Effect of Ionic Strength on the Sedimentation of Glycogen Phosphorylase a*

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Glycogen phosphorylase has been isolated from rabbit muscle in two different molecular forms, phosphorylase a, a tetramer with a molecular weight of 495,000 (2-4), and phosphorylase b, a dimer with a molecular weight of 242,000 (3-5). The dimerization of the protein that occurs in the conversion of phosphorylase b to a with phosphorylase kinase, Mg++ and ATP has been found to result from the incorporation of four phosphoryl groups per mole of phosphorylase a (6). Although dimerization appears to follow phosphorylation closely (6), the exact relation ship between phosphorylation and dimerization is not clear. The possibility that dimerization occurs through formation of a phosphodiester bond in the protein, hydrolysis of such a linkage were present in phosphorylase a. If there existed a phosphodiester bond, it would be expected to result in the incorporation of four phosphoryl groups or some other ionic groups of phosphorylase a. The possibility that dimerization occurs through formation of a phosphodiester bridge appears remote since tryptic attack of phosphorylase a liberates all the protein-bound phosphate as a monoester with serine (7). Furthermore, modification of cysteinyl residues of phosphorylase a by p-mercuribenzoate results in the cleavage of the molecule into four subunits (4). If the phosphoryl groups are directly involved in the dimerization process, this cannot be explained by chelation of four phosphoryl groups by a metal introduced in the phosphorylase b to a reaction (6). Tryptic attack of phosphorylase a shows that the portion of the peptide chain at the site of phosphorylation is highly positively charged, and it has been suggested that dimerization follows phosphorylation by neutralization of the charge of this positive site, which would allow an interaction between interpeptide chains that were previously electrostatically repulsed (i).

The present work was undertaken to determine whether the phosphoryl groups or some other ionic groups of phosphorylase a were important for the stabilization of the tetrameric form of this enzyme. The results reported here show that phosphorylase a is sensitive to ionic strength and at high salt concentrations dissociates into a dimeric form of the enzyme that is not identical with phosphorylase b. The degree of structural change produced by solutions of high ionic strength is dependent upon pH, protein concentration, and the type of salt employed.

EXPERIMENTAL PROCEDURE

Phosphorylase a was prepared with the use of phosphorylase b kinase (8) and crystalline phosphorylase b (5). Third or fourth crystals of phosphorylase a pretreated with Norit A to remove AMP were then dialyzed against different buffers for 24 hours at 3-4o before ultracentrifuge examination. The pH of the buffers used was adjusted at room temperature before addition of solid NaCl or KCl. AMP, ATP, IMP, IDP, and UMP were purchased from Pabst Laboratories, Milwaukee, Wisconsin. Phosphorylase concentration was determined spectrophotometrically with the use of an absorbancy index of 11.7 for 1% solution of protein (9). Enzyme activities were measured according to the method of Jillingworth and Cori (10). Analysis for pyridoxal phosphate was carried out as described by Kent, Krebs, and Fischer (11) on enzyme samples obtained by dialysis against 0.02 m glyceraldehyde-0.03 m cysteine with NaCl followed by dialysis against 0.02 m Tris-0.0015 m EDTA containing NaCl.

Sedimentation coefficients were determined with a Spinco model E analytical ultracentrifuge employing a 12-mm single sector cell at a rotor speed of 59,780 r.p.m. The temperature of the rotor during most runs was maintained at 20 ± 0.2 o. Movement of boundaries was calculated from direct micro-comparator measurements of the schlieren diagram. Corrections for viscosity and density of the various buffers were applied in calculation of sedimentation coefficients, although reported sedimentation coefficients were not extrapolated to zero protein concentration. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components as described by Ogston (12) from schlieren diagrams 45 to 50 minutes after centrifugation. Error in the estimation of areas of poorly resolved components was as high as 20%.

The O of inorganic phosphate released in the conversion of phosphorylase a to b was determined by isolation of KH2PO4 reaction with guanidine-HCl (13), and analysis of CO2 produced by mass spectrometry.

RESULTS

Conversion of Phosphorylase a to b in H2O—The reaction was run to provide additional evidence that no phosphodiester bonds were present in phosphorylase a. If there existed a phosphodiester bond in the protein, hydrolysis of such a linkage might be expected to result in the incorporation of 2 oxygen atoms from medium H2O (14). The results depicted in Table I show that only 1 oxygen atom from medium H2O is introduced into each inorganic phosphate liberated from phosphorylase a by phosphorylase phosphatase.

Effect of Ionic Strength on Sedimentation Coefficients of Phos-
phorylase a and b—In order to assess whether the tetrameric form of phosphorylase a was stabilized by electrostatic interactions, enzyme was first dialyzed against solutions of high ionic strength and then analyzed for possible structural changes in the ultracentrifuge. Fig. 1 shows that native phosphorylase a with an $\alpha_{20, w}$ of 13.2 S (3) is converted into a slower sedimenting component by solutions of increasing concentration of NaCl. At 2.8 m NaCl, only the slow moving component with an $\alpha_{20, w}$ of 8.4 S could be detected in the ultracentrifuge. Increase in NaCl to 3.5 m did not further alter the sedimentation coefficient. At concentrations of NaCl at which both forms of the enzyme could be detected, phosphorylase a and the slow moving component were found to have average sedimentation coefficients of 12.4 S and 8.3 S, respectively.

The complete reversibility of transformation of phosphorylase a to a slow moving component could be demonstrated after removal of NaCl by dialysis against 0.03 M cysteine-0.02 M glycerophosphate, pH 7.4. Ultracentrifugal examination showed the presence of a symmetrical component with the sedimentation coefficient of native phosphorylase a. Although no enzyme activity could be detected in 2.8 m NaCl, 90% of initial activity was regained after dialysis. Phosphorylase a in NaCl was found to contain 90% of the pyridoxal phosphate that was present in control samples.

The degree of transformation of phosphorylase a in salt solutions is not merely dependent upon the ionic strength. At pH 7.4, 2.0 m NaCl gave a 33% conversion, but 2.0 m KCl yielded only 8% conversion of phosphorylase a to a slow sedimenting component, and potassium phosphate at equal ionic strength was without effect.

Phosphorylase b, a dimer, in contrast to phosphorylase a is not transformed to a slower moving component in the ultracentrifuge by NaCl. The sedimentation coefficients of the enzyme in the absence and presence of 3.2 m NaCl were found to be 8.2 S and 8.4 S, respectively.

Molecular Weight of Phosphorylase a in NaCl—The molecular weight of the enzyme (8 mg per ml) in 3.0 m NaCl containing 0.02 m glycerophosphate-0.03 m cysteine, pH 7.4, was determined by the approach to equilibrium in the ultracentrifuge as described by Ehrenberg (17). The partial specific volume of phosphorylase a in 3.2 m NaCl was found to be 0.74, and the average molecular weight of the slow sedimenting component was calculated as 258,000 ± 7,000. Since phosphorylase a is a tetramer with subunits of a molecular weight of 125,000 (3), this new form of phosphorylase a may be considered to be a dimer.

Effect of pH—The stability of phosphorylase a to high ionic strength at different pH values has been examined. Ultracentrifugal analysis shows that phosphorylase a tetramer can be transformed completely to a dimer with an $\alpha_{20, w}$ of 8.4 S at pH 7.0 in 2.5 m NaCl (Fig. 2A).

At pH 6.8, phosphorylase a appears to be even more unstable to high ionic strength as large amounts of inhomogeneous material with an $\alpha_{20, w}$ of 22 S appear in addition to small amounts of the 8.4-S form (Fig. 2B). In contrast to results obtained with phosphorylase a at pH 7.4, dialysis at pH 6.8 resulted in 90%

![Image](http://www.jbc.org/)

**Table I**

Conversion of phosphorylase a to phosphorylate b in H$_2$O$^{18}$

<table>
<thead>
<tr>
<th>Source of P,</th>
<th>Amount of P, released</th>
<th>Atom % excess</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>Reaction mixture</td>
<td>4.14</td>
<td>4*</td>
</tr>
</tbody>
</table>

* Four moles of P, were expected to be released in the complete conversion of 1 mole of phosphorylase a to phosphorylase b (15).

† Calculated for transfer of 1 oxygen atom from H$_2$O per mole of inorganic phosphate formed.

![Image](http://www.jbc.org/)

**Fig. 1.** Conversion of phosphorylase a to a slower sedimenting component by NaCl. Phosphorylase a (6 mg per ml) in different concentrations of NaCl in 0.02 m glycerophosphate-0.03 m cysteine, pH 7.4.

**Fig. 2.** Effect of pH on the ultracentrifuge schlieren diagrams of phosphorylase a in NaCl. Phosphorylase a (6 mg per ml) in 2.5 m NaCl in 0.02 m glycerophosphate-0.03 m cysteine. A (left), pH 7.0, 30 minutes at 59,780 r.p.m., sedimentation coefficient 8.4 S; B (right), pH 6.8, 20 minutes, sedimentation coefficients from meniscus 8.4 S and 22 S, respectively.
loss of pyridoxal phosphate from the enzyme and a complete lack of enzyme activity after removal of NaCl. The recovered phosphorylase α showed in the ultracentrifuge 27% of a component with an $s_{20}$ of 5.5 S (uncorrected for salt) and 73% of a component with an $s_{20}$ of 12.8 S (uncorrected for salt), similar to results described by Illingworth et al. (18) with apophosphorylase α. Incubation of recovered phosphorylase α, pH 6.8, with $5 \times 10^{-4}$ M pyridoxal phosphate restored the enzyme to its native tetrameric form with almost full recovery of enzyme activity. The ultracentrifuge pattern of phosphorylase α at pH 6.8 in 0.02 M cysteine-0.02 M glycerophosphate containing 2.5 M NaCl (Fig. 2B) is not altered by addition of $5 \times 10^{-4}$ M pyridoxal phosphate.

Increase of pH from 7.0 to 9.3 in 2.5 M NaCl results in the decrease of dimer formed from phosphorylase α tetramer (Fig. 3). No heavy inhomogeneous material was formed at these pH values.

Effect of Protein Concentration—Fig. 4 shows the conversion of the tetrameric form of the enzyme into a dimeric form as a function of protein concentration in 2.5 M NaCl. As might be expected from protein reactions involving aggregation, higher protein concentration favors tetramer formation. The sedimentation coefficients of dimeric and tetrameric phosphorylase α were found to be constant in 2.5 M NaCl at all protein concentrations indicated.

Effect of Nucleotides and Inorganic Phosphate—AMP, a specific activator of muscle phosphorylase (19), has been found to prevent the dissociation of phosphorylase α in NaCl. Addition of AMP to a solution of phosphorylase α in 2.5 M NaCl (Fig. 5A) yielded only one symmetrical ultracentrifugal component (Fig. 5B) with an $s_{20}$,w of 13.2 S. The protective effect afforded by AMP to dissociation of phosphorylase α was not dependent on the order of addition of NaCl and nucleotide. ATP, IDP, and UMP at $10^{-3}$ M all were without effect, whereas a $10^{-3}$ M solution of IMP provided a 5 to 10% protection to dissociation. A solution of 0.1 M potassium phosphate, a substrate, in 3.2 M NaCl with 0.02 M glycerophosphate-0.03 M cysteine, pH 7.4, did not prevent dissociation of the enzyme.

**DISCUSSION**

The present studies suggest that the quaternary structure of phosphorylase α is stabilized by electrostatic forces since solutions of high ionic strength result in dissociation of the enzyme. The dimer formed in this reaction is not identical with phosphorylase β, a nonphosphorylated dimer. "Nonphosphorylated," in this case, refers to the absence of 4 phosphorylserine residues present in phosphorylase α (7). Madsen and Gurd, in their studies with phosphorylase α inhibited by p-mercuribenzoate, showed experimental evidence for the existence of a transient dimeric form of phosphorylase α (20). The fact that, in this work, no monomeric form of phosphorylase α was detected in solutions of high ionic strength suggests that the types of interaction between the individual monomeric units of phosphorylase α are not identical.

In view of the complex nature of the dissociation of phosphorylase α, no attempt has been made to relate the degree of
dissociation with pH for tentative identification of an amino acid residue involved in this reaction. The marked change in structure of phosphorylase a observed in the ultracentrifuge from pH 7.0 to 6.8 with resultant loss of pyridoxal phosphate and reversal of structural changes of the enzyme upon readdition of pyridoxal phosphate provides further information that this prosthetic group influences markedly the stability of this enzyme (18).

Of the nucleotides tested, only AMP and IMP provided any stabilization of the quaternary structure of phosphorylase a. It is interesting to note that the degree of stabilization provided by these nucleotides roughly parallels their degree of activation of muscle phosphorylase (21). The possible relationship between activation and stabilization of protein structure afforded by these nucleotides is not known. It is clear, however, that AMP, a constituent of muscle phosphorylase (22), cannot bridge individual monomeric units of phosphorylase a together because removal of AMP from enzyme by treatment with Norit A yields no dissociation. As a working hypothesis, it has been assumed that binding of AMP to phosphorylase a produces an alteration of secondary and tertiary structure of the protein to a more favorable state for interaction between dimeric units. In support of the view that AMP affects the structure of phosphorylase a, release of a phosphohexapeptide from phosphorylase a by trypsin is greatly hindered in the presence of this nucleotide (7). More recently, Appelman and Fischer presented evidence that AMP alters the structure of muscle phosphorylase b (23).

The present experiments do not allow any conclusion in regard to the role of AMP in stabilization of phosphorylase a structure or the possible catalytic activity of the dimeric form of phosphorylase a. Experiments are in progress to answer these questions.

**SUMMARY**

Attack of phosphorylase a by phosphorylase phosphatase in H₂O resulted in the transfer of 1 oxygen atom from medium H₂O to each mole of inorganic phosphate released in the conversion of phosphorylase a to b. Phosphorylase a, a tetramer, in 2.8 M NaCl, pH 7.4, is transformed into a slower moving component with an s₂₀,w of 8.4 S. The reaction was reversed after dialysis, and 90% of the enzyme activity was recovered. The molecular weight of the slow sedimenting component by approach to equilibrium was found to be 258,000 ± 7,000. In contrast to phosphorylase a, phosphorylase b showed no change in sedimentation coefficient in solutions containing NaCl.

The degree of dissociation of phosphorylase a in NaCl was found to be dependent upon the protein concentration and pH values above 7.0. At equal ionic strength KCl was not as effective as NaCl. Adenosine 5'-phosphate, an activator of this enzyme, prevented dissociation of phosphorylase a in 3.0 M NaCl. Slight protection was afforded by inosine 5'-phosphate, but adenosine 5'-triphosphate, inosine 5'-diphosphate, and uridine 5'-phosphate had no effect.

At pH 6.8 in 2.5 M NaCl, pyridoxal phosphate is released from phosphorylase a with formation of rapidly sedimenting materials having average s₂₀,w of 22 S. Removal of NaCl and readdition of pyridoxal phosphate restore the activity and native form of the enzyme.

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