Chemistry of the Adenosine Monophosphate Site of Rabbit Muscle Glycogen Phosphorylase. II. Properties of 8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine-Modified Phosphorylase†

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ABSTRACT: The enzymic and physical properties of rabbit skeletal muscle phosphorylase *b* in which the allosteric binding site has been covalently modified with 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine were investigated. When assayed at high glucose 1-phosphate levels (75 mM), this form of phosphorylase *b* exhibited 30% of the activity that would be observed with the native enzyme at saturating levels of adenosine monophosphate. The amount of activity of the modified enzyme was proportional to the amount of analog bound, reaching maximal activity when 1 mol of analog was bound per mol of enzyme. The irreversibly modified phosphorylase *b* displayed a decreased affinity for its substrates, glucose 1-phosphate (K_m = 35 mM) and glycogen (K_m = 0.05%). It also was more heat stable than native phosphorylase and more difficult to resolve free of pyridoxal phosphate. Adenosine monophosphate had no affect on enzymic activity of the modified enzyme. The irreversibly modified enzyme could be converted to phosphorylase *a* by phosphorylase kinase and back to phosphorylase *b* by phosphorylase phosphatase. Modified phosphorylase *a* displayed 50% of the activity of native enzyme and also had affinities similar to those of the native enzyme for the substrates, glucose 1-phosphate and glycogen. A pentapeptide to which 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine was bound was isolated; the peptide contained 2 mol of glycine, 2 mol of alanine, and 1 mol of tyrosine. The site of attachment is suggested to be the tyrosyl residue.

Over the past 50 years, three facts have emerged to serve as the basis for active site directed irreversible inhibitors that can specifically label an enzyme at or near the active site by covalent modification: (a) enzymes are macromolecules that can form complexes with substrates and inhibitors, (b) enzymes have functional groups on their surfaces that can be attacked by chemical reagents with the formation of a covalent linkage, and (c) neighboring group reactions can be accelerated as much as 10,000-fold over the same chemical reaction by a bimolecular process (Baker, 1967).

In the preceding paper (Anderson and Graves, 1973), we presented evidence that 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine binds covalently to the allosteric site of glycogen phosphorylase *b*, yielding an enzyme form that is active in the absence of AMP.[1] In this manuscript, the properties of the 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]-adenine-modified phosphorylase and the role of this analog in the interconversion reactions of phosphorylase were investigated.

A preliminary report of this research has been presented (Graves *et al.*, 1972).

Materials and Methods

Isolation of phosphorylase *b*, preparation of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b* and determination of protein concentration have been described in the preceding paper (Anderson and Graves, 1973).

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 β-mercaptoethanol, glucose-6-phosphate dehydrogenase, phosphoglucomutase, and phosphorylase (pH 7.5). Glucose-6-phosphate dehydrogenase, phosphoglucomutase, and NADP were obtained from Calbiochem.

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°. Sedimentation coefficients were determined with a Nikon Model 6C microcomparator and were corrected for viscosity of the buffer to water at 20°.

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-labeled 8-[*m*-(*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

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[1] Abbreviations used are: glycerol-P, β-glycerophosphate; AMP, adenosine 5′-monophosphate; NADP, nicotinamide adenine dinucleotide phosphate; pyridoxal-P, pyridoxal 5′-phosphate.

buffer previously equilibrated at 52°. 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°. A zero time point was obtained by making a separate dilution into buffer at 30°.

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}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}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° for 24 hr. Tryptophan analysis was determined by the method of Matsubara and Sasaki (1969).

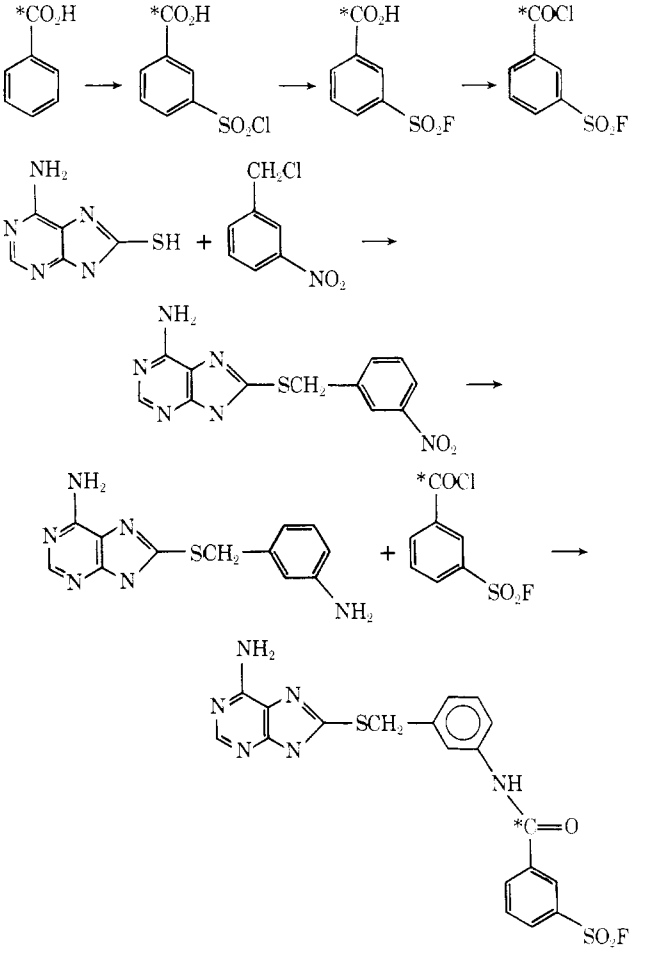
High-voltage electrophoresis was performed with Whatman No. 3MM paper in pyridine–acetic acid–water buffer (1:3.4:409) (pH 4.0) with a Gilson Model D high-voltage electrophorator.

Thin-layer chromatography was performed on precoated silica gel sheets obtained from Eastman Organic.

Synthesis of ^{14}C-Labeled 8-[m-(m-Fluorosulfonylbenzamido)-benzylthio]adenine. [^{14}C]Benzoic acid was purchased from New England Nuclear. 6-Amino-8-purinethiol was purchased from Aldrich Chemical Co. α-Chloro-*m*-nitrotoluene was purchased from Eastman. [^{14}C]*m*-Chlorosulfonylbenzoic acid was synthesized from [^{14}C]benzoic acid (Smiles and Stewart, 1921). [^{14}C]*m*-Fluorosulfonylbenzoyl chloride was prepared in two steps from [^{14}C]*m*-chlorosulfonylbenzoic acid (De Cat *et al.*, 1965). [^{14}C]8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine was prepared from 6-amino-8-thioadenine, α-chloro-*m*-nitrotoluene, and [^{14}C]*m*-fluorosulfonylbenzoyl chloride in three steps (Baker and Kozma, 1968). The overall synthetic scheme is shown in Scheme I. [^{14}C]-8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine showed all the properties of the published material and showed only one radioactive spot with thin-layer chromatography. The specific activity of [^{14}C]-8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]-adenine was 0.011 Ci/mol.

Isolation of Peptide that Binds 8-[m-(m-Fluorosulfonylbenzamido)benzylthio]adenine. Phosphorylase *b* at 2–2.5 mg/ml in 0.04 M glycerophosphate–0.002 M EDTA (pH 7.8) was reacted with an 0.9 molar ratio of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine to native phosphorylase *b* for 45 min at 30°. The enzyme was then precipitated with an equal volume of saturated ammonium sulfate. After centrifugation, the pellet was dissolved in 0.05 M glycerophosphate–0.05 M β-mercaptoethanol (pH 6.8) and dialyzed against the same buffer for 8 hr. The enzyme crystallized during dialysis; the crystals were centrifuged and dissolved in 0.02 M glycerophosphate–0.02 M β-mercaptoethanol 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 hr. At this time, the pH was adjusted again to pH 1.9, and the same amount of pepsin was added. After 15 hr, the digested enzyme solution was lyophilized to dryness. The sample was dissolved in water and applied to a charcoal–Celite column. A charcoal–Celite column (0.5 × 2.5 cm) was prepared by mixing Celite 560–acid-washed Norit A (2:1, wt). Quantitative binding of the

SCHEME I



^{14}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}C-labeled peptide was eluted with 3% ammonia in 50% acetone (Shapiro and Stadtman, 1968) and evaporated to dryness. At this point, the ^{14}C-labeled peptide appeared as one spot on ascending chromatography (butanol–acetic acid–H_2O, 4:1:1) on precoated, 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-X2 column (1 × 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 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-[*m*-(*m*-fluorosulfonyl-

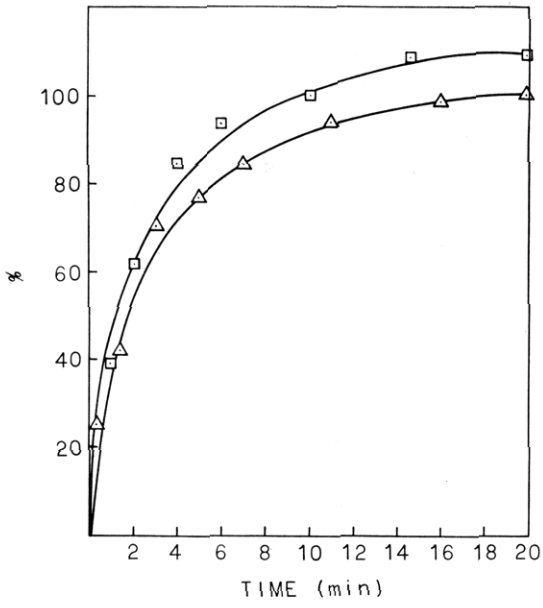


FIGURE 1: Effect of the amount of incorporation of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine on enzymatic activity. Phosphorylase *b* (9.53×10^{-5} M) in 0.04 M glycerol-P–0.002 M EDTA (pH 7.8) in the presence of 1.6×10^{-4} M 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine. (□) Per cent maximal incorporation of [^{14}C]-8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine (maximal incorporation was defined as 1 mol of analog incorporated per mol of enzyme); (△) per cent maximal phosphorylase activity.

benzamido)benzylthio]adenine was covalently bound was composed of 2 mol of alanine, 2 mol of glycine, and 1 mol of tyrosine. The peptide to which the adenine derivative is bound absorbs maximally at 285 nm in 10% acetic acid. This spectrum is almost identical with the spectrum of free 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine in 10% acetic acid ($A_{285} = 2.04 \times 10^5$).

Results

Incorporation of [^{14}C]-*8-[m-(m-Fluorosulfonylbenzamido)benzylthio]adenine into Phosphorylase b.* Incorporation of [^{14}C]-8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine corresponded with the increase in phosphorylase activity when the modified enzyme was assayed in the absence of AMP (Figure 1). Phosphorylase *b* activity increased to a specific activity of 20.8 μmol of P_i/min per mg of protein (0.075 M glucose 1-phosphate in assay). Native enzyme, when assayed under similar conditions, had a specific activity of 62.1 μmol of P_i/min per mg of protein in the presence of saturating level of AMP (1×10^{-3} M). The activity of the isolated modified enzyme was not increased by the addition of AMP from 2.5×10^{-5} to 2×10^{-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 I, 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; Kastenschmidt *et al.*, 1968), ultra-

TABLE I: Effect of Glucose 6-Phosphate on Activity of Native and 8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine-Modified Phosphorylase *b*.[a]

	Specific Activity	
	10 mM Glucose-6-P	No Glucose-6-P
Native phosphorylase *b*	4.4	37.9
Modified phosphorylase *b*	9.4	9.3

[a] Specific activity (μmol of P_i/min per mg of protein) was determined in 0.016 M glucose 1-phosphate, 1% glycogen, 1×10^{-3} M AMP, 0.025 M β-glycerophosphate, and 0.025 M β-mercaptoethanol (pH 6.8).

centrifugation experiments were performed to determine whether 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine and AMP had similar effects on the state of aggregation of phosphorylase *b*. The adenine derivative modified phosphorylase *b*, prepared according to the procedure of Anderson and Graves (1972), sediments primarily with an $s_{20,w}$ of 12.7 S (Figure 2a) in the absence of AMP. The quaternary structure of the modified phosphorylase *b* is similar to native phosphorylase *b*, which, in the presence of 1×10^{-3} M AMP, has an $s_{20,w}$ of 12.8 S (Figure 2b, lower curve). The upper curve (Figure 2b) 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 5×10^{-5} M AMP, the ultracentrifugal pattern of the modified enzyme remained unchanged, whereas native phosphorylase *b* showed significant amounts of the tetrameric form. With 1×10^{-3} M AMP (Figure 2a, 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 of modified phosphorylase *b* also is

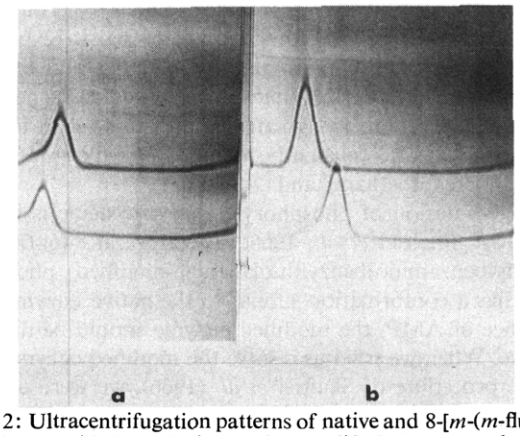


FIGURE 2: Ultracentrifugation patterns of native and 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b*. Enzymes were centrifuged at 52,000 rpm at 12.8° in 0.05 M glycerol-P–0.05 M β-mercaptoethanol buffer (pH 6.8). Lower patterns with 1×10^{-3} M AMP; upper patterns, no additions. (a) Modified phosphorylase *b* (5.59 mg/ml); (b) native enzyme (5.55 mg/ml). Direction of sedimentation is from left to right.

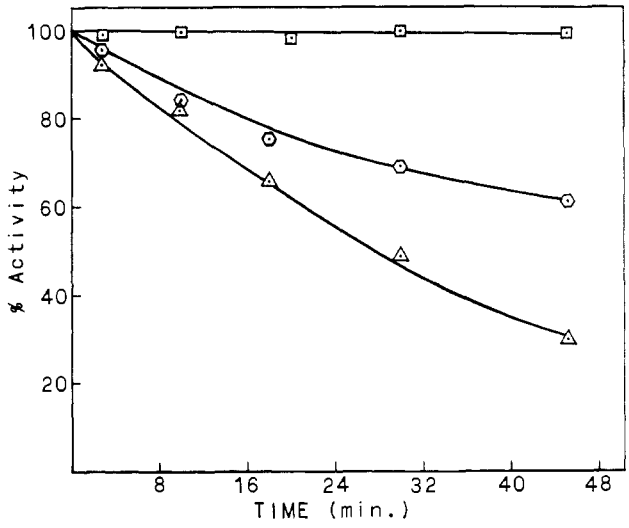


FIGURE 3: Heat stability of native and 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b*. Native phosphorylase *b* 11.85 mg/ml (△) was diluted 1:100 in 0.05 M glycerol-P–0.05 M β-mercaptoethanol at 52°; (⊙) same but buffer contained 0.001 M AMP. Modified phosphorylase *b* 13.00 mg/ml (⊡) diluted 1:100 in 0.05 M glycerol-P–0.05 M β-mercaptoethanol (pH 6.8). Aliquots were removed at designated times and assayed for inorganic phosphate released at 30°. Time on the abscissa refers to time of incubation at 52°.

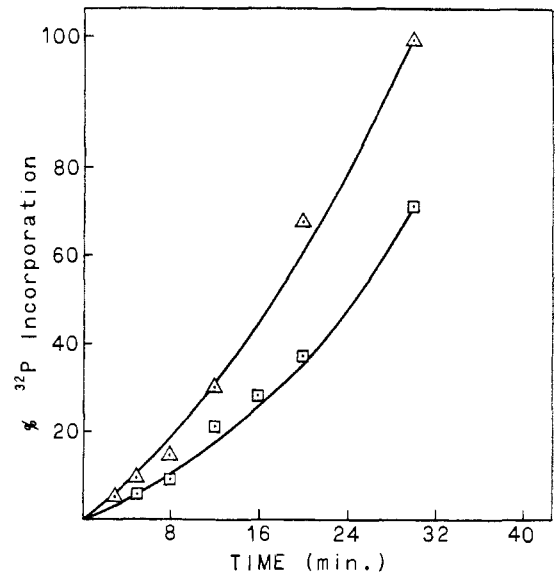


FIGURE 4: Rate of phosphorylation by phosphorylase *b* kinase of native and 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b*. The reaction mixture contained 0.006 M ATP, 0.020 M Mg^{2+}, 0.020 M glycerol-P, 0.020 M Tris, 0.4 μg of phosphorylase *b* kinase and native phosphorylase *b* (7.60 mg/ml) (△), or modified phosphorylase *b* (7.68 mg/ml) (⊡).

temperature dependent; at 23°, 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 3 is a comparison of the heat stability of modified phosphorylase *b* and native phosphorylase *b* in the presence (1×10^{-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 β-mercaptoethanol, presumably because L-cysteine can remove the pyridoxal-P from the holoenzyme, but β-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 rate of inactivation was greater in L-cysteine than in β-mercaptoethanol. The covalently modified enzyme was stable in both β-mercaptoethanol and L-cysteine.

The resolution of phosphorylase by L-cysteine is inhibited by AMP (Shaltiel *et al.*, 1966). Therefore, if 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b* has a conformation similar to the native enzyme in the presence of AMP, the modified enzyme should be diffcult 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-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]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°, it had no activity when assayed at 52°. In this experiment, we incubated modified enzyme and substrate (0.032 M glucose-phosphate–2% glycogen, pH 6.8) separately at 52° 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°, 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°.

Interconversion of Modified Phosphorylase b. The conversion of phosphorylase *b* to phosphorylase *a* is relatively insensitive to adenosine monophosphate; AMP at 2×10^{-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*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified and native phosphorylase *b*. This is demonstrated in Figure 4, where the conversion of modified phosphorylase *b* is compared with native phosphorylase *b*. The specific activity of the modified enzyme increased from 9.2 μmol of P_i/min per mg of protein in the nonphosphorylated form to 29.6 μmol of P_i/min per mg 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 II). 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 μmol of P_i/min per mg of protein. Thus, upon

TABLE II: Kinetic Characteristics of Native and 8-[*m*-(*m*-Fluorosulfonylbenzamido)benzylthio]adenine-Modified Phosphorylase.

	Analog *b*	Analog *a*	Native *b*	Native *a*
K_m (glucose 1-phosphate (mM)	35	5	6	5.5[a]
K_m (glycogen) (%)	0.05	0.02	0.02	0.021[a]
Specific activity: μmol of P_i/min per mg of protein				
16 mM glucose-1-phosphate	9.9	29.6	37.9	43.8
75 mM glucose-1-phosphate	20.8	36.3	62.1	69.8

[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 β-mercaptoethanol–0.025 M glycerophosphate (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, 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 1×10^{-3} M AMP. All other measurements were in the absence of AMP.

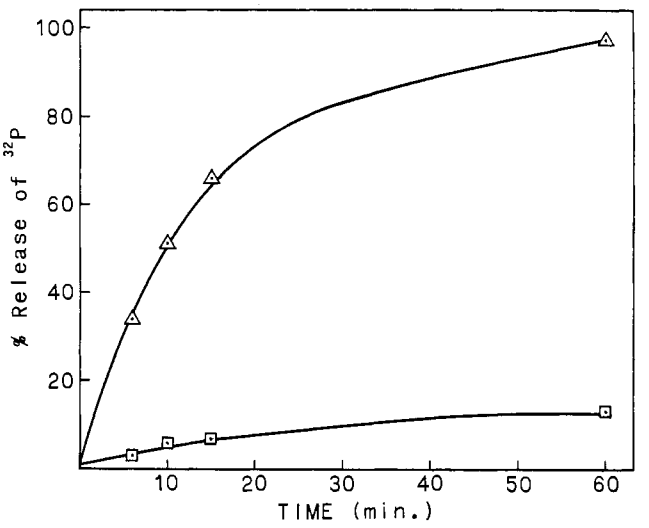


FIGURE 5: Rate of dephosphorylation by phosphorylase phosphatase of native and 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *a*. The reaction mixture contained native phosphorylase *a* (△) (0.24 mg/ml), modified phosphorylase *a* (□) (0.3 mg/ml), in 0.05 M Tris-acetate–0.001 M dithiothreitol (pH 7.5). Aliquots were removed at designated times and assayed for ^{32}P released.

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 phosphorylase *b* should be observed. This is demonstrated in Figure 5, where the dephosphorylation of the modified phosphorylase occurs at a much slower rate than that of the native enzyme.

Discussion

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 (Figure 2) of the modified phosphorylase *b* is nearly identical with 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°, 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 analog. It can be seen in Figure 3 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°. 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 5, 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 in our laboratory 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*-(*m*-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*-(*m*-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-phos-

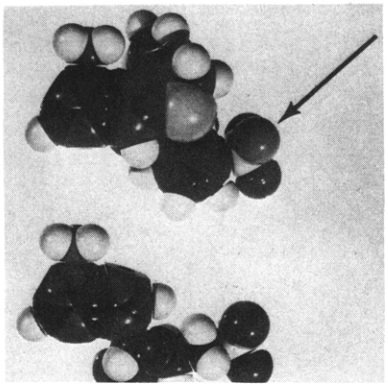


FIGURE 6: Space-filling models of 8-[*m*-(*m*-fluorosulfonylbenzamido)-benzylthio]adenine and adenosine monophosphate. Arrow denotes the sulfonyl fluoride group.

phate 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*-fluorosulfonylbenzamido)benzylthio]adenine may be intermediate between the conformation induced by inosine monophosphate (Black and Wang, 1968) and AMP. Both 8-[*m*-(*m*-fluorosulfonylbenzamido)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*-fluorosulfonylbenzamido)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 II).

In Figure 6 are space-filling models of 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine (upper figure) and AMP (lower figure). Although the two molecules possess obvious differences, it can be seen that, in 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine, the SO_2F 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 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 [^{14}C]-8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine-modified phosphorylase *b* contained 2 mol of glycine, 2 mol of alanine, and 1 mol of tyrosine. The analysis of the sequence of this peptide will be the subject of a separate communication. 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-P (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. In addition to its effect on rabbit glycogen phosphorylase, this analog also has been shown to activate lobster glycogen phosphorylase *b* (R. A. Anderson and D. J. Graves, 1972, unpublished data) and sheep heart phosphofructokinase (T. Mansour, 1972, personal communication).

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