Conformational Changes and the Mechanism of Resolution of Glycogen Phosphorylase *b**

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ABSTRACT: This study purports to define the role of deforming agents in promoting the release of pyridoxal 5′-phosphate from rabbit muscle phosphorylase *b* and the changes in structure undergone by the latter during this process. Deformation (localized reversible denaturation) of the enzyme is seen as a highly specific reaction whose course is influenced by both the nature of the cations and anions involved. While neither ammonia nor primary, secondary, or tertiary amines allowed resolution to occur, imidazole and several of its derivatives including 1-, 2-, or 4-methylimidazole were highly effective. By contrast, 4-imidazole acetic acid, histidine, was or histamine either poorly active or totally inactive. Di-, tri-, or tetracarboxylic acids which, by themselves, were quite ineffective in promoting resolution greatly accelerated this phenomenon when used in conjunction with imidazole. Specificity with respect to anions was further demonstrated when resolution was followed as a function of temperature: removal of pyridoxal 5′-phosphate increased sharply with temperature in imidazole citrate but decreased in imidazolium chloride. Data presented indicate that deforming agents cause dissociation of the enzyme into monomers and that this process unmasks the cofactor, allowing it to interact with carbonyl reagents such as L-cysteine.

Rapid resolution is also observed in the presence of hydroxylamine following monomerization of the enzyme with *p*-mercuribenzoate according to the procedure of Madsen and Cori (Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1075). Changes in structure upon addition of deforming agents are further evidenced by changes in ultraviolet and fluorescence spectra due to the bound cofactor; these changes are blocked by addition of effectors such as adenosine monophosphate which oppose resolution of the enzyme. No evidence was obtained that resolution proceeds through the formation of a Schiff base between the protein and the cofactor which, because of its high reactivity, could spontaneously decompose into apoenzyme and free pyridoxal 5′-phosphate. A possible scheme for the resolution of phosphorylase consistent with the data is presented.

The ease with which prosthetic groups can be removed from enzymes varies greatly with the affinity of the cofactor for the protein, and the nature of bonding between them (Åkeson *et al.*, 1963; Rossi-Fanelli *et al.*, 1964; Fasella, 1967). In the case of tightly bound prosthetic groups, extremes of pH or precipitation of the protein have been utilized, and often partial irreversible denaturation of the protein occurs (Reed *et al.*, 1958; Nawa *et al.*, 1960; Kosower *et al.*, 1962). The binding of pyridoxal 5′-phosphate to glycogen phosphorylase[1] involves covalent bonds (Kent *et al.*, 1958; Fischer, 1964) which contribute to the extremely tight binding of the prosthetic group to the protein. However, by the use of specific "deforming agents" the enzyme could be resolved without irreversible damage to the structure of the protein (Shaltiel *et al.*, 1966).

It was previously shown that in the presence of a deforming buffer, phosphorylase displayed a low enzymatic activity, exchanged its bound pyridoxal 5′-phosphate with free pyridoxal 5′-phosphate added to the medium, and readily released the cofactor in the presence of aldehyde reagents. It was therefore proposed that two distinct steps were involved in the process of resolution: (a) distortion of the protein that exposes the otherwise "buried" pyridoxal 5′-phosphate residues, and (b) removal of the unmasked cofactor by combination with a carbonyl reagent. Since neither the deforming agent nor the carbonyl reagent alone promoted resolution, it was suggested that resolution proceeded through the formation of an intermediate with properties different from those of the native enzyme.

The present paper deals with the mechanism of resolution of phosphorylase; more particularly, an attempt was made to define the role of the deforming agent by characterizing the enzyme in its presence and absence. It was shown as a whole that deformers promote dissociation of the oligomeric protein: compounds or reaction conditions that favored dissociation usually enhanced the rate of resolution while reactants such as AMP, G-6-P, UDPG, or caffeine that opposed dissociation also retarded resolution. Whether or not monomer formation in itself allowed resolution to occur or whether dissociation brought about an alteration of the pyridoxal 5′-phosphate site which then permitted rapid reaction of the cofactor with a carbonyl reagent was difficult to ascertain. The latter alternative probably holds since deforming agents quenched the pyridoxal 5′-phosphate fluorescence of the protein and this quenching was prevented by AMP which blocks resolution. This manuscript is the fourth of a series on the role of pyri-

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

doxal 5′-phosphate in phosphorylase. For the previous publication, see Hedrick *et al.* (1966).

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared by the method of Fischer *et al.* (1958) with the modification of Krebs *et al.* (1964). The enzyme was measured, resolved, and reconstituted by methods described earlier in this series (Hedrick and Fischer, 1965; Shaltiel *et al.*, 1966; Hedrick *et al.*, 1966). Molecular weights of 185,000 and 92,500 were used for phosphorylase *b* and the enzyme monomer, respectively, as recently determined (Seery *et al.*, 1967; De Vincenzi and Hedrick, 1967; Ullmann *et al.*, 1968). All chemicals used were of the highest grade available. Commercial imidazole was usually found to contain a fluorescent impurity (emission maximum at 385 mμ) that could be removed by dissolving the material in a minimum volume of ethyl acetate and treating this solution twice with charcoal. Imidazole was then crystallized twice from the same solvent and a third time from water. The resulting crystals were colorless and had no fluorescence even at the highest concentrations used.

Absorption spectra were taken with a Beckman DK-1 recording spectrophotometer. Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge using either double-sector or wedge window cells. The temperature of the rotor during the runs was maintained within ±0.2° of the indicated value. Movement of boundaries was calculated from direct microcomparator measurements of the schlieren diagrams; corrections for viscosity and density of the various buffers were applied in calculating the sedimentation coefficients. High-speed sedimentation equilibrium runs were carried out in multiple cells with 2-mm light path according to the procedure of Yphantis (1964). Speeds of 11,000 rpm were used for phosphorylase *a* and 13,000 rpm for phosphorylase *b* and runs were carried out until equilibrium was attained; the protein concentration was of the order of 0.5 mg/ml.

Results

Several lines of evidence reported earlier in this series suggested that phosphorylase *b* is structurally altered by the deforming buffers used to bring about the resolution (*i.e.*, removal of pyridoxal 5′-phosphate) of the enzyme. In the presence of imidazolium chloride, for example, there was a remarkable enhancement in the rate of exchange of pyridoxal 5′-phosphate with ^{32}P-labeled pyridoxal 5′-phosphate. Also, it was shown that in the presence of the deforming buffers, phosphorylase *b* dissociated into monomers and its catalytic activity was drastically lowered (Shaltiel *et al.*, 1966; Hedrick *et al.*, 1966).

Compounds Related to Imidazole and Citric Acid as Deforming Agents. Since earlier studies indicated rather stringent structural requirements for compounds to act as deforming agents, a number of nitrogenous bases were tested as to their ability to promote resolution. As seen in Table I, neither ammonia nor primary, secondary, or tertiary alkylamines will act as deformers under standard sets of conditions, *i.e.*, in the presence of 0.1 M cysteine, 0°, and pH 6.5. On the other hand, no absolute specificity for imidazole itself could be established since some of its structural analogs or derivatives were also effective. For example, 4- (or 5-) methylimidazole was just as potent a deformer as imidazole itself, while methylation of the imino nitrogen (1-methylimidazole) or of position 2 of the imidazole ring was still appreciably effective ($t_{0.5}$ for resolution = 22 min *vs.* 8 min). On the other hand, if an additional carboxyl group was added in position 4 (or 5), *e.g.*, imidazole 4- (or 5-) acetic acid, the resulting derivative became totally inactive. Larger imidazole derivatives such as L-histidine, histamine, or carnosine (β-alanyl-L-histidine) were only poorly effective, if at all, while heterocyclic bases such as pyridine or piperidine (the cyclic alipathic analog of pyridine) had intermediate efficiencies. As previously stated, distortion of the enzyme depended upon the nature of both the cation and the anion. For example, among carboxylic acids, the rate of resolution increased with the number of carboxyl groups per molecule (acetate < succinate < citrate). In view of the possibility that deformation and subsequent release of pyridoxal 5′-phosphate could constitute a physiological means by which phosphorylase activity or availability of pyridoxal 5′-phosphate could be controlled, resolution of phosphorylase was investigated in the presence of citric acid cycle intermediates such as oxalacetic acid, α-ketoglutaric acid, fumaric acid, and malic acid. When these were tested at 0.1 M concentration in the presence of 0.1 M L-cysteine, pH 7.0, 23°, none proved to be more effective than citrate itself ($t_{0.5}$ = 20 min). On the other hand, they were effective deformers when used in conjunction with imidazole. Solutions containing 0.4 M imidazole and 0.1 M L-

TABLE I: Effect of Various Nitrogenous Bases on the Rate of Resolution of Phosphorylase *b*.[a]

Nitrogen Base	$t_{0.5}$ for Loss of Act. (min)
Ammonia	Stable[b]
Ethylamine	Stable
Diethylamine	Stable
Triethylamine	Stable
Imidazole	8
1-Methylimidazole	22
2-Methylimidazole	23
4- (or 5-) Methylimidazole	8
4- (or 5-) Imidazole acetic acid	Stable
Histidine	75
Histamine	101
Carnosine	Stable
Pyrrole	28[c]
Pyridine	35
Piperidine	67

[a] Reaction mixtures contained 1 mg/ml of AMP-free phosphorylase *b*, 0.1 M L-cysteine hydrochloride, and 0.4 mole of each of the nitrogen bases listed except 4- (or 5-) imidazole acetic acid (0.2 M) and carnosine (0.1 M). The pH was adjusted to 6.5 with HCl. Resolutions were carried out at 0°. Control experiments were carried out in which L-cysteine was replaced by 2-mercaptoethanol; in every instance the controls were stable. [b] Less than 5% loss of activity observed within 60 min. [c] Some precipitation of protein occurred during the reaction.

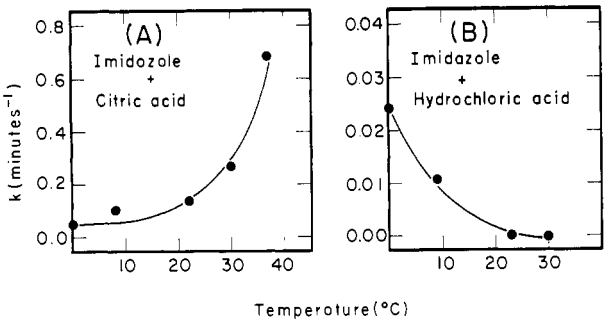


FIGURE 1: Effect of temperature on the resolution of phosphorylase *b*. The resolution medium contained final concentrations of 2.75 mg/ml of protein, 0.4 M imidazole, and 0.1 M L-cysteine hydrochloride. The pH was adjusted to 7.0 with citric acid (A) or with HCl (B).

cysteine, adjusted to pH 6.0 with either malic acid, citric acid, or EDTA, gave half-lives for resolution of 30, 12, and 11.5 min, respectively. Of course, the concentration of the di- and tricarboxylic acid intermediates that was used in these experiments was totally outside the physiological range.

The contribution of both the cations and anions to deformation is also a function of temperature. In an imidazolium citrate buffer, the rate of resolution increased with increasing temperature. In contrast, the opposite was found in an imidazolium chloride buffer of the same concentration, *i.e.*, the rate of resolution *decreased* with increasing temperature (Figure 1). At pH 7.0 and 30°, resolution proceeded with a half-life of 2.6 min in imidazolium citrate while no resolution was detected after 40 min in imidazolium chloride.

Urea and Guanidine as Deformers. The deforming ability of urea and guanidine, two classical protein denaturants, was also tested under various experimental conditions. Although some irreversible loss of activity occurred during resolution, urea did act as a deformer when used at a concentration between 1 and 1.8 M; within this range, phosphorylase *b* lost its activity when L-cysteine was present but not in the presence of 2-mercaptoethanol (Figure 2). Such a cysteine-dependent inactivation of phosphorylase *b* at low molarities of urea was reported by Appleman (1962); the conclusion reached that this phenomenon could be attributed to resolution of the enzyme was confirmed and extended here. In a preparative experiment

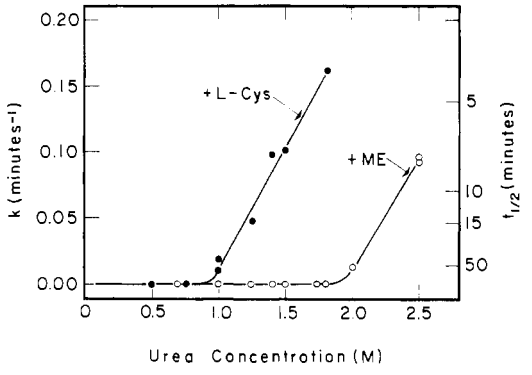


FIGURE 2: Effect of urea on the rate of resolution of phosphorylase *b* at 0°. The reaction mixtures contained 2.3 mg/ml of enzyme and 0.1 M L-cysteine (free base) (●) or 0.1 M 2-mercaptoethanol and 0.01 M sodium β-glycerophosphate (○). The pH was adjusted to 6.5 with HCl.

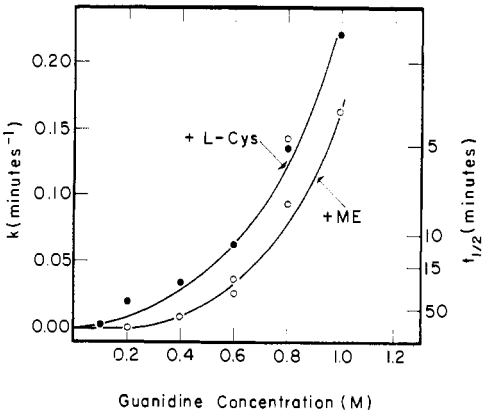


FIGURE 3: Effect of guanidine on the rate of resolution of phosphorylase *b*. Resolution of the enzyme was performed exactly as described in Figure 2 except that guanidine hydrochloride replaced urea.

involving 1.5 M urea, 0.1 M L-cysteine, and 0.01 M sodium β-glycerophosphate (pH 6.5), resolution of phosphorylase *b* (2 mg/ml) occurred with a half-life of 5.3 min at 0°. After 45 min the apoenzyme was precipitated with $(NH_4)_2SO_4$ and then subjected to gel filtration on Sephadex G-25 as described previously (Shaltiel *et al.*, 1966). The isolated apoenzyme preparation had a residual activity of less than 1% and could be reactivated to the extent of 78% by restoration of pyridoxal 5′-phosphate.

Treatment of phosphorylase *b* with guanidine also resulted in inactivation of the enzyme (Figure 3). However, the loss of activity occurred both with L-cysteine and 2-mercaptoethanol; it was only slightly less pronounced in the latter instance, indicating that irreversible denaturation (rather than deformation) was mainly involved. As expected, loss of activity in guanidine

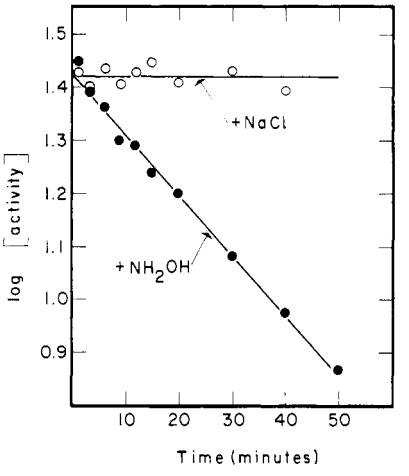


FIGURE 4: Resolution of phosphorylase *b* in the presence of *p*-mercuribenzoate and hydroxylamine. A stock solution of the enzyme (31 mg/ml) was dialyzed overnight against 0.05 M sodium β-glycerophosphate and 0.001 M EDTA (pH 7.0) to remove the 2-mercaptoethanol. The dialyzed solution was diluted and reacted with *p*-mercuribenzoate (final concentration, 3.8 mg/ml of protein and 5×10^{-4} M *p*-mercuribenzoate). The reaction was allowed to proceed for 2 hr at pH 6.0 and 0°; then an equivalent volume of 0.2 M hydroxylamine (HCl salt) was added. In the control experiment hydroxylamine hydrochloride was replaced by an equal concentration of NaCl.

TABLE II: Effect of Various Compounds on the Rate of Resolution of Phosphorylase *b*.[a]

Compound Added	Final Concn (M)	$t_{0.5}$ for Loss of Act. (min)
None		7
AMP	1×10^{-3}	25
AMP	1×10^{-2}	Stable[b]
ADP	1×10^{-3}	9
ATP	1×10^{-4}	10
Cyclic 3′,5′-AMP	1×10^{-3}	8
UMP	5×10^{-4}	7
UMP	1×10^{-2}	9
UDPG	5×10^{-4}	27
UDPG	1×10^{-2}	42
Glucose-1-P	1×10^{-2}	12
Glucose-6-P	1×10^{-2}	27
Fructose-6-P	1×10^{-2}	8
Glucose	1×10^{-2}	18
Glyceraldehyde	1×10^{-2}	8
Glycolaldehyde	1×10^{-2}	7
Glycogen	1%	9
Caffeine	1×10^{-2}	Stable
$Mg(CH_3CO_2)_2$	1×10^{-2}	6

[a] Reaction mixtures contained 2 mg/ml of AMP-free phosphorylase *b*, 0.4 M imidazole, 0.1 M L-cysteine hydrochloride, and the effectors at the concentration indicated. The pH was adjusted to 6.5 with citric acid; the reactions were carried out at 0°. Control experiments containing 0.1 M 2-mercaptoethanol instead of L-cysteine were stable in all instances. [b] Less than 5% loss of activity observed within 50 min.

occurred at lower concentrations than in urea, which is in good agreement with the accepted view that guanidine is a more potent protein denaturant than urea (Greenstein, 1938; Reithel *et al.*, 1964; Kawahara and Tanford, 1966; Tanford *et al.*, 1966).

Dissociation of the Enzyme and Rates of Resolution. In the presence of imidazolium citrate, phosphorylase *b* ($s_{20,w}$ = 8.4 S) dissociated into monomer units ($s_{20,w}$ = 5.5 S) in a reaction reminiscent of that previously observed following *p*-mercuribenzoate treatment of the enzyme (Madsen and Cori, 1956; Madsen and Gurd, 1956). It was therefore of interest to determine whether or not in the presence of an aldehyde reagent the *p*-mercuribenzoate-treated enzyme would also undergo resolution, *i.e.*, would behave as a deformed molecule. This was indeed the case: in the presence of 0.2 M hydroxylamine[2] resolution ensued with a half-life of 27 min (Figure 4). Removal of hydroxylamine and the mercuribenzoate groups attached to the enzyme by addition of an excess of 2-mercaptoethanol followed by gel filtration on Sephadex G-25, yielded an apo-

[2] L-Cysteine could not be used in this case as the pyridoxal 5′-phosphate reagent since it is known to remove the *p*-mercuribenzoate from the cysteinyl residues of the protein and consequently reassociate the enzyme (Boyer, 1954; Madsen and Cori, 1956).

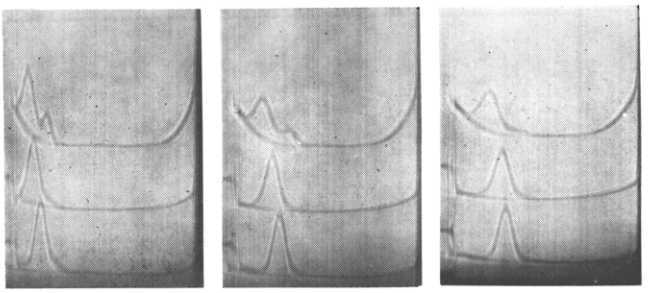


FIGURE 5: Ultracentrifuge studies of phosphorylase *b* in the presence of deforming and nondeforming buffers as a function of temperature. Sedimentation velocities were run at 52,640 rpm at 1.2°, A; 10.0°, B; and 20.0°, C. Phosphorylase *b* (5 mg/ml) was dissolved in 0.4 M imidazole neutralized with citric acid (upper pattern); 0.4 M imidazole neutralized with HCl (middle pattern); 0.05 M sodium β-glycerophosphate neutralized with HCl (lower pattern). All buffers contained 0.1 M β-mercaptoethanol.

enzyme preparation that could be reactivated to the extent of 66% after reconstitution with pyridoxal 5′-phosphate.

In general, there appeared to be a close relationship between the extent of dissociation and the rate of resolution of the enzyme. Compounds and reaction conditions that favored dissociation of the enzyme also allowed resolution to occur when aldehyde trapping agents were included in the medium. On the other hand, compounds that prevented dissociation of the enzyme in the presence of the deformer also lowered the rate of resolution. This is exemplified in Table II in which a number of compounds (effector nucleotides, substrates, substrate analogs, etc.) known to affect the enzymatic properties of phosphorylase were tested as to their ability to influence resolution. As can be seen, nucleotides that activate (AMP) or inhibit (UDPG; Madsen, 1961) phosphorylase or compounds such as glucose, glucose-6-P, or caffeine that affect either the catalytic or the structural properties of the enzyme opposed resolution. Increasing the concentration of imidazolium citrate or the temperature, or lowering the protein concentration or the pH, increased both the extent of dissociation of the enzyme and the rate of resolution. Even for the resolutions carried out in imidazolium citrate *vs.* imidazolium chloride, which were shown to be affected in opposite ways by temperature, removal of pyridoxal 5′-phosphate paralleled dissociation of the enzyme. This is seen in Figure 5 where it can be calculated from the relative areas of the two sedimenting species that in imidazolium citrate, dissociation increases with temperature (74% monomer at 1–2°, 82% at 10.0°, and 90% at 20.0°), whereas in imidazolium chloride the extent of dissociation decreased with increasing temperatures (10% monomer at 1.2°, 4% at 10.0°, and 0% at 20.0°). No dissociation occurred at these three temperatures in the presence of the nondeforming, sodium glycerophosphate buffer. Similarly, considerable dissociation was observed in 1.5 M urea (pH 6.5) conditions that promoted resolution.

Dissociation of phosphorylase *b* by 0.4 M imidazole–0.1 M 2-mercaptoethanol (pH 6.5) was also demonstrated by high-speed sedimentation equilibrium (Yphantis, 1964). Using 2-mm solution columns to decrease the time required to reach equilibrium, values of $145 \pm 6 \times 10^3$ and $159 \pm 7 \times 10^3$ were obtained for the weight-average and *z*-average molecular weights, respectively. Since the molecular weight of the enzyme monomer is 92,500 (Seery *et al.*, 1967; De Vincenzi and Hedrick, 1967; Ullmann *et al.*, 1968), this result was interpreted as

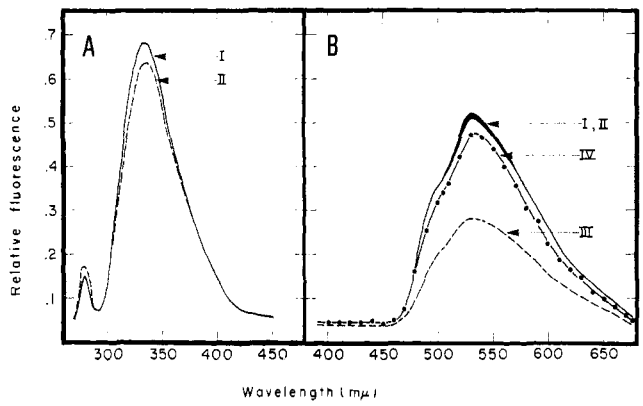


FIGURE 6: Effect of the imidazolium citrate buffer on the fluorescence properties of phosphorylase *b*. (A) Emission spectra of the protein (excitation at 280 mμ). The enzyme (0.085 mg/ml) was dissolved in 0.1 M sodium β-glycerophosphate adjusted to pH 6.0 with HCl (I) or in 0.4 M imidazole adjusted to pH 6.0 with citric acid (II). (B) Emission spectra due to bound pyridoxal 5′-phosphate (excitation at 330 mμ). The enzyme solutions (5.2 mg/ml) were dissolved in either 0.1 M sodium β-glycerophosphate adjusted to pH 6.0 with HCl (I), 0.1 M sodium β-glycerophosphate and 10^{-3} M AMP adjusted to pH 6.0 with HCl (II), 0.4 M imidazole adjusted to pH 6.0 with citric acid (III), or the same as III but with 10^{-3} M AMP (IV). All solutions contained also 0.1 M 2-mercaptoethanol; spectra were taken at 4°, 5 min after addition of the enzyme.

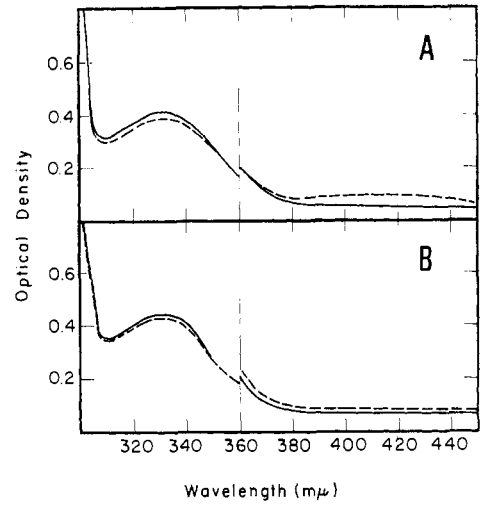


FIGURE 7: Influence of AMP on the absorption spectra of phosphorylase *b* in a nondeforming (sodium β-glycerophosphate) and deforming (imidazolium citrate) buffer. The enzyme (5.2 mg/ml) was dissolved in each of the following media: (A) sodium β-glycerophosphate (0.1 M) adjusted to pH 6.0 with HCl (——) and 0.4 M imidazole (free base) adjusted to pH 6.0 with citric acid (– – –); (B) same as above but in the presence of 10^{-3} M AMP. All reaction mixtures also contained 0.1 M 2-mercaptoethanol; spectra were taken at 4°, 5 min after addition of the enzyme.

reflecting a rapid equilibrium between the monomeric and dimeric species of phosphorylase, in agreement with the conclusions that dissociation is required for resolution.

Fluorescence Measurements. In addition to its protein fluorescence (excitation at 280 mμ and emission maximum at 335 mμ; Figure 6A, curve I), glycogen phosphorylase *b* possesses a fluorescence in the visible range of the spectrum associated with the presence of bound pyridoxal 5′-phosphate (Shaltiel and Fischer, 1967). This fluorescence (excitation at 330 mμ, emission maximum at 535 mμ; Figure 6B, curve I) was quenched to the extent of 46% when an imidazolium citrate buffer was added to the enzyme (Figure 6B, curve III), but unaffected by AMP (Figure 6B, curve II *vs.* curve I). On the other hand, when 10^{-3} M AMP was added to the imidazolium citrate buffer, fluorescence was restored almost completely (curve IV), in line with the earlier observation that this nucleotide blocks resolution (Shaltiel *et al.*, 1966). As expected, the 335-mμ fluorescence due to the tryptophanyl residues in the protein was quenched less than 5% by the presence of the deforming buffer (Figure 6A, curve II).

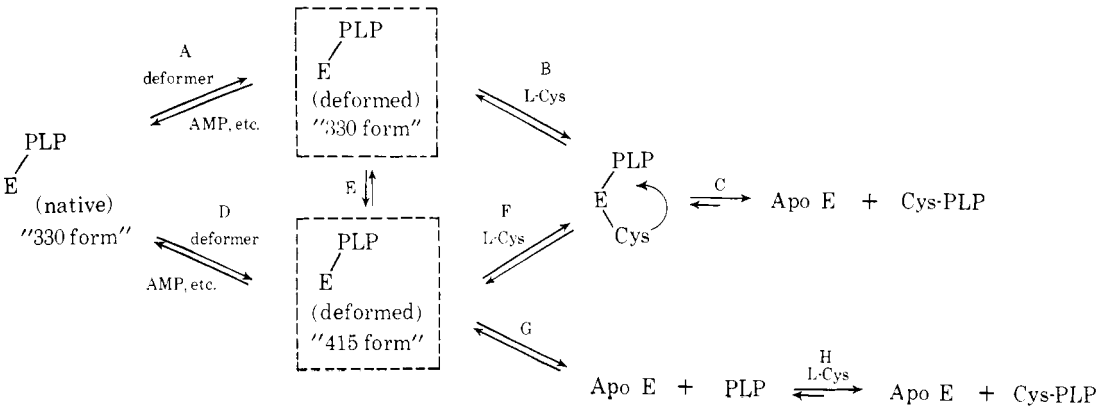
Absorption Spectra Studies. At neutral pH phosphorylase displays a small absorption peak at 330 mμ due to bound pyridoxal 5′-phosphate in addition to the large absorption maximum at 280 mμ due to the protein itself. At acidic or basic pH values (below 4.5 or above 9.5) the 330-mμ absorption maximum shifts to 415 mμ (Kent *et al.*, 1958; Fischer, 1964). At temperatures approaching 0° and particularly in the presence of imidazolium citrate, there was a small but noticeable conversion of the "330 form" of phosphorylase into the "415 form" even at pH 6.0 (Figure 7A). Once again, this shift was prevented when 10^{-3} M AMP was added to the enzyme solution (Figure 7B). Under similar conditions, no "415 absorption" was observed when the enzyme was dissolved in a sodium glycerophosphate buffer whether or not AMP was present. It should be emphasized, however, that the formation of the "415 form" of phosphorylase was not observed at higher temperatures (*e.g.*, 22° either in the presence or absence of the deforming buffer), casting some doubt on the possible formation of a 415-mμ-absorbing Schiff base intermediate during resolution.

Discussion

The search for deforming agents that would bring about the resolution of phosphorylase was based on the following premise. Compounds should exist that would specifically affect the structure of the pyridoxal 5′-phosphate binding site without disrupting other regions of the protein. It was assumed that denaturation does not occur as a sudden, total collapse of the structure of a protein but proceeds in a stepwise fashion initially affecting certain more susceptible regions of the molecule. Whether or not a particular region would be affected would depend upon the chemical properties of the denaturant, the reaction conditions and the dominant type of interactions maintaining the conformation of that particular site. Conversely, the same compound could act either as a deformer or a denaturing agent depending upon conditions of pH, temperature, or concentration. An ideal deformer would be one that would "loosen" the structure of a certain site without damage to the rest of the molecule; when resolution of an enzyme is involved, an ideal deformer would be one that would produce an apoenzyme that could be quantitatively reactivated by restoration of the coenzyme. The term "deformer" was therefore introduced as a purely operational one; to designate those compounds that produced localized denaturation of a protein that could be totally reversed by their removal.

The present study indicates that several compounds were efficient deformers for the removal of pyridoxal 5′-phosphate from phosphorylase. Among cationic agents, imidazole was highly effective whereas ammonia and alkylamines or other

SCHEME I



imidazole derivatives were either poorly or totally ineffective. Even urea could serve somewhat as a deforming agent when used within narrow limits of pH, temperature, and concentration (pH 6.5, 0°, and 1.5 M). Guanidine, on the other hand, could not be used in this respect; under all conditions tested, irreversible denaturation accompanied resolution.

Two pathways can be proposed for the resolution of phosphorylase (Scheme I), where E designates the enzyme, PLP, pyridoxal 5′-phosphate, and the numbers in quotes, the spectral species. In the upper pathway, the enzyme is "deformed" and this localized reversible denaturation unmasks the cofactor that can now react with cysteine. In the lower pathway, deformation leads to the formation of a Schiff base between the cofactor and protein. Since this Schiff base is very reactive (or unstable), it can either react with cysteine (F) or with water (G) to yield apoenzyme and free pyridoxal 5′-phosphate. The equilibrium of this latter reaction would then be displaced toward resolution by reaction of free pyridoxal 5′-phosphate with cysteine (H). Two steps are common to both pathways: (a) deformation of the enzyme (reactions A or D); (b) reaction of the deformed enzyme or free pyridoxal 5′-phosphate with an appropriate aldehyde reagent to yield the apoenzyme (B–C or H). These two steps can be studied separately since neither the deforming agents nor the aldehyde reagent alone will promote resolution. The aim of the present paper was to establish which of the above pathways was operative, and that "deformed phosphorylase" was a molecular entity structurally distinct from the native, catalytically active enzyme.

From earlier studies on the optical properties of phosphorylase, pyridoxal 5′-phosphate was shown to be bound to the protein in two spectrally distinct forms: one (ascribed to a substituted aldamine) absorbing maximally at 330 mμ and the other (ascribed to the formation of a Schiff base between the cofactor and the protein) absorbing at 415 mμ (Kent *et al.*, 1958). Under acidic (pH 4.5) or basic (pH 9.5) conditions, the "415 form" is produced and pyridoxal 5′-phosphate readily dissociates from the enzyme. However, the apoenzyme produced under these conditions could not be quantitatively reactivated by restoration of the coenzyme due to irreversible denaturation of the enzyme. A first possibility, therefore, was to assume that the deformers shifted the equilibrium toward the "415 form" (susceptible to attack by the aldehyde reagent) allowing it to appear even at neutral pH. In support of this alternative was the observation that in the presence of imidazolium citrate, one can observe the appearance of a yellow color corresponding to the 415-mμ absorption, though most of the protein remains in the "330 form" (Figure 7). This "415 form" can be reduced by $NaBH_4$ (Kent *et al.*, 1958; Strausbauch *et al.*, 1967); AMP, which blocks resolution, also prevents its formation.

In spite of these observations there is reason to believe that the formation of the 415-mμ-absorbing Schiff base (steps D or E) is not required for the removal of pyridoxal 5′-phosphate. First, the yellow intermediate produced in imidazolium citrate can be seen only at temperatures below 7°; no 415-mμ absorption is observed at higher temperatures even though the rate of resolution increases considerably with increasing temperature. Secondly, direct dissociation of the cofactor (G) and displacement of this equilibrium toward resolution by interaction of free pyridoxal 5′-phosphate with the carbonyl reagent (H) is excluded: as shown in the following paper of this series, resolution proceeds rapidly when L-cysteine is used as the aldehyde reagent but not at all if L-cysteine is replaced by its D isomer; yet, the two enantiomorphs have identical association constants with free pyridoxal 5′-phosphate (Shaltiel *et al.*, 1969). Therefore, L-cysteine must interact with a particular site on the protein molecule at which pyridoxal 5′-phosphate is bound rather than with the free coenzyme (B or F).

On the other hand, the deformer has some other distinct effects on the properties of phosphorylase. It lowers considerably the catalytic activity of the enzyme, accelerates the rate of pyridoxal 5′-phosphate exchange, quenches the pyridoxal 5′-phosphate fluorescence, and promotes dissociation of the enzyme into monomers. Compounds or reaction conditions that counteract these changes also oppose resolution. For example, AMP which blocks resolution prevents dissociation of the enzyme and quenches the 535-mμ fluorescence. All these observations are consistent with the view that the deformer affects specifically the structure of the pyridoxal 5′-phosphate site and "exposes" the cofactor to attack by carbonyl reagents without gross alterations of the structure of the molecule.

Much experimental data support the conclusion that the deformed enzyme exists in a monomeric state. Lowering the pH and the protein concentration or increasing the concentration of the imidazolium citrate buffer or the temperature resulted in both dissociation of the enzyme and resolution. Urea,

at a concentration that brought about resolution in the presence of L-cysteine, also promoted dissociation even when L-cysteine was replaced by 2-mercaptoethanol. In this instance, it can be assumed that partial denaturation of the enzyme resulted in spontaneous dissociation of the cofactor according to reaction G. Madsen and Cori (1956) and Madsen and Gurd (1956) showed that titration of the free SH group of phosphorylase with *p*-mercuribenzoate resulted in monomerization of the protein; addition of an aldehyde reagent such as hydroxylamine to the *p*-mercuribenzoate-treated enzyme caused rapid resolution. Finally, AMP, caffeine, G-6-P, and UDPG, which block dissociation of the enzyme, also inhibit resolution.

It is currently assumed that the structure of a monomeric protein becomes constrained when it associates to an oligomeric form (Monod *et al.*, 1965). If this holds true for phosphorylase, one could understand that dissociation of an oligomeric structure might result in a conformational change that would specifically affect certain regions of the protein; in this instance, the pyridoxal 5′-phosphate site appears to be affected.

The mode of action of deforming agents is not yet clear. It may involve labilization of the native state or, conversely, stabilization of an intermediate distorted conformation. Deformers probably act by a mechanism similar to that of denaturants. From the thorough studies of Robinson and Jencks (1963) and Gordon and Jencks (1963) on the chemical basis of denaturation, the activity of denaturating agents cannot be ascribed simply to their hydrogen-bonding capacity, their hydrophobic character, or their effectiveness in altering the polarity of the solvent. It is probable that both denaturants and deformers act by a combination of these factors. Nozaki and Tanford (1963) suggested that urea stabilizes both the exposed peptide backbone and the hydrophobic groups. In the case of imidazole, several types of interactions could be visualized. It was shown earlier in this series (Shaltiel *et al.*, 1966) that the rate coefficients for resolution were not proportional to the total concentration of imidazole, but rather, to the concentration of the imidazolium ion. The latter could act as an acceptor for hydrogen bonds or in forming charge transfer complexes (for example, with tryptophanyl residues as described by Shinitzky and Goldman, 1967). Of course, it could also interfere with charge–charge interactions. The assumption that more than one factor may be involved is supported by the finding that both the cation and the anion component of a deforming buffer appear to contribute to denaturation. Simple replacement of the citrate ion by chloride completely reverses the effect of temperature on the rate of reaction (Figure 1). If imidazolium ions alone were to act by increasing certain hydrophobic interactions, one could rationalize the increase in rate observed with decreasing temperatures. However, when citrate ions are introduced in the reaction mixture, the rate of resolution increases with increased temperature, which would lead to the opposite explanation.

In conclusion, the results presented indicate that resolution of phosphorylase proceeds through the formation of an intermediate that is structurally distinct from the native enzyme. Pyridoxal 5′-phosphate, originally "buried" in the native enzyme, is still bound to the protein in the deformed state but "exposed" to the surrounding medium. In this state it can interact readily with aldehyde reagents and thereby be removed from the enzyme.

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

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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).