Ultraviolet difference spectroscopy studies with glycogen phosphorylase

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ULTRAVIOLET DIFFERENCE SPECTROSCOPY
STUDIES WITH GLYCOGEN PHOSPHORYLASE.

Iowa State University, Ph.D., 1969
Biochemistry

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ULTRAVIOLET DIFFERENCE SPECTROSCOPY STUDIES
WITH GLYCOGEN PHOSPHORYLASE

by

Mary Louise Shonka Bartlett

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1969
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### LIST OF ABBREVIATIONS

<table>
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<th>Abbreviation</th>
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<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>$E_{280}$</td>
<td>Molar extinction coefficient at 280 nm</td>
</tr>
<tr>
<td>G-HCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>GP</td>
<td>Sodium glycerophosphate</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5'-phosphate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) aminomethane</td>
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DEDICATION

To my husband and parents
INTRODUCTION

Glycogen phosphorylase is the enzyme which attacks the glycogen molecule from the terminus of each chain, releasing successive molecules of glucose residues as glucose-1-phosphate. Although this reaction is reversible, it is thought that in vivo this enzyme functions primarily in the breakdown of glycogen. Two forms, phosphorylase a and b have been isolated from rabbit muscle (1,2). These forms were found to be interconvertible as shown below (3,4):

\[
\begin{align*}
4 \text{ H}_2\text{O} & \quad \text{Phosphorylase phosphatase} \quad 4 \text{ P}_1 \\
\text{Phosphorylase a} & \quad \text{Phosphorylase b} \\
4 \text{ ADP} & \quad \text{Phosphorylase b kinase} \quad 4 \text{ ATP}
\end{align*}
\]

Another species, called phosphorylase b', can be made from limited tryptic attack of phosphorylase a. This reaction releases a phosphohexapeptide, which contains as a phosphomonoester the seryl residue phosphorylated in the b to a reaction, with the following sequence: Lys-Glu(NH\_2)-Ileu-Ser(OP)-Val-Arg (5).

In some respects phosphorylase a, b and b' are very different. Phosphorylase b and b' are essentially inactive in the absence of 5'-AMP, whereas phosphorylase a is active without it. Phosphorylase b and a have molecular weights of 185,000 and 370,000 respectively (6). Phosphorylase b has two pyridoxal 5'-phosphate (PLP) groups and two combining
sites for AMP, whereas phosphorylase $a$ has four (7). Phosphorylase $a$ tetramer can be converted to a dimer either in the presence of glycogen (8), or glucose (9), or 3 M NaCl (10), or at low protein concentrations (11). Phosphorylase $b$ is normally a dimer but may exist as a tetramer at high concentrations at low temperatures in the presence of Mg$^{++}$ and AMP (12). Other differences between phosphorylase $a$ and $b$ are found in their solubility and thermostability (13), and rotational strength of their dinitrophenyl derivatives (14).

Because of these differences it is reasonable to assume that phosphorylase $a$ and $b$ have different conformations. The approach taken in this thesis research was to determine whether this difference could be related to the exposure of the aromatic chromophores in these two proteins. One method used to investigate their exposure is solvent perturbation spectroscopy.

The principle behind this technique is that the spectra of tyrosine and tryptophan residues located on the surface of proteins are usually perturbed by changes in the physical properties of the solvent with which they are in contact. The aromatic residues deeply buried in the protein are shielded from the solvent and its perturbing effect. Consequently their spectra are not affected by the change in solvent properties. Mild substances, which do not alter the conformation of the protein but are known to produce
measurable spectral shifts are employed as perturbants.

This technique was developed by Herskovits and Laskowski (15) and first applied to ribonuclease. They found, using polyhydroxyl compounds as perturbants, that the perturbation was twice as great for the denatured fully exposed enzyme as for the native enzyme, showing that half of the six tyrosyl residues were buried in the protein.

So far the technique of solvent perturbation has been confined to studying the environment of aromatic residues. It was thought that extension of this method to the PLP chromophore of phosphorylase might reveal something about its environment. PLP is a cofactor indispensable to the activity of phosphorylase. The PLP is bound to an ε amino group of a lysyl residue adjacent to a phenylalanyl residue (16). Removal of the PLP leads to an apoenzyme with significantly different structural properties. Therefore PLP's presence is needed for maintaining phosphorylase's structure and catalytic activity, but what role it plays is very obscure. Previous workers had posited that the PLP residue was buried (17), and that certain reagents would distort the protein and expose the PLP. This theory could be tested with the solvent perturbation technique, and perhaps some insight could be gained into the mode of binding of PLP to phosphorylase.

Different proteins with the same aromatic chromophores
do not necessarily have the same absorption in the ultraviolet. Because the conformation of phosphorylase a differs from that of phosphorylase b, their absolute absorptions probably differ. A second approach to this problem was to try to accurately measure the absorption of phosphorylase a as compared to that of phosphorylase b. In order to experimentally detect these differences, it is essential that the concentrations of phosphorylase a and b be the same. This problem was solved by various methods in this thesis, and the spectral differences between the forms of phosphorylase were seen.
EXPERIMENTAL

Materials

Crystalline phosphorylase b was prepared according to the procedure of Fischer and Krebs (18) from commercial frozen rabbit muscle obtained from Pel-Freeze Biologicals, Inc., Rogers, Arkansas. Phosphorylase a was prepared from phosphorylase b and partially purified phosphorylase b kinase (19). Third or fourth crystals which were treated with Norit A to remove AMP were used for all experiments.

Potassium glucose-1-phosphate, sodium glycerophosphate, cysteine-HCl, shellfish glycogen and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Missouri. Glycogen was made AMP-free by treatment with Norit A. AMP and ATP were purchased from Pabst Laboratories, Milwaukee, Wisconsin.

Carrier-free $^{32}$P-phosphoric acid in 1 N HCl was obtained from International Chemical and Nuclear Corp., City of Industry, California.

G-HCl with low absorbance in the UV range was obtained from Mann Research Laboratories, New York, N. Y.

Trypsin was obtained as salt-free crystals from Calbiochem. Los Angeles, California. Phosphorylase b kinase (20) and phosphorylase phosphatase (21) used in the spectral studies were isolated from rabbit muscle.
Methods

Phosphorylase concentrations were determined spectrophotometrically with the use of an absorbancy index of 1.24 for a 0.1% solution of protein (22).

Enzyme activities were measured according to the procedure of Illingworth and Cori (23). The concentrations of reactants in the assay were 1% glycogen, 0.016 M glucose-1-phosphate and 0.001 M AMP unless otherwise stated.

$^{32}\text{P}$-labelled phosphorylase $a$ was prepared from phosphorylase $b$ with phosphorylase $b$ kinase using $^{32}\text{P}$-ATP prepared by the method of Lowenstein (24). The $^{32}\text{P}$-ATP was not purified but was used directly from the reaction mixture. Counting of $^{32}\text{P}$-radioactivity was done on a Nuclear Chicago gas flow counter.

Refractive increments were measured by means of a Brice-Phoenix differential refractometer, model BP-2000-V.

All difference spectral measurements were carried out in a Cary model 15 double beam recording spectrophotometer by employing either pairs of matched cylindrical tandem double cells, each containing 2, 1 cm compartments, or four matched 1 cm cuvettes in four separate cuvette holders. These four separate compartments were needed in order to subtract the solvent contribution to the difference spectrum in the case of solvent perturbation, or in the case of an enzyme acting on phosphorylase, to subtract...
RESULTS

Specific Refractive Increments of Phosphorylase a and b

Since the absorption of tyrosine and tryptophan is sensitive to their environment (27), the conformational difference between phosphorylase a and b might be expressed in their $A_{280}^{1\text{mg/ml}}$. The specific refractive increment or $(n-n_0)/c$ where $n$ and $n_0$ are the refractive indices of the solution and solvent and $c$ is the protein concentration in g/ml was calculated for phosphorylase from the amino acid composition and the values for the specific refractive increments of amino acid residues (28). It was found to be 0.185±0.007. Assuming a value of 0.185 and measuring the absorption of a solution and its $n-n_0$ in a differential refractometer, the $A_{280}^{1\text{mg/ml}}$ may be calculated.

Phosphorylase a and b were dialyzed against 0.001 M GP, 0.001 M DTT pH 6.8 for 24 hours. Binding of salt to protein might cause a difference in the buffer concentrations in the two cells when measuring $n-n_0$, so very low buffer concentrations were used in order that the salt would not make a significant contribution to the $n-n_0$ of the protein. Figure 1 shows a plot of $n-n_0$ versus absorbance for phosphorylase a and b. The points appear to fit one straight line. Using the slope of this line and assuming that the specific refractive increment is 0.185, an $A_{280}^{1\text{mg/ml}}$ of
Figure 1. Refractive increments of phosphorylase a and b. Enzyme dialyzed 24 hours in 0.001 M GP, 0.001 M DTT, pH 6.8, the same solution being used for dilutions. (x) represents values for phosphorylase a from two experiments. (o) are values for phosphorylase b from two experiments.
1.28±4% was calculated. If the assumption that the specific refractive increment is 0.185 is incorrect, then this absolute value is incorrect. However since phosphorylase a and b differ only by a phosphate group per monomer in their amino acid composition, their value for this increment is the same and consequently their $A_{280}^L$'s appear very close.

Absorbances of Phosphorylase a and b in Guanidine-HCl

Studies of phosphorylase a and b in water and in guanidine hydrochloride (G-HCl) can also give an idea of their relative $A_{280}^L$'s. When the protein is diluted identically into water and into G-HCl, dividing the former absorption by the latter corrects for protein concentration, giving a ratio of extinction coefficients, $E$. This is shown below:

$$\frac{A_{\text{H}_2\text{O}}}{A_{\text{G-HCl}}} = \frac{(Eg_{\lambda})_{\text{H}_2\text{O}}}{(Eg_{\lambda})_{\text{G-HCl}}}$$

This is done for both phosphorylase a and b. The ratio of the values obtained for b and a are simply the ratio of their $E$'s in water, since their $E$'s in G-HCl cancel. These latter $E$'s cancel since the chromophores of phosphorylase a and b are the same and would be expected to be in the same environment when the protein is converted to a random chain by G-HCl. Tanford (29) has shown by various physical
studies that 6 M G-HCl was sufficient to convert protein polypeptide chains to random coils.

Experiments were performed in 0.04 M GP, 0.001 M DTT pH 6.8 buffer, using 7 M G-HCl. The absorption in G-HCl was seen to increase slightly after two hours and then remain constant. The absorption at 280 nm of the protein in buffer was taken after 15 minutes and in G-HCl, after 2 hours. The ratio of the absorbances of phosphorylase a to b was found to be 1.01±1%, again showing that the absorptions of these two forms at 280 nm are very similar.

The amount of tyrosine and tryptophan in phosphorylase was determined from the E at 280 and 288 nm of phosphorylase in 7 M G-HCl by the method of Edelhoch (30). Assuming a molecular weight of 195,000 for dimer (28) and $A_{280}^{1 \text{mg/ml}} = 1.24$ (22), 63±2% moles of tyrosine and 27±3% moles of tryptophan were determined per mole of dimer. This differs slightly from the values of 68 moles of tyrosine and 23 moles of tryptophan determined by Appleman (31) and corrected for this molecular weight and $A_{280}^{1 \text{mg/ml}}$.

Exposure of Aromatic Residues of Phosphorylase to Ethylene Glycol

The spectra of chromophoric residues coming freely in contact with the solvent are sensitive to changes in the physical properties of the solvent, such as refractive index and dielectric constant, and to solvent-solute interactions.
This sensitivity is expressed in small spectral shifts which can be seen in a difference spectrum. On the basis of these facts Herskovits and Laskowski (15) developed the method of solvent perturbation.

Perturbation spectra are usually reported as \( \Delta \text{OD/OD} \) which is equal to \( \Delta \text{E/E} \), a constant, where \( \Delta \text{E} \) is the molar difference absorption coefficient of the protein in the presence of perturbant minus the protein in its absence. \( E \) is the molar absorption coefficient and in this paper, is taken at 280 nm when measuring the extent of exposure of tyrosinyl and tryptophanyl residues.

The protein environment itself perturbs the chromophore and the transfer of a chromophore from the interior of the protein to an aqueous environment gives rise to a difference spectrum 3-10 times greater than that caused by solvent perturbation by a perturbant. Therefore it is necessary to get a perturbant which does not cause large conformational changes in the protein. Polyhydroxyl solvents were found to be safe perturbants (25), and of this series, ethylene glycol was chosen for the experiments in this paper.

Figure 2 shows the effect of various concentrations of ethylene glycol on the activity of phosphorylase b. Although the activity of the enzyme is affected by this perturbant, the amount of exposed aromatic residues does not seem to be affected. This is shown in Figure 3 where a plot of
Effect of ethylene glycol on activity of phosphorylase b. Enzyme, final concentration, 0.02 mg/ml, in 0.02 M GP, pH 6.8, 30°.
Figure 3. Perturbation of phosphorylase b. Enzyme, 1.4 mg/ml in 0.02 M GP, 0.001 M ethylenediaminetetraacetic acid, 0.1 M KCl, pH 6.8.
\[ \text{\% ethylene glycol} \]

\[ \Delta \text{OD/OD} \]

\[ 5 \quad 10 \quad 15 \quad 20 \]

Graph showing the relationship between % ethylene glycol and \( \Delta \text{OD/OD} \).
\( \Delta E_{285}/E_{280} \) versus percent concentration of the perturbant, ethylene glycol, is linear and passes through zero. Any large conformational changes caused by the perturbant would be expected to cause a deviation from linearity. Figures 4 and 5, solid lines, show the perturbation curves for phosphorylase \( a \) and \( b \). The curves are very similar, indicating that they are similar in their exposure of aromatic residues. The figures are an average of 5 experiments on phosphorylase \( a \) and \( b \), and vary about \( \pm 10\% \).

An estimate of the number of tyrosines and tryptophans exposed can be obtained by solving the following two equations (32):

\[
\begin{align*}
\Delta E_{291-293}^{\text{protein}} &= x\Delta E_{291-293}^{\text{trp}} + y\Delta E_{291-293}^{\text{tyr}} \\
\Delta E_{286-288}^{\text{protein}} &= x\Delta E_{286-288}^{\text{trp}} + y\Delta E_{286-288}^{\text{tyr}}
\end{align*}
\]

where coefficients \( x \) and \( y \) represent the number of tryptophans and tyrosines exposed, and \( \Delta E_\lambda \) is the molar perturbation difference at the wavelength designated by the subscripts. \( \Delta E_\lambda \) for phosphorylase was calculated using the \( E \) for the dimer of phosphorylase \( a \) and \( b \). This value was obtained for the model compounds, \( N\text{-acetyl-L-tyrosine ethyl ester} \) and \( N\text{-acetyl-L-tryptophan ethyl ester} \), from the data given by Herskovits and Sorensen (26). From these equations, phosphorylase \( b \) was found to have 30.1 tyrosines and 10.3 tryptophans exposed, while phosphorylase \( a \) dimer had 28.6 tyrosines and 9.7 tryptophans exposed.
Figure 4. Perturbation of phosphorylase a by 20\% ethylene glycol. Solid line, phosphorylase a, OD = 0.95 at 280 nm in 0.03 M GP, 0.1 M KCl, pH 6.8. Dashed line, calculated curve for 35 N-acetyl-L-tyrosine ethyl esters and 6.6 N-acetyl-tryptophan ethyl esters.
Figure 5. Perturbation of phosphorylase b by 20% ethylene glycol. Solid line, phosphorylase b, OD = 1.39 at 280 nm in 0.03 M GP, 0.1 M KCl, pH 6.8. Dashed line calculated curve for 43 N-acetyl-L-tyrosine ethyl esters and 5 N-acetyl-tryptophan ethyl esters.
Using these values for tyrosine and tryptophan, and replacing x and y in the equations with them, and using the published values for $\Delta E_{\text{trp, tyr}}$ (26) for various wavelengths, the $\Delta E_{\text{model}}$'s for various wavelengths were calculated, and a solvent perturbation spectrum of the model compound mixture was obtained. These calculated curves did not agree very well with the experimental curves for phosphorylase. One reason for these values not being correct is that the model compounds for tyrosine and tryptophan have their perturbation maxima at different wavelengths than tyrosine and tryptophan would have if they were in a protein. Another reason is that it was hard to evaluate the $\Delta E_{291-293}$ (protein) for phosphorylase since there was a shoulder and not a peak. Other values for x and y were tried and calculated curves to better fit the perturbation spectra were obtained. The solid line in Figure 4 shows the experimental curve for phosphorylase a and the dashed line shows the curve calculated for 35 tyrosines and 6.6 tryptophans. The solid line in Figure 5 shows the experimental curve for phosphorylase b and the dashed line shows the curve calculated for 43 tyrosines and 5 tryptophans.

It is an oversimplification to say that a certain number of residues are exposed. It is perhaps better to say that there is approximately a 50% exposure of tyrosine and a 20% exposure of the tryptophans in phosphorylase. The similarity
in the exposure of aromatic residues in phosphorylase a and b supports the finding of their similar $\frac{1 \text{ mg/ml}}{280}$.

Effect of Trypsin on Phosphorylase a and b

Limited trypptic attack of phosphorylase a will release various peptides, one of the main ones being a phosphohexapeptide. The amino acid sequence of this peptide was found to be Lys-Glu(NH$_2$)-Ileu-Ser(OP)-Val-Arg (5). The seryl residue is phosphorylated in the phosphorylase b to a reaction. The new protein formed upon trypptic attack, called phosphorylase b', is catalytically similar to phosphorylase b and is dependent on AMP for activity. This allows the trypptic attack to be followed by the loss in activity measured in the absence of AMP. Spectral changes were observed during the conversion of phosphorylase a to b'. Figure 6 shows that after 10 minutes, when there was very little phosphorylase b' since phosphorylase had 85% of its original activity without AMP, there was a spectral change. The dashed line shows the difference spectrum when only 20% of the phosphorylase a activity remained. These spectra show some differences between phosphorylase a and b' but their interpretation is complicated by the release of other peptides from trypptic attack as shown by the large changes observed after very little attack.

Phosphorylase b is also thought to be converted to b' upon limited trypptic attack (33). Since both phosphorylase
Figure 6. Effect of trypsin on the spectrum of phosphorylase a. Phosphorylase a, 1.4 mg/ml and trypsin, 7.5 μg/ml in 0.03 M GPP, 0.1 M KCl, pH 6.8. Solid line, 10 minutes of reaction (15% loss of enzyme activity measured in the absence of AMP). Dashed line, 2 hours of reaction (80% loss of enzyme activity measured in the absence of AMP).
b and b' require AMP, the progress of the reaction cannot be followed by the loss in activity without AMP. High concentrations of phosphorylase b can form a tetramer in the cold with AMP and Mg\(^{++}\), whereas phosphorylase b' cannot. The tetramer is less active than the dimer (8), so if trypsin does convert phosphorylase b to b', one would expect an increase in activity upon tryptic attack. Figure 7 shows this increase and is a further proof of the conversion of phosphorylase b to b'. Figure 8 shows the spectral effect of tryptic attack on phosphorylase b dimer. The spectrum was taken right before any appreciable drop in the activity was found in the presence of AMP. The spectral differences between phosphorylase b and b' seem smaller than those between phosphorylase a and b', but again the interpretation is complicated by the release of peptides other than the hexapeptide.

Effect of Phosphorylase Kinase on Phosphorylase b

The difference between the absorption of phosphorylase a and b can be seen more directly if the same concentration of phosphorylase b is placed in the reference and the sample beam, and then the latter is converted to phosphorylase a with phosphorylase kinase, ATP and Mg\(^{++}\). Because of the ATP absorption in the ultraviolet, various concentrations of ATP were tested to determine the minimal concentration
Figure 7. Activation of phosphorylase b tetramer by trypsin. Phosphorylase b, 5 mg/ml, incubated at 10° with 0.1 M Mg++, 0.01 M AMP in 0.06 M KCl, 0.01 M GP at pH 6.8 for 1 hour. At zero time 45 µg/ml of trypsin were added. At various times 0.2 ml aliquots were removed and added to 0.2 ml soybean trypsin inhibitor, 0.32 µg/ml, and assayed for phosphorylase activity.
Figure 8. Effect of trypsin on the spectrum of phosphorylase b. Phosphorylase b, 1.6 mg/ml, and trypsin, 15 μg/ml, in 0.03 M GP, 0.1 M KCl, pH 6.8. Spectrum taken 15 minutes after mixing, with 97% of phosphorylase's original activity remaining as measured in the presence of AMP.
at which a good \( b \) to \( a \) conversion would occur. This was found to be \( 10^{-4} \) M. The phosphorylase \( b \) to \( a \) reaction was complete within 20 minutes as shown by equal activity in the absence and presence of AMP. Figure 9 shows the resultant difference spectrum. The part of the spectrum below 270 nm seems not to be due solely to the difference between phosphorylase \( a \) and \( b \) because it was seen to change 10 minutes after total conversion to phosphorylase \( a \). When this experiment was repeated using boiled kinase, no phosphorylase \( b \) to \( a \) conversion occurred, but a large negative difference spectrum was seen again below 270 nm as shown by the dashed line in Figure 9. Since the absorption of ATP at 260 nm is about three times that of phosphorylase, these changes are probably due to this nucleotide. ATP may be hydrolyzing differently in the two reaction mixtures in the sample and reference beams. Alternatively the kinase or some contaminant associated with it may be affecting the absorption of ATP.

**Effect of Phosphorylase Phosphatase on Phosphorylase \( a \)**

The spectral differences between phosphorylase \( a \) and \( b \) were best seen using phosphorylase phosphatase. The same concentration of phosphorylase \( a \) was placed in both the sample and reference cells and a small amount of phosphatase added to the sample cell converted the phosphorylase \( a \) to
Figure 9. Spectrum after conversion of phosphorylase b to a by phosphorylase b kinase. Phosphorylase b, OD = 1.17, kinase OD = 0.05 at 280 nm in 0.04 M Tris pH 7.4. ATP 10⁻⁴ M OD = 1.5 at 260 nm, Mg⁺⁺⁺ 10⁻⁴ M. Solid line, in sample beam phosphorylase b, ATP, Mg⁺⁺⁺ and kinase in one cell. Kinase in separate cell in reference beam. Spectrum taken after 20 minutes with complete conversion to phosphorylase a as seen by full activity when measured in the absence of AMP. Dashed line, same as above except the kinase was boiled and filtered.
b. Figure 10 shows the spectral differences between phosphorylase $a$ and $b$. The total OD of the phosphorylase is much greater than that of the phosphatase so the spectral changes seen are attributable to the former. When repeating the experiment but starting with phosphorylase $b$ instead of $a$, no change in the spectrum was observed as seen by the dashed line in Figure 10. Therefore it is reasonable to conclude that the difference spectrum seen with phosphorylase $a$ is due to its conversion to phosphorylase $b$ in the sample cell and not to the effect of some contaminant in the phosphatase affecting the spectrum of phosphorylase $a$.

During the conversion of phosphorylase $a$ to $b$, the change in the spectrum with time was correlated with the release of phosphate. $^{32}\text{P}$-phosphorylase $a$ was made from $^{32}\text{P}$-ATP, phosphorylase $b$ and phosphorylase $b$ kinase. At various times during phosphatase attack, aliquots were removed, the protein precipitated with 5% trichloroacetic acid, and the supernatant solutions obtained after centrifugation were plated and counted. When the spectral changes at 289 nm were followed with time and compared to phosphate release it was found that the spectral change was complete when about 70% of the phosphate was released as seen in Figure 11. Hurd et al. (34) have reported that when assaying in the absence of AMP and at high concentrations of glucose-1-phosphate, no loss in the activity of phosphorylase $a$ could be seen until approximately 50% of the protein-bound
Figure 10. Spectrum after conversion of phosphorylase a to b by phosphorylase phosphatase. Solid line, difference between phosphorylase b (sample cell) and phosphorylase a, OD = 1.4 at 280 nm phosphatase OD = 0.02 at 280 nm in 0.04 M Tris, pH 7.3. Dashed line, control same as above except phosphorylase b, OD = 1.21 at 280 nm in sample and reference.
Figure 11. Conversion of phosphorylase a to b. Phosphorylase a, OD = 1.05, phosphatase OD = 0.013 at 280 nm, in 0.04 M Tris, 0.001 M DTT, pH 7.45. (•) percent phosphate, (x) percent spectral change and (o) percent activity remaining. Enzyme assayed 1 minute pH 6.5 in 0.05 M malate and 0.075 M glucose-1-phosphate.


$^{32}$P had been released. Although attempts were made to exactly reproduce the assay conditions showing this effect, this retention of activity was not found as shown in Figure 11.

As would be expected, doubling the phosphorylase phosphatase concentration doubled the rate of spectral change and of phosphate release. Glucose-6-phosphate, which was reported to have accelerated the phosphorylase $a$ to $b$ conversion (34), accelerated the spectral change along with the phosphate release, as seen in Figure 12. Again the spectral changes were complete when about 70% of the phosphate was removed.

Glucose, which is known to dissociate the tetramer of phosphorylase $a$ to a dimer (9), also accelerated the phosphatase reaction. Since 0.05 M glucose was added to phosphorylase $a$ in the reference and sample at the beginning of the reaction, the difference spectrum at the end of the reaction give the comparison of a phosphorylase $b$-glucose complex with a phosphorylase $a$-glucose complex. As seen in Figure 13, this difference was much less than the difference between phosphorylase $a$ and $b$. Glucose is known to be an allosteric inhibitor of phosphorylase $a$ (35) and may be causing phosphorylase $a$ to form a less active conformation where the environment of the aromatic residues may be more like that of the less active species, phosphorylase $b$. 

Figure 12. Conversion of phosphorylase a to b. Phosphorylase a, OD = 0.91
phosphatase OD = 0.01 at 280 nm in 0.04 M Tris, 0.001 M DTT,
pH 7.45. Solid line, (o), percent phosphate, (x), percent spectral
change remaining. Dashed line, (o), percent phosphate, (Δ), per-
cent spectral change remaining, same experiment as above except
in the presence of 0.001 M glucose-6-phosphate.
Figure 13. Spectrum after conversion of phosphorylase a to b in the presence of glucose. Phosphorylase b (sample cell) OD = 1.4, phosphatase OD = 0.012 at 280 nm in 0.05 M glucose, 0.04 M Tris, pH 7.3.
Phosphorylase a Concentration Difference Spectrum

Since phosphorylase a, at the concentrations used in this study, is a tetramer, and phosphorylase b, a dimer, it was interesting to see whether the difference seen between phosphorylase a and b could be due in part to the fact that phosphorylase a was a tetramer and phosphorylase b a dimer. By light scattering measurements it has been seen that phosphorylase a dissociates to a dimer upon dilution (28). 0.02 mg/ml is mainly in the dimeric form at 22° whereas 0.2 mg/ml is mainly tetramer (8). In 0.02 M phosphate pH 6.8, 0.02 mg/ml in a 5 cm cell versus 0.2 mg/ml in a 0.5 cm length cell gave no difference spectrum from 340 nm to 270 nm as shown in Figure 14. A serious limitation of this experiment is that to form a dimer, a very low protein concentration is needed. The total OD is essentially 0.1 while the other difference spectra were performed at approximately 1.5 OD. It is quite possible that this protein concentration is too low to see the differences between phosphorylase a tetramer and dimer.

Exposure of Pyridoxal Phosphate to Ethylene Glycol

Phosphorylase contains one mole of pyridoxal phosphate (PLP) per mole of enzyme monomer. The function of PLP is obscure, however removal of this prosthetic group from phosphorylase causes a loss of activity and of structural integrity (23). Reduction of the protein with NaBH₄ fixes
Figure 14. Protein concentration difference spectrum of phosphorylase a. Enzyme, 0.02 mg/ml in a 5 cm cell versus 0.2 mg/ml in a 0.5 cm cell, 0.02 M phosphate, pH 6.8.
the PLP to the protein and leads to a molecule which still retains 60% or more of maximum activity. The PLP is covalently bound in reduced phosphorylase as a secondary amine derivative of a lysyl residue. The PLP behaves as if it were buried within the protein molecule since it will not react with aldehyde reagents, nor be reduced unless the protein is modified by changes in pH, or distorted by salts or denaturing agents (16). It was of interest therefore to use the technique of solvent perturbation spectroscopy on PLP in phosphorylase and to see if it is exposed or buried to the perturbant.

Native phosphorylase b, pH 6.8, OD = 0.32 at 333 nm due to the bound PLP, shows no difference spectrum from 460 nm to 305 nm as seen in Figure 15, dashed curve. 5, 10 and 20% ethylene glycol were used, and since there was no perturbation in the region of PLP absorption, it appears that PLP is inaccessible to ethylene glycol. It also shows that a concentration as high as 20% ethylene glycol does not distort the protein structure so as to change the PLP environment.

Shaltiel et al. (17) showed that removal of the PLP from phosphorylase b by L-cysteine can occur at 22° in 0.4 M imidazole adjusted to pH 7 with citric acid. This resolution does not occur without the imidazole. From their studies, the authors proposed that the imidazole
Figure 15. Perturbation of phosphorylase b by 10% ethylene glycol. Dashed line, native phosphorylase b 6 mg/ml, OD = 0.32 at 333 nm, in 0.1 M KCl 0.03 M GP, pH 6.8. Solid line, native phosphorylase b 4.5 mg/ml, OD = 0.24 at 333 nm in 0.4 M imidazole-citrate, pH 6.8.
distorts the protein and exposes the otherwise buried PLP residue so that the cysteine can then remove it. It was therefore interesting to see whether the PLP could be perturbed by ethylene glycol when the enzyme was in imidazole. Surprisingly, 10% ethylene glycol showed no effect on the spectrum of phosphorylase b in 0.4 M imidazole-citrate pH 6.8, as shown in Figure 15, solid line. This was after two hours in the imidazole buffer and the enzyme still had its full activity.

NaBH₄ reduced phosphorylase b has many properties like those of native phosphorylase b (16), but it no longer has an absorption maximum at neutral pH at 333 nm. As would be expected, its spectrum is not affected by ethylene glycol in this region. It was found, however, that in 0.4 M imidazole, a peak appears at 333 nm which has a lower E than the native enzyme. In contrast to the native, the reduced enzyme gave the difference spectrum shown in Figure 16, dotted line. This was after two hours in imidazole-citrate buffer with only 60% of its original activity left.

PLP is thought to be exposed at low pH since it will react with aldehyde reagents under these conditions. However at low pH, native enzyme looses the PLP, making it difficult to do solvent perturbation of this protein-PLP complex. This difficulty can be overcome by using the reduced enzyme. At pH 4.6 the reduced enzyme shows an E_max
Figure 16. Perturbation of reduced phosphorylase b by 10% ethylene glycol. Reduced phosphorylase b, 10 mg/ml, OD = 0.31 at 333 nm in 0.4 M imidazole-citrate, pH 6.8. Solid line, enzyme in reference cell, dashed line, enzyme and perturbant in sample cell, dotted line, difference spectrum.
at 333 nm, about 1.2 times that of the native enzyme. The perturbation by 10% ethylene glycol is shown in Figure 17.

10 mg/ml of reduced enzyme also shows a peak at 328 nm OD = 0.75 in 0.37% sodium dodecyl sulfate. This too was found to be a form which could be perturbed by 10% ethylene glycol as shown in Figure 18.

Exposure of Pyridoxal Phosphate to Deuterium Oxide

PLP in native phosphorylase b was found not to be exposed to ethylene glycol, so it was of interest to see if it would be exposed to a smaller perturbant, namely D$_2$O. 80% D$_2$O was used as the perturbant and a correction of 0.28 pH units was added to the pH meter reading to obtain pD (36). Surprisingly, D$_2$O does have an effect on the PLP spectrum as seen in Figure 19.

It was interesting to see whether along with the effect on the spectrum, D$_2$O also had an effect on the activity of phosphorylase b. 5 µl of a concentrated protein solution was added to 2 ml of substrate in 80% D$_2$O and in H$_2$O. As seen in Figure 20 D$_2$O slightly accelerated the release of phosphate from glucose-1-phosphate. Each curve is an average of two runs.
Figure 17. Perturbation of reduced phosphorylase b by 10% ethylene glycol. Enzyme, 10 mg/ml; OD = 0.65 at 333 nm, in 0.05 M acetate, pH 4.6. Solid line, enzyme in reference cell, dashed line, enzyme and perturbant in sample cell, dotted line, difference spectrum.
Figure 18. Perturbation of reduced phosphorylase b by 10% ethylene glycol. Enzyme, 10 mg/ml, OD = 0.75 at 328 nm in 0.37% sodium dodecyl sulfate, 0.05 M GP-0.05 M mercaptoethanol, pH 6.8. Solid line, enzyme in reference cell, dashed line, enzyme and perturbant in sample cell, dotted line, difference spectrum.
Figure 19. Perturbation of phosphorylase b by 80% D.O. Phosphorylase b, 6.6 mg/ml, in 0.01 M GP, 2x10^-4 M DTT, pK, pD 6.74. Solid line, enzyme in reference cell, dashed line, enzyme and perturbant in sample cell, dotted line, difference spectrum.
Figure 20. Effect of 80% D$_2$O on the activity of phosphorylase b. Enzyme, final concentration 0.016 mg/ml, (x) assayed in 80% D$_2$O and (•) assayed in H$_2$O pH and pD 6.83, 23°.
DISCUSSION

Although phosphorylase a and b are very different in other respects, it appears as though they have the same or very similar $E_{280}$. This is shown by the slight spectral difference at 280 nm upon conversion of phosphorylase a to b by phosphorylase phosphatase and by approximately the same spectral difference upon conversion of phosphorylase b to a by phosphorylase kinase. The various procedures used in the isolation and storage of phosphorylase a and b were seen not to cause a large difference in their $E_{280}$ as seen by the measurement of their specific refractive indices and by the measurements of their absorptions with and without guanidine-HCl.

Since there is no large difference in the $E_{280}$ of phosphorylase a and b it appears that the aromatic chromophores are in very similar environments in these two species. This is an indication of their structural similarity and agrees with the finding that the ordered structure as measured by ORD appears to be the same for these two proteins (37).

Solvent perturbation shows that the exposure of the aromatic residues in phosphorylase a and b is very similar. However the interpretation of these results is complicated since phosphorylase b has as many as 63 tyrosines and 27 tryptophans. The environment of a few of these residues
may be very different in phosphorylase a and b, but these differences could be masked by the large absorbance of all the other residues. Since phosphorylase a is a tetramer and phosphorylase b, a dimer, it appears as though very few if any tyrosines and tryptophans are excluded from the solvent when two dimers bind to form a tetramer, because there is no large difference in the exposure of residues. This finding agrees with the similarity in the absorption of aromatic residues in phosphorylase a and b.

Although the differences between the ultraviolet absorption of phosphorylase a and b are very small, they may be seen best in the difference spectrum taken right after the conversion of phosphorylase a to b by phosphorylase phosphatase. From this curve the difference between phosphorylase a and b absorption at 280 nm is seen to be approximately 0.8%.

When following the spectral changes at 289 nm with time and correlating these with the release of phosphate, it was surprising to find that the spectral change was complete when only about 70% of the phosphate was released. This gives some idea as to the action of phosphatase attack. The fully phosphorylated species gives the spectrum of phosphorylase a and the totally dephosphorylated species gives the phosphorylase b spectrum. If the attack by phosphorylase phosphatase were all or none, then at 50% phosphate release, there would be a spectrum due to 50%
phosphorylase \( a \), 50% phosphorylase \( b \), and the spectral change would be 50%. This must not be the case since the spectral change is actually about 75%. The results seem to be consistent with the existence of partially phosphorylated intermediates. A possible explanation for the loss in activity measured in the absence of AMP not being complete when the spectral change is complete, is that the partially phosphorylated species, although they are spectrally like phosphorylase \( b \) may assume a partially active conformation when in the presence of substrates.

The existence of the partially phosphorylated species agrees with the earlier results of Hurd et al. (34). They found that during the conversion of phosphorylase \( a \) to \( b \) there were species which had a catalytic character and sedimentation behavior different from that of phosphorylase \( a \) and \( b \).

The spectral differences between phosphorylase \( a \) and \( b \) were found to be smaller when the phosphatase reaction was completed in the presence of glucose. This may be because glucose, an inhibitor of phosphorylase \( a \) and \( b \), is refolding both \( a \) and \( b \) into less active conformations, which might be more like one another in regard to the environment of their aromatic groups. Since this experiment was performed when both forms of phosphorylase were dimers, and there was still some spectral change observed, it appears
that this changes is not due solely to the tetramer going
to dimer, but also to a change in the monomer upon dephos-
phorylation.

Some of the difference spectra of PLP in reduced
phosphorylase, notably Figure 16, are negative which is
contrary to what would be expected with ethylene glycol if
it were simply a case of solvent perturbation. If the chromo-
phore were exposed, bringing it to a region of higher
refractive index, that is, from water to ethylene glycol,
would cause a higher degree of stabilization of the excited
state and subsequently a red shift. A possible explanation
of the negative spectra is that ethylene glycol, when it is
in contact with the PLP, could stabilize a form other than
the 333 nm form, perhaps a 290 nm form, which might be in
equilibrium with the 333 nm form as shown in Figure 21. This
would cause a decrease in the absorption of the sample beam
and subsequently a negative spectrum.

PLP in native phosphorylase b appears not to be exposed
to ethylene glycol. However, it was surprising to find that
in imidazole buffer, the PLP was still found to be inaccessible
to ethylene glycol. This points to some kind of specific
effect by cysteine on the enzyme structure. That is, in
order to resolve the enzyme, cysteine would either be able
to gain access to the PLP, or to distort the enzyme structure
so as to expose the PLP. The finding of Johnson and Graves
Figure 21. Possible forms of PLP in reduced phosphorylase b.
290 nm (?) \quad \Leftrightarrow \quad 333 \text{ nm}
(37), that in the resolution of phosphorylase b by L-cysteine, a loss of enzyme activity proceeds faster than formation of the cysteine-PLP compound, supports the latter theory. On the other hand, the lack of perturbation could be reconciled with the theory that PLP is buried in that there might be some exposed PLP in equilibrium with the buried PLP but in too small a concentration to be detected by solvent perturbation. The cysteine could then shift the equilibrium by continually removing the unmasked cofactor, as fast as the imidazole-distorted enzyme could form it.

The reduced phosphorylase b was seen to exhibit the same heat-stability and urea stability as the native enzyme (16). However, it appears to be more susceptible to the distorting effects of imidazole, as seen by its loss of activity and the exposure of its PLP to ethylene glycol. This seems to indicate that reduction of the PLP-enzyme complex and subsequent conversion of this reduced PLP form to a 333 nm absorbing form causes this conformation of the reduced enzyme to be less stable than the native enzyme. This has also been shown by other experiments in this lab. The reduced enzyme at pH 6.0 was seen to have a broader peak in the ultracentrifuge than the native enzyme at pH 6.0 (38).

It appears that in the reduced enzyme at pH 4.6 the PLP is exposed to some extent. If an analogy can be made to the native enzyme, then this exposure would explain why
PLP is easily attacked by aldehyde reagents at this pH. Since the PLP of the reduced enzyme also appears to be exposed in 0.37% sodium dodecyl sulfate, it is probable that resolution of the native enzyme by cysteine would readily occur when it is exposed to this detergent.

Although ethylene glycol cannot perturb the major absorption band of PLP in native enzyme, D$_2$O was seen to have an effect on the spectrum. The blue shift is what is expected when D$_2$O is used as a perturbant. A possible explanation of this perturbation is that D$_2$O is a smaller perturbant than ethylene glycol and can better penetrate into crevices of the protein, close enough to perturb PLP. An alternative explanation is that there is an equilibrium between various enzyme forms and D$_2$O affects this equilibrium. This is feasible since deuterium is known to form more stable bonds than hydrogen (39). PLP may be exposed, and D$_2$O is affecting the equilibrium between forms of PLP which absorb at slightly different wavelengths. However, for these results it need not be assumed that PLP is exposed to D$_2$O. D$_2$O may affect the strength of interaction of specific groups in the protein which result in different enzyme conformations. The difference spectrum could be a result of slight difference in the absorption of PLP in these enzyme forms. It should be noted that this latter explanation is also applicable to the solvent perturbation work done on various other
proteins.

The slight increase in activity seen in the presence of D\textsubscript{2}O shows that D\textsubscript{2}O has some effect either on the equilibrium constants before the rate determining step, or on the rate determining step itself.
SUMMARY

Many catalytic differences are known between phosphorylase a and b but not much work has been done on their relative conformational states. Because of these differences it is probable to think that the two forms have different conformations. These conformations were seen to cause a slight difference in the environment of tyrosine and tryptophan and subsequently a slight difference in their ultraviolet absorption.

No differences were detectable when comparing the $E_{280}$'s of phosphorylase a and b as they are normally isolated and stored. A more sensitive method namely, placing the same concentration of phosphorylase a in each of 2 cells, one in the sample and one in the reference beam of a double beam spectrophotometer and then converting the phosphorylase a in one cell to b by means of phosphorylase phosphatase, gave an ultraviolet difference spectrum between the two forms with the $E_{280}$ of phosphorylase a approximately 0.8% larger than the $E_{280}$ of phosphorylase b. This spectrum was in good agreement with the one seen in the reverse reaction, converting phosphorylase b to a using phosphorylase kinase.

When following spectral changes at 289 nm during the phosphorylase phosphatase reaction, the spectral changes were seen to be complete when approximately 70% of the
phosphate was released from phosphorylase \(a\). Glucose and glucose-6-phosphate were seen to accelerate the rate of phosphorylase \(a\) to \(b\) conversion and the rate of spectral change.

The effects of trypsin on the spectrum of phosphorylase \(a\) and \(b\) were also seen and evidence was presented for the conversion of phosphorylase \(b\) to \(b'\) by trypsin. The difference spectrum seen between phosphorylase \(a\) and \(b'\) was not like the one seen between phosphorylase \(a\) and \(b\) but its interpretation is complicated because trypsin releases peptides other than the phosphohexapeptide lost in the conversion of phosphorylase \(a\) to \(b'\).

The number of tyrosines and tryptophans in phosphorylase was redetermined and found to be respectively, 63 and 27 moles per mole of dimer. Solvent perturbation spectroscopy showed the percent exposures of tyrosine and tryptophan were 67 and 19 for phosphorylase \(b\) and 56 and 24 for phosphorylase \(a\) respectively. This similarity in the exposure of aromatic groups agrees with the similarity in absorptions between phosphorylase \(a\) and \(b\). It also shows that there are not many more aromatic groups exposed when the tetramer of phosphorylase \(a\) goes to a dimer of phosphorylase \(b\).

Solvent perturbation spectroscopy, which has so far only been applied to the study of tyrosine, tryptophan and phenylalanine, was used to study pyridoxal phosphate which
absorbs at 333 nm in the native enzyme. This cofactor was found to be buried to ethylene glycol and to be perturbed by 80% D₂O in the native enzyme at neutral pH. The PLP in the reduced enzyme was found to be exposed either in imidazole-citrate buffer, or at low pH, or in sodium dodecyl sulfate.
BIBLIOGRAPHY


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