On the Role of Pyridoxal 5′-Phosphate in Phosphorylase. II. Resolution of Rabbit Muscle Phosphorylase *b**

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ABSTRACT: A method for the removal of pyridoxal 5′-phosphate (PLP) from rabbit muscle phosphorylase *b* is described. The procedure involves two simultaneous operations, namely, distortion of the protein by an appropriate deforming agent which exposes the covalently bound PLP, and removal of the cofactor by interaction with a PLP reagent. A number of deforming buffers were tested, of which imidazolium citrate proved to be the most effective. Using this buffer and L-cysteine as the carbonyl reagent, resolution of phosphorylase occurred with a half-life of *ca.* 5 min at pH 6.2, 0°, <1 min at pH 7.0 and 37°; the energy of activation at pH 7.0 was 11.7 kcal/mole. Resolution was specific with respect to the PLP reagent. Of a number of cysteine analogs tested, L-cysteine was found to be the most effective; penicillamine was barely active and cysteamine totally inactive. Under these same conditions neither imidazolium citrate nor L-cysteine alone brought about resolution. The deforming buffer probably facilitates removal of PLP by causing gross conformational changes in the enzyme: even in the absence of L-cysteine, it caused dissociation of phosphorylase *b* into monomers ($s_{20,w}$ = 5.5 S) and a rapid exchange of the protein-bound PLP with free [^{32}P]PLP. Resolution of phosphorylase *b* was blocked by addition of adenosine 5′-phosphate or by phosphorylation of the protein as it occurs during the conversion of phosphorylase *b* to *a*.

Apophosphorylase *b* prepared by this procedure has a residual activity of <1% and a correspondingly low PLP content. There is no indication that the protein has undergone any irreversible denaturation during resolution since the apoenzyme can be fully reactivated by incubation with PLP.

In the first paper of this series (Hedrick and Fischer, 1965) phosphorylase (EC 2.4.1.1 α-1,4-glucan: orthophosphate glucosyltransferase) was shown to display none of the enzymatic activities characteristic of B_6-dependent enzymes (for a recent review, see Meister, 1965). It was also reported that in nonenzymatic model systems, PLP[1] and several of its analogs or derivatives had no catalytic effect on the reaction between glucose 1-phosphate and glycogen. Removal of PLP from phosphorylase was shown to yield an enzymatically inactive protein (Cori and Illingworth, 1957). On the other hand, reduction of phosphorylase *b* with sodium borohydride, resulting in a fixation of the cofactor onto the protein, yielded a modified enzyme in which most of the original activity was retained (Fischer *et al.*, 1958b). All other classical PLP-enzymes would have been inactivated by such a treatment, which irreversibly reduces the aldehyde group of PLP. It was therefore proposed that in phosphorylase PLP might have a structural rather than a catalytic function.

Evidence obtained in this and other laboratories indicates that PLP is very strongly held by phosphorylase (Brown and Cori, 1961; Krebs and Fischer, 1962). At neutral pH and under moderate conditions of temperature and ionic strength, PLP cannot be removed by common aldehyde reagents such as hydroxylamine, semicarbazide, etc. From the spectral properties of phosphorylase together with a total absence of reduction or fixation of PLP by sodium borohydride at neutral pH, it was concluded that the cofactor is covalently bound to the protein, possibly as a substituted aldamine derivative (Kent *et al.*, 1958; Fischer, 1964).

Removal of PLP from phosphorylase (resolution) was first achieved by Cori and Illingworth (1957) by precipitation of the enzyme with ammonium sulfate at pH 3.4. Under these conditions, considerable denaturation occurred and hence a modified procedure was introduced, involving 12 hr of dialysis *vs.* sodium glycerophosphate and cysteine at pH 6.0 (Illingworth *et al.*, 1958). Apophosphorylase *b*, prepared by the latter procedure, could regain a considerable percentage of its original activity when incubated with PLP.

In an attempt to establish the contribution of PLP

* From the Department of Biochemistry, University of Washington, Seattle, Washington. *Received March 28, 1966.* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM 7902), and from the National Science Foundation (GB-3249). For the first publication in this series, see Hedrick and Fischer (1965).

† On leave of absence from the Weizmann Institute of Science, Rehovoth, Israel. Recipient of a U. S. Government grant under the Fulbright–Hays program.

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[1] Abbreviations used: AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate; ME, 2-mercaptoethanol; PLP, pyridoxal 5′-phosphate; Im, imidazole; Cit, citrate.

to the structure and function of phosphorylase, and to study the physicochemical properties of the apoenzyme, a systematic reinvestigation of the resolution of phosphorylase *b* was undertaken. In view of the evidence cited above, it was felt that removal of PLP with minimal denaturation of the protein would require two distinct operations: first, slight deformation of the molecule that would "expose" the PLP site, then cleavage of the covalent bonds linking the cofactor to the protein by attacking the PLP residue with an appropriate aldehyde reagent.

The purpose of this study was first, to determine the conditions controlling the removal of PLP from phosphorylase *b*, then to establish a procedure for the preparation of apophosphorylase with minimal structural damage. This paper describes a new approach to the resolution of phosphorylase *b* through the use of specific deforming agents; the apophosphorylase *b* obtained displays a very low residual activity and can be fully reactivated by restoration of the cofactor.

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared according to Fischer *et al.* (1958a) with the modification of Krebs *et al.* (1964). Phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase (Krebs *et al.*, 1964).

Phosphorylase activity was determined by a zero-order kinetics assay, as described by Hedrick and Fischer (1965). In this assay both phosphorylase *a* and *b* have a specific activity of 80 unit/mg when measured in the presence of AMP, and <0.5 unit/mg (phosphorylase *b*) and 54 units/mg (phosphorylase *a*) in its absence. A unit of activity is defined as the amount of enzyme causing the release of 1 μmole of inorganic phosphate from glucose 1-phosphate/min. Protein concentration was determined spectrophotometrically using an absorbancy index $A_{278}^{1\%}$ 11.9 both for phosphorylase *a* and *b* (Appleman *et al.*, 1963).

The PLP content of phosphorylase was measured spectrophotometrically after release of PLP from the enzyme by precipitation of the protein with perchloric acid added to a final concentration of 0.3 N (Baranowski *et al.*, 1957). A molar extinction coefficient of 6250 l. mole^{-1} cm^{-1} at 295 mμ was found in 0.3 N perchloric acid as compared to 6700 l. mole^{-1} cm^{-1} in 0.1 N HCl (Peterson and Sober, 1954).

Sodium tripolyphosphate and sodium polyphosphate were obtained from Blockson Chemical Co. Sodium glycerophosphate, obtained from Nutritional Biochemical Co. as an unspecified mixture of the α and β isomers, was recrystallized from water–ethanol (2:1) before use. L-Cysteine·HCl was purchased from Nutritional Biochemical Co.; the free base was prepared according to the procedure described by Greenstein and Winitz (1961). Cysteamine·HCl and 2,3-dimercaptopropanol were obtained from Calbiochem, DL-homocysteine from Nutritional Biochemical Co., DL-penicillamine (free amine) from Mann Research Laboratories, and citric acid from Merck. Imidazole

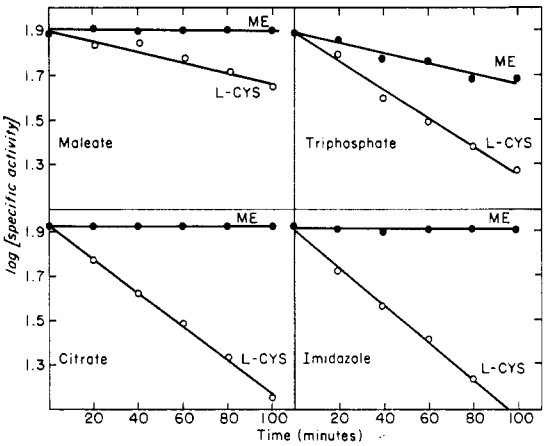

FIGURE 1: Kinetics of resolution of phosphorylase *b*. Resolution was performed at pH 6.0 and 0° at a protein concentration of 3.3 mg/ml. Each of the resolution mixtures contained the indicated buffers at a final concentration of 0.1 M and either 0.1 M L-cysteine or ME in the controls. The pH of the buffers was adjusted with HCl (for imidazole) or NaOH. The reaction was initiated and followed as described under Methods.

was purchased from Eastman Organic Chemicals; it was recrystallized from ethyl acetate and dried overnight *in vacuo* before use. ATP and AMP were purchased from Pabst Research Biochemicals, bovine serum albumin from Sigma Chemical Co., and Sephadex G-25 from Pharmacia. [^{32}P]PLP was synthesized by Dr. Anna Pocker according to the method of Peterson *et al.* (1953).

Stock solutions of phosphorylase *b* for resolution were freed from AMP by passage through a small charcoal–cellulose column (Fischer and Krebs, 1958) and kept at 0° in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with HCl. The protein concentration of these solutions was usually kept at 20–25 mg/ml. Unless otherwise stated, resolution of phosphorylase *b* was initiated by diluting a sample of the stock enzyme solution 1:10 with the appropriate deforming agent and cysteine. For kinetic experiments, aliquot samples were removed from the reaction mixture at eight different times between 1 and 60 min, and diluted 1:100 with a 0.1 M maleate buffer containing 0.04 M 2-mercaptoethanol and 1 mg/ml of albumin, pH 6.5. Resolution was found to be stopped by this dilution. The diluted solutions were kept at room temperature, rather than in the cold, to avoid cold inactivation of the enzyme (Graves *et al.*, 1965). Duplicate samples were then removed and assayed for phosphorylase activity within 2 hr. The loss of activity with time was normally found to follow first-order kinetics over the first 80% of the process (see examples given in Figure 1). The first-order rate coefficient (k) was calculated from the slope of the line obtained when the logarithm of the specific activity was plotted *vs.* time. The half-life of the process was calculated from

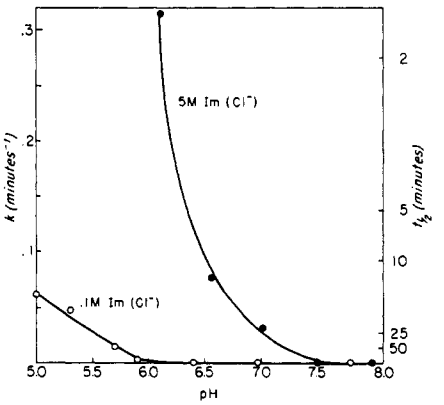

FIGURE 2: pH dependence for resolution of phosphorylase *b* in imidazole buffers. Resolution of phosphorylase *b* (2.8 mg/ml) was followed as described under Methods at two imidazole concentrations (0.5 and 0.1 M) in the presence of 0.1 M cysteine and at 0°. The pH of the buffers was adjusted with HCl. Control experiments (not illustrated) in which L-cysteine was replaced by 0.1 M ME showed no loss of activity within 30 min.

the equation $t_{0.5} = 0.693/k$. Rates of resolution are reported as either first-order rate coefficients or half-lives. Reconstitution of phosphorylase *b* with PLP was performed as described in the following publication in this issue (Hedrick *et al.*, 1966).

Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge employing a double-sector cell. The temperature of the rotor during the run was maintained within ±0.2° of the indicated temperature. Movement of boundaries was calculated from direct microcomparator measurements of the Schlieren diagrams.

Results

In an attempt to find compounds which would act as deforming agents, *i.e.*, which would facilitate the release of PLP from phosphorylase *b*, the enzyme was incubated with a variety of organic and inorganic salts, in the presence of 0.1 M L-cysteine. As can be seen in the examples illustrated in Figure 1, loss of activity occurred readily in many instances. Rates followed first-order kinetics and, in general, the reaction was cysteine dependent, *i.e.*, it did not occur in the control runs in which L-cysteine was replaced by an equal molar concentration of 2-mercaptoethanol.

Resolution of phosphorylase was followed by activity measurements rather than by direct determinations of PLP (released or still bound to the enzyme) because of the simplicity of the procedure. The assumption was made that all losses of activity that could be reversed by incubation with PLP after removal of the deforming agent were due to resolution. Losses of activity that could not be reversed by addition of PLP were attributed to irreversible denaturation. In all cases of partial resolution, the residual activity was found to be directly proportional to the amount of PLP remaining on the protein.

Table I summarizes the half-lives of resolution of phosphorylase *b* observed in the presence of various deforming agents. Under this set of conditions the reaction was found to be the fastest in sodium triphosphate, sodium citrate, or imidazolium chloride. Of these, citrate and imidazole were chosen for further study, since in both instances there was no detectable loss of activity over a period of 100 min when L-cysteine was replaced by 2-mercaptoethanol.

TABLE I: Resolution of Phosphorylase in the Presence of Various Buffers.[a]

Buffer[b]	$t_{0.5}$ for Loss of Act. (min)	
	L-Cysteine	2-Mercapto-ethanol
Glycerophosphate	Stable[c]	Stable
Phosphate	78	Stable
Pyrophosphate	44	Stable
Triphosphate	37	96
Polyphosphate[d]	68	151
ATP[e]	Stable	Stable
Maleate	87	Stable
Citrate	40	Stable
EDTA	88	Stable
Imidazole	36	Stable

[a] Resolutions were carried out at pH 6.0 and 0°. Each of the reaction mixtures contained final concentrations of 3.3 mg/ml of AMP-free phosphorylase *b*, 0.1 M L-cysteine·HCl or ME, and 0.1 M of one of the buffers listed. The half-lives for resolution were determined as described under Methods. [b] The pH of imidazole was adjusted with HCl. In all other cases the pH was adjusted with NaOH. [c] No significant loss of activity observed within 100 min. [d] Average mol wt 1080. [e] The concentration of this buffer was 0.05 M.

Effect of pH and Composition of the Medium. Resolution of phosphorylase *b* was greatly affected by the pH and composition of the medium. In general, as reported by Illingworth *et al.* (1958), the rate of resolution increased as the pH was lowered. However, strikingly different pH-dependence curves were obtained with different deforming agents.

The resolution of phosphorylase *b*, carried out in two series of imidazole buffers, is depicted in Figure 2. The marked differences in reaction rates could not be attributed simply to the difference in ionic strength between the two buffers, but to a specific effect of imidazole at high concentration. This was demonstrated by comparing the rates of resolution in two series of 0.1 M citrate buffers adjusted to various pH values either with imidazole or with NaOH. As seen in Figure 3, the rate

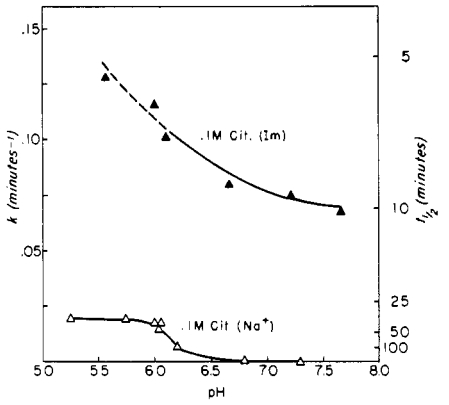

FIGURE 3: pH dependence for resolution of phosphorylase *b* in citrate buffers. Resolution of phosphorylase *b* was followed as described under Methods with two series of 0.1 M citrate (Cit) buffers adjusted to the various pH values with either NaOH or imidazole (Im). Each buffer contained also 0.1 M L-cysteine·HCl. The protein concentration was 2.8 mg/ml and the temperature 0°. Control experiments (not illustrated) in which L-cysteine was replaced by 0.1 M ME showed no significant loss of activity except in the imidazole series below pH 6.0. The dashed curve in this pH region indicates that loss of activity might be due in part to irreversible denaturation rather than to resolution.

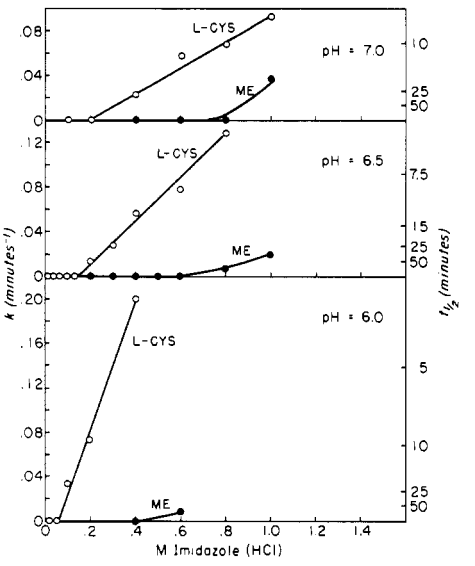

FIGURE 4: The effect of imidazole concentration on the rate of resolution of phosphorylase *b* as a function of pH. Resolution was followed as described under Methods in three series of imidazole buffers. Each of the buffers contained either 0.1 M L-cysteine·HCl or 0.1 M ME; pH values were adjusted with HCl. The protein concentration was 2.8 mg/ml and the temperature 0°.

of reaction increased considerably when the sodium ions were replaced by imidazolium ions. At pH 6.0, *e.g.*, the rate of resolution in imidazolium citrate was nine times faster than in sodium citrate. In yet another experiment (Table II) the rate of the reaction even *decreased* when NaCl was added to increase the ionic strength. When resolution of phosphorylase *b* was followed in 0.4 M imidazole solutions, adjusted to the same pH values with different acids, marked differences in rate were observed (Table III). In a series of carboxylic acids, the rate increased with the number of carboxyl groups (acetate < succinate < citrate). On the other hand, chloride, an inorganic monovalent anion, was more effective than the acetate or succinate ions.

The rate of resolution was found to be greatly affected by the concentration of the deforming agent. This is

TABLE II: Effect of Ionic Strength on Resolution of Phosphorylase *b*.[a]

Resolution Medium (M)	$t_{0.5}$ for Loss of Act. (min)
Imidazole[b] (0.1)	
L-Cysteine (0.1)	15
+ NaCl (0.5)	25
+ NaCl (1)	57
Citrate[c] (0.1)	
L-Cysteine (0.1)	36
+ NaCl (0.5)	50
+ NaCl (1)	133

[a] Resolutions were carried out at pH 5.3, 0°, and a protein concentration of 2.75 mg/ml. No significant loss of activity was detected during 100 min in control runs, containing 0.1 M 2-mercaptoethanol instead of L-cysteine·HCl. [b] pH adjusted with HCl. [c] pH adjusted with NaOH.

TABLE III: Contribution of Various Anions to the Resolution of Phosphorylase *b* in the Presence of Imidazole.[a]

Imidazole Salt	$t_{0.5}$ for Loss of Act. (min)
Acetate	41
Succinate	18.7
Citrate	5.2
Chloride	9.3

[a] The resolution medium contained a final concentration of 0.4 M imidazole and 0.1 M L-cysteine (free base). The pH was adjusted in each case to 6.5 using the appropriate acid. Resolution was carried out at 0° with a protein concentration of 2.8 mg/ml. No significant loss of activity occurred during 50 min in the control runs, in which L-cysteine was replaced by an equal concentration of 2-mercaptoethanol.

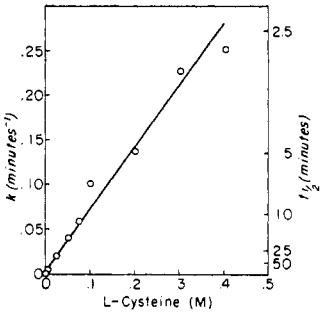

FIGURE 5: Effect of cysteine concentration on the rate of resolution of phosphorylase *b*. Resolution of the enzyme (2.8 mg/ml) was followed at 0° as described under Methods with imidazole buffers (0.4 M) containing various concentrations of L-cysteine (free base) and adjusted to pH 6.5 with citric acid.

clearly illustrated in Figure 4, where resolution was followed with increasing concentrations of imidazolium chloride at three pH values. Lowering the pH effectively lowered the concentration at which imidazolium chloride promoted resolution. At high concentrations of the deforming agent the enzyme lost activity even in the absence of cysteine (Figure 4). The cysteine-independent inactivation of the enzyme could not be reversed by incubation with PLP and was often accompanied by precipitation of the protein; it was therefore attributed to an irreversible denaturation of the enzyme. At lower pH values, denaturation started even at low concentrations of the deforming agent. In the preparation of the apoenzyme, care should be taken to work within the "safe" range of concentrations of the deforming agent, where loss of activity occurs in the presence of cysteine but not in its absence.

Effect of Temperature. The effect of temperature on resolution of phosphorylase *b* was followed in imidazolium citrate at pH 6.5 and 7.0. As seen in Table IV the rate of resolution increased with increasing temperature. From an Arrhenius plot of the temperature dependence of the reaction rate at pH 7.0, an energy of activation of 11.7 kcal/mole was calculated.

Effect of Various PLP Reactants. The rate of resolution of phosphorylase *b* was found to vary linearly with the concentration of L-cysteine (Figure 5). However, when L-cysteine was replaced by equimolar concentrations of other compounds also known to react with PLP, unexpected results were obtained. While hydroxylamine was found to be as effective as cysteine ($t_{0.5}$ = 20 min under the conditions described in Table V), other compounds such as cysteamine or DL-homo-

TABLE IV: Effect of Temperature on the Resolution of Phosphorylase *b*.[a]

Temp[b]	pH	$t_{0.5}$ for Loss of Act. (min)	
		L-Cysteine	2-Mercapto-ethanol
0	6.5	3.2	Stable[d]
22		<1.2[c]	31
30		<0.9[c]	62
0	7.0	13.4	Stable
8		6.8	Stable
22		5.3	Stable
30		2.6	Stable
37		<1.0[c]	93

[a] The resolution medium contained final concentrations of 0.4 M imidazole and 0.1 M L-cysteine·HCl or ME. The pH was adjusted to 6.5 or 7.0 with citric acid. Resolution was carried out at the indicated temperatures with a protein concentration of 2 mg/ml. [b] At temperatures of >30° some turbidity was observed in the reaction mixtures and in the control runs. [c] The rate of the reaction was too fast for accurate determination of the half-life. [d] No significant loss of activity was observed within 50 min.

TABLE V: Resolution of Phosphorylase *b* in the Presence of Various Sulfhydryl Compounds.[a]

Sulfhydryl Compd	Formula	$t_{0.5}$ for Loss of Act. (min)
L-Cysteine	$CH_2CHCOOH$ $SH\ NH_2$	21
2-Mercapto-ethanol	CH_2CH_2 $SH\ OH$	Stable[b]
Cysteamine	CH_2CH_2 $SH\ NH_2$	Stable
DL-Penicillamine	$(CH_3)_2C(SH)CH(NH_2)COOH$	149
2,3-Dimercapto-propanol (British Anti-lewisite)	CH_2CHCH_2OH $SH\ SH$	76
DL-Homocysteine	$CH_2(CH_2SH)CH(NH_2)COOH$	Stable

[a] The resolution medium contained a final concentration of 3 mg/ml of protein, 0.1 M imidazole, and 0.1 M of the sulfhydryl compound. The pH was adjusted with HCl to 6.0 at 0°. Resolution was allowed to proceed at 0° and followed as described under Methods. [b] No significant loss of activity observed within 50 min.

cysteine, structurally related to cysteine, were totally ineffective (see Table V). The specificity of aldehyde reagents in resolution will be dealt with in detail in a forthcoming publication.

Isolation of Apophosphorylase b. Various procedures were investigated for rapid isolation of the apoenzyme from the resolution reaction mixture. Prolonged exposure to the deforming agent caused irreversible denaturation. When attempts were made to separate the protein from the reaction mixture by dialysis, residual activities as high as 10–20% were observed. This could be attributed to the fact that the deforming agent was removed faster than the cofactor, allowing the latter to be trapped by the apoenzyme. On the other hand, precipitation of the apoenzyme with ammonium sulfate followed by gel filtration on Sephadex G-25 led to apoenzyme preparations displaying <1% residual activity. These preparations could be fully reactivated upon reconstitution with PLP. The best medium for storage of the apoenzyme and subsequent reconstitution was 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with HCl (Hedrick *et al.*, 1966).

Deforming Effect of Imidazolium Citrate. As mentioned above, imidazolium citrate or L-cysteine alone did not bring about resolution of the enzyme and, therefore, preparation of apophosphorylase *b* could be visualized as occurring in two successive steps: structural changes induced in the enzyme by the deforming agent and removal of PLP by an appropriate carbonyl reagent.

Table VI summarizes the specific activities displayed by phosphorylase *b* in the presence of various buffers. The highest specific activity (80 units/mg) was obtained in the maleate buffer used for assay, and this value was designated as 100%. In general, compounds that promoted resolution also lowered the activity of the enzyme (compare Tables I and VI). However, if the enzyme was incubated with the deforming agent for 60 min at 0°, then diluted 1:100 in the maleate buffer used for assay, the activity was fully restored in most instances (Table IV) indicating that the interaction of the enzyme with the deforming agents was reversible.

To demonstrate the structural changes caused by the deforming agents, the sedimentation properties of phosphorylase *b* were studied in a medium similar to that used for resolution, but in which L-cysteine was replaced by an equimolar concentration of 2-mercaptoethanol. Under these conditions PLP was not removed from phosphorylase but the enzyme structure was found to be remarkably altered. In the presence of 0.4 M imidazolium citrate and at pH 6.0, phosphorylase *b* had an $s_{20,w}$ value of 5.5 as compared to 8.4 (see also Keller and Cori, 1953) in the presence of 0.1 M sodium glycerophosphate (Figure 6). These results indicate that in imidazolium citrate phosphorylase *b* dissociated into monomer units similar to those obtained by treatment of this enzyme with *p*-mercuribenzoate (Madsen and Cori, 1956; Madsen and Gurd, 1956). The dissociation of phosphorylase *b* in imidazolium citrate was reversible. When the deforming agent was removed by dialysis *vs.* sodium glycerophosphate, phosphorylase regained its original sedimentation characteristics ($s_{20,w}$ = 8.4S).

TABLE VI: Effect of Various Salts on the Activity of Phosphorylase *b*.[a]

Buffer (M)	pH Adjusted with	% Act. in the Presence of the Buffer[b]	% Act. after Exposure to the Buffer
Maleate (0.1)	NaOH	100	100
Citrate (0.1)	NaOH	69	100
EDTA (0.1)	NaOH	100	100
Pyrophosphate (0.1)	NaOH	2	100
Triphosphate (0.1)	NaOH	3	57
Polyphosphate[c] (0.1)	NaOH	1	42
Glycerophosphate (0.1)	NaOH	81	100
ATP (0.05)	NaOH	47	100
Chloride (0.3)	Imidazole	24	97
Acetate (0.3)	Imidazole	37	98
Citrate (0.1)	Imidazole	20	96
Imidazole (0.1)	HCl	69	98

[a] Phosphorylase *b* (25 mg/ml) was diluted 1:1000 with each of the buffers indicated (all at pH 6.5) and assayed immediately. In the parallel experiment, phosphorylase *b* (2.5 mg/ml) was first incubated for 1 hr at pH 6.0 (0°) in each of the buffers listed, and then diluted 1:100 with the maleate buffer (pH 6.5) used for the assay of phosphorylase (Hedrick and Fischer, 1965). [b] Each of the buffers indicated contained also 0.04 M ME and 1 mg/ml of bovine serum albumin. [c] Average mol wt 1080.

The assumption that the deforming buffer promoted resolution by exposing the otherwise buried PLP residue was further supported by the following experiment. Phosphorylase *b* (0.09 μmole in 4.4 ml) was incubated with 0.57 μmole of [^{32}P]PLP at pH 6.5 in the presence of 0.05 M 2-mercaptoethanol and either 0.25 M imidazolium chloride or 0.05 M sodium glycerophosphate. After 30 min at 30° the protein was precipitated with saturated ammonium sulfate and redissolved in the glycerophosphate buffer three times in succession; 40% PLP was exchanged in the imidazole buffer and <6% in the glycerophosphate buffer.

Effect of AMP and Phosphorylation of the Protein on Resolution. As shown in Figure 7, resolution of phosphorylase *b* was blocked by the presence of AMP, a nucleotide which is known to affect many of the properties of the enzyme (Brown and Cori, 1961; Krebs and Fischer, 1962). Likewise, no resolution of phosphorylase occurred when phosphorylase *b* was converted to phosphorylase *a* by phosphorylase *b* kinase, Mg^{2+}, and ATP (Figure 7). However, apophos-

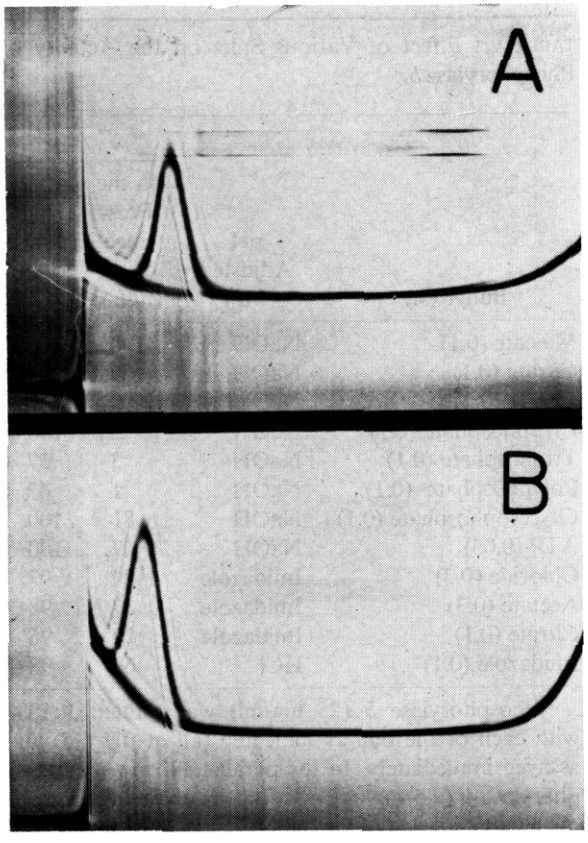

FIGURE 6: Effect of imidazolium citrate on the sedimentation properties of phosphorylase *b*. (A) Phosphorylase *b* (3.6 mg/ml) in 0.1 M sodium glycerophosphate, 0.1 M ME, adjusted to pH 6.0 with HCl at 0°, $s_{20,w}$ 8.4. (B) Phosphorylase *b* (5.0 mg/ml) in 0.4 M imidazole, 0.1 M ME, adjusted to pH 6.0 with citric acid at 0°, $s_{20,w}$ 5.5. The temperature of the rotor was kept at 4° during the run and the rotor speed was 59,780 rpm. The pictures given were taken 24 min after attainment of maximum speed.

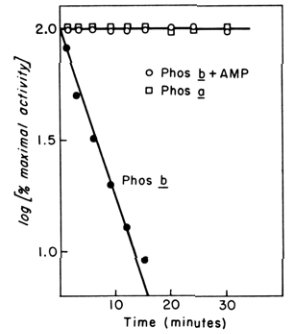

FIGURE 7: Effect of AMP and phosphorylation of the protein on resolution of phosphorylase. Resolution of phosphorylase *b* (3.1 mg/ml) was carried out at 0° in 0.4 M imidazole and 0.1 M L-cysteine·HCl adjusted to pH 6.5 with citric acid. The same reaction was carried out in the presence of 10^{-2} M AMP or with phosphorylase *a*.

phorylase *a* could be prepared from apophosphorylase *b* in the regular *b* to *a* conversion reaction (Hedrick *et al.*, 1966).

Procedure for the Preparation of Apophosphorylase b. On the basis of the findings mentioned above the following procedure was established for the preparation of apophosphorylase *b*. A suspension of phosphorylase *b* crystals is centrifuged (5 min at 15,000 rpm) and the pellet is dissolved at 30° in a 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol buffer adjusted to pH 7.0 with HCl to a final protein concentration of 20–30 mg/ml. This solution is passed through a small charcoal–cellulose column to remove the bound AMP (Fischer and Krebs, 1958) and kept at 0°.

The stock phosphorylase *b* solution is diluted to 4 mg/ml in the glycerophosphate–mercaptoethanol buffer described above. Resolution is initiated by dilution of this solution with an equal volume of a freshly prepared solution containing 0.8 M imidazole–0.2 M L-cysteine·HCl adjusted to pH 6.2 with citric acid at 0°. The reaction is allowed to proceed for 35 min (7–9 half-lives) at 0°, and terminated by addition of 1 volume of saturated ammonium sulfate, pH 6.2. The precipitated apoenzyme is dissolved at 22° in a minimum volume of 0.05 M glycerophosphate–0.05 M 2-mercaptoethanol neutralized to pH 7.0 with HCl. The remaining ammonium sulfate, PLP, and cysteine carried over from the pellet are removed by passing the solution through a 1.5 × 25 cm column of Sephadex G-25 equilibrated and eluted at 22° with the same glycerophosphate buffer. The ultraviolet absorbancy of the effluent determined at 280 mμ and the protein fractions are pooled and characterized, as indicated under Methods.

The over-all yield of the apoenzyme for batches of 5–250 mg is 80–95%. It usually displays 0.1 –1% of the specific activity of the original holoenzyme and contains an equivalent PLP content as determined spectrophotometrically.

For reconstitution, the apoenzyme is diluted to a protein concentration of *ca.* 2 mg/ml and incubated for 10 min at pH 7.0, 37°, with an equal volume of 2×10^{-4} M PLP (Hedrick *et al.*, 1966). Restoration of 95–100% of the specific activity of phosphorylase *b* is usually obtained, but values as low as 85% have been occasionally observed, probably due to unspecific denaturation during isolation of the apoenzyme.

Discussion

This paper describes a new procedure for the preparation of apophosphorylase *b* by which total removal of PLP from the enzyme can be achieved with essentially no irreversible damage to the protein. The apoenzyme thus prepared can be fully reactivated upon restoration of PLP, and the reconstituted enzyme is indistinguishable from native phosphorylase *b* (Hedrick *et al.*, 1966).

Two distinct steps are involved in the process of

TABLE VII: Effect of Protonated and Nonprotonated Imidazole on the Rate of Resolution.[a]

Series	pH	$[Im^+] + [Im]$ (M)	k (min^{-1})	$k/[ImH^+]$	$k/[Im]$	$k/[ImH^+Im]$
I	6.00	0.38	0.108	0.32	2.84	0.83
	6.25	0.39	0.102	0.30	1.85	0.55
	6.50	0.40	0.094	0.29	1.16	0.36
	6.75	0.43	0.085	0.32	0.51	0.19
	7.00	0.47	0.077	0.28	0.39	0.14
	7.25	0.53	0.069	0.36	0.20	0.11
	7.50	0.62	0.060	0.38	0.13	0.08
II	6.50	0.50	0.100	0.25	1.00	2.50
	6.75	0.50	0.086	0.28	0.45	1.46
	7.00	0.50	0.077	0.27	0.37	1.26
	7.25	0.50	0.069	0.38	0.22	1.19

[a] Rate coefficients for resolution were obtained from pH-dependency curves (–●–) in Figure 2 and (–▲–) in Figure 3. The total concentration of imidazole in series I was determined experimentally by the amount of imidazole (free base) needed to adjust the pH of the buffers to the indicated value; the imidazole concentration was constant in series II. The molar concentrations of imidazolium ions $[ImH^+]$ and imidazole free base [Im] were obtained by multiplying the total imidazole concentration by the molar fraction of each species. The molar fractions were calculated from a titration curve of imidazole (see also Bruice and Topping, 1963).

resolution: distortion of the protein that exposes the otherwise "buried" PLP residue and removal of the unmasked cofactor following reaction with an appropriate carbonyl reagent. These two steps can be studied separately since neither the deforming agent nor the carbonyl reagent alone can promote resolution of the enzyme.

The over-all process is affected by many factors such as the nature of the deforming agent and its concentration, pH of the medium, temperature, structure of the PLP reagent, and even the procedure followed to isolate the apoenzyme. The effect of imidazole on the process of resolution is quite remarkable. This was best demonstrated when two series of citrate buffers were compared: between pH 6.0 and 7.0 the rate of resolution was one to two orders of magnitude greater when imidazole rather than sodium hydroxide was used to adjust the pH of the buffer (Figure 3). The rate of resolution was directly related to the concentration of imidazole present in the reaction mixture. At lower pH values, lower concentrations of imidazole were needed to promote resolution (Figure 4). It should be emphasized, however, that high concentrations of imidazole (>1 M at pH 7.0 or 0.6 M at pH 6.0) resulted in irreversible denaturation of the enzyme.

Around neutrality, imidazole exists in two molecular species: the nonionized free base and the protonated imidazolium ion. Since Bruice and Topping (1963) suggested that both imidazole and imidazolium ions are catalytically involved in the reaction between pyridoxal and α-amino acids, the possibility was considered that the reaction described here also involved concerted catalysis by these two molecular species. However, as seen in Table VII, the rate coefficients for resolution were roughly proportional to the concentration of the imidazolium ions rather than to the concentration of the uncharged imidazole molecules or to the product of concentrations of both species; this indicates that the protonated form of imidazole is indeed involved in this process. The increased rates of resolution observed at lower pH values can, therefore, be attributed, in part, to an increase in the concentration of the imidazolium ion. The main effect of pH is probably on the protein itself, altering its state of ionization and conformation, and also the mode of binding of PLP.

In contrast to L-cysteine, compounds with analogous structure such as cysteamine or penicillamine were either very poor or totally ineffective in promoting resolution. This phenomenon could not be attributed to a lack of reactivity toward the cofactor since cysteamine, *e.g.*, binds to PLP just as well as cysteine. These results imply that a very specific interaction occurs between the functional groups of cysteine and those of the PLP site, and that the aldehyde reagent probably directly participates in the bond-breaking step required for resolution.

Little is presently known as to the mode of action of imidazolium citrate in promoting the removal of PLP from phosphorylase *b*. Gross conformational alterations in the architecture of the enzyme, structural modifications of the PLP binding site, and catalysis of the bond-breaking steps could all be visualized. The lowering of enzyme activity, the accelerated rate of PLP exchange, and the dissociation of the enzyme in the presence of the imidazole buffer alone strongly suggest a reversible deformation of the protein that exposes the PLP site to extraneous carbonyl reagents. It is difficult

to decide whether monomerization is a prerequisite for resolution. Consistent with this view are the findings that both monomerization and resolution are increased at high temperature, and blocked by AMP or phosphorylation of the protein as will be detailed in a later publication. Against it, however, is the observation that a high concentration of NaCl which favors the dissociation induced by the deformer (J. L. Hedrick, S. Shaltiel, and E. H. Fischer, unpublished data) inhibits the resolution of the enzyme (Table II).

The conditions described above for the resolution of phosphorylase are of course far from physiological. Nonetheless, the findings that under rather specific conditions, phosphorylase *b* can undergo structural changes that expose PLP to outside reactants, and ultimately allow its removal from the enzyme, raise the possibility that a phenomenon of this nature may take place in biological systems as part of a regulatory mechanism.

The approach described in this paper for the resolution of phosphorylase *b* may find a variety of applications in the selective modification of proteins and the preparation of other apoenzymes. It may provide an additional tool in studies correlating the structure of proteins with their biological function.

Acknowledgments

The authors are greatly indebted to Mr. Alan J. Smith and Mr. Robert Sorensen for their excellent technical assistance. Sincere thanks are also due to Mrs. Wen Ling T. Chu for performing the ultracentrifuge analysis and to Dr. Anna Pocker for the synthesis of ^{32}P-labeled PLP, and many stimulating discussions.

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