with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole leads to complete inactivation of the enzyme, while adenine nucleotide substrates produce a differential effect on the rate of reaction of the two groups reminiscent of the reactivity of the two SH groups of myosin in the presence of ADP and ATP.

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Oxidation and Sulfonation of the Highly Reactive Sulfhydryl Groups of Muscle Phosphorylase *b*†

Alfred Schwab‡ and Aaron Lukton*

ABSTRACT: The reaction of muscle phosphorylase *b* with a threefold molar excess of *o*-iodosobenzoate leads to aggregation of the enzyme and a 94% loss of enzyme activity. Substrates and AMP have little or no effect on the inactivation of phosphorylase *b* by *o*-iodosobenzoate. Dithiothreitol restores the activity of *o*-iodosobenzoate-inactivated phosphorylase *b* to 92% of the control activity and causes reformation of a dimeric structure which is similar to that of the native enzyme. Treatment of oxidized phosphorylase *b* with $^{35}SO_3^{2-}$ results in the sulfonation of 1.3–1.6 sulfhydryl groups/molecule of phosphorylase *b* dimer. The S-sulfonated enzyme has 1.3–2.0 fewer sulfhydryl groups available for rapid reaction with iodoacetamide than native phosphorylase *b*. Phosphorylase *b* that has 3.0–3.2 rapidly reacting sulfhydryl groups alkylated, by reaction with iodoacetamide, retains 73–85% of its activity after reaction with *o*-iodosobenzoate. It is concluded that those sulfhydryl groups of phosphorylase *b* that react rapidly with iodoacetamide can be oxidized by *o*-iodosobenzoate and subsequently sulfonated by reaction with sulfite ion. The inactivation of phosphorylase *b* by *o*-iodosobenzoate is attributed to the change in the state of aggregation of the enzyme that occurs when the highly reactive sulfhydryl groups are oxidized. S-Sulfonated phosphorylase *b* has 59–68% of the control activity and, in the absence of sulfite ion, sediments in the ultracentrifuge as a mixture of dimer (major component) and tetramer. S-Sulfonation of the highly reactive sulfhydryl groups of phosphorylase *b* leads to a loss in the homotropic cooperativity of AMP sites and a threefold increase in the K_m for AMP; these effects can be reversed by dithiothreitol.

Battell *et al.* (1968a) found that the reaction of two sulfhydryl groups of rabbit skeletal muscle phosphorylase *b* with iodoacetamide, or with various other sulfhydryl reagents, does not cause any loss of enzyme activity. Recently, Zarkadas *et al.* (1970) have reported that 3.1 sulfhydryl groups of freshly prepared phosphorylase *b* can react rapidly with iodoacetamide without loss of enzyme activity.

Gold and Blackman (1970) have shown that up to 3.6 sulfhydryl groups of phosphorylase *b* are exceptionally reactive with 2,4-dinitrochlorobenzene and have concluded that these sulfhydryl groups are identical with those that react rapidly with iodoacetamide. However, dinitrophenylation of the rapidly reacting sulfhydryl groups of phosphorylase *b* leads to a decrease in the affinity of the enzyme for AMP and glucose 1-phosphate, and a small decrease in the V_{max} (Gold, 1968). Apparently, modification of the same sulfhydryl groups of phosphorylase *b* with different reagents can lead to different effects on enzyme activity.

Aggregates of phosphorylase *b* that disappear upon addition of mercaptoethanol are sometimes seen in the ultracentrifuge (Madsen and Cori, 1956; Seery *et al.* (1967)). Battell *et al.* (1968b) have suggested that such aggregates are due to the formation of intermolecular disulfides by the highly reactive sulfhydryl groups. These investigators also suggested that the reactive sulfhydryl groups may play a role in regulating the ac-

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tivity of phosphorylase *b*.

The data presented here indicate that incubation of phosphorylase *b* with *o*-iodosobenzoate results in oxidation of the highly reactive sulfhydryl groups, aggregation of the enzyme, and a 94% decrease in enzyme activity; these effects are reversed by dithiothreitol.

Materials and Methods

Oyster glycogen was purchased from Calbiochem and purified by the method of Somogyi (1957). Glucose 1-phosphate, AMP, Nbs_2,[1] iodoacetamide, and dithiothreitol were also obtained from Calbiochem. *o*-Iodosobenzoic acid, from Mann Research Laboratories, was purified as described by Chinard and Hellerman (1954). Stock solutions of *o*-iodosobenzoate (0.1 M) were prepared by dissolving *o*-iodosobenzoic acid in enough 1 M NaOH to give a final concentration of 0.1 M. ^{35}S-Labeled sodium sulfite (lot 659-152), which had an initial specific activity of 27 Ci/mol, and [1-^{14}C]iodoacetamide (lot 252-152), with a specific activity of 3.1 Ci/mol, were purchased from New England Nuclear Corp. The ^{14}C-labeled iodoacetamide was dissolved in hot water and crystallized with an excess of nonradioactive iodoacetamide which had previously been recrystallized from water.

Phosphorylase *b* was prepared from frozen rabbit skeletal muscle (Pel-Freez Biologicals, Inc.) by the method of Fischer and Krebs (1962), as modified by DeLange *et al.* (1968), except that 0.005 M dithiothreitol replaced mercaptoethanol. The enzyme was recrystallized three times after preparation and weekly thereafter as described by Gold and Blackman (1970). Immediately before use, enzyme crystals were centrifuged out of suspension, dissolved in the appropriate buffer, and passed through a Sephadex G-25 column (0.9 × 23 cm) which had previously been equilibrated with the same buffer. The protein concentration was determined from the absorbance at 280 nm, using a value of 13.1 for $E_{1cm}(1\%)$; molar concentrations of phosphorylase *b* are based on a molecular weight of 200,000 (Cohen *et al.*, 1971).

Phosphorylase activity was measured by the zero-order method of Hedrick and Fischer (1965), except the 0.001 M EDTA replaced mercaptoethanol.

S-Sulfonated phosphorylase *b* was prepared from enzyme that had been oxidized with *o*-iodosobenzoate as described in the legend to Table I.

The reaction mixture contained oxidized phosphorylase *b*, 3.2 mg/ml, and 0.01 M $Na_2{}^{35}SO_3$. The buffer was 0.05 M sodium barbital–0.001 M EDTA–0.8 M NaCl (pH 8.0). After incubation for 30 min at 25°, the enzyme was precipitated by addition of an equal volume of 0.05 M sodium β-glycerophosphate–0.001 M EDTA saturated with ammonium sulfate at pH 6.8. Following centrifugation at 0°, the enzyme was dissolved in the glycerophosphate buffer and precipitated again with ammonium sulfate. The precipitate was centrifuged, dissolved in the glycerophosphate buffer, and passed through a Sephadex G-25 column (0.9 × 23 cm) equilibrated with the same buffer. Parallel samples of unoxidized phosphorylase *b* were carried through the same procedure and usually contained 0.04 mol of $^{35}SO_3{}^{2-}$/mol of enzyme.

The incorporation of radioactive reagents into phosphorylase *b*, or modified phosphorylase *b*, was determined by pipetting aliquots of enzyme solutions into filter funnels containing 5 ml of cold 5% trichloroacetic acid. After filtration through a Millipore HAWP 02400 filter, the precipitated protein was washed four times with 5-ml portions of cold 5% trichloroacetic acid.

[1] Abbreviation used is: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE I: The Effect of Reducing Agents on Native and *o*-Iodosobenzoate–Inactivated Phosphorylase *b*.[a]

Reducing Agent	Enzyme Activity: Native Phosphorylase *b*	Enzyme Activity: *o*-Iodosobenzoate-Treated Phosphorylase *b*
None	100	6
Dithiothreitol, 0.002 M	105	97
Glutathione, 0.02 M	99	90
NaCN, 0.02 M	104	10
Na_2SO_3, 0.02 M	103	76

[a] Phosphorylase *b*, 4.0 mg/ml, was incubated for 10 min with, and without, *o*-iodosobenzoate, 6.0×10^{-5} M, in 0.05 M sodium barbital–0.001 M EDTA–1.0 M NaCl at pH 8.0 and 25°. Aliquots containing 200 μg of enzyme were then diluted to 1.0 ml with 0.05 M sodium barbital–0.001 M EDTA which contained the indicated concentration of reducing agent at pH 8.0. After 30 min at 25°, aliquots were diluted tenfold in the assay buffer described in the legend to Figure 1.

The filters were placed in scintillation vials and air-dried. After addition of 10 ml of scintillation fluid, samples were counted to an error of 1% in a Picker Nuclear Liquimat 220 liquid scintillation counter. When ^{35}S was the isotope being counted, the scintillation fluid was a toluene solution containing 4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene per liter; Bray's solution (Bray, 1960), without ethylene glycol, was used when samples of ^{14}C were counted. The cpm were corrected using quench correction curves obtained by the channels-ratio method (Bush, 1963).

Titrations of phosphorylase *b* and S-sulfonated phosphorylase *b* with Nbs_2 were carried out according to Ellman (1959). The concentrations of enzyme and Nbs_2 were 5.0×10^{-6} and 1.0×10^{-4} M, respectively. The buffer was 0.05 M sodium β-glycerophosphate–0.001 M EDTA (pH 6.8) at 24°.

Sedimentation velocity experiments were carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics and an RTIC unit for temperature control. Generally, two single sector cells, one of which contained a +1° wedge window, were used in an An-D rotor. When solutions of phosphorylase *b* contained high concentrations of salt, a partial specific volume of 0.765 was used in the calculation of the sedimentation coefficient (Cohen *et al.*, 1971). Sedimentation coefficients were corrected for the viscosity and density of buffer relative to water.

Results

Inactivation of Phosphorylase b by o-Iodosobenzoate. *o*-Iodosobenzoate is known to oxidize low molecular weight thiols to disulfides (Hellerman *et al.*, 1941) and has been frequently used as an inhibitor of sulfhydryl enzymes (Webb, 1966). The inactivation of phosphorylase *b* by *o*-iodosobenzoate is complete in 10 min at 25°. Figure 1 shows that the loss of enzyme activity is linear with *o*-iodosobenzoate concentration until the concentration of this reagent is 1.5 times the concentration of the enzyme. Further inactivation requires more than a stoichio-

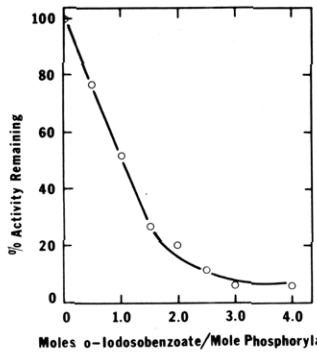

FIGURE 1: The stoichiometry of *o*-iodosobenzoate inactivation of phosphorylase *b*. Reaction mixtures contained phosphorylase *b*, 1.0×10^{-5} M, 0.05 M sodium barbital, 0.001 M EDTA, 1.0 M NaCl, and *o*-iodosobenzoate at pH 8.0. After incubation for 10 min at 25°, aliquots were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, and 1 mg/ml of bovine serum albumin at pH 6.5 for assay of phosphorylase *b* activity.

metric amount of *o*-iodosobenzoate. Incubation of phosphorylase *b* with a threefold molar excess of *o*-iodosobenzoate results in the loss of about 94% of the enzyme activity.

Reactivation of o-Iodosobenzoate-Inactivated Phosphorylase b by Reducing Agents. As shown in Table I, the inactivation of phosphorylase *b* by *o*-iodosobenzoate is almost completely reversed by dithiothreitol or glutathione. Treatment of oxidized phosphorylase *b* with sodium sulfite also resulted in considerable reactivation of the enzyme, while sodium cyanide was almost ineffective in restoring enzyme activity. The reactivation of *o*-iodosobenzoate-inactivated phosphorylase *b* by reducing agents suggests that enzyme inactivation is associated with disulfide bond formation.

Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b. To determine whether inactivation of phosphorylase *b* by *o*-iodosobenzoate leads to changes in quaternary structure, the sedimentation behavior of native and *o*-iodosobenzoate-inactivated phosphorylase *b* was determined after gel filtration as described in the legend to Figure 2a. The activity of the *o*-iodosobenzoate-treated enzyme did not change after gel filtration. As shown in Figure 2a, the sedimentation velocity pattern of *o*-iodosobenzoate-inactivated phosphorylase *b* is considerably altered compared with that of the native enzyme. The polydisperse nature of the inactivated enzyme is apparent from the broad sedimentation pattern (Figure 2a, lower pattern). The sedimentation coefficient, $s_{20,w}$, was 8.1 S for the control enzyme, and was estimated as 12.2 S for the oxidized enzyme.

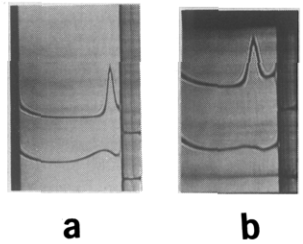

FIGURE 2: Sedimentation velocity patterns of native and *o*-iodosobenzoate-inactivated phosphorylase *b*. Ultracentrifugation was carried out at 48,000 rpm and 20°. Sedimentation proceeds to the left. Phosphorylase *b*, 4.0 mg/ml, was oxidized with *o*-iodosobenzoate as indicated in the legend to Table I and immediately treated as described below. (a) The native and oxidized enzymes were subjected to gel filtration on a Sephadex G-25 column (0.9 × 23 cm) equilibrated with 0.05 M sodium barbital–0.001 M EDTA–1.0 M NaCl (pH 8.0) before ultracentrifugation. Upper pattern, phosphorylase *b*, 3.3 mg/ml; lower pattern, *o*-iodosobenzoate-inactivated phosphorylase *b*, 3.5 mg/ml. The picture was taken 16 min after the rotor reached speed. (b) Aliquots (0.9 ml) of oxidized phosphorylase *b* were incubated with, and without, dithiothreitol for 30 min at 25° before ultracentrifugation. Upper pattern, o-iodosobenzoate-inactivated phosphorylase *b*, 3.6 mg/ml, with 0.002 M dithiothreitol; lower pattern, *o*-iodosobenzoate-inactivated phosphorylase *b*, 3.6 mg/ml. The picture was taken 39 min after the rotor reached speed.

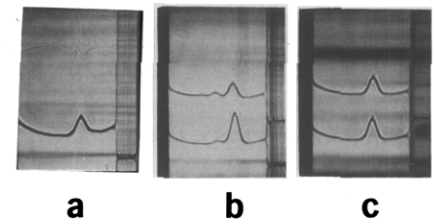

FIGURE 3: Sedimentation velocity patterns of native and S-sulfonated phosphorylase *b* with, and without, dithiothreitol. Phosphorylase *b*, 4.0 mg/ml, was oxidized with *o*-iodosobenzoate as described in the legend to Table I; the final volume was 3.0 ml. The oxidized enzyme and control samples were incubated with Na_2SO_3 for 30 min as described under Materials and Methods. (a) *o*-Iodosobenzoate-inactivated phosphorylase *b*, 3.2 mg/ml, in 0.05 M sodium barbital–0.001 M EDTA–0.8 M NaCl containing 0.01 M Na_2SO_3 at pH 8.0. (b) Upper pattern, S-sulfonated phosphorylase *b*, 3.2 mg/ml; lower pattern, native phosphorylase *b*, 3.2 mg/ml. (c) Aliquots of the enzyme solutions used for Figure 3b were treated with 0.005 M dithiothreitol for 1 hr at 25° before ultracentrifugation. Upper pattern, S-sulfonated phosphorylase *b*, 2.7 mg/ml; lower pattern, native phosphorylase *b*, 2.7 mg/ml. The buffer in b and c was 0.05 M sodium β-glycerophosphate–0.001 M EDTA (pH 6.8). Ultracentrifugation was carried out at 56,000 rpm and 20° in each experiment; the direction of sedimentation is to the left. The photographs in a, b, and c were taken 34, 33, and 37 min after the rotor reached speed.

Effect of Dithiothreitol on the Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b. After incubation of *o*-iodosobenzoate-inactivated phosphorylase *b* with dithiothreitol, the sedimentation velocity pattern of the oxidized enzyme resembles that of the native enzyme (Figure 2b, upper pattern). The $s_{20,w}$ was 11.6 S for the oxidized enzyme. After incubation with dithiothreitol, the oxidized enzyme had an $s_{20,w}$ of 8.5 S. The specific activity of oxidized phosphorylase *b* increased from 4 to 90% of the control activity following treatment with dithiothreitol.

Sedimentation Velocity Pattern of o-Iodosobenzoate-Inactivated Phosphorylase b in the Presence of Sulfite Ion. Incubation of *o*-iodosobenzoate-inactivated phosphorylase *b* with 0.01 M Na_2SO_3 changes the sedimentation velocity pattern of the oxidized enzyme (Figure 3a) and restores its activity to 68–78% of the control activity. While the *o*-iodosobenzoate–inactivated enzyme sedimented as a single boundary in the presence of sulfite ion, the $s_{20,w}$ was 11.2 S in the barbital buffer described in the legend to Figure 3a. The $s_{20,w}$ of native phosphorylase *b* was 8.2 S under identical conditions. This suggests that the sulfite-reactivated enzyme associates under conditions in which the native enzyme does not.

Incorporation of $^{35}SO_3^{2-}$ into o-Iodosobenzoate-Inactivated Phosphorylase b. The reaction of sulfite ion with a disulfide proceeds as indicated in equation 1 (Cecil, 1963). The reactivation of *o*-iodosobenzoate-inactivated phosphorylase *b* by sulfite ion supports the view that inactivation involves disulfide bond formation.

$$RSSR + SO_3^{2-} = RSSO_3^- + RS^- \quad (1)$$

To gain information regarding the number of sulfhydryl groups of phosphorylase *b* that are modified by reaction with *o*-iodosobenzoate, the oxidized enzyme was incubated with $Na_2{}^{35}SO_3$ as described under Materials and Methods. Prelimi-

TABLE II: The Effect of *o*-Iodosobenzoate on [1-^{14}C]Iodoacetamide–Phosphorylase *b*.[a]

moles of [1-^{14}C]Iodoacetamide Incorporated / mole of Phosphorylase *b*	Activity after *o*-Iodosobenzoate Treatment (%)
None	7
3.2	85[b]
3.0	73[b,c]

[a] Phosphorylase *b*, 10 mg/ml, was incubated with 0.001 M [1-^{14}C]iodoacetamide for 60 min in 0.02 M sodium β-glycerophosphate–0.0015 M EDTA at pH 6.8 and 30°. The reaction was terminated by addition of mercaptoethanol to a final concentration of 0.05 M. The ^{14}C-labeled enzyme was passed through a Sephadex G-25 column (0.9 × 23 cm) which had been previously equilibrated with 0.05 M sodium barbital–0.001 M EDTA at pH 8.0. Phosphorylase *b*, or iodoacetamide-treated phosphorylase *b*, 1.0×10^{-5} M, was reacted with *o*-iodosobenzoate, 3.0×10^{-5} M, described in the legend to Table I. [b] Activities are expressed as a percentage of the activity of iodoacetamide-treated phosphorylase *b* that had not been oxidized with *o*-iodosobenzoate. [c] After reaction with *o*-iodosobenzoate for 1 hr, this enzyme retained 61% of the control activity.

nary experiments indicated that no further incorporation of ^{35}S into oxidized phosphorylase *b* occurred after 30 min. After separation from the reaction mixture, the S-sulfonated enzyme contained from 1.3 to 1.6 mol of $^{35}SO_3^{2-}$/mol of phosphorylase *b* (five experiments). The enzyme activity of S-sulfonated phosphorylase *b* varied from 59 to 68% of the control activity.

Sedimentation Velocity Pattern of S-Sulfonated Phosphorylase b in the Absence of Sulfite Ion. The sedimentation velocity pattern shown in the upper part of Figure 3b indicates that S-sulfonated phosphorylase *b* is a mixture of two components in the absence of sulfite ion. The major, slowly moving component has an $s_{20,w}$ of 9.3 S, while the more rapidly sedimenting species has an $s_{20,w}$ of 13.0 S. These sedimentation coefficients were obtained using ^{35}S-labeled phosphorylase *b* and a control sample that had been stored for 2 days at 4° prior to ultracentrifugation. The ^{35}S content of the S-sulfonated enzyme decreased during this period from an initial value of 1.3 mol to 1.0 mol of ^{35}S/mol of phosphorylase *b* on the day of centrifugation. This decrease in the ^{35}S content is probably due to the attack of enzyme sulfhydryl groups on the $S^{35}SO_3^{2-}$ group (Cecil, 1963).

Figure 3b (lower pattern) shows the sedimentation velocity pattern of native phosphorylase *b* which, except for treatment with *o*-iodosobenzoate, was treated exactly like the ^{35}S-labeled enzyme. In addition to the major peak ($s_{20,w}$ = 8.7 S), a small amount of rapidly sedimenting material ($s_{20,w}$ = 11.6 S) is also present.

To determine whether the rapidly sedimenting components shown in Figure 3b were the result of intermolecular disulfide bond formation between phosphorylase *b* dimers, S-sulfonated phosphorylase *b* and the control enzyme were treated with dithiothreitol. The dithiothreitol-treated enzymes sediment as dimers, having $s_{20,w}$ values of 8.7 S and 8.8 S, respectively (Figure 3c).

Titration of the Sulfhydryl Groups of Native and S-Sulfonated Phosphorylase b with Nbs$_2$. The titration of native and S-sulfonated phosphorylase *b* with Nbs_2 was carried out as indicated under Materials and Methods. Based on a molar extinction coefficient of 13,600 (Ellman, 1959), 4.1 sulfhydryl groups of native phosphorylase *b* and 1.9 sulfhydryl groups of the S-sulfonated enzyme reacted with Nbs_2. These data suggest that Nbs_2 and sulfite ion modify similar sulfhydryl groups on phosphorylase *b*.

Effect of o-Iodosobenzoate on the Activity of Iodoacetamide-Treated Phosphorylase b. Zarkadas *et al.* (1970) have reported that 3.1 highly reactive sulfhydryl groups of freshly prepared phosphorylase *b* can be alkylated with iodoacetamide, without loss of enzyme activity. As shown in Table II, phosphorylase *b* that has 3.0 to 3.2 highly reactive sulfhydryl groups alkylated, by reaction with iodoacetamide, retains 73–85% of its activity after reaction with *o*-iodosobenzoate. These data suggest that *o*-iodosobenzoate reacts with those sulfhydryl groups of phosphorylase *b* that react rapidly with iodoacetamide.

Effect of Substrates and AMP on the Inactivation of Phosphorylase b by o-Iodosobenzoate. Phosphorylase *b*, 2.0 mg/ml, was oxidized with *o*-iodosobenzoate, 3.0×10^{-5} M, in the presence and absence of substrates or AMP, as described in the legend to Table I. Neither 0.02 M glucose 1-phosphate, 1% glycogen, or 0.001 M AMP significantly protected the enzyme against inactivation by *o*-iodosobenzoate. Phosphorylase *b* was 88% inactivated when oxidized in the presence of both glucose 1-phosphate and AMP.

Incorporation of [1-^{14}C]Iodoacetamide into S-Sulfonated Phosphorylase b. To determine whether the highly reactive sulfhydryl groups of S-sulfonated phosphorylase *b* are blocked, the modified enzyme and native enzyme, 2.7–3.2 mg/ml, were alkylated with [1-^{14}C]iodoacetamide as described in the legend to Table II. S-Sulfonated phosphorylase *b* incorporated 1.2–1.4 mol of ^{14}C/mol of enzyme without loss of enzyme activity, while control samples incorporated 2.7–3.2 mol of ^{14}C/mol of enzyme.

Kinetic Properties of S-Sulfonated Phosphorylase b. Gold (1968) observed that dinitrophenylation of 3.6 highly reactive sulfhydryl groups of phosphorylase *b* results in a decreased affinity of the modified enzyme for AMP. Kastenschmidt *et al.* (1968) found that modification of up to 3.2 sulfhydryl groups of phosphorylase *b* with Nbs_2 increased the K_m for AMP and abolished the homotropic cooperativity of AMP sites without changing the V_{max}.

In view of these observations, the activity response of S-sulfonated phosphorylase *b* to AMP was determined. We found that double reciprocal plots for the activation of S-sulfonated phosphorylase *b* by AMP, 2×10^{-6}–2×10^{-4} M, were linear while identical plots for the native enzyme showed upward curvature. The kinetic parameters shown in Table III indicate that S-sulfonation of phosphorylase *b* leads to a threefold increase in the K_m for AMP and a decrease in the homotropic cooperativity of AMP sites with little change in V_{max}. Kastenschmidt *et al.* (1968) reported similar results for Nbs_2-treated phosphorylase *b*.

After incubation with dithiothreitol for 17 hr (Table III), S-sulfonated phosphorylase *b* still contained 0.1 mol of ^{35}S/mol of phosphorylase *b* dimer. The Hill coefficient, *n*, and the K_m for AMP of dithiothreitol-treated, S-sulfonated phosphorylase *b* are comparable to the values obtained for the native enzyme. However, prolonged treatment of S-sulfonated phosphorylase *b* with dithiothreitol results in a decrease in the V_{max}.

Discussion

It has been suggested that the rapidly reacting sulfhydryl

TABLE III: Kinetic Constants of Native and S-Sulfonated Phosphorylase *b*.

Enzyme Form	$K_m \times 10^5$ (M)	n	V_{max} (μmol min^{-1} mg^{-1})
Native	1.9	1.3	42
S-Sulfonated	6.1	1.1	40
Native + DTT[a,b]	1.9	1.3	44
S-Sulfonated + DTT[b]	1.5	1.4	34

[a] Native or S-sulfonated phosphorylase *b*, 1.5 mg/ml, was incubated with 0.01 M dithiothreitol for 17 hr in 0.05 M sodium β-glycerophosphate–0.001 M EDTA at pH 6.8 and 23°. Aliquots were then diluted for assay of phosphorylase activity. Activity measurements of dithiothreitol-treated enzymes were done in 0.008 M sodium β-glycerophosphate–0.003 M EDTA–0.001 M dithiothreitol, which contained 0.6% glycogen, 0.048 M glucose 1-phosphate, and various amounts of AMP at 23° and pH 6.8. The enzyme concentration was 25 μg/ml. Native and S-sulfonated phosphorylase *b* were assayed in the absence of dithiothreitol. V_{max} values were obtained from activity measurements in the presence of 0.001 M AMP. The K_m values and Hill coefficients, n, were obtained from Hill plots (see Atkinson, 1966). [b] DTT, dithiothreitol.

groups of phosphorylase *b* may form intermolecular disulfides and may play a role in regulating phosphorylase activity *in vivo* (Battell *et al.*, 1968b). The data presented in this study indicate that oxidation of 2.6–3.2 sulfhydryl groups of phosphorylase *b* with *o*-iodosobenzoate leads to a 94% loss of enzyme activity and enzyme aggregation. The fact that thiols or sulfite ion can reactivate the partially inactivated enzyme and cause reformation of a dimeric structure resembling that of native phosphorylase *b* suggests that inactivation is associated with the formation of intermolecular disulfide bonds.

Alkylation of the rapidly reacting sulfhydryl groups of phosphorylase *b* with iodoacetamide strongly protects the enzyme against inactivation by *o*-iodosobenzoate (Table II). Furthermore, the S-sulfonated enzyme obtained by treating *o*-iodosobenzoate-inactivated phosphorylase *b* with sulfite ion has 1.3–2.0 fewer sulfhydryl groups available for rapid reaction with iodoacetamide than native phosphorylase *b*. These data strongly suggest that *o*-iodosobenzoate and sulfite ion modify the rapidly reacting sulfhydryl groups of phosphorylase *b*.

Since modification of two rapidly reacting sulfhydryl groups of phosphorylase *b* with various reagents does not change enzyme activity (Battell *et al.*, 1968a), it is unlikely that oxidation of these sulfhydryl groups with *o*-iodosobenzoate is the direct cause of enzyme inactivation. It seems more probable that the change in the state of aggregation of phosphorylase *b* which accompanies intermolecular disulfide formation affects the active site, or the AMP binding site, and is responsible for the loss of catalytic activity. This interpretation is supported by the fact that dithiothreitol reactivates the oxidized enzyme and causes formation of a dimeric structure similar to that of native phosphorylase *b* (Figure 3c).

The kinetic data on the activation of S-sulfonated phosphorylase *b* by AMP (Table III) indicate that S-sulfonation of the rapidly reacting sulfhydryl groups leads to a decrease in the affinity of the enzyme for AMP and a loss in the homotropic cooperativity of AMP sites. The Nbs_2-treated phosphorylase *b* studied by Kastenschmidt *et al.* (1968) showed a similar activity response toward AMP. This suggests that Nbs_2 and sulfite ion modify the same type of sulfhydryl groups of phosphorylase *b*. The observation that S-sulfonated phosphorylase *b* has two fewer sulfhydryl groups available for rapid reaction with Nbs_2 is consistent with this hypothesis.

Although S-sulfonation of the highly reactive sulfhydryl groups of phosphorylase *b*, or modification with Nbs_2, influences the interaction of the enzyme with AMP, alkylation of these sulfhydryl groups with iodoacetamide does not lead to changes in the kinetic parameters (Battell *et al.*, 1968b; Zarkadas *et al.*, 1970). This suggests that a secondary effect, perhaps a change in enzyme conformation, is responsible for the modified response of Nbs_2-treated phosphorylase *b* or S-sulfonated phosphorylase *b* to AMP.

The following observations suggest that conformational changes do occur when the highly reactive sulfhydryl groups of phosphorylase *b* are modified by certain reagents. (i) Incubation of Nbs_2-treated phosphorylase *b* with dithiothreitol causes rapid removal of thionitrobenzoate groups, but several hours are required for restoration of the homotropic cooperativity of AMP sites and the V_{max} (Kastenschmidt *et al.*, 1968). (ii) S-Sulfonated phosphorylase *b* has an $s_{20,w}$ of 11.2 S in the presence of sulfite ion (Figure 3c), while native enzyme has an $s_{20,w}$ of 8.3 S under identical conditions.

We suggest that the changes in enzyme conformation which occur when the highly reactive sulfhydryl groups of phosphorylase *b* are modified by *o*-iodosobenzoate, sulfite ion, or Nbs_2 are responsible for the observed effects on enzyme activity. Similar changes in enzyme conformation may occur *in vivo* as a result of modification of the highly reactive sulfhydryl groups and may serve to regulate phosphorylase activity (Battell *et al.*, 1968b).

Routine assays of S-sulfonated phosphorylase *b* indicate that the modified enzyme has 59–68% of the control activity. The low activity of the modified enzyme can be attributed in part to the presence of intermolecular disulfide bonds, since incubation with dithiothreitol restores 80% of the control activity and a dimeric structure similar to that of native phosphorylase *b* (Figure 3c). Furthermore, the S-sulfonate group is known to be labile in the presence of thiols (Cecil, 1963). Thus, it is possible that sulfhydryl groups on the S-sulfonated enzyme react with the S-sulfonate group under assay conditions. Such reactions could lead to disulfide formation and a consequent decrease in V_{max}. We have found that the ^{35}S content of S-sulfonated phosphorylase *b* and the activity of the enzyme decrease after storage for several days at 4° (see Results).

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Inhibition of Bound Enzymes. I. Antienergistic Interaction of Chemical and Diffusional Inhibition†

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ABSTRACT: When the diffusion of the substrate to bound enzymes is relatively slow the substrate concentration is lower in the microenvironment than in the macroenvironment and the resulting reduction in the enzyme activity is referred to as diffusional inhibition. On the other hand, an inhibitor, which reduces the enzyme activity *per se*, attenuates the magnitude of diffusional inhibition. Because of this antienergistic interaction between chemical and diffusional inhibition the activity of the bound enzyme at steady state is less affected by an inhibitor in the presence than in the absence of diffusion limitations for the substrate. As diffusional inhibition frequently occurs in heterogeneous enzyme systems, the antienergism has to be taken into account when the action of inhibitors is considered on enzymes artificially immobilized or bound to membranes in the cellular milieu.

The inhibition of enzymes has been extensively treated in the literature (Webb, 1963) because inhibitors are valuable tools in the biological sciences for the study of isolated enzymes and of the various aspects of cellular metabolism. They also play a well-established regulatory role in living systems. Most studies have been concerned with the inhibition of enzymes in free solution and little attention has been paid to the action of inhibitors on enzymes in heterogeneous media. The cellular milieu, however, is compartmented and most enzymes are embedded in or bound to membranes (Greville, 1969; Lehninger, 1970). In such heterogeneous systems the rate of the reaction is usually attenuated by the relative slowness of the substrate diffusion to the enzyme (Engasser and Horvath, 1973) and this phenomenon is referred to as diffusional inhibition. As a result the effect of an inhibitor on the enzymic reaction, *i.e.*, chemical inhibition, in heterogeneous media manifests itself in a way different from that in a free solution where diffusional resistances are negligible.

In this study the interplay between diffusional and chemical inhibition is treated quantitatively. Both external and internal diffusion resistances are considered (Horvath and Engasser, 1974), and it is assumed that the noncompetitive inhibitor is neither a substrate nor a product of the enzymic reaction. The results shed light on the inhibition of naturally and artificially immobilized enzyme systems.

Theoretical Analysis

Inhibition with External Diffusion Resistance. When the enzyme is attached to a surface and the substrate is transported from the macroenvironment to the enzyme through an "unstirred layer" of liquid or across a membrane, the external diffusion resistance in the nonreactive medium may result in a depletion of the substrate at the enzymic surface. Then the surface concentration of the substrate in the microenvironment of the bound enzyme, $[S_0]$, is lower than that in the macroenvironment, [S].

For Michaelis-Menten kinetics the rate of enzymic reaction, v, with noncompetitive inhibition is given by

$$v = \frac{V_{max}[S_0]}{[1 + ([I]/K_I)][K_M + [S_0]]} \quad (1)$$

where V_{max} is the saturation rate in the absence of the inhibitor, [I] is the concentration of the inhibitor, and K_M and K_I are the Michaelis constant and inhibition constant, respectively. It is assumed that the inhibitor concentration is uniform throughout the system.

In most cases the transport of substrate to the reactive surface can be expressed by the product of a transport coefficient, h_s, and the driving force given by the difference between the substrate concentration in the macro- and the microenvironment, $([S] - [S_0])$. At steady state both the consecutive transport and consumption of the substrate by the chemical reaction

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