Chemistry of the Adenosine Monophosphate Site of Rabbit Muscle Glycogen Phosphorylase. I. Hydrophobic Nature and Affinity Labeling of the Allosteric Site†

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ABSTRACT: To obtain a better understanding of the function and site of interaction of AMP, several adenine and hypoxanthine derivatives were tested as inhibitors of rabbit skeletal muscle phosphorylase *b*. Adenine derivatives substituted in the 8 position with hydrophobic groups were the most effective inhibitors tested. Substituent constants, π, were used to evaluate the hydrophobic nature of the adenine derivatives. Derivatives with increasing π values, and therefore of increasing hydrophobic character, were correspondingly more effective competitive inhibitors with respect to AMP. In an attempt to irreversibly modify the allosteric site of phosphorylase *b*, 11 sulfonyl fluoride derivatives of adenine were tested. One, 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]-adenine, not only bound irreversibly, but also yielded an enzyme form that had approximately 24% of the activity obtained with phosphorylase *b* at saturating levels of AMP. This is the first report of the activation of glycogen phosphorylase *b* by an analog that contains neither a ribose ring nor a phosphate group. The 8-[*m*-(*m*-fluorosulfonylbenzamido)-benzylthio]adenine–phosphorylase *b* complex crystallizes in the absence of AMP and Mg^{2+}. One mole of analog is bound per mol of enzyme. AMP does not increase the activity of the modified enzyme. The sulfonyl fluoride derivative of adenine bound covalently since this derivative was not released by dialysis, Sephadex chromatography, or perchloric acid precipitation. An apparent binding constant for 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine, $K_D = 1.8 \times 10^{-4} \pm 0.4$ M, and a first-order rate constant for the chemical reaction at saturation, $k_2 = 0.72 \pm 0.11$ min^{-1}, were observed. When 8[*m*-(*p*-fluorosulfonylbenzamido)benzylthio-adenine is incubated with phosphorylase *b*, only enzyme inactivation occurs. An apparent binding constant, $K_D = 1.25 \times 10^{-3} \pm 0.2$ M, and a first-order rate constant at saturation, $k_2 = 0.05 \pm 0.01$ min^{-1}, were determined for the para derivative.

Rabbit muscle glycogen phosphorylase *b* exists in two interconvertible forms: phosphorylase *b*, which has an absolute requirement for adenosine monophosphate, and phosphorylase *a*, which is enzymically active in the absence of AMP.[1] Adenosine monophosphate has been shown to affect enzyme conformation and interconversion, subunit interactions, and the binding of substrates and inhibitors. These effects have been explained through models that describe the binding of adenosine monophosphate as an allosteric effector. References to the original literature can be found in a review by Graves and Wang (1972).

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; Hulla and Fasold, 1972), which have shown that a large number of nucleotides and related compounds can bind at the AMP binding site. 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. Protamine was shown to enhance nucleotide activation of phosphorylase *b*, but the mechanism of enhancement was not evident (Mott and Bieber, 1968).

In the present study, several adenine derivatives were used to gain a better understanding of the nature of the allosteric site of phosphorylase *b*. A number of potential, irreversible binding adenine derivatives bearing a sulfonyl fluoride group also were tested for their ability to affinity label the allosteric site of rabbit glycogen phosphorylase *b*. Evidence is presented that indicates that the allosteric binding site in glycogen phosphorylase *b* is in a relatively large hydrophobic region and also that the allosteric site of phosphorylase *b* can be affinity labeled by using 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]-adenine.

A preliminary account of this study has been presented (Anderson and Graves, 1972).

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° from pH 6.8 buffer consisting of 0.05 M glycerol-P, 0.05 M β-mercaptoethanol, 0.001 M AMP, and 0.01 M Mg^{2+}. No additional AMP or Mg^{2+} 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.

Enzyme activity measurements were carried out in the direction of glycogen synthesis (Illingworth and Cori, 1953). Enzyme concentration was determined spectrophotometri-

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[1] Abbreviations used are: glycerol-P, β-glycerophosphate; AMP, adenosine 5′-monophosphate.

TABLE I: Inhibition of Phosphorylase *b* by Substituted Purines.

Compound No.	Compound	% Inhibn
I	Adenine	7
II	Adenosine	5
III	9-Phenyladenine	17
IV	9-Methyladenine	24
V	8-Methyladenine	22
VI	8-Phenyladenine	35
VII	8-Benzyladenine	13
VIII	8-Phenylvinyladenine	12
IX	8-Phenylethyladenine	3
X	1-Benzyladenine	13
XI	Hypoxanthine	3
XII	9-Phenylhypoxanthine	23
XIII	9-Methylhypoxanthine	23
XIV	1-Benzylhypoxanthine	11
XV	9-Benzylhypoxanthine	13
XVI	3-Benzylhypoxanthine	18
XVII	7-Benzylhypoxanthine	12

[a] 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 (4.5×10^{-5} M), glucose-1-P (0.016 M), glycogen (1%), and inhibitor (1.8×10^{-4} M). Reaction mixtures contained 10% dimethyl sulfoxide to solubilize the inhibitors. After 5 min of reaction at 30°, the inorganic phosphate released was determined by method of Illingworth and Cori.

cally at 280 nm. An absorbancy index (1% × cm^{-1}) of 13.2 was used (Kastenschmidt *et al.*, 1968). A molecular weight of 100,000 for monomer phosphorylase *b* was used in calculations involving molecular weight (Cohen *et al.*, 1971).

Inhibitor constants for the adenine analogs 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% dimethyl sulfoxide to solubilize the inhibitor, AMP (5.3×10^{-5} to 3.7×10^{-4} M), and adenine analogs (7.5×10^{-4} to 7.5×10^{-5} M). The adenine analogs were first dissolved in 100% dimethyl sulfoxide and then diluted into the assay mixture. Twenty-five per cent dimethyl sulfoxide had no effect on phosphorylase maximal activity or affinity for AMP.

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, Calif.

The amount of irreversible inhibition was determined by incubating phosphorylase *b* at $2\text{–}2.3 \times 10^{-5}$ M in 0.05 M glycerophosphate–0.002 M (ethylenedinitrilo)tetraacetic acid (pH 7.8) with irreversible inhibitor. At prescribed times, aliquots were diluted 50-fold in 0.05 M glycerophosphate–0.05 M β-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 Co. Glucose 1-phosphate was purchased from Calbiochem. Imidazole and *N*-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Eastman Organic.

Glycogen was purified by precipitating a 10% solution of glycogen in water with 100% trichloroacetic acid. CCl_3COOH (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 acid-washed Norit A–Celite 560 (2:1, wt). The glycogen solution was then passed 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.

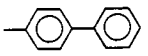
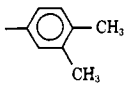
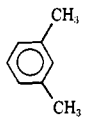
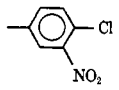
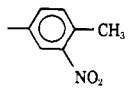
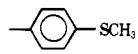
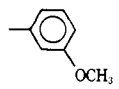
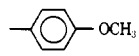
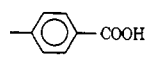
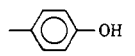
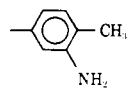
Determination of the Amount of 8-[m-(m-Fluorosulfonylbenzamido)benzylthio]adenine Bound. The amount of adenine derivative bound per mole of enzyme was determined by a modification of the procedure of Daniel (1961) for aromatic amines. An aromatic amine could be generated by hydrolysis of the amide linkage of 8-[*m*-(*m*-fluorosulfonylbenzamido)-benzylthio]adenine. Phosphorylase *b*, which had been reacted with the adenine derivative, was recrystallized twice, dialyzed against water for 8 hr, 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 hr at 110°. After hydrolysis, 1.25 ml of H_2O 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 min, 0.25 ml of 2.5% ammonium sulfamate and 0.25 ml of *N*-(1-naphthyl)ethylenediamine were added. After 15 min, 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 analog to native protein before acid hydrolysis. The blank consisted of native hydrolyzed protein, plus the usual reaction mixture. This assay will detect 10^{-8} mol of aromatic amine in the assay mixture.

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–XVII) to act as inhibitors of phosphorylase *b*. Substitution of a phenyl group at the 8 position of adenine (VI) resulted in the largest inhibition (35%).

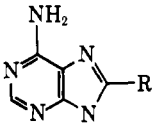
Since 8-phenyladenine proved to be the best inhibitor tested, further studies were performed on derivatives of this compound. In Table II, it can be seen that compounds with increasing substituent constants, π, and therefore of increasing hydrophobic character, are more effective inhibitors of glycogen phosphorylase *b* than compounds less hydrophobic in nature. Π is defined as: $\pi = \log P_X - \log P_H$, 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 exhibit corresponding increases in hydrophobicity. Replacement of the hydrogen at the 8 position of adenine by a phenyl group (VI) results in a compound that binds approximately four 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 (π), are cor-

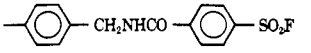
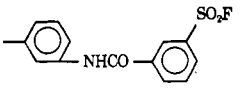
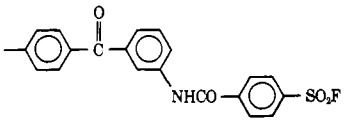
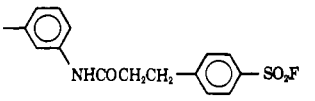
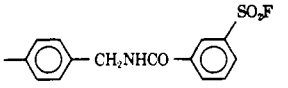
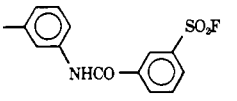
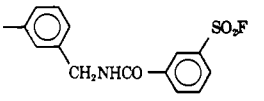
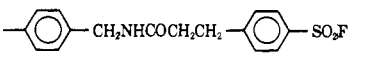
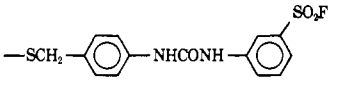
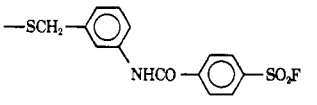
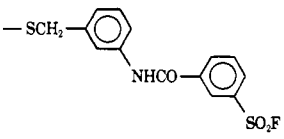
TABLE II: Correlation of Apparent K_i's of Adenine Derivatives with Their Substituent Constants.

Compound	Substitution at 8 Position of Adenine	K_i (M)	π[a]
I (adenine)		2.3×10^{-3}	
XVIII		1.3×10^{-4}	1.89[b]
XIX		3.2×10^{-4}	1.03
XX		2.2×10^{-4}	1.02
XXI		2.1×10^{-5}	0.81
XXII		4.0×10^{-4}	0.68
XXIII		6.23×10^{-4}	0.63
XXIV		4.9×10^{-4}	0.62
XXV		1.2×10^{-3}	0.12
VII		6.0×10^{-4}	0
XXVI		7.2×10^{-4}	−0.04
XXVII		2.0×10^{-3}	−0.28[c]
XXVIII		2.1×10^{-3}	−0.61
XXIX		1.6×10^{-3}	−0.77[d]

[a] The π values in this table are derived from phenylacetic acid (Fujita *et al.*, 1964). [b] Substituent constant is for a three substituted group. [c] π constant is taken from benzene. [d] π value for NH_2 is taken from phenol. Assay conditions are described in Materials and Methods.

TABLE III: Irreversible Inhibition of Phosphorylase *b* by 8-Substituted Adenine Derivatives.[a]



Compd No.	R	% Irrev Inhibn	% Activn
XXX		0	0
XXXI		<5	0
XXXII		0	0
XXXIII		0	0
XXXIV		<5	0
XXXV		<5	0
XXXVI		30	0
XXXVII		0	0
XXXVIII		22	0
XXXIX		<5	0
XL		76	24

[a] Incubation mixture contained 2.21×10^{-5} M phosphorylase *b*, 0.04 M glycerophosphate, 0.002 M EDTA, 4.0×10^{-5} M inhibitor, and 2% dimethyl sulfoxide, at pH 7.8. Reaction was allowed to proceed 63 minutes, diluted 1:50 in 0.05 M glycerophosphate–0.05 M β-mercaptoethanol (pH 6.8) and then assayed in the presence and absence of AMP.

respondingly better competitive inhibitors. Compound XXI binds approximately twofold more tightly than the native activator, AMP ($K_{i,XXI} = 2 \times 10^{-5}$ M, $K_{a,AMP} = 5 \times 10^{-5}$ 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 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

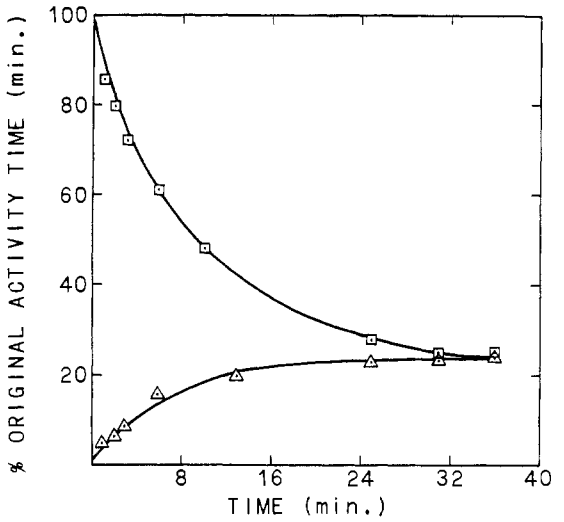


FIGURE 1: Inhibition and activation of phosphorylase *b* (2×10^{-5} M) by 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine (3.45×10^{-5} M) in 0.04 M glycerol-P–0.002 M EDTA (pH 7.8) and 11% dimethyl sulfoxide. Enzyme was diluted 50-fold in 0.05 M glycerol-P–0.05 M β-mercaptoethanol (pH 6.8) and assayed in 1% glycogen, 0.016 M glucose 1-phosphate, and 1×10^{-3} M AMP (upper curve □). Lower curve (△) did not contain AMP in the assay mixture.

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, 8-[*m*-(*m*-fluorosulfonylbenzamido)-benzylthio]adenine (XL) 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 XL was incubated with phosphorylase *b*. This time-dependent inactivation was demonstrated by removing aliquots from the incubation mixture, diluting 50-fold, and assaying in the presence of 10^{-3} M AMP, 0.016 M glucose 1-phosphate, and 1% glycogen at pH 6.8 (Figure 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 (Figure 1, lower curve).

Kinetics of Activation and Inhibition of Phosphorylase by Irreversible Inhibitors. A model for irreversible inhibition is shown in eq 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 (Kitz and Wilson, 1962).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_2} E\text{–}I \tag{1}$$

$$K_I = \frac{[E][I]}{[E\cdot I]} \tag{2}$$

$$V_{sat} = k_2[E\cdot I] \tag{3}$$

Under nonsaturation conditions, the integrated rate equation is

$$\ln \frac{[E']}{[E_0]} = \frac{-k_2 t}{K_I/[I]} + 1 \tag{4}$$

where $[E_0]$ is equal to total enzyme and $[E']$ equals concentration of enzyme not covalently modified.

The rate of inhibition can be obtained by plotting the log of the per cent activity *vs.* time. Compound XXXIX shows only inactivation (Table III) and therefore should display an inactivation pattern that can be predicted by the kinetic equations previously outlined. The pseudo-first-order rate constants are obtained from the slope of each line with five inhibitor concentrations. Equation 5 can be derived by substituting into eq 4 and rearranging (Kitz and Wilson, 1962)

$$\frac{1}{k_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \tag{5}$$

When the pseudo-first-order rate constants were plotted according to eq 5, and apparent binding constant, $K_D = 1.25 \times 10^{-3} \pm 0.20$, and a first-order rate constant at saturation, $k_2 = 0.05 \pm 0.01$ min^{-1} were evaluated for 8-[*m*-(*p*-fluorosulfonylbenzamido)benzylthio]adenine (XXXIX).

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 because these equations do not account for the ability of the meta analog 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 α, and by β for the specific activity of the modified enzyme. Then

$$A_0 = \alpha([E] + [E\cdot I]) \tag{6}$$

$$A_t = \alpha([E] + [E\cdot I]) + \beta[E\text{–}I] \tag{7}$$

$$A_t' = \beta[E\text{–}I] \tag{8}$$

where A_0 is equal to the activity of the enzyme not modified irreversibly, A_t equals total activity at any time, and A_t' is equal to the activity due to incorporation of analog measured in the absence of AMP.

$$[E'] = [E] + [E\cdot I] \tag{9}$$

$$A_t = \alpha[E'] + A_t \tag{10}$$

$$[E'] = \frac{A_t - A_t'}{\alpha} \tag{11}$$

$$\ln \frac{A_t - A_t'}{\alpha/(A_0/\alpha)} = \ln \left[\frac{A_t - A_t'}{A_0}\right] = \frac{k_2 t}{1 + K_I/[I]} \tag{12}$$

A plot of log $[A_t - A_t'/A_0]$ *vs.* time can be used to evaluate k_{app}. This is shown in Figure 2 for 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine binding to phosphorylase *b*. Linear lines are obtained at all inhibitor concentrations. In Figure 3, the pseudo-first-order rate constants evaluated from Figure 2 are plotted according to eq 4. 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 satura-

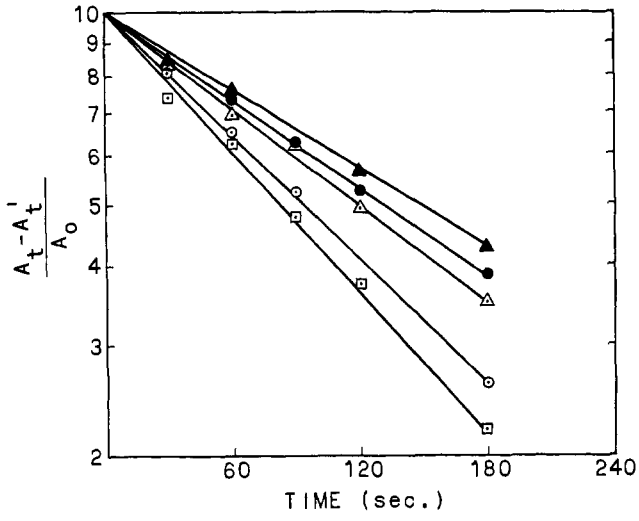


FIGURE 2: Rate of inactivation of phosphorylase *b* (2.10×10^{-5} M) as a function of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]-adenine concentration in 0.04 M glycerol-P–0.002 M EDTA (pH 7.8), and 11% dimethyl sulfoxide at 30° (▲) 9.0×10^{-5} M inhibitor, (●) 1.22×10^{-4} M, (△) 1.45×10^{-4} M, (⊙) 1.81×10^{-4} M, (⊡) 2.43×10^{-4} M.

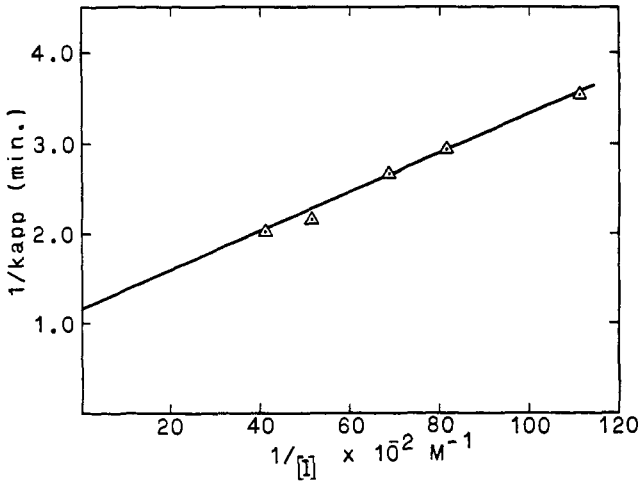


FIGURE 3: Reciprocal of the pseudo-first-order rate constants evaluated from Figure 2 as a function of the reciprocal 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine concentration. k_2 and K_D were evaluated from the *y* intercept and slope, respectively.

tion, $k_2 = 0.72 \pm 0.11$ min^{-1}, can be calculated from the slope and intercept of Figure 3. 8-[*m*-(*m*-fluorosulfonylbenzamido)benzythio]adenine binds approximately 14 times more tightly than the para-substituted derivative, XXXIX. The first-order rate constant at saturation also is approximately 14 times greater for meta derivative (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 min for XL and 13.86 min for XXXIX.

Preparation of 8-[m-(m-Fluorosulfonylbenzamido)benzylthio]-adenine-Modified Phosphorylase b and Determination of the Amount of Bound Adenine Analog. Reaction of phosphorylase *b* 2–2.3 $\times 10^{-5}$ M in 0.04 M glycerol-P–0.002 EDTA (pH 7.8) was reacted with 1.2–1.4 molar excess of 8-[*m*-(*m*-fluorosulfonlybenzamido)benzylthio]adenine. The reaction was allowed to proceed for 50 min at 30°. 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 (Figure 1). After 50 min, an equal volume of saturated ammonium sulfate was added. Following centrifugation, the pellet was dissolved in 0.05 M glycerol-P–0.05 M β-mercaptoethanol (pH 6.8) and dialyzed against the same buffer at 4° to remove excess adenine derivative. The modified enzyme crystallized during dialysis in the absence of AMP or Mg^{2+}. The modified enzyme was then recrystallized before analysis for the amount of analog bound. A 1.2–1.4 molar excess of analog to enzyme gives 0.93 ± 0.09 mol bound per mol of enzyme (Table IV). Reaction of phosphorylase *b* with a five-fold excess of analog to enzyme for an extended time will result in more than 1 mol of adenine derivative bound per mol of enzyme. When more than 1 mol of analog was bound per mol of enzyme, there was a loss of enzymic activity to a level lower than the enzymatic activity observed when 1 mol of analog is bound per mol 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. At an inhibitor concentration of 3.6×10^{-4} M and in the absence of AMP, 57% of the enzymic activity was lost in 6 min. Under similar conditions, but in the presence of 7.26×10^{-3} M AMP in the preincubation mixture with the adenine derivative, less than 2% loss in activity was observed.

Discussion

The use of structural analogs to study the role of activators is a useful and well-established technique. In this paper, with the aid of adenine analogs, we have attempted to establish the nature of the AMP binding site in glycogen phosphorylase *b*. The binding of adenine and also hypoxanthine analogs 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 estab-

TABLE IV: Binding of 8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine to Phosphorylase *b*.

Molar Ratio[a]	Amount Bound[b]	Sp Act. (μmol of P_i/min per mg)[c]
1.4	0.93 ± 0.1	9.91
5	1.8 ± 0.1	7.00

[a] Molar ratio of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine to phosphorylase *b* in the incubation mixture. [b] Moles of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine bound per mole of enzyme. [c] Assay mixture consisted of 0.016 M glucose 1-phosphate, 1% glycogen, and 0.025 M β-mercaptoethanol–0.025 M β-glycerophosphate (pH 6.8). Incubation conditions and assay methods are described in text. Aliquots were removed after incubation for 1 hr at 30° and assayed for enzymic activity.

lishing the hydrophobic nature of the adenine analogs. 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 π values from seven different parent compounds (systems) and concluded that, while π 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 π 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 π 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 π 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 affect the binding of the adenine analogs 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_2F group with the enzyme must be formed before the SO_2F group will react and form a covalent linkage (Baker *et al.*, 1966). Two sulfonyl fluoride derivatives, 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*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine (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 analog 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 XXXIX, which is similar to 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*-(*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 analog from the protein.

Activation of phosphorylase *b* by 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine is good evidence that this analog 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 1 mol of analog is bound per mol of enzyme, maximal activity is observed.

Hulla and Fasold (1972) also have affinity labeled the allosteric site of glycogen phosphorylase *b*. Hulla and Fasold used 6-(purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid)thioether to label the AMP binding site, but modification could not be carried further than 35% because of precipitation of the enzyme. Modification with this compound did yield an enzyme form that was enzymically active.

A detailed characterization of the 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified enzyme, including isolation and purification of the peptide to which the derivative is bound, will be discussed in the following paper (Anderson *et al.*, 1973).

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