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THE FREE ENERGY OF HYDROLYSIS OF SERINE PHOSPHATE AND *N*-ACETYLETHANOLAMINE PHOSPHATE AND ITS RELATION TO PHOSPHATASE ACTIVITY

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SUMMARY

The equilibrium constant for the hydrolysis of *N*-acetylethanolamine phosphate was found to be 2.0 at alkaline pH and estimated to be 2.7 at pH 7.0, and 25°. When the concentration of water in dilute solution is included in the equilibrium constants, as is customary, the constants become $1.1 \cdot 10^2$ and $1.5 \cdot 10^2$, respectively, corresponding to standard free energies of hydrolysis of 2.7 and 2.9 kcal/mole. The equilibrium constant for the hydrolysis of serine phosphate could not be evaluated, but from our measurements we conclude that it must be greater than 1 (or 55). Since the free-energy change is unfavorable the finding of others that labelled phosphate is incorporated into phosphatase and ultimately isolated as serine phosphate indicates that other changes must be involved.

INTRODUCTION

The chemical properties of serine phosphate and its derivatives are important for protein and enzyme chemistry. Phosphoproteins contain serine phosphate side chains, and a specific serine residue is very probably involved in the activity of a number of hydrolytic enzymes such as chymotrypsin (EC 3.4.4.5), trypsin (EC 3.4.4.4) and cholinesterase (EC 3.1.1.8). Organophosphorus anhydrides such as diisopropyl fluorophosphates irreversibly inhibit these enzymes by reacting with a serine residue to yield an inactive phosphoryl enzyme derivative[1–5]. A serine residue is phosphorylated in the conversion of phosphorylase *a* to phosphorylase *b*[6]. A phosphoryl enzyme is involved as an intermediate in the catalytic activity of phosphoglucomutase (EC 2.7.5.1), and the enzyme derivative which contains a serine phosphate side chain has, in fact, been isolated[7–10]. Finally, ENGSTRÖM[11,12] and others[13] have suggested that a phosphoryl enzyme is formed during the activity of phosphatases. They recovered radioactive serine phosphate in high yield from the protein hydrolysates of enzyme preparations that were incubated with low concentrations of ^{32}P-labelled phosphate. These results are surprising since one would expect the equilibrium constant for the reaction,

$$\text{serine phosphate} + H_2O \rightleftharpoons \text{serine} + \text{phosphate}$$

to have a value large enough to preclude the possibility of obtaining extensive phosphorylation of serine residues.

Since no value for the equilibrium constant for the hydrolysis of serine phosphate or related compounds has been reported, it seemed important to make this measurement.

Measurements of the equilibrium constant for the hydrolysis of phosphate esters have been determined by KAY[14] and others[15] in media containing high concentrations of alcohol and relatively low concentrations of water (with the aid of phosphatases to bring the system to equilibrium). With known initial concentrations of all reactants, equilibrium concentrations can be evaluated by a measurement of the phosphate concentration alone. We found that in the hydrolysis of serine phosphate the equilibrium position lies so far in the direction of hydrolysis, that even with a nearly saturated solution of serine, the measured amount of phosphate did not differ, within our experimental error, from the amount corresponding to complete hydrolysis. Going in the direction of synthesis, there was no measurable uptake of phosphate. We could assign, therefore, only a minimum value for the equilibrium constant. The compound *N*-acetylethanolamine, a good analogue for serine residues in proteins, proved suitable for experimental work, and the equilibrium constant for hydrolysis of its phosphate ester was determined. The enzyme alkaline phosphatase (EC 3.1.3.1) was active in solutions containing 70 and 80% of the alcohol, and under these conditions measurable amounts of phosphate were taken up.

EXPERIMENTAL

Alkaline phosphatase from *E. coli*, chromatographically purified, 5 mg/ml suspended in saturated ammonium sulfate, was purchased from Worthington Biochemical Corp. Ammonium sulfate was removed by centrifuging the suspension and dissolving 5–10 mg of the enzyme in 1 ml of water.

Serine phosphate was purchased from California Corp. for Biochem. Research.

N-acetylethanolamine from Aldrich Chemical Company, was distilled twice under reduced pressure, b.p. at 0.01 mm, 103°. Sp.gr. 1.115 (see ref. 16).

Phosphate was determined by the method of DRYER, TAMMES AND ROUTH[17].

Determination of equilibrium constants

The constant for serine phosphate hydrolysis was measured by incubating at 25°, 2.00 ml of 1 M serine, 0.200 ml of phosphatase (5 mg/ml) in 1 M serine, and either 0.200 ml of 0.057 M KH_2PO_4 (brought to pH 8.0 with NaOH) or 0.200 ml of 0.057 M serine phosphate (brought to pH 8.0 with NaOH) depending upon the direction of approach to equilibrium. After suitable time intervals 0.200 ml portions of the reaction mixture were added to 2.00 ml of 10% trichloracetic acid. Protein was removed by centrifugation and 1 ml of the supernatant fluid was transferred to a 5 ml volumetric flask for phosphate determination. Ammonium molybdate, 0.008 M, 0.2 ml, and 2 ml of semidine reagent (0.5 mg/ml in 1% sodium bisulfite) were added and diluted to the mark. The absorbancy at 770 mμ was read after 10 min with a Beckman model DU spectrophotometer set at a slit width of 0.19 mm. Phosphate standards in 1 M serine and controls of serine phosphate in serine and enzyme in serine were run simultaneously. The enzyme was shown to be completely active after 24 h incubation with 1 M serine by determining the rate of hydrolysis of serine phosphate.

The equilibrium constant for the hydrolysis of *N*-acetylethanolamine phosphate was measured by incubating at 25.0° 0.400 ml of phosphatase (5 mg/ml), 0.400 ml of 0.0470 M KH_2PO_4 (adjusted to pH 8.0 with NaOH) with either 0.400 ml of water and 3.122 g (2.80 ml) of *N*-acetylethanolamine or with 3.57 g (3.20 ml) of *N*-acetylethanolamine. The final volumes were approx. 4.04 ml, about 1% greater than the sums of the volumes of the component liquids. Samples of 0.200 ml were assayed for phosphate, as described above, until constant values were obtained.

RESULTS

The results obtained with serine phosphate are summarized in Table I. There was no measurable uptake of phosphate in a system intially containing $4.73 \cdot 10^{-3}$ M phosphate and 1 M serine but no serine phosphate. A system intially containing no phosphate, but $4.73 \cdot 10^{-3}$ M serine phosphate and 1 M serine, was found to contain at equilibrium $4.73 \cdot 10^{-3}$ M phosphate within the limits of experimental error. The equilibrium

TABLE I

DETERMINATION OF EQUILIBRIUM CONSTANT FOR SERINE PHOSPHATE HYDROLYSIS

Reaction mixture at 25° contained 2.00 ml of 1 M serine, 0.200 ml of phosphatase (5 mg/ml) in 1 M serine, and either 0.200 ml of 0.057 M KH_2PO_4 or 0.200 ml of 0.057 M serine phosphate. Constant readings were obtained in Expt. 3 after 1.5 h. All experiments were carried out for 120 h.

Expt.	*Concentration of enzyme (mg/ml)*	*Initial molar concentration*				*Equilibrium concentration of Phosphate* $\times 10^3$
		Serine	*Serine phosphate* $\times 10^3$	*Phosphate* $\times 10^3$	H_2O	
1	0.42	1	0	4.73	52.3	4.77
2	—	1	0	4.73	—	4.73
3	0.42	1	4.73	0	52.6	4.67

position evidently lies so far towards hydrolysis that the differences in phosphate concentrations at equilibrium were too small to measure. A change of 2% in the concentration of phosphate would fall within the limits of our experimental error, and would be undetected. If we assume that such a change did in fact occur, we can calculate a minimum value of 1 for the equilibrium constant.

The results obtained with *N*-acetylethenolamine are summarized in Table II. In these experiments, about 19–28% of the phosphate was taken up when phosphate was incubated with very high concentrations of *N*-acetylethanolamine in the presence of enzyme. When water was added after the phosphate uptake had ceased, phosphate was released, indicating that the enzyme was still active and that equilibrium had been achieved. The equilibrium constant for this reaction,

N-acetylethanolamine phosphate + $H_2O \rightleftharpoons$ *N*-acetylethanolamine + phosphate

was calculated from the data summarized in Table II. The concentrations used for phosphate and the phosphate ester were the total values, *i.e.* the sums of all ionic forms. The resulting equilibrium constant is therefore a function of the pH:

$$\frac{(N\text{-acetylethanolamine})\ (\text{phosphate})}{(N\text{-acetylethanolamine phosphate})\ (H_2O)} = \frac{K(1 + (H^+)/K_2)}{(1 + (H^+)/K_2')}$$

where K is the equilibrium constant expressed in terms of the doubly charged anions, K_2 is the second ionization constant of phosphoric acid, and K_2' is the second ionization constant of N-acetylethanolamine phosphate. Only the second ionization constants are important in the pH range 4 to 9. Since phosphate esters in general are more acidic than phosphoric acid[18], we can assume that the second ionization constant of N-acetylethanolamine phosphate is larger than the second ionization constant of phosphoric acid.

TABLE II

DETERMINATION OF EQUILIBRIUM CONSTANT FOR HYDROLYSIS OF N-ACETYLETHANOLAMINE PHOSPHATE

Reaction mixture at 25° contained 0.400 ml of phosphatase, 0.400 ml of 0.0470 M KH_2PO_4 with either 0.400 ml of water and 3.122 g (2.80 ml) of N-acetylethanolamine or with 3.57 g (3.20 ml) of N-acetylethanolamine. Constant readings were obtained after 2 days for Expts. 1 and 2 and after 13 days for Expt. 3.

Expt.	*Concentration of enzyme (mg/ml)*	*Initial molar concentration*				*Equilibrium molar concentration*		*K*
		N-acetyl-ethanol-amine	*Phosphate* $\times 10^3$	H_2O	*Ester*	*Phosphate* $\times 10^3$	*Ester* $\times 10^2$	
1	0.05	7.58	4.73	16.38	0	3.88	0.85	2.11
2	0.1	7.58	4.73	16.36	0	3.88	0.85	2.11
3	0.05	8.66	4.73	10.94	0	3.41	1.32	2.05
							Average:	2.09

The thermodynamic second ionization constant for phosphoric acid is $pK = 7.1$ and the apparent constant, which is more appropriate for dilute salt solutions, is $pK = 6.8$. Values for N-acetylethanolamine phosphate are not listed, but we can make a rough estimate of the constant based upon the value of $pK = 5.8$ for ethanolamine phosphate[18]. Since ethanolamine phosphate bears a positively charged function and N-acetylethanolamine phosphate does not, the latter must be decidedly less acidic than the former. An estimate of about $pK = 6.3$ for the apparent constant would seem reasonable for N-acetylethanolamine phosphate. A precise value is not actually needed because the equilibrium constant is only weakly dependent upon this value.

Our measurements were well in the alkaline region, so that the value of the equilibrium constant we have tabulated is equal to the value of K. If our estimate of the second ionization constant of the ester is correct, the value for the equilibrium constant at pH 7.0 in dilute salt solution is about 2.7. The value of the equilibrium constant calculated for pH 7.0 must lie between 2.1 and 3.2, no matter what the value of K_2', provided $K_2' > K_2$. The interest in the equilibrium constant lies in its value in dilute aqueous solution, and our measurements shares with all similar measurements the uncertainty that comes from using concentrations rather than activities.

For use in dilute aqueous solutions it is customary to include the concentration of water, 55.5 M, in the equilibrium constant. Our K then becomes $1.1 \cdot 10^2$ and our estimate for pH 7.0 becomes $1.5 \cdot 10^2$. The corresponding standard free energies of hydrolysis are 2.7 kcal/mole and 2.9 kcal/mole.

DISCUSSION

The standard free energy of hydrolysis of *N*-acetylethanolamine phosphate is small; it is about the same as those of other esters such as α-glycerophosphate and hexose 6-phosphates[11].

N-Acetylethanolamine is clearly a reasonable analogue for serine side chains in proteins, and the fact that its phosphorylation is decidedly unfavorable raises the perplexing question of how it was obtained by incubating low concentrations of phosphate, 10^{-4}–10^{-6} M, with alkaline phosphatase preparations. We should estimate that the fraction of serine residues phosphorylated under these conditions would be no more than 10^{-6}–10^{-8} M. Yet serine phosphate is found and in some cases the fraction of a particular serine side chain that was phosphorylated was estimated to be 0.6 (see ref. 11). Possibly the formation of serine phosphate is a coupled reaction. The driving force might perhaps be obtained from a concomitant change in protein structure or a change in the structure of the side chain. Possibly there is an exchange reaction. An exchange reaction would be evidence against a phosphoryl enzyme intermediate in the catalytic reaction. The possibility that an exchange reaction occurred is discussed but rejected by ENGSTRÖM.[11] In the light of this discussion, the finding that labelled phosphate is incorporated into protein of active phosphatase preparations as serine phosphate side chains is very difficult to explain. The explanation possibly is intimately related to the catalytic mechanism. Considerations of this kind have been noted by SCHWARTZ AND LIPMANN[19]. LIPMANN AND RABINOWITZ[20] found that the free energy of hydrolysis of some of the phosphate bonds in phosvitin was very high, perhaps about as high as in ATP. Here too it appears that some special explanation is required.

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