The Free Energy of Hydrolysis of Phosphoryl-Phosphatase*

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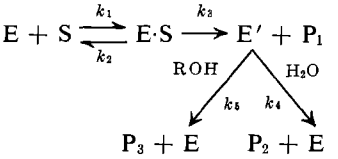
ABSTRACT: The equilibrium constant for the reaction of *O*-phosphorylethanolamine with alkaline phosphatase from *Escherichia coli* is 2×10^5 and the equilibrium constant for the hydrolysis of the phosphoryl enzyme is 3×10^{-6}. These values are for the total concentrations of reactants at *p*H 8.0, 25°. The value given for the second constant requires the explicit use of the concentration of water. These values were obtained by a new kinetic method which assumes the formation of a phosphoryl enzyme as an intermediate. The method is described. The values of phosphate uptake calculated from the equilibrium constant were compared with the phosphate uptake measurements of other workers. The equilibrium constant for the hydrolysis of *O*-phosphorylethanolamine was found to be 0.56.

Engstrom and Agren (1958) and others (Engstrom, 1961, 1962a; Schwartz and Lipmann, 1961; Agren, 1959) have found radioactive *O*-phosphorylserine in the protein hydrolysate from purified preparations of alkaline phosphatase which had been incubated with phosphate containing ^{32}P. Similar results were obtained with ^{32}P-labeled glucose-1-phosphate (Engstrom, 1962b). These findings suggest that a phosphoryl enzyme may be formed as a catalytic intermediate in the hydrolysis of phosphate esters. This is an attractive hypothesis since analagous intermediates have been satisfactorily demonstrated for other hydrolytic enzymes, acetylcholinesterase and chymotrypsin.

Engstrom (1961), assuming the phosphorylation of only one specific serine side chain, found the surprising result that the fraction phosphorylated was large (0.6) even though the medium contained only 10^{-4}–10^{-6} M phosphate. This forces us to conclude that the phosphoryl enzyme is exceedingly more stable than related simple phosphate esters such as *O*-phosphoryl-*N*-acetylethanolamine (Dayan and Wilson, 1963). This stability may not be intrinsic to the covalent bond involved but may arise from a concurrent process such as interaction of the phosphate structure with the enzyme.

The isolation of serine phosphate does not demonstrate that a phosphoryl enzyme is formed as a catalytic intermediate, for, if the enzyme or other protein in the preparation contains a serine side chain capable of forming a thermodynamically stable *O*-phosphoryl derivative, its phosphorylation would be catalyzed by the enzyme. The isolation experiment shows only that such a side chain exists. If we postulate the formation of a catalytic phosphoryl enzyme intermediate, it is possible to evaluate the equilibrium constant for its hydrolysis from kinetic measurements. The importance of the isolation of serine phosphate as evidence for the formation of a catalytic phosphoryl enzyme intermediate would be immeasurably strengthened if the value of the equilibrium constant for its hydrolysis obtained from kinetic measurements should turn out, at least in approximation, to be as small as that found in the isolation experiments. In explaining the kinetic method, we consider the scheme for the solvolysis of the substrate, *p*-nitrophenyl phosphate, in the presence of an added phosphate acceptor, ethanolamine (Wilson *et al.*, 1964), involving the formation of a phosphoryl enzyme, E′,



Scheme 1

where S is the substrate, *p*-nitrophenyl phosphate, P_1 is *p*-nitrophenol, ROH is ethanolamine, P_2 is phosphate, and P_3 is *O*-phosphorylethanolamine. This scheme is formulated for negligible concentrations of P_1, P_2, and P_3 so that the reverse reactions do not have to be considered. The scheme, in anticipating the experimental result that the rate of formation of P_3 is proportional to the concentration of ethanolamine, presents this step as a second-order reaction.

It is true that this pathway is in actuality more than a single step and involves a complex between E and P_3, but this detail is without significance for our purposes. The effect is to lump a number of constants together as k_5; we require only the composite constant k_5.

The steady-state solution for the initial rate of solvolysis of substrate, *v*, has the form of the Michaelis-Menten equation

$$v = \frac{kE^o}{1 + K_m/(S)} = \frac{V}{1 + K_m/(S)} \qquad (1)$$

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with

$$k = \frac{k_3}{1 + k_3/[k_4 + k_5(\mathrm{ROH})]} \quad (2)$$

and

$$K_m = \frac{\frac{k_2}{k_1} + \frac{k_3}{k_1}}{1 + k_3/[k_4 + k_5(\mathrm{ROH})]} \quad (3)$$

where E^o is the total concentration of enzyme in all its forms.

The concentration of H_2O has been included in k_4. Note that k/K_m is independent of k_4 and k_5 (ROH). The ratio of the initial rates of formation of phosphate and *O*-phosphorylethanolamine, f, is an easily measured quantity and is given by

$$f = \frac{k_4}{k_5(\mathrm{ROH})} \quad (4)$$

From equations (1), (2), and (4),

$$k_4E^o = \frac{V_oV}{(1 + f)(V - V_o)} \quad (5)$$

and

$$k_5E^o = \frac{V_oV}{(1 + f)(V - V_o)f(\mathrm{ROH})} \quad (6)$$

where V_o is the maximum velocity measured in the absence of ethanolamine, and V is the maximum velocity in the presence of ethanolamine.

For the purpose of deriving the equations needed for evaluating the equilibrium constants, consider now the equilibrium between phosphatase and *very low* concentrations of *O*-phosphorylethanolamine. At substrate concentrations which are much lower than the Michaelis constant nearly all the enzyme is free, $(E) \rightarrow E^o$, and the Michaelis-Menten form approaches a second-order rate expression

$$v = \lim_{(S)/Km \to 0} \frac{kE^o}{1 + K_m/(S)} = \frac{k}{K_m}(S)(E)$$

with the second-order rate constant $k_1^o = k/K_m$ or $k_1^oE^o = V/K_m$. Therefore the equilibrium may be written

$$\mathrm{E} + \mathrm{P_3} \underset{k_5}{\overset{k_1^o}{\rightleftharpoons}} \mathrm{E'} + \mathrm{ROH} \qquad \text{Scheme 2}$$

and the equilibrium constant is given by

$$K_1 = k_1^o/k_5$$

Since V and K_m can be measured and since k_5E^o can be obtained by applying equation (6), it is possible to evaluate the equilibrium constant for the reaction of *O*-phosphorylethanolamine with the enzyme. Although we have discussed equilibrium at low concentrations of P_3, no experiment need be done at low concentrations.

The equilibrium constant for the hydrolysis of the phosphoryl enzyme, K_2, is given by

$$K_2 = \frac{K_3}{K_1}$$

where K_3 is the equilibrium constant for the hydrolysis of *O*-phosphorylethanolamine. The following experimental measurements have to be made: (1) V, V_o, and f for the solvolysis of *p*-nitrophenyl phosphate; (2) V and K_m for *O*-phosphorylethanolamine; (3) K_3.

The value of K_m was determined indirectly by using *O*-phosphorylethanolamine as a competitive inhibitor in the hydrolysis of *p*-nitrophenyl phosphate. This indirect procedure was adopted because phosphate is an extremely potent inhibitor of the enzyme. The dissociation constant of the enzyme-phosphate complex while comparable to the K_m for *p*-nitrophenyl phosphate is far smaller than the K_m for *O*-phosphorylethanolamine. The direct measurement of K_m for *O*-phosphorylethanolamine is complicated therefore by extensive product inhibition. The indirect experiment is feasible because very low concentrations of *p*-nitrophenol can be measured.

Experimental

Chromatographically purified alkaline phosphatase (Garen and Levinthal, 1960) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1.) (*Escherichia coli*) was obtained as a suspension, 5 mg/ml in saturated ammonium sulfate, from Worthington Biochemical Corp. All enzyme dilutions were made with 0.02 M Tris, *p*H 8.0.

The hydrolysis of *p*-nitrophenyl phosphate at 25°, *p*H 8.0, was used extensively in this study because the high extinction coefficient of *p*-nitrophenol at 400 mμ facilitated the difficult measurement of the very low concentrations of products demanded by the extremely small values of K_m and K_I.

V and K_m of p-Nitrophenyl Phosphate. The reaction was initiated by the addition of 0.01 ml of enzyme solution (0.125 μg) to 13 ml of incubation mixture, consisting of 0.1 M Tris (*p*H 8.0), 1×10^{-4}–1×10^{-6} M *p*-nitrophenyl phosphate in the presence or absence of 1 M NaCl, contained in 5-cm cuvets. The rate of liberation of *p*-nitrophenol at 25° was followed by taking OD readings at 400 mμ every minute with a Zeiss PMQ 11 spectrophotometer. Initial rates, for six concentrations in the range 1×10^{-6}–1×10^{-4} M, were obtained from measurements taken during the hydrolysis of up to 10% of the initial substrate concentration. Reciprocal plots, v^{-1} versus $(S)^{-1}$, using the average value of (S), yielded good straight lines.

K_I for Phosphate. Initial rates of hydrolysis of *p*-nitrophenyl phosphate in the concentration range 1×10^{-3}–5×10^{-6} M were measured as before in the presence of 1×10^{-5} and 3×10^{-6} M phosphate with and without 1 M NaCl and 0.125 μg of enzyme. Reciprocal plots, v^{-1} versus $(S)^{-1}$, yielded good straight lines intercepting the ordinate at $1/V_0$.

K_m for O-Phosphorylethanolamine. The direct determination of K_m for *O*-phosphorylethanolamine by the rate of liberation of phosphate is difficult because K_m is

TABLE I: Equilibrium Constant for the Hydrolysis of *O*-Phosphorylethanolamine.[a]

Substance	Molar Concentration					
	Initial	Equilibrium		Initial	Equilibrium	
	Expt 1, 2	Expt 1	Expt 2	Expt 3, 4	Expt 3	Expt 4
Ethanolamine	1.00	1.00	1.00	1.00	1.00	1.01
Phosphate	0.02	0.0193	0.0193	0.02	0.029	0.029
O-Phosphorylethanolamine	0	0.00068	0.00068	0.01	0.00091	0.00097
Water	53.4					
K_3 (total concentrations, *p*H 8.0)		0.54	0.54		0.60	0.56

[a] Only the concentration of *O*-phosphorylethanolamine was measured at equilibrium. The equilibrium concentrations of phosphate and ethanolamine were calculated from the initial values and the change in the concentration of *O*-phosphorylethanolamine. The concentration of water was computed from the density of the solution in experiment 1 and taken to be the same in all others. The equilibrium constant is

$$K = \frac{(\text{phosphate})(\text{ethanolamine})}{(O\text{-phosphorylethanolamine})(H_2O)}$$

The predominant species at *p*H 8.0 are $NH_3C_2H_4OH^+$, HPO_4^{2-}, and $NH_3C_2H_4PO_4^-$. The ionic strength is about 1 M. The average value of K in terms of total concentrations is 0.56 ± 0.03 and 0.49 in terms of the predominant species.

small yet very much larger than K_I for phosphate. Since nitrophenol can be measured at much lower concentrations than phosphate, and K_m for *p*-nitrophenyl phosphate is comparable to K_I for phosphate, the K_m of *O*-phosphorylethanolamine can be determined indirectly in an experiment in which it acts as a competitive inhibitor of *p*-nitrophenyl phosphate hydrolysis. Initial rates of hydrolysis of *p*-nitrophenyl phosphate, in the concentration range 1×10^{-4}–5×10^{-6} M, were measured as above in the presence of 6×10^{-5} and 3×10^{-4} M *O*-phosphorylethanolamine. Reciprocal plots, v^{-1} versus $(S)^{-1}$, yielded good straight lines intercepting the ordinate at $1/V_o$. *O*-phosphorylethanolamine acts as a competitive inhibitor in this experiment. The constant obtained, however, is K_m.

V for O-Phosphorylethanolamine and p-Nitrophenyl Phosphate. The maximum velocity, V, for the hydrolysis of *O*-phosphorylethanolamine and *p*-nitrophenyl phosphate was determined by measuring the rate of liberation of phosphate at a substrate concentration of 2×10^{-3} M [$(S) >> K_m$]. The reaction was initiated by the addition of 0.01 ml of enzyme solution (2.5 μg) to 1.3 ml of reaction mixture containing 0.1 M Tris (±1 M NaCl) and 2×10^{-3} M *O*-phosphorylethanolamine or *p*-nitrophenyl phosphate in a 10-ml volumetric flask. The reaction was stopped after 3 minutes by the addition of 2 ml of 5 N H_2SO_4. Phosphate (Dryer *et al.*, 1957) was determined by reading the absorbancy of 10 ml of solution in 5-cm cuvets at 770 mμ with a Zeiss spectrophotometer. A control containing no enzyme and a phosphate standard containing 0.08 μmole PO_4 and no enzyme were run simultaneously.

V, V_o, and f for the Hydrolysis of p-Nitrophenyl Phosphate. Initial rates of formation of *p*-nitrophenol and phosphate were measured at 25°, *p*H 8.0, 1 M NaCl, with 10^{-2} M *p*-nitrophenyl phosphate, in the presence of varying concentrations (0.1–0.6 M) of ethanolamine. The reaction was initiated by the addition of 0.1 ml of enzyme solution (12.5 μg) to 1.2 ml of reaction mixture, and stopped after 3 minutes by the addition of 1 ml 5 N H_2SO_4. A 1-ml aliquot was withdrawn for phosphate determination (Dryer *et al.*, 1957) and a 1-ml aliquot was added to 9 ml of 1 M ethanolamine HCl at *p*H 10.7 to measure *p*-nitrophenol spectrophotometrically in 10-mm cells at 400 mμ. Phosphate and *p*-nitrophenol standards, in which enzyme was replaced by known amounts of the two substances, and controls containing no enzyme were run concurrently.

Equilibrium Constant for the Hydrolysis of O-Phosphorylethanolamine. A solution of 1 M ethanolamine hydrochloride and 0.02 M phosphate were incubated at *p*H 8.0 and 25° in the presence of alkaline phosphatase, 0.5 mg/ml, for 5 days. One ml was diluted with 1.5 ml of citrate buffer containing phosphoserine as a marker and 2 ml of this solution was assayed for *O*-phosphorylethanolamine by column chromatography using the Beckman-Spinco amino acid analyzer. Samples were applied to the column in 0.2 M sodium citrate buffer, *p*H 2.1, and eluted with 0.2 M sodium citrate buffer, *p*H 3.28.

Equilibrium was approached in the reverse direction by including 0.01 M *O*-phosphorylethanolamine in the incubation mixture. The reactions were run in duplicate and the procedure was standardized with known concentrations of *O*-phosphorylethanolamine.

Results

The initial and equilibrium concentrations of the reactants and products in the hydrolytic reaction of *O*-phosphorylethanolamine at *p*H 8.0, 25°, are summarized in Table I, along with the values of the apparent equilibrium constants calculated from them. The constant obtained by using total concentrations of the reactants is *p*H dependent. At the *p*H of this experiment *O*-phosphorylethanolamine is nearly completely in the form of the multipolar, singly charged anionic species, ethanolamine is 97% cation, and phosphate is about 90% doubly charged anion. In terms of these, the predominant species, the equilibrium constant is 12% lower than the value for total concentrations and is independent of *p*H. Only the concentration of *O*-phosphorylethanolamine was measured at equilibrium and changes in the other concentrations were calculated from it.

If pure solvent is taken as the reference state of water, the equilibrium constant in terms of the distinct molecular species, specified above, is 27 and the corresponding standard free energy of hydrolysis is 2.0 kcal/mole.

The value of K_m for *O*-phosphorylethanolamine and K_I for phosphate given in Table II are based upon the values of K_m for *p*-nitrophenyl phosphate. Because this latter quantity is very small and therefore difficult to measure accurately, the value is only approximate and may be in error by as much as 50% in either direction. The ratios of the constants are much more accurate, however, since they come from measurements made at much higher concentrations where accuracy is better and where the ratio is not so greatly influenced by the uncertainty in K_m for *p*-nitrophenyl phosphate.

The value for phosphate is taken as K_I rather than K_m on the assumption that, similar to the intestinal enzyme (Stein and Koshland, 1952), the bacterial enzyme does not readily catalyze oxygen exchange with water. The binding of phosphate is so pronounced that with many substrates the direct measurement of K_m is likely to be severely affected by product inhibition.

The apparent maximum velocity for the hydrolysis of *p*-nitrophenyl phosphate was decidedly higher than for *O*-phosphorylethanolamine. This result is in large measure caused by substantial phosphate inhibition in the hydrolysis of *O*-phosphorylethanolamine. When this is taken into account, the corrected maximum velocities are only slightly lower. For our calculations, we have taken the maximum velocities as equal. We had previously concluded that dephosphorylation is the rate-controlling step in the hydrolysis of *p*-nitrophenyl phosphate (Dayan and Wilson, 1964; Wilson *et al.*, 1964). This is confirmed by results of a similar nature presented in Table III. The constant rate of formation of phosphate in the face of increasing rates of formation of *p*-nitrophenol immediately indicates that dephosphorylation is rate controlling. In more formal terms substitution in equation (5) shows that $k_4E^\circ = V_o$. Under these special conditions

$$K_1 = \frac{(f)(\text{ethanolamine})}{K_m}$$

TABLE II: Kinetic Parameters.

Substance	M NaCl	K_m or K_I	V'[a]	V
p-Nitrophenyl-phosphate[b]	0	1×10^{-6}	8.4	8.6
	1	2×10^{-6}	15.4	15.7
Phosphate	0	6×10^{-7}		
	1	2.5×10^{-6}		
O-Phosphoryl-ethanol-amine[b]	0	5×10^{-6}	5.4	7.0
	1	1.3×10^{-5}	11.9	14.4

[a] Our *O*-phosphorylethanolamine contains 2.4% phosphate; *p*-nitrophenyl phosphate contains 0.5% phosphate. The velocities are in μmoles/min per mg of enzyme preparation. V' is the apparent maximum velocity, measured at 2×10^{-3} M substrate. V is the maximum velocity corrected for phosphate inhibition according to the equation for competitive inhibition. The value for V measured by liberation of *p*-nitrophenol in K_m experiment for *p*-nitrophenyl phosphate was 15.5. [b] Percentages of substrate hydrolyzed in the course of the measurement were: *p*-nitrophenyl phosphate (V' = 8.4) 2.4, (V' = 15.4) 4.4; *O*-phosphorylethanolamine (V' = 5.4) 1.6, (V' = 11.9) 3.4.

TABLE III: Rates of Formation of *p*-Nitrophenol and Phosphate as a Function of Ethanolamine Concentration.

Ethanolamine (M)	Rates μmoles/mg E, min Nitrophenol	Phosphate	f[a]	fx (ethanolamine)
0.10	15.5	14.8	21	2.1
0.20	16.0	14.9	12.3	2.5
0.30	16.4	14.7	9.2	2.8
0.40	17.1	14.9	6.4	2.6
0.50	17.9	14.9	4.8	2.4
0.60	18.2	14.6	4.3	2.6
Extrapolated to 0	14.8			
		av. 14.8		av. 2.5

[a] Calculated using 14.8 as the rate of formation of phosphate in all cases.

Using values from the tables, $K_1 = 2 \times 10^5$ for total concentrations of the reactants at *p*H 8.0, 25°, and 1 M NaCl. In the absence of added salt, K_1 is about 6×10^5. These values indicate that the transfer of a phosphate group from *O*-phosphorylethanolamine to the enzyme is very favorable. The equilibrium constant for the hydrolysis of the phosphoryl enzyme is

$$K_2 = 0.56/2 \times 10^5 = 3 \times 10^{-6}$$

for total concentrations of reactants at 1 M ionic strength, 25°, *p*H 8.0. If pure solvent is taken as the reference state for water $K_2 = 1.5 \times 10^{-4}$. The corresponding free energy of hydrolysis is +5.3 kcal/mole; the reaction is quite unfavorable. The results are obtained in terms of total concentrations because the prototropic enzymic species and the equilibrium constants are not known. The "equilibrium constant" given depends upon the *p*H.

Discussion

Our results show that the phosphoryl enzyme in the thermodynamic sense is surprisingly stable toward hydrolysis; it is, of course, kinetically labile. Roughly half the enzyme should be phosphorylated in 10^{-4} M phosphate at *p*H 8.0. In a general sense, by predicting considerable uptake of phosphate from 10^{-4} M phosphate solutions, our results are in remarkably good agreement with the $^{32}P_i$ incorporations in the *p*H range 4–6 found by Engstrom (1961). In a more precise sense our results are in disagreement with the incorporation studies because those studies show only very slight $^{32}P_i$ incorporation at *p*H 8.0.

We have noted that the transfer of a phosphoryl group from *O*-phosphorylethanolamine to the enzyme is very favorable. This requires some explanation since in the absence of a concurrent effect the equilibrium constant of this reaction should have the order of magnitude one, if we assume that a serine side chain in the enzyme is the site of phosphorylation. We are off by a factor of about 2×10^5. It is not hard to see how this extra binding might arise. The binding constant for phosphate, K_I^{-1}, is 4×10^5. If much of the binding interaction between phosphate and the enzyme also occurs in the phosphoryl enzyme the additional driving force for the phosphoryl transfer would be very large. To make a quantitative estimate, we have to take into account that relative to the phosphoryl transfer reaction the binding constant of phosphate is diminished by an adverse entropy effect arising from the decrease in the number of reactants. This effect decreases the binding by a factor of about 50. The binding interaction of the phosphate group is, very roughly, 100 times larger than we need to account for the measured equilibrium constant. This difference is probably a little too large to dismiss as arising from our approximations and it appears more likely that there is more "noncovalent" binding in the enzyme-phosphate complex than in the phosphoryl enzyme. It is clear that the equilibrium constant for the transfer of a phosphoryl group from *O*-phosphorylethanolamine to the enzyme is consistent with the supposition that a serine side chain in the enzyme is the phosphoryl acceptor in the catalytic intermediate. This constant is not consistent with proposals that the side-chain acceptor is one such as the imidazole group of histidine, which would form a high energy phosphate "bond."

References

Agren, G. (1959), *Acta Chem. Scand. 13*, 1047.

Dayan, J., and Wilson, I. B. (1963), *Biochim. Biophys. Acta 77*, 446.

Dayan, J., and Wilson, I. B. (1964), *Biochim. Biophys. Acta 81*, 620.

Dryer, R. L., Tammes, A. R., and Routh, G. J. (1957), *J. Biol. Chem. 225*, 177.

Engstrom, L. (1961), *Biochim. Biophys. Acta 52*, 49.

Engstrom, L. (1962a), *Biochim. Biophys. Acta 56*, 606.

Engstrom, L. (1962b), *Arkiv Kemi 19*, 129.

Engstrom, L., and Agren, G. (1958), *Acta Chem. Scand. 12*, 357.

Garen, A., and Levinthal, C. (1960) *Biochim. Biophys. Acta 38*, 470

Schwartz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1996.

Stein, S. S., and Koshland, D. E. (1952), *Arch. Biochem. Biophys. 39*, 229.

Wilson, I. B., Dayan, J., and Cyr, K. (1964), *J. Biol. Chem. 239*, 4182.