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Stereospecific Requirements for Carbonyl Reagents in the Resolution and Reconstitution of Phosphorylase *b**

Shmuel Shaltiel,† Jerry L. Hedrick,‡ and Edmond H. Fischer

ABSTRACT: Removal of pyridoxal 5′-phosphate from phosphorylase by means of deforming agents and carbonyl reagents is a stereospecific process. Resolution occurs with L-cysteine but not with D-cysteine, although the two isomers have identical association constants with pyridoxal 5′-phosphate.

D-Cysteine does not compete with L-cysteine in the removal of pyridoxal 5′-phosphate from the enzyme. Stereospecificity also is observed in the reconstitution of apophosphorylase *b* with pyridoxal 5′-phosphate to yield the holoenzyme; however, this reaction is inhibited by the presence of a large excess of D-cysteine due to competition of the two enantiomorphs for the free cofactor. Evidence is presented for the existence of a cysteine binding site in phosphorylase. Upon incubation of the enzyme with radioactively labeled cysteine and gel filtration on Sephadex G-25, binding of *ca.* 1 mole of cysteine per mole of enzyme protomer (mol wt 92,500) was observed. The bound cysteine can be removed by exhaustive dialysis or urea denaturation indicating that it is probably linked to the protein by a noncovalent bond or a bond that is easily hydrolyzed. Data indicate that during both resolution and reconstitution of phosphorylase the cofactor leaves and reenters the protein as a pyridoxal 5′-phosphate–L-cysteine complex.

In a previous publication (Shaltiel *et al.*, 1966) a new method for the removal of pyridoxal 5′-phosphate from phosphorylase *b*[1] was described. This procedure involved two distinct operations, namely, deformation of the enzyme with an imidazolium citrate buffer followed by reaction of pyridoxal 5′-phosphate with L-cysteine. The deforming action of imidazolium citrate on the enzyme was discussed in detail in the preceding paper (Hedrick *et al.*, 1969) where it was suggested that the deformer "loosens" reversibly the structure of the enzyme and renders the bound cofactor readily available to chemical interactions.

One of the striking findings in this study was that while resolution of the enzyme (removal of pyridoxal 5′-phosphate) occurred readily with L-cysteine, it did not take place with a number of cysteine analogs which react just as well as cysteine with free pyridoxal 5′-phosphate (Shaltiel *et al.*, 1966, 1967). A systematic study was therefore undertaken in an attempt to establish which structural features of the cysteine molecule were required for resolution to occur. It was found that both resolution of phosphorylase and reconstitution of the apophosphorylase with pyridoxal 5′-phosphate are stereospecific processes, *i.e.*, they occur with L- but not with D-cysteine. Furthermore, there appears to be a cysteine binding site in phosphorylase. These findings further elucidate the structure of the pyridoxal 5′-phosphate site of the enzyme and its possible involvement in the control of phosphorylase activity. For previous publication, see Hedrick *et al.* (1969).

Materials and Methods

Rabbit muscle phosphorylase *b* and apophosphorylase *b* were prepared, assayed, and characterized as described previously (Fischer and Krebs, 1958; Hedrick and Fischer, 1965; Shaltiel *et al.*, 1966; Hedrick *et al.*, 1966). These publications also describe the determination of rates of resolution and reconstitution of the enzyme. Molecular weights of 370,000, 185,000, and 92,500 were used for phosphorylase *a*, *b*, and the enzyme monomer, as recently determined (Seery *et al.*, 1967; DeVincenzi and Hedrick, 1967; Ullmann *et al.*, 1968). Reduced phosphorylase *b* was prepared according to Fischer *et al.* (1958) and Strausbauch *et al.* (1967).

D- and L-cysteine as well as the other amino acids and their analogs were purchased from Calbiochem, Eastman Organic Chemicals, or Mann Research Laboratories. All other chemicals were the best available from commercial sources. L-[^{35}S]-Cystine was obtained from the Radiochemical Center, England, and DL-[3-^{14}C]cystine from Nuclear-Chicago. Each disulfide was reduced to the corresponding sulfhydryl derivative

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[1] α-1,4-Glucan:orthophosphate glucosyltransferase (EC 2.4.1.1).

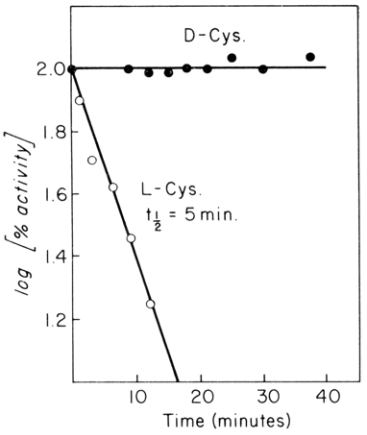


FIGURE 1: Stereospecificity in the resolution of phosphorylase *b* with cysteine. The resolution medium contained final concentrations of 2.3 mg/ml of protein, 0.4 M imidazole, and 0.1 M cysteine hydrochloride (D or L). The pH was adjusted to 6.5 at 0° with citric acid and resolution was allowed to proceed at this temperature.

by incubation with a 100-fold excess of nonlabeled, L- or DL-cysteine, respectively, for 15 min at 37°, pH 8.5.

^{35}S or ^{14}C radioactivity was measured in a Packard liquid-scintillation spectrometer. Sedimentation coefficients were determined in a Spinco Model E ultracentrifuge employing wedge window cells. The temperature during the run was maintained within ±0.2° of the indicated value.

Results

Sterospecific Requirement of L-*Cysteine during Resolution.* Previous investigation of the resolution of phosphorylase indicated that the over-all reaction displayed a distinct specific-

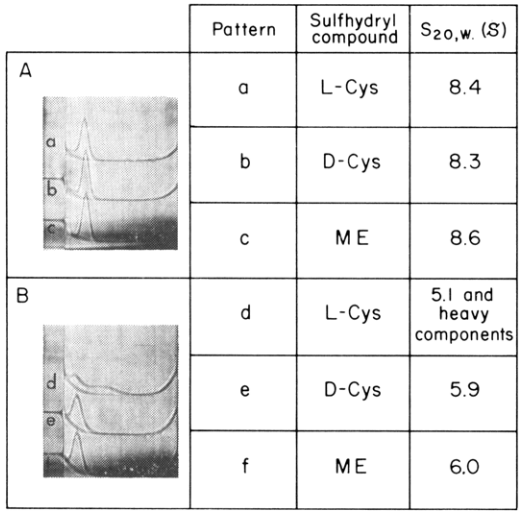


FIGURE 2: Effect of L-cysteine, D-cysteine, or 2-mercaptoethanol on the sedimentation properties of phosphorylase *b*. (A) Phosphorylase *b* (5.4 mg/ml) in 0.1 M sodium glycerophosphate, 0.1 M of the sulfhydryl compound, adjusted at 0° to pH 6.0 with HCl. (B) Phosphorylase *b* (5.3 mg/ml) in 0.4 M imidazole, 0.1 M sulfhydryl compound, adjusted at 0° to pH 6.0 with citric acid. The temperature of the rotor was kept at 2.4° during the run and the rotor speed was 52,670 rpm. The photographs were taken 32 min after attainment of maximum speed.

TABLE I: Resolution of Phosphorylase *b* in the Presence of Various Aldehyde Reagents.[a]

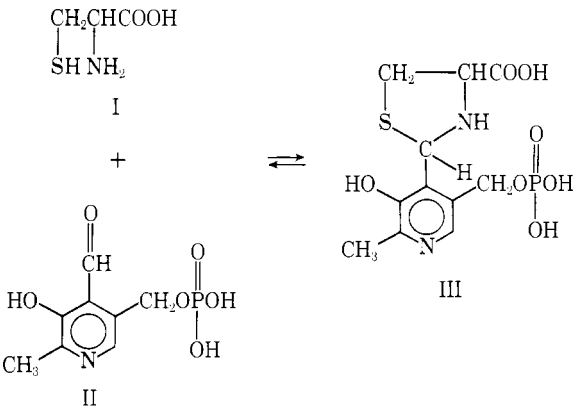
Aldehyde Reagent	$t_{0.5}$ for Loss of Act. (min)
Hydroxylamine	3.4[b]
Semicarbazide	21.0
Hydrazine	39.0
Isonicotinylhydrazine	40.0
Bisulfite (Na^+)	Stable[c]
L-Cysteine	4.4[d]

[a] The resolution medium contained a final concentration of 1.5 mg/ml of protein, 0.4 M imidazole, and 0.1 M of the aldehyde reagent. The pH was adjusted with HCl to 6.0 at 0° and resolution was allowed to proceed at this temperature. [b] In a separate experiment, the protein obtained following hydroxylamine treatment was isolated after precipitation with ammonium sulfate and gel filtration through Sephadex G-25 (see Shaltiel *et al.*, 1966). The material freed of pyridoxal 5′-phosphate regained 80% of its original activity following restoration of the cofactor (Hedrick *et al.*, 1966), indicating that loss of activity resulted from resolution and not from irreversible denaturation. [c] No loss of activity was observed within 40 min. [d] The rates of resolution varied slightly from one enzyme preparation to the other, perhaps due to trace contamination by AMP. When aldehyde reagents were compared with one another, experiments were performed with the same enzyme preparation; L-cysteine was always included as a control.

ity not only toward the deforming agent but also toward the carbonyl reagent used to remove pyridoxal 5′-phosphate from the enzyme (Shaltiel *et al.*, 1966; Hedrick *et al.*, 1967). This is exemplified in Table I, in which it can be seen that the rate of resolution of the enzyme varies somewhat from one pyridoxal 5′-phosphate reagent to the other. Of the nonsulfhydryl reagents tested, hydroxylamine was the most effective with a half-life of resolution similar to that obtained with L-cysteine. On the other hand, amazingly strict structural requirements became apparent when analogs or derivatives of cysteine were examined. Loss of any one of the three functional groups of cysteine (as in cysteamine, β-mercaptopropionic acid, or alanine in which the carboxyl, amino, and sulfhydryl groups, respectively, are replaced by a hydrogen atom) or substitution of any one of these groups (as in cysteine ethyl ester[2] or *S*-methylcysteine) abolished resolution. Similarly, no resolution was observed when cysteine was replaced by 2-mercaptoethanol, 2-aminoethanol, ethylenediaminethioglycolic acid, γ-mercaptobutyric acid, DL-homocysteine, DL-methionine, cystathionine, reduced glutathione, ergothionine, β-alanine, histidine, histamine, or cycloserine. These experiments were carried out on a reaction mixture containing final concentrations of 5 mg/ml of phosphorylase *b*, 0.1 M imidazole, and 0.1 M amine or

[2] A very slow rate of resolution observed (half-life of over 2.5 hr) would be attributed to trace amounts of free L-cysteine contaminating the ester in spite of two recrystallizations.

SCHEME I



sulfhydryl compound tested; the pH was adjusted with HCl to 6.0 at 0° and resolution was allowed to proceed at this temperature. Mild conditions were intentionally chosen (0.1 M imidazole *vs.* 0.4 M in the regular deforming buffer) in case one of the analogs tested proved to be more effective than cysteine in promoting resolution of the enzyme; half-life of resolution with L-cysteine under these conditions was 21 min. The differences just described could not be explained in terms of the chemical reactivity of the various analogs toward the aldehyde group of pyridoxal 5′-phosphate. In fact, and most unexpectedly, resolution did not occur when L-cysteine was replaced by D-cysteine, indicating that the process was stereospecific (Figure 1).

The striking difference in the effectiveness of D- and L-cysteine in promoting resolution of phosphorylase was also reflected in the behavior of the enzyme in the ultracentrifuge. Phosphorylase *b* has an $s_{20,w}$ of 8.3–8.6 S when dissolved in a sodium glycerophosphate buffer (pH 7.0; 0°) containing either D-cysteine, L-cysteine, or 2-mercaptoethanol (Figure 2A). However, when the ultracentrifugations were carried out in an imidazolium citrate buffer, totally different sedimentation patterns were observed depending upon whether D- or L-cysteine was present (Figure 2B). In the presence of L-cysteine, resolution occurred in the ultracentrifuge cell and the sedimentation pattern indicated the presence of heavy aggregates characteristic of the apoenzyme when held at temperatures close to 0° (Hedrick *et al.*, 1966). With D-cysteine, on the other hand, the sedimentation pattern was similar to that obtained with 2-mercaptoethanol, indicating that dissociation to monomers, but not resolution, had occurred (Shaltiel *et al.*, 1966).

Cysteine (I) reacts with pyridoxal 5′-phosphate (II) through its amino and thiol groups to form a thiazolidine derivative (III) (Heyl *et al.*, 1948; Buell and Hansen, 1960) (Scheme I). Since the spectral properties of the thiazolidine derivative (III) (λ_{max} 335 mμ) differ markedly from those of pyridoxal 5′-phosphate (II) (λ_{max} 395 mμ), the equilibrium constant of this reaction could be determined spectrometrically; a $K_{assocn} = 2.2 \times 10^3$ M^{-1} was found for both D- and L-cysteine in 0.1 M sodium citrate phosphate buffer (pH 6.8) (Gomori, 1955). Obviously, the inability of D-cysteine to promote resolution was not due to a lower reactivity toward free pyridoxal 5′-phosphate.

D-Cysteine not only failed to promote resolution of phosphorylase, but even failed to inhibit this process. In a series of experiments in which resolution was carried out at various L-cysteine/D-cysteine (or L-cysteine/2-mercaptoethanol) ratios, keeping the total concentration of SH compounds constant at 0.1 M, rates obtained were essentially identical with those afforded by L-cysteine alone (Figure 3).

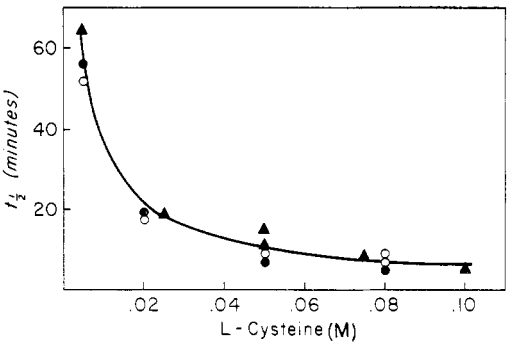


FIGURE 3: Rate of resolution of phosphorylase *b* as a function of L-cysteine concentration: effect of added D-cysteine or mercaptoethanol. The resolution mixtures contained final concentrations of 2 mg/ml of protein, 0.4 M imidazole, and the indicated concentration of L-cysteine (—▲—). When other thiols were added, the total concentration of sulfhydryl compounds was maintained at 0.1 M by addition of either 2-mercaptoethanol (—○—) or D-cysteine (—●—). In each case, the pH of the resolution medium was adjusted to 6.6 with citric acid at 0° and resolution was performed at this temperature.

Stereospecificity during Reconstitution of the Enzyme. Another remarkable effect of SH compounds on the interaction of pyridoxal 5′-phosphate with apophosphorylase was observed during the reconstitution of the enzyme. As expected, no reconstitution of phosphorylase occurred in the presence of a large excess (1000-fold over the concentration of pyridoxal 5′-phosphate) of high-affinity aldehyde reagents such as hy-

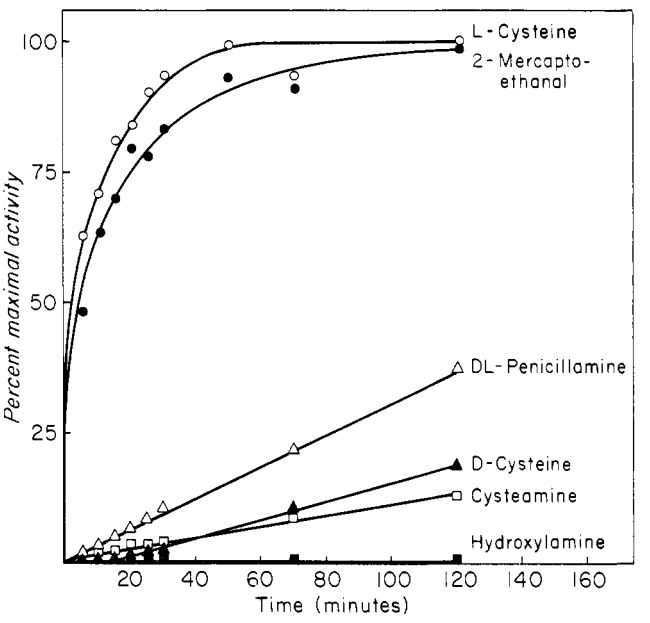


FIGURE 4: Reconstitution of apophosphorylase *b* in the presence of cysteine analogs and aldehyde reagents. The reconstitution mixtures were composed of 1.3 mg/ml of apophosphorylase *b*, 2.5×10^{-5} M pyridoxal 5′-phosphate, 2.5×10^{-2} M sulfhydryl compound or aldehyde reagent indicated, and 2.5×10^{-2} M sodium glycerophosphate. Reactivation was allowed to proceed at pH 7.0, 17°.

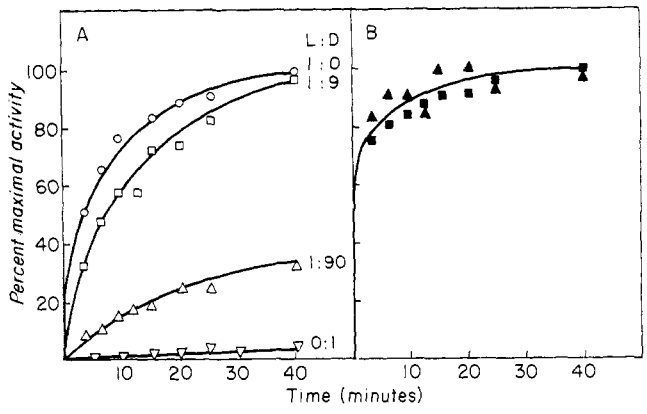


FIGURE 5: Reconstitution of apophosphorylase *b* with pyridoxal 5′-phosphate in the presence of D- and L-cysteine. The reaction mixtures contained 2 mg/ml of apophosphorylase *b*, 1.7×10^{-4} M pyridoxal 5′-phosphate, and 0.05 M sodium glycerophosphate at pH 7.0. In A, the total concentration of D- plus L-cysteine was maintained at 5×10^{-2} M, in the ratios indicated. In B, reconstitution was performed with L-cysteine alone at 5×10^{-3} M (—■—) and 5×10^{-4} M (—▲—). Temperature during reconstitution was $20 \pm 1°$.

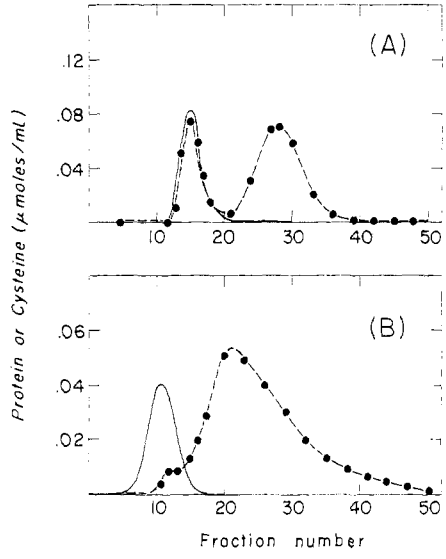


FIGURE 6: Binding of labeled cysteine to phosphorylase *b*. The AMP-free enzyme was dissolved in 0.05 M sodium glycerophosphate (pH 7.0) and diluted to a concentration of 3 mg/ml. This solution (2.5 ml) was diluted with 10 ml of 0.05 M sodium glycerophosphate containing 5×10^{-3} DL-[^{14}C]cysteine (pH 8.5). After standing for 15 min at 37°, the protein was precipitated by addition of an equal volume of saturated ammonium sulfate containing 0.05 M sodium glycerophosphate (pH 7.0). After centrifugation, the pellet was dissolved in 1 ml of 0.05 M sodium glycerophosphate (pH 6.5) and subjected to gel filtration at 22° on a Sephadex G-25 column (1.5 × 25 cm) previously equilibrated (A) with 0.05 M sodium glycerophosphate (pH 6.5) or (B) 0.05 M sodium glycerophosphate containing 4 M urea (pH 6.5). Fractions (1 ml) were collected and characterized for protein concentration (———) and radioactivity (- - ● - -).

droxylamine (Figure 4), isonicotinylhydrazine, or sodium cyanide (not illustrated). Similarly, reconstitution was very slow in the presence of D-cysteine or cysteamine. However, when the same excess of L-cysteine was used, very rapid reconstitution occurred; in fact, the reaction was even faster than in the presence of 2-mercaptoethanol (Figure 4) even though this latter compound binds pyridoxal 5′-phosphate 30 times more weakly than cysteine (K_{assoc} = 70 M^{-1} for 2-mercaptoethanol).

When reconstitution was carried out with mixtures of D- and L-cysteine (Figure 5A), again keeping the total concentration of cysteine constant at 0.1 M, the reaction was greatly inhibited by a large excess (*e.g.*, 90-fold) of D-cysteine. Inhibition was definitely due to competition between the two isomers of cysteine for the free cofactor, not to the low concentration of L-cysteine present (5×10^{-4} M in the case cited above): L-cysteine alone allowed for a rapid reconstitution of the enzyme even at this low concentration (Figure 5B).

Stoichiometric Binding of Cysteine to Phosphorylase. The above results could be interpreted by assuming that during resolution or reconstitution of phosphorylase, the cofactor leaves or reenters the protein as a pyridoxal 5′-phosphate–L-cysteine complex, such as the thiazolidine derivative (III). If this were the case, an L-cysteine binding site might be present in the enzyme. To investigate this point, phosphorylase *b* was incubated with either L-[^{35}S]cysteine or DL-[3-^{14}C]cysteine, precipitated with ammonium sulfate, and then subjected to gel filtration on Sephadex G-25 (Figure 6A). Stoichiometric amounts of labeled cysteine (*ca.* 1 mole of cysteine/mole of enzyme protomer mol wt 92,500) were bound to the protein (Table II).

The stoichiometric binding of cysteine to the holoenzyme was not altered significantly when the gel filtration was performed in the presence of glucose 1-phosphate (substrate) or AMP (an allosteric effector) which otherwise affect dramatically the structure and catalytic function of the enzyme (for review, see Brown and Cori, 1961, and Krebs and Fischer, 1962). It was even observed in the presence of the deforming medium (0.4 M imidazole adjusted to pH 6.0 with citric acid) used to bring about resolution of phosphorylase *b;* in this instance, resolution did not occur during passage through the column because of the low concentration of cysteine present (see Figure 5 in Shaltiel *et al.*, 1966). Binding of cysteine was also observed with $NaBH_4$-reduced phosphorylase *b* to nearly the same extent as with the native enzyme (Table II).

Little binding occurred when gel filtration was performed in the presence of 4 M urea where phosphorylase *b* loses its native conformation and releases pyridoxal 5′-phosphate (Figure 6B and Table II). The bound labeled cysteine could also be removed from the enzyme by 48-hr dialysis at 0° against 0.05 M sodium glycerophosphate buffer (pH 6.0) or by simple exchange with a number of SH compounds. In the former instance, removal of the labeled cysteine probably resulted from a slight dissociation of the cysteine protein complex and shifting of the equilibrium by air oxidation of cysteine to cysteine. Most conveniently, the enzyme was dissolved in a buffer containing 2-mercaptoethanol, L-, or even D-cysteine, and filtered through a Sephadex G-25 column (Table II).

The binding of cysteine to apophosphorylase *b* proved to be more difficult to study since the protein fractions often became turbid, apparently due to partial denaturation of the apoenzyme; the turbid solutions could not be fully reactivated with pyridoxal 5′-phosphate even in the presence of L-cysteine or 2-mercaptoethanol. In these instances, the apoenzyme bound only small amounts of cysteine varying from 0.15 to 0.18 mole per mole of enzyme protomer. In some experiments where precipitation did not occur, binding of cysteine to the

TABLE II: Binding of Labeled Cysteine to Phosphorylase *b*, $NaBH_4$-Reduced Phosphorylase *b*, and Apophosphorylase *b*.[a]

Enzyme	Medium	Cysteine Isomer	Moles of Cysteine Bound/ Mole of Enzyme Protomer	Sp Act. Measured: In the Absence of Added Thiols[b]	Sp Act. Measured: In Regular Assay Buffer[c]
Phosphorylase *b*	Sodium glycerophosphate[d]	^{14}C, DL	0.82	22	55
	Sodium glycerophosphate	^{35}S, L	1.03	23	53
	Sodium glycerophosphate + glucose 1-phosphate (5×10^{-2} M)	^{14}C, DL	0.96	31	81
	Sodium glycerophosphate + AMP (10^{-3} M)	^{14}C, DL	0.90	40	62
	Imidazole (citrate; 0.4 M)	^{14}C, DL	0.96	41	70
	Sodium glycerophosphate + urea (4 M)[e]	^{14}C, DL	0.22	0	0
	Sodium glycerophosphate (exchange with 2-mercaptoethanol)	^{14}C, DL	0	22	53
	Sodium glycerophosphate (exchange with L-cysteine)	^{14}C, DL	0	24	56
	Sodium glycerophosphate (exchange with D-cysteine)	^{14}C, DL	0	20	60
Reduced phosphorylase *b*[f]	Sodium glycerophosphate	^{14}C, DL	0.74	10	17
Apophosphorylase *b*	Sodium glycerophosphate	^{14}C, DL	0.15 precipitate[g]		
	Sodium glycerophosphate	^{35}S, DL	0.18 precipitate		
	Sodium glycerophosphate	^{14}C, DL	0.37 no precipitate		

[a] For the procedure used in these experiments, see legend to Figure 6. The Sephadex G-25 column was equilibrated in each case with the solution described under Medium at pH 6.5. In the exchange experiments, the pellets obtained after ammonium sulfate precipitation were taken up in 1 ml of sodium glycerophosphate buffer (pH 6.5) containing 0.1 M sulfhydryl compound listed; these solutions were incubated for 30 min at 22° and then subjected to gel filtration. [b] The specific activity was determined by diluting the material in the protein peak fraction with 0.05 M sodium glycerophosphate (pH 6.5). [c] Determined in 0.1 M maleate–0.04 M mercaptoethanol buffer (pH 6.5) containing 1 mg/ml of bovine serum albumin (Hedrick and Fischer, 1965). [d] Average of four experiments with deviations within ±10%. [e] This material was completely inactive due to urea denaturation. [f] $NaBH_4$ reduction was achieved to the extent of 98.5%; the reduced enzyme had a specific activity of 30 units/mg. [g] These protein fractions were turbid as they emerged from the column.

extent of 0.48 mole/mole of phosphorylase protomer was observed.

There is no indication that cysteine is chemically modified during its binding to phosphorylase. In one experiment, the bound [^{14}C]cysteine was first displaced with an excess of unlabeled L-cysteine, then the reaction mixture was deproteinized by perchloric acid precipitation.[3] Paper chromatography and electrophoresis of the supernatant solution indicated that the labeled cysteine had not been modified (Figure 7). Similarly, no loss of radioactivity from L-[^{35}S]cysteine was observed, as would have been expected if H_2S had been released.

Sulfhydryl Compounds and Phosphorylase Activity. In view of the known activation of phosphorylase by sulfhydryl compounds (Cori *et al.*, 1943; Green and Cori, 1943; Buell and Hansen, 1961), it was of interest to establish whether: (a) this phenomenon also exhibited a stereospecific requirement for the L isomer of cysteine, (b) the stoichiometric amount of cysteine bound to the protein sufficed to activate the enzyme, and (c) activation could be afforded by compounds other than thiols.

Phosphorylase to which [^{14}C]cysteine had been added was freed from sulfhydryl compounds by dialysis, precipitation with ammonium sulfate, and then gel filtration on Sephadex G-25. Total removal of thiols was followed by loss of radioactivity. When the cysteine-free enzyme was further diluted with an SH-free buffer and assayed, a low specific activity was observed (8–12 units/mg, Table III). However, dilution buffers containing either 2-mercaptoethanol or D- or L-cysteine produced a six- to sevenfold enhancement in activity (Table III).

[3] Exchange with an excess of unlabeled cysteine was necessary prior to acid precipitation of the protein to prevent some coprecipitation of the labeled material.

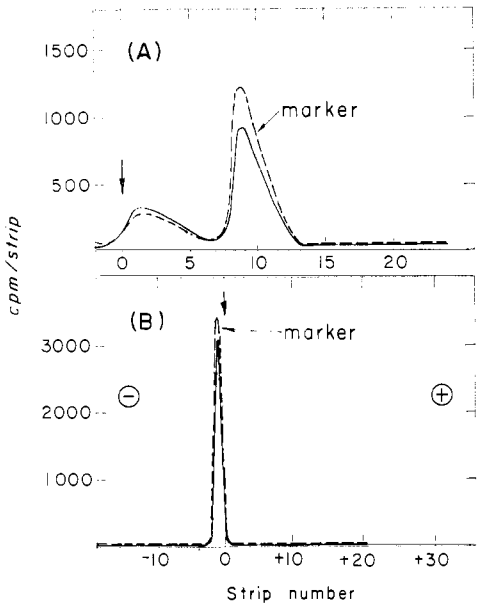


FIGURE 7: Characterization of labeled cysteine bound to phosphorylase. The protein fractions containing stoichiometric amounts of [^{14}C]cysteine as obtained in the experiment illustrated in Figure 6A were pooled, then incubated 10 min at 22° with unlabeled L-cysteine (final concentration 10^{-2} M). Perchloric acid (6 N) was added to a final concentration of 0.3 N and the nonradioactive precipitate formed was spun. After removal of the perchloric acid by precipitation with KOH, the supernatant was freeze dried, dissolved in 0.2 ml of water, and 25 μl was applied on paper for (A) descending chromatography in butanol–acetic acid–water (4:1:5, v/v) and (B) electrophoresis in pyridine acetate (pH 6.5), 40 V/cm for 1 hr. The paper was cut into equal strips (1.5 cm wide) and radioactivity was monitored (——). A labeled cysteine solution identically prepared except that phosphorylase was omitted from the incubation reaction mixture was used as marker (— —). Arrows indicate the starting points on the chromatogram and electropherogram.

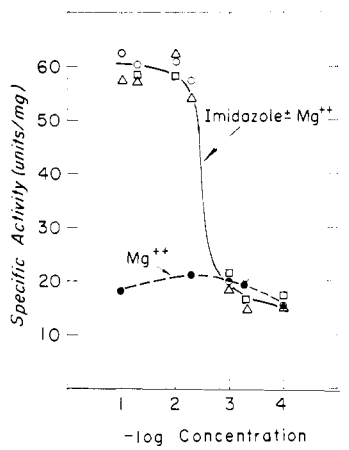


FIGURE 8: Effect of imidazole and Mg^{2+} ions concentration on the specific activity of phosphorylase *b*. Phosphorylase *b* (6.6 mg/ml) was freed from AMP and sulfhydryl compounds (see legend to Table III). Aliquots were diluted 1:40 with various concentrations of: (—○—) imidazole (Cl^-); (– –●– –) $MgCl_2$; (—△—) imidazole–$MgCl_2$ (1:1 molar ratio); (—□—) imidazole–$MgCl_2$ (1:5 molar ratio). All assays were performed at pH 6.5.

TABLE III: Effect of Various Compounds on the Activity of Phosphorylase *b*.[a]

Additives	Sp Act.
None	11
0.05 M 2-mercaptoethanol	69
0.05 M cysteamine	68
0.05 M L-cysteine	68
0.05 M D-cysteine	63
0.05 M EDTA	70
0.05 M 8-hydroxyquinoline	58
0.05 M imidazole	55
1 mg/ml of albumin	72
Assay buffer (control)[b]	77

[a] AMP-free phosphorylase *b* (1.1 mg/ml in 0.02 M sodium glycerophosphate, pH 6.5) was freed from SH compounds to a level below 0.1 mole/mole of enzyme protomer as indicated in the text. This solution was diluted 1:40 with each of the indicated solutions (all at pH 6.5), preincubated for 10 min at 30°, and then assayed. [b] As described by Hedrick and Fischer (1965).

In the presence of stoichiometric amounts of protein-bound cysteine, the specific activity of phosphorylase *b* was 20–23 units/mg (Table II), *i.e.*, about twice that obtained in the total absence of SH compounds. If AMP or glucose 1-phosphate were present during the preparation of the cysteine–phosphorylase (1:1) complex, the enzyme exhibited even higher specific activities reaching 40 and 31 units per mg, respectively. In all instances, much higher specific activities were observed when the various enzyme preparations were assayed in the regular dilution buffer containing an excess of sulfhydryl compounds.

Phosphorylase did not display an absolute requirement for SH compounds for maximum activity. As seen in Table III, albumin, EDTA, 8-hydroxyquinoline, and imidazole (see also Figure 8) were all capable of restoring enzyme activity; maximum values of 75–85 units/mg were obtained in the maleate–albumin–2-mercaptoethanol buffer described by Hedrick and Fischer (1965).

Activation of phosphorylase by imidazole is reminiscent of that described for phosphoglucomutase where two- to six-fold increases in specific activity were observed following preincubation with imidazole and Mg^{2+} (Robinson and Najjar, 1960; Harshman *et al.*, 1965). However, in the case of phosphorylase, activation occurred with imidazole alone and was not enhanced by addition of magnesium ions (Figure 8).

Discussion

As discussed in the preceding paper, two possible mechanisms can be visualized for the involvement of cysteine in the resolution of phosphorylase. In the first pathway, deformation of the enzyme unmasks the pyridoxal 5′-phosphate site and allows uptake of cysteine; the latter attacks the bonds connecting the cofactor to the protein, weakened by distortion of the molecule, and brings about their cleavage. In the second pathway, deformation of the enzyme labilizes the bonds between phosphorylase and pyridoxal 5′-phosphate and the

latter spontaneously dissociates from the enzyme; cysteine merely shifts the equilibrium to the fully dissociated state.

An apparent paradox is that cysteine accelerates both resolution and reconstitution of the enzyme. The difference is, of course, that resolution is carried out in the presence of the deforming buffer whereas reconstitution is carried out in glycerophosphate. A further complication results from the experiment reported in Table II; it indicates that even when the enzyme contains 1 mole of bound cysteine/mole of enzyme protomer, addition of 0.4 M imidazole citrate (a classical deforming buffer) will not bring about resolution. Clearly, removal of pyridoxal 5′-phosphate requires both the deforming agent and a *high* concentration of cysteine (*e.g.*, greater than 10^{-2} M) as previously determined (Shaltiel *et al.*, 1966). The reason for the excess cysteine requirement and whether or not this amino acid acts synergistically with imidazole citrate to further distort the pyridoxal 5′-phosphate site is not known.

The finding that resolution proceeds rapidly with L-cysteine and not with D-cysteine (even though the two isomers have identical association constants with free pyridoxal 5′-phosphate) clearly excludes the second alternative. Therefore, L-cysteine must interact with the cofactor while the latter is still bound to the protein and forms the thiazolidine derivative (III) *in situ;* the latter then dissociates from the protein. The fact that stereospecificity is observed during reconstitution strongly supports the view that pyridoxal 5′-phosphate also reenters the protein as a thiazolidine derivative.

A large variety of cysteine analogs do not promote resolution and reduce dramatically the rate of reconstitution of phosphorylase with pyridoxal 5′-phosphate. This suggests that all three functional groups of L-cysteine are involved in the processes of resolution and reconstitution, as if there were some sort of functional recognition on behalf of the enzyme for this amino acid, and a specific site for its binding. This hypothesis is strongly supported by the findings that phosphorylase does indeed bind equimolar amounts of cysteine, presumably at the pyridoxal 5′-phosphate binding site.

How is cysteine bound to phosphorylase? Its removal from the enzyme by exhaustive dialysis or by gel filtration in the presence of 4 M urea strongly suggests that either noncovalent bonds or very easily hydrolyzable covalent bonds are involved. This would seem to exclude the formation of a mixed disulfide, unless one assumed that a mixed disulfide could be displaced by a sulfhydryl residue on the protein, to give an inter- or intramolecular disulfide derivative of the protein and free cysteine. There is no evidence for the presence of such disulfide bonds in phosphorylase.

In view of the proposal that at neutral pH, pyridoxal 5′-phosphate is bound to phosphorylase as a substituted aldamine derivative involving the ε-amino group of a lysyl residue and an additional group X (Kent *et al.*, 1958; Fischer *et al.*, 1963; Fischer, 1964), the possibility was considered that the extraneous cysteine molecule taken up by the enzyme provides this group X, for instance, by forming a bond between its sulfur atom and the 4′-carbon of pyridoxal 5′-phosphate. However, $NaBH_4$-reduced phosphorylase which could not form such a bond was found to bind almost stoichiometric amounts of cysteine.

Attempts to determine the binding of cysteine to the apoenzyme were complicated because of denaturation of the latter. Nonetheless, binding of up to 0.48 mole/mole of enzyme protomer was observed indicating that the cysteine site might be operative even after removal of pyridoxal 5′-phosphate.

There does not seem to be a connection between the stereospecific requirement of L-cysteine during resolution and reconstitution of phosphorylase, and the need of SH compounds for maximum activity. Phosphorylase can be activated by various SH compounds including L- or D-cysteine. Furthermore, it was shown above that imidazole, EDTA, 8-hydroxyquinoline, etc., also activate the enzyme in the absence of thiols (see also Buell and Hansen, 1961).

The reason for the presence of a cysteine binding site in phosphorylase is not apparent. It is unlikely that binding of cysteine is associated with another catalytic activity of the enzyme since none was found under a variety of reaction conditions (Hedrick and Fischer, 1965). As shown here, the cysteine molecule bound to phosphorylase is not modified by the enzyme. However, the fact that the binding of cysteine is stoichiometric and stereospecific may indicate that this phenomenon is of physiological significance; conceivably, it could provide a means by which the activity of phosphorylase could be regulated through resolution and reconstitution of the enzyme.

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Isolation and Characterization of a Phytohemagglutinin from the Lentil*

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ABSTRACT: A hemagglutinin from the common lentil has been isolated from crude extracts by precipitation in slightly acidified water followed by either DEAE-cellulose chromatography or elution from a Sephadex G-100 column with 0.1 M D-glucose. The hemagglutinin thus prepared exhibited a single precipitin arc on immunoelectrophoresis and a single symmetrical peak on ultracentrifugation. The sedimentation coefficient did not vary appreciably over a range of concentration from 2 to 8 mg per ml, and the $s^0_{20,w}$ = 4.0 S. Despite the homogeneity indicated by the above criteria, *Lens culinaris* hemagglutinin consistently migrated as a smear upon prolonged electrophoresis on cellulose acetate at pH 8.6. Red cells of different animal species were agglutinated to specific extents by the hemagglutinin; some strongly, others moderately, still others not at all. A saccharide binding site was implicated in the agglutination reaction by the following observations: (a) several soluble saccharides inhibited agglutination to various degrees; (b) D-glucose, a good inhibitor of agglutination, could protect the hemagglutinin against heat denaturation; (c) the hemagglutinin adhered to columns of dextran gels in which the pore size was such that the agglutinin could reach the interior of the gel; (d) the hemagglutinin could be eluted from such gels with D-glucose but not with buffer alone.

Hemagglutinins are proteins which combine with red cell surface structures causing visible clumping. Although the most familiar ones are antibodies (*e.g.*, human blood-group isoagglutinins), there are a large number whose occurrence does not appear to be immunologically induced. Such "naturally occurring" hemagglutinins have been noted in the *Limulus* crab (Marchalonis and Edelman, 1968a) and the lamprey eel, *Petromyzon* (Marchalonis and Edelman, 1968b). Many hemagglutinins are found in plants and are known as phytohemagglutinins or lectins (Boyd, 1954). Structural studies have been performed on only a few phytohemagglutinins (Agrawal and Goldstein, 1968; Olson and Liener, 1967b; Rigas *et al.*, 1966) and little is known about their *in vivo* function or about the nature of the hemagglutinin–erythrocyte bond(s). A number of phytohemagglutinins appear to combine specifically with saccharide-containing structures of the red cell surface (Mäkelä, 1957). It has been suggested that phytohemagglutinins react with their receptor molecules in a manner similar to antibodies (So and Goldstein, 1967). It has also been suggested (Agrawal *et al.*, 1968) that some lectins may serve as ideal models for studying protein–carbohydrate interactions because, unlike antibodies, phytohemagglutinins can be readily obtained in a homogeneous state.

The common lentil, *Lens culinaris* or *Lens esculenta*, was first reported to contain a hemagglutinin, *L. culinaris* hemagglutinin, by Landsteiner and Raubitschek (1908). *L. culinaris* hemagglutinin has been termed an "unspecific" phytohemagglutinin because it agglutinated red cells of all human blood groups equally well (Mäkelä, 1957). The receptor site(s) for *L. culinaris* hemagglutinin probably contained a saccharide moiety since a number of sugars (D-glucose, D-mannose, D-fructose, etc.) specifically interfered with hemagglutination. These sugars fell into the group III saccharides of Mäkelä (1957).

Because *L. culinaris* hemagglutinin was easily obtained from a common source and reacted with simple sugars, it appeared to be a useful material for studying the structure and

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