Protein Hydration Changes in the Formation of the Nicotinamide Adenine Dinucleotide Complexes of Glyceraldehyde 3-Phosphate Dehydrogenase of Yeast

I. BUOYANT DENSITIES, PREFERENTIAL HYDRATIONS, AND FLUORESCENCE-QUENCHING TITRATIONS*

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SUMMARY

The preferential hydrations of the glyceraldehyde 3-phosphate dehydrogenase of yeast and its (NAD) complex have been calculated from buoyant densities measured by an isopycnic sedimentation method in a concentration series of potassium phosphate solutions and by sedimentation equilibrium in cesium chloride gradients. In potassium phosphate solution at the isopycnic concentration and pH 7.4 and 25°, the preferential hydration of the apoenzyme is 0.480 g of water per g of protein. There is a 15.7% decrease in preferential hydration when the NAD complex is formed. This corresponds to a 6.3% decrease in volume of the solvated protein and is in accord with a reported 7% volume contraction measured by x-ray small angle scattering. The buoyant density increase associated with NAD complex formation is attributed to the elimination of water from cavities in the protein structure that exclude the salt component of the solvent. Essentially the same hydration change occurs at pH 8.5, where the protein binds NAD with a high degree of positive cooperativity, and at pH 7.4, where the binding appears to occur at noninteracting sites.

Chloride binding to the apo- and holoenzymes has been estimated from the pH shift that occurs when cesium chloride at its isopycnic concentration is added to a solution of the isoionic protein. The buoyant density increase associated with NAD complex formation does not involve an increased binding of salt, the heavy component of the solvent.

It has been observed by x-ray small angle scattering that the glyceraldehyde 3-phosphate dehydrogenase of yeast undergoes a particle volume contraction when it combines with NAD (1). As measured by this method, a volume contraction is associated with the elimination of an equivalent volume of solvent from cavities in the protein structure. It seemed likely that a solvation change of this type would also be expressed in terms of protein buoyant density in concentrated salt solutions provided that the salt were one that favored a high degree of preferential hydration of the protein. Aside from a purely methodological interest, our purpose in making buoyant density measurements was 2-fold. The x-ray measurements had been made under conditions in which NAD binding exhibited a high degree of positive cooperativity. We wished to determine whether the dehydration occurred under conditions in which the cooperative response of the enzyme to NAD was zero or negative, as previously inferred from a calorimetric analysis of the binding reaction (2). It was also desired to provide a more extended basis for the interpretation of a parallel set of proton spin lattice relaxation measurements described in a following report (3).

MATERIALS AND METHODS

The preparation and treatments of the enzyme crystallized from Fleischmann's bakers' yeast at 4-week intervals have been described (2). Only enzyme with specific activity between 140 and 180 mmoles per min per mg, measured in the direction of NADH oxidation (a), was employed. The NAD was a product of Sigma Chemical Co., supplied in the lyophilized form in precalibrated vials. These samples were reassayed at intervals by enzymic reduction.

Sedimentation Velocities in Potassium Phosphate Solutions—The solvents for these measurements were a concentration series of potassium phosphate solutions prepared by the mixing and dilution of standard 2.00 M di- and monopotassium phosphates in a 4:1 molar ratio for the pH 7.4 series and in a 100:1 molar ratio for the pH 8.5 series. No adjustments were made for the concentration dependence of pH. The solvent densities over the concentration range examined were determined pycnometrically and agreed well with values in the International Critical Tables. Capillary viscometers with outflow times in
the range of 100 to 200 s at 25° were employed for the measurements of relative viscosities. Depending upon the salt concentration, the protein was equilibrated with solvent by passage through a column (40 cm) of Sephadex G-25 or by dialysis. Sedimentation velocities were measured in a double sector capillary synthetic boundary cell. The relative amounts of solvent and enzyme solution were selected to place the solution boundary near the center of the cell. Runs at 5.5 mg of enzyme per ml were made at 44,700 rpm in the AN-D rotor at 5.0 and 25.0° and were kept as short as possible to further minimize effects of salt redistribution. Boundary movement was monitored with the schlieren optical system, and the coefficients were calculated from the movement of the boundary maxima.

Equilibrium Sedimentation in Cesium Chloride Gradients—These measurements were made in a cell with a double sector, filled Epon centerpiece and sapphire windows at 44,770 rpm and 25°. The densities of the stock cesium chloride solutions were measured by weighing a calibrated micrometer syringe (Gilmont) filled with the salt solution and then reweighing after removal of a known volume of the solution. The initial concentration was 2.12 M, ρ = 1.250 g ml⁻¹. Sedimentation equilibria of the apo- and holoenzymes were obtained within about 16 hours after boundary formation, and the results were recorded by schlieren photographs after 22 hours. The schlieren curves and base-lines were measured in a microcomparator, and large scale plots were made.

Fluorescence Titrations—Binding equilibria were measured in a temperature-controlled Turner spectrofluorimeter in the fluorescence mode which corrected for the small changing attenuation of the incident light by the titrant. Excitation was at 300 nm and emission was measured at 350 nm. The band widths in both cases were 10 nm. Enzyme solutions of 2.0 ml in a 1-cm square cuvette were titrated with concentrated NAD solutions from a micrometer syringe that was calibrated to 0.1 μl. After each addition the solution was stirred with a Teflon rod, and the output was measured on the strip chart recorder for a sufficient amount of time to ensure completion of slow transitions. The latter were observed only at low temperatures.

RESULTS

Equilibrium Titrations—There have been some conflicting reports concerning details of the NAD-binding isotherms of the yeast enzyme at pH 8.5 (4–6). The extent to which these differences are due to the analytical methods employed, differences in the yeast strains from which the enzymes were prepared, or variations in the isolation and storage of the sensitive oligomeric protein has not been determined. Since our methods with enzyme from still another yeast strain are at variance with those cited above, we have characterized the NAD-binding curves of the present enzyme over a temperature and pH range by fluorescence-quenching titrations. Results by this method at pH 7.4 and 5–40° have been described (2). Under these conditions, to a good approximation, the nucleotide was observed to bind at four equivalent noninteracting sites, and this behavior has been confirmed with the present enzyme preparations. Titrations monitored by this method over the same temperature range but at pH 8.5 are shown in Fig. 1. These curves illustrate the positive cooperativity of NAD binding at pH 8.5 and its strong temperature dependence. Scatchard plots of the data are shown in Fig. 2. The deviations from the form expected for the concerted allosteric model are much smaller than those observed by Cook and Koshland (6) and may be within the limits of error of the measurements. In particular, the stoichiometry is approximately correct, and there is no upward deflection as saturation is approached.

![Fig. 1. Fluorescence-quenching titration of glyceraldehyde 3-phosphate dehydrogenase of yeast (4.4 μM) with NAD at pH 8.5 in 0.06 M sodium pyrophosphate, 2 mM EDTA, and three temperatures.](image-url)
Preferential Hydration Changes in Potassium Phosphate Solutions—The buoyant density, \( \rho_b \), of a protein in an aqueous concentrated salt solution is described in Equation 1 (7)

\[
\rho_b = \frac{1 + \Gamma}{V_p + \Gamma V_w}
\]

where \( V_w \) is the partial specific volume of water, \( V_p \) is the partial specific volume of the protein in dilute salt solutions, and \( \Gamma \) is the preferential hydration. \( \Gamma \) is expressed in grams of water per g of protein and corresponds to the effective fraction of the total solvation domain which, as a result of preferential hydration, may be viewed as salt-free. It thus represents a lower limit of the total hydration since it does not include any solvation fraction which is of the same effective density as that of the free multicomponent solvent. From the work of Ifft and Vinograd (8) with bovine serum mercaptoalbumin, \( \Gamma \) is minimal with salts with small hydrated ionic radii, such as cesium iodide, and large with salts with a large oxy anion, such as sodium sulfate. For the present purposes, the salt had to be of the latter type and sufficiently soluble to give solutions of the required density with no precipitating or denaturing the protein. The recent work of Aune and Timasheff (9), with a glyceraldehyde 3-phosphate dehydrogenase of unspecified NAD content from skeletal muscle, indicated that potassium phosphate in the neutral pH range has the required properties and that \( \rho_b \) could be obtained by a very short extrapolation in a plot of \( S_0 \) against solvent density, where \( S \) is the sedimentation coefficient of the protein and \( \eta \) is the relative viscosity of the solvent.

The results with the holo- and yeast apoenzyme at pH 7.4 and 5° are depicted in Figs. 3 to 5. The extrapolations to \( S_0 = 0 \) are quite short. In fact, the buoyant density of the apoprotein at pH 7.4 and 25° could be exceeded without precipitating the protein and resulted in an inverted schlieren boundary that ascended in the centrifugal field. However, the NAD complex at this temperature, and both forms of the enzyme at 5°, crystallized before or during a centrifuge run when the buoyant densities were too closely approached. This might have been avoided by working at lower enzyme concentrations, but for present purposes there was no need to do so. The pairs of curves under the three sets of conditions examined are consistently displaced, and the buoyant densities are greater for the enzyme-NAD complex than for the apoprotein. The same factor, namely the protein volume contraction, is responsible for the curve displacements at both low and high salt concentrations. However, in low salt concentrations the preferential hydration is negligible and the effect of the volume contraction on \( S \) is expressed by its influence on the frictional coefficient, whereas in high salt concentrations at \( S = 0 \) the system is in thermodynamic equilibrium and the buoyant density rather than the size and shape of the protein is the determining factor.

Numerical results are summarized in Table I. The preferential hydrations and the differences in preferential hydrations between the holo- and apoenzyme are quite large. At 25° the change in \( \Gamma \) associated with NAD addition is -0.075, corresponding to a 15.6% \( \Gamma \) decrease in the preferential hydration of...
the protein. It should be noted that the same decrease at 25°
occurs at pH 8.5 and 7.5 and that the dehydration is considerably
diminished at 5°.

**Solution in Cesium Chloride Solutions**—The buoyant density
of a macropolyelectrolyte involves interactions with both ions
and the water component of the solvent. A strong anion binding
property of the yeast and muscle enzymes was observed several
years ago by shifts in the isoelectric points, measured electrophoretically, and by direct measurements of phosphate binding
(10). Such measurements are not practical at the high salt
concentrations of the present work, but a reasonable approxima-
tion of the extent of salt binding and changes thereof may be
obtained by measurement of the pH shift that occurs when salt is added to the protein in distilled water at its isoionic point.
For this purpose, phosphate is not suitable in the pH range of
interest because of its buffering action. Fluorescence-quenching
titrations indicated that NAD binding was not strongly affected
in concentrated solutions of cesium chloride, and we selected this
salt for measurements of the pH shift. Since Ifft and Vinograd
(8) had established the method for calculating solvent density
as a function of radial distance in a cesium chloride gradient at
sedimentation equilibrium in potassium phosphate gradients at pH
6.8 and 25°. A, holoenzyme; B, apoenzyme.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature</th>
<th>Buoyant density</th>
<th>Preferential hydration</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Enzyme</td>
<td>NAD complex</td>
</tr>
<tr>
<td>7.4</td>
<td>5°</td>
<td>1.228</td>
<td>1.234</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.213</td>
<td>1.227</td>
</tr>
<tr>
<td>8.5</td>
<td>25</td>
<td>1.224</td>
<td>1.230</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated for \( V_p = 0.73 \) at 5° (13).

<sup>b</sup> Calculated for \( V_p = 0.74 \) at 25° (13).

measured small volume of a concentrated NAD solution (ad-
justed to the pH of the isoionic protein) was added at a concen-
tration calculated to saturate the binding sites, the \( ΔpH \) was
+0.10, and addition of cesium chloride to this solution resulted
in a \( ΔpH \) of +0.51. The increase in pH is a sign of anion bind-
ing, and the relation is given by Equation 2 (12)

\[
Δv = \frac{2.3}{2w} ΔpH
\]  

where \( v \) is the number of anions bound per molecule of protein
and \( w \) is the electrostatic free energy factor. The value of \( ν \)
calculated from these results is 104 for the apo- and 98 for the
holoenzyme. NAD addition decreases slightly the binding of
chloride and probably has a similar effect on the binding of phos-
phato. It is therefore not likely that an increase in salt binding
contributes to the buoyant density increase associated with NAD
complex formation.

The hydration, \( Γw \), necessary to produce the observed buoyant
density of the protein salt complex is given by Equation 3 (8)

\[
Γw = \frac{1 + Z + Γ*}{V_p + ZV_s + Γ*V_w}
\]  

where \( Γ* \) is the partial specific volume of cesium chloride (0.233)
and \( Z \) is the weight fraction of bound salt. It is assumed that
the bound chloride ions are accompanied by an equivalent num-
ber of cesium ions in an outer hydration sphere. The results for
cesium chloride are summarized in Table II.

**Discussion**

If the hydration of glyceraldehyde 3-phosphate dehydrogenase
is 0.5 g of water per g of protein, a minimum value, the net hy-
dration change of \(-15.6%\) on complex formation with NAD at
25° would correspond to a 6.3% volume contraction of the sol-
vent protein. This may be compared with the 7% volume contraction measured by x-ray scattering at a higher temperature.
Buoyant densities and preferential hydrations of aldehyde 3-phosphate dehydrogenase and its (NAD)_k complex in cesium chloride density gradients at 25°

<table>
<thead>
<tr>
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<th>p0</th>
<th>r</th>
<th>z</th>
<th>r_s</th>
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<tr>
<td>Enzyme</td>
<td>1.256</td>
<td>0.275</td>
<td>104</td>
<td>0.611</td>
</tr>
<tr>
<td>Complex</td>
<td>1.258+</td>
<td>0.267</td>
<td>98</td>
<td>0.581</td>
</tr>
<tr>
<td>Δ%</td>
<td>-2.9</td>
<td>-4.9</td>
<td></td>
<td></td>
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</table>

The degree of agreement indicates that the same phenomenon is observed by the two independent methods and that the preferential hydration change derived from buoyant density measurements does, in fact, result from the elimination of an internal hydration component in a protein contraction. The simplest model that satisfies the x-ray results is a single cavity in a hollow cylinder of small axial ratio. However, the relative absence of phosphate from the solvent that is extruded is in accord with a model in which water is eliminated from several small cavities that exclude phosphate, presumably because of its size. From the results depicted in Table II, cesium chloride penetrates the solvation domain to a greater extent than does potassium phosphate. The contraction with NAD may be as great as with phosphate but involves the elimination of a larger amount of the salt component, hence the smaller change in buoyant density.

The preferential hydration in phosphate solution is less at 5° than at 25° (Table I) and the magnitude of the contraction, as measured by the change in preferential hydration, is less than half as large at the lower temperature. A model consistent with these results and with the thermodynamic parameters of NAD binding is an apoprotein that expands more rapidly with temperature than does the NAD complex. The calorimetric ΔH of complex formation changes linearly from -1.9 to -20.1 Cal per mole of NAD over the temperature range of 5 to 40°, and the ΔS over the same temperature range falls from +19.9 to -41 e.u. (2). These changes are in the direction expected for bond formation and a tightening of the protein structure. A positive contribution to the entropy change from water release is more than compensated at temperatures above 15° but should contribute to the positive ΔS at 5°.

The same change in preferential hydration occurred at pH 8.5, where there is a substantial degree of positive cooperativity in NAD binding, and at pH 7.4 where, to a first approximation, the subunit responses to NAD binding appear independent. If the cooperativity at pH 8.5 involves a concerted conformational transition from a low affinity form of the protein to a form of high NAD affinity, then the increase of the association constant and the lack of cooperativity at pH 7.4 may be due to a great predominance, at that pH, of the high affinity form of the protein. The equivalent changes in preferential hydration at the two pH values should then be the expression of the effect of NAD upon the high affinity form of the protein and should not be associated directly with the allosteric transition.

REFERENCES

Protein Hydration Changes in the Formation of the Nicotinamide Adenine Dinucleotide Complexes of Glyceraldehyde 3-Phosphate Dehydrogenase of Yeast:
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