)benzylthio}]\) adenine modified phosphorylase \( b \). The reaction mixture contained 0.006 M ATP, 0.020 M Mg\(^{++}\), 0.020 M glycerol-P, 0.020 M Tris, 0.4 \( \mu \)g/\( \mu \)l phosphorylase \( b \) kinase and native phosphorylase \( b \) 7.60 mg/ml (\( \Delta \)), or modified phosphorylase \( b \) 7.68 mg/ml (\( \square \)).
of protein in the phosphorylated form. One of the reasons for the more than threefold activation of modified phosphorylase b (assayed at 0.016 M glucose-1-phosphate) upon phosphorylation is due to the decrease in the $K_m$ for glucose-1-phosphate. The nonphosphorylated form of the modified enzyme has a $K_m$ of 35 mM for glucose-1-phosphate, while the phosphorylated form has a $K_m$ of 5 mM (Table VI). AMP had no affect on the $K_m$ values of the modified enzyme. If the conversion of modified phosphorylase b was followed by assaying at 75 mM glucose-1-phosphate, the specific activity increased from 19.1 to 36.3 μmoles $P_i$/min/mg of protein. Thus, upon phosphorylation, there was an increase in the affinity of the modified enzyme for its substrate and a corresponding increase in specific activity.

Unlike phosphorylase b kinase, which is relatively insensitive to AMP, the phosphorylase phosphatase reaction is very sensitive to AMP (Sutherland, 1951). AMP inhibition of the phosphorylase phosphatase reaction is due to binding to phosphorylase a, not to phosphorylase phosphatase (Nolan et al., 1964). Assuming that 8-$[m(m$-fluorosulfonylbenzamido)benzylthio]adenine induces a conformation similar to the native activator, AMP, a much slower rate of dephosphorylation of the modified
Table VI. Kinetic characteristics of native and 8-[m(m-fluorosulfonylbenzamido)benzylthio]adenine modified phosphorylase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Analogue b</th>
<th>Analogue a</th>
<th>Native b</th>
<th>Native a</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose-1-phosphate</td>
<td>35 mM</td>
<td>5 mM</td>
<td>6 mM</td>
<td>5.5 mM</td>
</tr>
<tr>
<td>glycogen</td>
<td>0.05%</td>
<td>0.02%</td>
<td>0.02%</td>
<td>0.021%</td>
</tr>
</tbody>
</table>

Specific Activity: μmoles/P_i/min/mg of protein

<table>
<thead>
<tr>
<th>Glucose-1-phosphate</th>
<th>Analogue b</th>
<th>Analogue a</th>
<th>Native b</th>
<th>Native a</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 mM</td>
<td>9.9</td>
<td>29.6</td>
<td>37.9</td>
<td>53.8</td>
</tr>
<tr>
<td>75 mM</td>
<td>20.8</td>
<td>36.3</td>
<td>62.1</td>
<td>69.8</td>
</tr>
</tbody>
</table>

a $K_m$ values for native phosphorylase a are from Cori et al., 1943. Assay mixture for $K_m$ for glucose-1-phosphate contained 1% glycogen, 0.025 M 2-mercaptoethanol - 0.025 M glycero-phosphate, pH 6.8, and varying levels of glucose-1-phosphate (0.004-0.075 M). $K_m$ values for glycogen were determined using a coupled assay (Helmreich and Cori, 1964). The conditions for the specific activity measurements are the same as the conditions for the determinations of $K_m$ values for glucose-1-phosphate but were performed at the indicated glucose-1-phosphate concentrations. The assay mixture for native phosphorylase b always contained $1 \times 10^{-3}$ M AMP. All other measurements were in the absence of AMP.
phosphorylase \( b \) should be observed. This is demonstrated in Figure 11, where the dephosphorylation of the modified phosphorylase occurs at a much slower rate than that of the native enzyme.

Isolation of Peptide that Binds 8-\([m(m-\text{fluorosulfonylbenzamido})-\text{benzylthio}]\) adenine

Phosphorylase \( b \) at 2-2.5 mg/ml in 0.04 M glycero-phosphate - 0.002 M EDTA, pH 7.8, was reacted with an 0.9 molar ratio of 8-\([m(m-\text{fluorosulfonylbenzamido})-\text{benzylthio}]\) adenine to native phosphorylase \( b \) for 45 minutes at 30 degrees. The enzyme was then precipitated with an equal volume of saturated ammonium sulfate. After centrifugation, the pellet was dissolved in 0.05 M glycero-phosphate - 0.05 M 2-mercaptoethanol, pH 6.8, and dialyzed against the same buffer for 8 hours. The enzyme crystallized during dialysis; the crystals were centrifuged and dissolved in 0.02 M glycero-phosphate - 0.02 M 2-mercapto-ethanol and extensively dialyzed against 0.0015 M hydrochloric acid. After dialysis, the pH of the modified phosphorylase \( b \) solution was adjusted to pH 1.9 with 2 N hydrochloric acid. Pepsin (0.1\% by weight) was added and allowed to react for 12 hours. At this time, the pH was adjusted again to pH 1.9, and the same amount of pepsin was added. After 15 hours, the digested enzyme solution
Figure 11. Rate of dephosphorylation by phosphorylase phosphatase of native and 8-[m(m-fluorosulfonylbenzamido)benzylthio]adenine modified phosphorylase a. The reaction mixture contained native phosphorylase a (Δ) (0.24 mg/ml), modified phosphorylase a (□) (0.3 mg/ml), in 0.05 M tris-acetate - 0.001 M DTT, pH 7.5. Aliquots were removed at designated times and assayed for $^{32}$P released.
was lyophilized to dryness. The sample was dissolved in water and applied to a charcoal:
celite column. A charcoal:
celite column (0.5 x 2.5 cm) was prepared by mixing 2 parts by weight of celite 560 with 1 part of acid-washed Norit A. Quantitative binding of the $^{14}\text{C}$ labeled peptide occurred upon passage of the pepsin digest through the column. The column was then extensively washed with water to remove all loosely bound peptides. The $^{14}\text{C}$ labeled peptide was eluted with 3% ammonia in 50% acetone (Shapiro and Stadtman, 1968) and evaporated to dryness. At this point, the $^{14}\text{C}$ labeled peptide appeared as one spot on ascending chromatography (butanol:acetic acid:H$_2$O 4:1:1) on pre-coated, silica-gel plates, with only very slight amounts of contaminating peptides. Further purification of the peptide to which the adenine derivative was bound was performed by using Dowex 50 chromatography according to the method of Schroeder (1967). After evaporation to dryness, the partially pure peptide was dissolved in pyridine-acetic acid buffer pH 3.1 (0.2 M pyridine). The pH of this solution was then lowered to 2.3-2.5 with 2 N hydrochloric acid before application of the peptide to a Dowex 50 - x 2 column (1 x 18 cm) equilibrated with pyridine-acetic acid buffer, pH 3.1. The column was washed with pyridine-acetic acid (2 M pyridine) pH 5.0.
After thorough washing, a linear gradient between pyridine-acetic acid pH 5.0 and pH 5.6 (8.5 M pyridine) was employed to elute the purified peptide. A 60% yield of the purified peptide was obtained.

After Dowex 50 chromatography the peptide appeared as a single spot on thin-layer chromatography with butanol, acetic acid, water (4:1:1) as a solvent ($R_f = 0.33$). The purified peptide did not migrate on high-voltage electrophoresis at pH 4.0. An autoradiogram of the thin-layer chromatogram sheet displayed one radioactive spot. Amino acid analysis showed that the peptide to which 8-[[8-fluorosulfonylbenzamido]benzylthio] adenine was covalently bound was composed of 2 moles of alanine, 2 glycine, and 1 tyrosine. The peptide to which the adenine derivative is bound absorbs maximally at 285 nm in 10% acetic acid. This spectrum is almost identical to the spectrum of free 8-[[8-fluorosulfonylbenzamido]-benzylthio] adenine in 10% acetic acid ($A_{285} = 2.04 \times 10^5$). There was no tryptophan in the peptide which was determined by the procedure of Matsubara and Sasaki (1969).
Sequence of Isolated Peptide

The sequence of the peptide to which the adenine derivative was bound was determined using leucine amino peptidase (Light, 1972) and carboxypeptidase A (Ambler, 1972). In Table VII the amounts of individual amino acids released by leucine amino peptidase are given. From this data, it is apparent that 2 alanine molecules appear at the N-terminal end of the peptide followed by glycine and another glycine molecule or tyrosine. The order of the fourth and fifth amino acids, glycine and tyrosine, is difficult to determine from Table VII. The order of these amino acids was determined using carboxypeptidase A (Table VIII). Glycine is the first amino acid released by carboxypeptidase followed by tyrosine and then a second molecule of glycine. From this data, it can be concluded that the sequence is alanine•alanine•glycine•tyrosine•glycine.
Table VII: Release of amino acids by leucine amino peptidase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>10'</th>
<th>20'</th>
<th>30'</th>
<th>50'</th>
<th>125'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.016^a</td>
<td>0.021</td>
<td>0.022</td>
<td>0.022</td>
<td>0.025</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.003</td>
<td>0.010</td>
<td>0.017</td>
<td>0.022</td>
<td>0.026</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>—</td>
<td>0.002</td>
<td>0.005</td>
<td>0.010</td>
<td>0.012</td>
</tr>
</tbody>
</table>

^a μmoles of amino acids released.

2 × 10⁻⁷ μmoles of peptide in 10% acetic acid was lyophilized to dryness. 0.6 ml of 0.2 M tris-0.005 M magnesium acetate (pH 8.6) and 0.04 ml of leucine amino peptidase (5 mg/ml) were added. 0.1 ml aliquots were diluted into sodium citrate buffer (0.2 M; pH 2.2) at designated times for amino acid analysis.
Table VIII: Release of amino acids by carboxypeptidase A.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>5'</th>
<th>10'</th>
<th>40'</th>
<th>15 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.0168</td>
<td>0.0182</td>
<td>0.314</td>
<td>0.0446</td>
</tr>
<tr>
<td>Alanine</td>
<td>trace</td>
<td>0.0010</td>
<td>0.006</td>
<td>0.0074</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0070</td>
<td>0.0124</td>
<td>0.0184</td>
<td>0.0242</td>
</tr>
</tbody>
</table>

$4 \times 10^{-7}$ μmoles of peptide in 10% acetic acid was lyophilized to dryness. After lyophilization 0.6 ml of 0.4 M N-ethylmorpholine (pH 8.5) was added. 0.75 mg of carboxypeptidase A in 1% sodium bicarbonate was added at zero time. 0.1 ml aliquots were removed at designated times for amino acid analysis.
DISCUSSION

The use of structural analogues to study the role of activators is a useful and well-established technique. In this manuscript, with the aid of adenine analogues, we have attempted to establish the nature of the AMP binding site in glycogen phosphorylase b. The binding of adenine and also hypoxanthine analogues is increased by hydrophobic groups at several different positions of the purine ring (Table I). This may indicate that the adenine portion of the AMP binding site in glycogen phosphorylase b is a rather large hydrophobic region. To investigate this theory in greater detail, several 8-substituted adenine derivatives were examined for their ability to bind to phosphorylase b. The use of substituent constants was very useful in establishing the hydrophobic nature of the adenine analogues. The usefulness of substituent constants as a tool for structure-activity studies was first established by Hansch and colleagues (Fujita et al., 1964). Fujita et al. (1964) evaluated \( \pi \) values from seven different parent compounds (systems) and concluded that, while \( \pi \) values for individual groups may vary depending upon the parent compound, the variation is usually not large. Since substituent constants from system to
system usually vary only slightly, we used phenylacetic acid to evaluate $\pi$ for the adenine derivatives. From the data in Table II, it can be seen that there is good agreement between substituent constants and relative $K_i$ values. The experimental observation that compounds with increasing $\pi$ values and, therefore, increasing hydrophobicity are better inhibitors is further evidence that AMP binds in a hydrophobic region. Compound VI, 8-(3-nitro-4-chlorophenyl)adenine, is one of the most hydrophobic compounds and is the most effective competitive inhibitor tested. The slightly lower $\pi$ value than expected for the amount of inhibition may be due to the additional electron-releasing ability of the NO$_2$ group (Hansch, 1970). The size of the substituted groups did not effect the binding of the adenine analogues tested to glycogen phosphorylase $b$, indicating that steric effects do not play a major role in binding at the allosteric binding site.

The irreversible inhibition of phosphorylase $b$ was a very specific reaction (Table III). Sulfonyl fluoride derivatives were chosen as irreversible inhibitors because a specific reversible complex of the moiety bearing the SO$_2$F group with the enzyme must be formed before the SO$_2$F group will react and form a covalent linkage (Baker et al., 1966). Two sulfonyl
fluoride derivatives, compounds XXXVI and XXXVIII, significantly irreversibly modify phosphorylase b, but showed no activity in the absence of AMP. We did not study these compounds in detail because of the additional controls that need to be taken to show that a compound that is only an inhibitor is really binding at the allosteric site or some similar site. 8-[(m-fluorosulfonylbenzamido)benzylthio] adenine (compound XL) offered the advantage of being a more effective irreversible inhibitor than any of the compounds tested, but more importantly, this adenine derivative, when bound to phosphorylase b, yields enzymic activity in the absence of AMP. This is the first analogue that is an activator of glycogen phosphorylase b that does not contain a ribose ring or a phosphate group. The specificity of activation is demonstrated by the binding of compound XXXIX, which is similar to compound XL, except that the sulfonyl fluoride group is in the para instead of the meta position of the terminal benzene ring. Compound XXXIX, not only does not activate phosphorylase b, but also is a relatively poor inhibitor (Table III).

8-[(m-fluorosulfonylbenzamido)benzylthio] adenine was demonstrated to be covalently bound since it could not be removed by dilution, extensive dialysis, sephadex chromatography, or treatment of the enzyme with 0.3 N
perchloric acid. Treatment of the irreversibly modified enzyme with Norit A removed some of the protein from solution, but did not free the analogue from the protein.

Activation of phosphorylase b by 8- [m(m-fluorosulfonylbenzamido)benzylthio] adenine is good evidence that this analogue is binding at the allosteric site. Additional evidence that 8- [m(m-fluorosulfonylbenzamido)benzylthio] adenine is functioning in a manner similar to AMP is that, when one mole of analogue is bound per mole of enzyme, maximal activity is observed.

The reaction of 8- [m(m-fluorosulfonylbenzamido)benzylthio] adenine with glycogen phosphorylase b results in an irreversibly modified enzyme form similar to the native enzyme in the presence of the native allosteric activator, adenosine monophosphate. The ultracentrifugal pattern (Fig. 8) of the modified phosphorylase b is nearly identical to the native enzyme in the presence of AMP. Both native enzyme, in the presence of AMP, and modified enzyme sediment predominantly at 12.6 S at 12.8 degrees, indicating that the most abundant form of the enzyme is the tetrameric form.

The increased heat stability of the irreversibly modified phosphorylase b may be due to the fixed conformation that would be induced by the irreversible binding of the adenine analogue. It can be seen in
Figure 9 that AMP also increases the heat stability of phosphorylase b. In modified phosphorylase b, 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine would remain on the enzyme and therefore hold the enzyme in a fixed, more stable conformation. In the native enzyme, AMP would be shuttling on and off the enzyme surface, resulting in a finite time (when no AMP is bound) in which denaturation would be more likely to occur. Some change in conformation must occur in the modified enzyme at elevated temperatures because it has no catalytic activity at 52 degrees. Full activity is recovered upon cooling suggesting that the conformational change is fully reversible. Thus, the derivatized enzyme is well suited for a study of conformation at different temperatures and an analysis of thermodynamic parameters.

The binding of 8-[m(m-fluorosulfonylbenzamido)-benzylthio] adenine has little affect on the conversion of phosphorylase b to phosphorylase a. This was to be expected because AMP has little affect on the phosphorylase kinase reaction. This is in contrast to phosphorylase phosphatase, which is inhibited by low levels of AMP (Sutherland, 1951). As shown in Figure 11, the dephosphorylation of the adenine derivative modified phosphorylase a proceeds at a rate significantly slower
than that of native phosphorylase a. Studies are currently in progress using modified phosphorylase as an alternative substrate for phosphorylase kinase and phosphorylase phosphatase in studying the effects of nucleotides and other effectors presumed to bind to the AMP site of phosphorylase. If the allosteric site of phosphorylase is irreversibly modified with 8-[m(2-fluorosulfonylbenzamido)benzylthio]adenine, the effects due to addition of nucleotides and other effectors that can bind at the allosteric site of phosphorylase will be due to binding to phosphorylase kinase or phosphorylase phosphatase, not to the binding to the substrate, phosphorylase.

The somewhat lower affinity for glucose-1-phosphate and glycogen of the covalently modified form of phosphorylase b may indicate that the conformation induced by the binding of 8-[m(2-fluorosulfonylbenzamido)benzylthio]adenine to phosphorylase b is not identical to the conformation induced by AMP. Although modified phosphorylase does exhibit a decreased affinity for its substrates, glucose-1-phosphate and glycogen, it is enzymically active. For the modified enzyme to possess enzymic activity, it must be in a conformation similar to native phosphorylase b in the presence of the native allosteric activator, AMP. The conformation induced by
8-[\(m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}\)] adenine may be intermediate between the conformation induced by inosine monophosphate (Black and Wang, 1968) and AMP. Both 8-[\(m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}\)] adenine and inosine monophosphate, when bound to phosphorylase \(b\), yield enzymically active forms with similar affinities for glucose-1-phosphate. Inosine monophosphate alone, however, unlike 8-[\(m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}\)] adenine and AMP, does not induce tetramer formation of phosphorylase \(b\). The decreased affinities for substrates of the irreversibly modified phosphorylase \(b\) are not due to denaturation since, upon conversion to phosphorylase \(a\) by phosphorylase kinase, the phosphorylated form of the modified enzyme has a similar affinity for the substrates to native phosphorylase \(a\) (Table VI).

In Figure 12 are space-filling models of 8-[\(m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}\)] adenine (upper figure) and AMP (lower figure). Although the two molecules possess obvious differences, it can be seen that, in 8-[\(m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}\)] adenine, the \(\text{SO}_2\text{F}\) group (see arrow) occupies a position in relation to the adenine similar to that of the phosphate group in relation to adenine in adenosine monophosphate. It is
Figure 12. Space filling models of 8-[$^m$N($^m$-fluorosulfonyl-benzamido)benzylthio] adenine and adenosine monophosphate. Arrow denotes the sulfonyl fluoride group.
postulated that the sulfonyl fluoride group of 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine binds to a residue similar to that with which the phosphate group interacts in the binding of AMP.

The peptide isolated from 14C-8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine modified phosphorylase b contained 2 moles of glycine, 2 moles of alanine, and 1 mole of tyrosine. The sequence of this peptide is alanine•alanine•glycine•tyrosine•glycine. Interestingly, the composition of this peptide excludes it from part of the known sequences that contain the phosphorylated seryl residue (Nolan et al., 1964), the coenzyme, pyridoxal phosphate (Forrey et al., 1971) or the N-terminal peptide (Fischer et al., 1971). Because glycine and alanine have no reactive side groups and the peptide to which 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine is bound is not an N-terminal peptide, we presume that the sulfonyl group of the adenine derivative is bound to the phenolic hydroxyl group of tyrosine.

It is hoped that, through the use of 8-[m(m-fluorosulfonylbenzamido)benzylthio] adenine, the chemistry of the adenosine monophosphate site in a number of AMP binding enzymes can be studied and compared.
SUMMARY

In the present study we have demonstrated the following important aspects pertaining to the allosteric binding site of glycogen phosphorylase b from rabbit muscle.

1. The nucleotide binding site of glycogen phosphorylase is in a relatively hydrophobic region of the protein since compounds which are more hydrophobic in nature are correspondingly more effective inhibitors with respect to AMP.

2. The AMP binding site is in a readily accessible region. Bulk or steric effects have little influence on the binding of inhibitors.

3. Activation of phosphorylase can occur by a compound that contains neither a ribose ring nor a phosphate group.

4. The nucleotide binding site can be affinity labeled using 8-[[m(m-fluorosulfonylbenzamido)benzylthio]adenine which results in an enzyme form similar to native enzyme in the presence of saturating levels of adenosine monophosphate.
5. The covalently modified enzyme can be converted to phosphorylase \( a \) by phosphorylase kinase and back to phosphorylase \( b \) by phosphorylase phosphatase. The phosphorylase kinase reaction proceeds at a similar rate as the native enzyme but the phosphorylase phosphatase reaction occurs at a much slower rate when compared to the native enzyme.

6. \( 8-[m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}] \) adenine was shown to be covalently bound since this derivative was not released by extensive dialysis, sephadex chromatography, or precipitation of the enzyme with perchloric acid (0.3 N).

7. The peptide that \( 8-[m(m\text{-fluorosulfonylbenzamido})\text{benzylthio}] \) adenine was bound was isolated. The sequence of the peptide is alanine·alanine·glycine·tyrosine·glycine. The site of attachment of the adenine derivative is suggested to be the phenolic hydroxyl group of tyrosine.
Cohn, M., and Cori, G. T. (1948), J. Biol. Chem. 175, 89.


ACKNOWLEDGMENTS

I would like to express a sincere thank you to my major professor, Dr. Donald J. Graves, whose knowledge, wisdom, and understanding made this work possible.

I would like to thank Dr. Bernard White and Dr. John Robyt for their valuable discussions and assistance.

Thanks are extended to each of my laboratory colleagues: Dr. Todd Martenson, Mr. Jan Tu, Mr. Gerald Carlson, Mr. Richard Parrish, Mr. George Tessmer, and Dr. Thomas Carty.

I also would like to thank Ms. Dianna Leslie for performing the amino acid analysis.