ULTRAVIOLET SPECTRAL DIFFERENCES BETWEEN PHOSPHORYLASE a AND b*

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Summary

Phosphorylase a, a tetramer, and phosphorylase b, a dimer, were found to have very similar ultraviolet spectra. Essentially no difference was seen in the percentage exposure of tyrosinyl and tryptophanyl residues to solvent as measured by solvent perturbation spectroscopy. Differences (∿ 1% at 280 nm) were seen, however, when the conversion of phosphorylase a to phosphorylase b was followed spectrophotometrically. These spectral changes were faster than the rate of phosphate release from phosphorylase a and the rate of loss of activity as measured in the absence of AMP.

Phosphorylase a and b differ significantly in solubility, catalytic properties, stability, and subunit structure. This study attempts to determine whether conformational differences between these two proteins result in observable differences in the environment of the aromatic chromophores. Difference spectral measurements were employed in this work to examine the structures of phosphorylase a and b.

Methods

Crystalline phosphorylase b was prepared according to the procedure of Fischer and Krebs (1958). Phosphorylase a was prepared from phos-

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phorylase b with partially purified phosphorylase b kinase (Fischer and Krebs, 1962). Third or fourth crystals, which were treated with Norit A to remove AMP, were used for all experiments. ^{32}P-labelled phosphorylase a was prepared using ^{32}P-ATP (Lowenstein, 1960). All spectral measurements were carried out in a Cary model 15 double beam recording spectrophotometer by employing four matched 1 cm cuvettes in four separate cuvette holders. Phosphorylase with the perturbant or another enzyme is one cell in the sample beam, and the two are in separate cells in the reference beam. The second cell in the sample beam contains buffer. The enzyme solutions were put through 0.22μ millipore filters and then accurately pipetted, by using volumetric pipettes, into the same volume of either buffer, perturbant, or another enzyme. One pipette was used for phosphorylase additions, and another for buffer, perturbant or other enzymes.

Results and Discussion

Solvent perturbation spectroscopy (Herskovits, 1967) was used to see if there were large differences in the exposure of the aromatic amino acids in phosphorylase a and b. For these studies, it is important that the perturbant does not change the conformation of the protein. Ethylene glycol was found to have some effect on the activity of phosphorylase b (30% inhibition at 20% ethylene glycol). Since a plot of $\Delta\varepsilon_{285}/\varepsilon_{280}$ for phosphorylase b versus percent concentration of the perturbant, ethylene glycol, is linear and passes through zero, however, we concluded that the perturbant was not changing the exposure of the aromatic residues. The perturbation curves obtained showed phosphorylase a and b to be very similar. Published values of ethylene glycol perturbation of N-acetyl-L-tyrosine ethyl ester and N-acetyl-tryptophan ethyl ester (Herskovits and Sorensen, 1968) were used to obtain calculated curves resembling phosphorylase a and b perturbation. From this it was deduced that 50% of the tyrosyl residues and 20% of the tryptophanyl residues are exposed to ethylene glycol in phosphorylase a and b. Thus, it appears that the environment of

these residues in the two proteins is similar. A few of these residues may be very different but these differences could be masked by the large absorbance of all the other residues.

Ultraviolet spectral differences between phosphorylase a and b were best seen by placing the same concentration of phosphorylase a in both the sample and reference cells of a Cary double beam spectrometer and adding a small amount of phosphorylase phosphatase to the sample cell, which converted the phosphorylase a to b. Figure 1A 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 (Figure 1A, dashed line). 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.

A similar experiment was performed but starting with the same concentration of phosphorylase b in the reference and sample beam instead, and then the latter is converted to phosphorylase a with phosphorylase kinase, ATP and M_g^{++}. The spectrum obtained was in good agreement with the curve shown in Figure 1A from 340 nm to 270 nm. Since 10^{-4}M ATP was needed for the conversion and its absorption is approximately three times that of phosphorylase at 260 nm, the changes below 270 nm were not solely due to the differences between phosphorylase a and b.

From Figure 1A, it can be seen that the difference between phosphorylase a and b absorption at 280 nm is approximately 0.8%. Phosphorylase a was a tetramer and phosphorylase b a dimer under the conditions of this experiment. Thus, 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 absorptions of phosphorylase a and b.

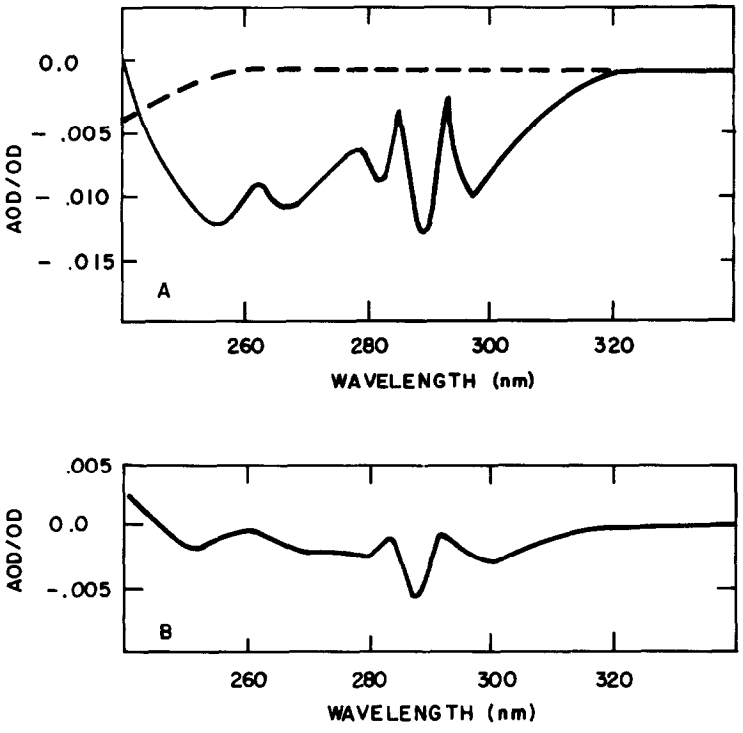

Figure 1

A. 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.

B. 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.

Glucose, which is known to dissociate the tetramer of phosphorylase a to dimer (Wang, et al., 1965), was found to accelerate the phosphatase reaction. Since 0.05M 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 gives the comparison of a phosphorylase b-glucose complex. As seen in Figure 1B, this difference was much less than the difference between phosphorylase a and b. Glucose is known to be an allosteric inhibitor of phosphorylase a (Helmreich, et al., 1967) 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.

During the conversion of phosphorylase a to b, the changes in the spectrum at 289 nm were followed with time and compared with the release of ^{32}P from ^{32}P-phosphorylase a. We found as shown in Figure 2 that the spectral change was complete when about 70% of the phosphate was released. The rate of loss of activity was measured in the absence of AMP was slightly slower than the loss of phosphate. 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 (Hurd, et al., 1966), accelerated the spectral change along with the phosphate release. Again, the spectral changes were complete when about 70% of the phosphate was removed. The difference in the rate of spectral change and of release of ^{32}P from ^{32}P-phosphorylase a gives some idea as to the action

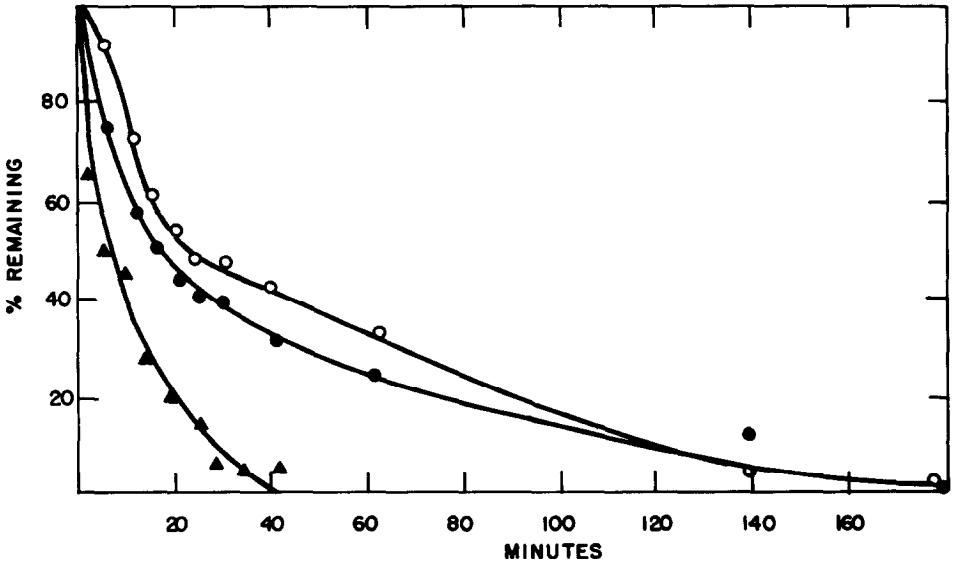

Figure 2

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, 0.075 M glucose-1-phosphate and 1% glycogen.

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%. These results seem consistent with the existence of partially phosphorylated intermediates, proposed by Hurd et al., (1966). These partially phosphorylated species may be spectrally like phosphorylase b, but may assume a partially active conformation when in the presence of substrates, explaining the loss in spectral differences when the enzyme is still active in the absence of AMP.

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