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Contribution of Water to Free Energy of Hydrolysis of Pyrophosphate†

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ABSTRACT: The energy of hydrolysis of phosphate compounds varies depending on whether they are in solution or bound to the catalytic site of enyzmes. With the purpose of simulating the conditions at the catalytic site, the observed equilibrium constant for pyrophosphate hydrolysis (K_{obsd}) was measured in aqueous mixtures of dimethyl sulfoxide, ethylene glycol, or polymers of ethylene glycol. The reaction was catalyzed by yeast inorganic pyrophosphatase at 30 °C. All the cosolvents used promoted a decrease of K_{obsd}. Polymers of ethylene glycol were more effective than dimethyl sulfoxide or ethylene glycol in decreasing K_{obsd}. The higher the molecular weight of the polymer, the lower the value of K_{obsd}. A decrease in K_{obsd} from 346 M ($\Delta G°_{obsd}$ = −3.5 kcal mol^{-1}) to 0.1 M ($\Delta G°_{obsd}$ = 1.3 kcal mol^{-1}) was observed after the addition of 50% (w/v) poly(ethylene glycol) 8000 to a solution containing 0.9 mM $MgCl_2$ and 1 mM P_i at pH 8.0. The association constants of P_i and pyrophosphate for H^+ and Mg^{2+} were measured in presence of different ethylene glycol concentrations in order to calculate the K_{eq} for hydrolysis of different ionic species of pyrophosphate. A decrease in all the K_{eq} was observed. The results are interpreted according to the concept that the energy of hydrolysis of phosphate compounds depends on the different solvation energies of reactants and products.

In aqueous solutions the hydrolysis of pyrophosphate, ATP, or an acyl phosphate residue is accompanied by a large change in free energy (George et al., 1970; Haynes et al., 1978). In contrast, during the catalytic cycle of enzymes involved in energy transduction, there are steps in which the hydrolysis of these compounds is accompanied by only a small energy change. This includes the yeast inorganic pyrophosphatase (Janson et al., 1979; Springs et al., 1981; Cooperman, 1982), myosin (Bagshaw & Trentham, 1974; Trentham et al., 1976), F_1-ATPase of mitochondria and chloroplasts (Boyer et al., 1973, 1977, 1982), the Ca^{2+}-ATPase of sarcoplasmic reticulum (Masuda & de Meis, 1973; Kanazawa, 1976; de Meis & Vianna, 1979), the (Na + K)ATPase of plasma membrane (Post et al., 1975; Taniguchi & Post, 1975), 3-phosphoglycerate kinase (Nageswara et al., 1978), and pyruvate kinase (Nageswara et al., 1979). At present, it is not known why the K_{eq}[1] for hydrolysis of these compounds varies depending on whether they are in solution or on the enzyme surface. This phenomenon appears to be crucial to the mechanism of energy transduction in the living cell.

A large decrease in the P_i concentration required for the spontaneous formation of an acyl phosphate residue at the catalytic site of the Ca^{2+}-ATPase has been observed when organic solvents such as dimethyl sulfoxide and glycerol are included in the assay medium (de Meis, 1981; de Meis et al., 1980, 1982; Dupont & Pougeois, 1983; Chiesi et al., 1984). This has also been shown for the spontaneous synthesis of "tightly" bound ATP at the catalytic site of F_1-ATPase (Sakamoto & Tonomura, 1983; Yoshida, 1983; Cross et al., 1984; Sakamoto, 1984). These findings raise the possibility that solvent structure might be involved in the decrease of the K_{eq} for hydrolysis of phosphate compounds (de Meis, 1981, 1982, 1984). Water possesses the most cooperative structure of all common solvents, but it is destroyed by organic cosolvents. The chemical reactivity of water molecules is scarcely affected by cosolvents. However, reactions that depend on solvent structure change markedly with cosolvent addition (Arnett & McKelvey, 1966; Arnett, 1967; Tan & Lovrien, 1972; Amis & Hinton, 1973).

The aim of this study was to ascertain whether or not a change of solvent structure might lead to a large change in the energy of hydrolysis of a phosphate compound. For this purpose, inorganic pyrophosphate was chosen. In the conditions prevailing in the cytosol, the $\Delta G°_{obsd}$ of pyrophosphate hydrolysis is about −4.0 kcal mol^{-1} (de Meis, 1984; Flodgaard

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[1] Abbreviations: K_{obsd}, observed equilibrium constant; K_{eq}, equilibrium constant; $\Delta G°_{obsd}$, observed standard free energy; PEG, poly(ethylene glycol); MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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& Fleron, 1974). In contrast, the $\Delta G°_{obsd}$ of the tightly bound pyrophosphate found on the surface of yeast inorganic pyrophosphatase is −0.9 kcal mol^{-1} (Springs et al., 1981; Cooperman, 1982). In a previous paper (de Meis, 1984) we have measured the K_{eq} of pyrophosphate hydrolysis in aqueous solutions containing 30–60% (v/v) dimethyl sulfoxide, glycerol, ethylene glycol, methanol, or ethanol. It was found that in the presence of these organic solvents the observed energy of hydrolysis of pyrophosphate was 0.6–1.5 kcal mol^{-1} smaller than in totally aqueous medium. This small decrease suggested that water could be involved but would not be the only factor contributing to the low energy of hydrolysis of the tightly bound pyrophosphate.

Recently, Ueberreiter (1980a,b, 1981) observed that polymers of ethylene glycol are more effective than ethylene glycol in breaking the water structure and that this effect increases with increasing molecular weight of the polymer up to about 10000. In this paper it is shown that at a concentration of 50% (w/v) PEG 8000 promotes a decrease of $\Delta G°_{obsd}$ of pyrophosphate hydrolysis of about 5 kcal mol^{-1}. In analogy with the effect on water structure, the effect of PEG in decreasing the energy of pyrophosphate hydrolysis increases with increasing molecular weight of the polymer. This new finding indicates that the difference in energies of hydrolysis between pyrophosphate in solution and the tightly bound pyrophosphate can be accounted solely by a change of water structure on the enzyme surface.

Materials and Methods

[^{32}P]P_i was obtained from the Brazilian Institute of Atomic Energy and purified as previously described (de Meis, 1984). Synthesis of pyrophosphate was assayed by measuring the formation of radioactive pyrophosphate from [^{32}P]P_i. In all experiments, controls were performed in order to detect precipitation of magnesium phosphate or magnesium pyrophosphate in the medium. There was no formation of precipitates in the conditions described under Results. Typically, before the reaction was arrested, samples of 0.8 mL were centrifuged at $1000g_{av}$ for 10 min. Immediately after centrifugation, a 0.020-mL sample of supernatant was diluted in order to measure the total radioactivity remaining in solution, 0.39 mL of supernatant was used to measure soluble pyrophosphate, and the remaining 0.39 ml was used to measure the total amount of radioactive Pi and pyrophosphate, soluble and insoluble. These two samples were quenched with 0.8 mL of a mixture containing 2 mM nonradioactive pyrophosphate and either 10 or 50% (w/v) trichloroacetic acid. Since PEG precipitates in the presence of excess trichloroacetic acid, these samples were quenched with the 50% (w/v) trichloroacetic acid solution. After centrifugation, 0.5 mL of the supernatant was mixed with 0.4 mL of a 2.5 N H_2SO_4 solution containing 5% (w/v) ammonium molybdate, followed by 0.3 mL of acetone and 1 mL of a mixture of isobutyl alcohol and benzene (1:1 v/v). The mixture was vigorously stirred on a Vortex mixer for 40 s. After phase separation, the organic phase was discarded, 0.015 mL of KH_2PO_4 carrier (20 mM) and 0.3 mL of acetone were added to the water phase, and this was reextracted with 1 mL of the isobutyl alcohol–benzene mixture. This procedure was repeated 7 times. The water phase was counted in a scintillation counter. After extraction, only about 0.001% of the original content of [^{32}P]P_i remained in the aqueous phase. This was negligible in the standard assay. In each set of experiments, controls were performed in which the enzyme was not added to the medium. The small radioactivity found after extracting these controls was subtracted from that found in the sample containing enzyme. Concentrations of radioactive pyrophosphate were calculated on the basis of two [^{32}P]P_i incorporated into each pyrophosphate. The specific activity of [^{32}P]P_i in the assay medium varied between 8×10^7 and 4×10^8 cpm/μmol of P_i.

Under two experimental conditions, the radioactive material formed during the incubation was isolated and characterized in order to confirm that it was pyrophosphate. The experiments were performed in a large volume of assay medium (6 mL) containing either 1 mM [^{32}P]P_i 8 mM MgCl, 50 mM Tris-HCl buffer (pH 8.0), and 66% (w/v) ethylene glycol or 1 mM [^{32}P]P_i, 0.9 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 8.0), and 50% (w/v) PEG 8000. The reaction was started by the addition of inorganic pyrophosphatase (1 μg of protein/mL), and after 8 h incubation at 30 °C it was stopped by addition of 4.5 mL of 100% (w/v) trichloroacetic acid and 0.12 mL of nonradioactive 0.1 M pyrophosphate. After centrifugation, 4 mL of the clear supernatant was mixed with 1 mL of a 60 mM ammonium molybdate solution in 0.01 N HCl and 0.48 mL of 12 N HCl. The radioactive P_i was extracted 6 times as described above with in each extraction 5 mL of isobutyl alcohol–benzene mixture, 0.5 mL of acetone, and 0.1 mL of nonradioactive 0.1 M P_i solution. After these extractions, most of the molybdate added was extracted together with the carrier P_i. The aqueous phase was alkalinized with 0.5 mL of concentrated NH_4OH, and pyrophosphate was precipitated as the magnesium salt by addition of 1 mL $MgCl_2$ (1 m). The white precipitate was separated by centrifugation and solubilized with 0.10 mL of HCl (6 N). After addition of 1 mL of water, the pH of the mixture containing the purified pyrophosphate was adjusted to 7.2 with Tris base. The percent of recovery of radioactive material and of nonradioactive pyrophosphate added when the reaction was quenched with trichloroacetic acid was practically the same and varied between 65 and 75% in the different experiments. Autoradiography of ascending thin-layer chromatograms performed as described by Penningroth et al. (1980) using 0.75 M KH_2PO_4 adjusted to pH 3.4 with phosphoric acid revealed that the radioactive material had the same R_f as inorganic pyrophosphate. Only a faint spot having the same mobility as P_i was detected (inset of Figure 1). Subsequently, different amounts of yeast inorganic pyrophosphatase were added to 0.4-mL samples of the purified material, which had been diluted with 4 mL of a mixture containing 100 mM MOPS buffer (pH 7.0) and 10 mM $MgCl_2$. Figure 1 shows that pyrophosphate and the radioactive material were hydrolyzed to yield P_i at the same rate. Autoradiography of chromatograms performed after incubation with enzyme revealed that all the radioactivity moved with the same R_f as that of P_i (inset of Figure 1). Essentially the same results were obtained whether the medium from which the radioactive material was isolated contained 66% (v/v) ethylene glycol or 50% (w/v) PEG 8000. These results demonstrate that the radioactive material measured in the experiments of Figures 2–5 was pyrophosphate.

Inorganic pyrophosphatase from bakers' yeast was purchased from Sigma Chemical Co. The specific activity was 500 units/mg of enzyme. PEG was purchased from Sigma Chemical Co. Concentrations of the ionic species and complexes were calculated as described by Fabiato & Fabiato (1979). The formulas used in calculations were as follows: $K_{obsd} = [P_i]^2/[PP_i]$; $\Delta G° = RT \ln K_{eq}$; molar fraction of solvent (X_{sol}) = (moles of solvent)/(moles of solvent + moles of H_2O).

Results

Criteria for Equilibrium. Yeast inorganic pyrophosphatase catalyzes the synthesis of pyrophosphate at a velocity that

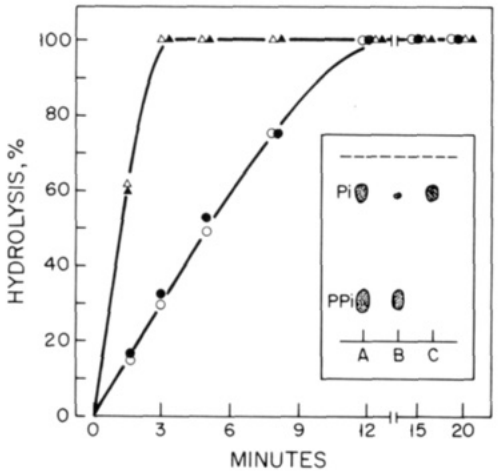

FIGURE 1: Characterization of radioactive pyrophosphate. Radioactive material formed in presence of 50% (w/v) PEG 8000 was copurified with nonradioactive pyrophosphate (see text). The inorganic pyrophosphatase concentrations were 0.1 (○, ●) and 0.4 μg/mL (△, ▲). After different incubation intervals at 30 °C, 0.3-mL samples were quenched with 0.3 mL of ice-cold 10% (w/v) TCA. To each sample was added 0.4 mL of a 2.5 N H_2SO_4 solution containing 5% (w/v) ammonium molybdate, followed by 1 mL of isobutyl alcohol and benzene. The mixture was vigorously stirred on a Vortex for 30 s. After phase separation, a sample of the organic phase was counted in a scintillation counter (○, △), and another sample was used to measure P_i (●, ▲) as described by Ernster et al. (1950). After 20-min incubation, all the radioactive material in the aqueous phase was cleaved and was recovered as phosphomolybdate in the organic layer. The inset shows a drawing copied from an autoradiogram of an ascending thin-layer chromatogram where (A) is a control containing [^{32}P]P_i and [^{32}P]PPi and (B) and (C) are samples of the purified radioactive material before (B) and after (C) 20-min incubation with 0.4 μg/mL inorganic pyrophosphatase.

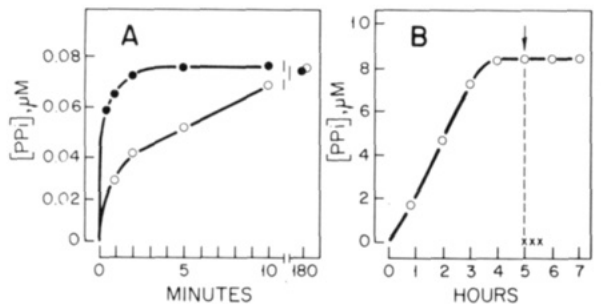

FIGURE 2: Rate of pyrophosphate synthesis. (A) The assay medium consisted of 50 mM MOPS buffer (pH 7.0), 1 mM [^{32}P]P_i, 8 mM $MgCl_2$, 55% (w/v) ethylene glycol, and either 0.1 (○) or 1.0 μg/mL (●) yeast inorganic pyrophosphatase. (B) The assay medium consisted of 50 mM Tris-HCl buffer (pH 8.0), 1 mM [^{32}P]P_i, 0.9 mM $MgCl_2$, 50% (w/v) PEG 8000, and 1 μg/mL inorganic pyrophosphatase. The arrow indicates dilution of 1 volume of the assay medium with 9 volumes of solution containing 50 mM Tris-HCl buffer (pH 8.0), 1 mM [^{32}P]P_i, and 0.9 mM $MgCl_2$. After dilution, the concentration of PEG 8000 decreased from 50 to 5%. The concentration of pyrophosphate remaining in the medium after dilution was 0.005 μM. The experiments were performed at 30 °C.

depends on the concentration of enzyme used (Figure 2A). Synthesis of pyrophosphate could not be detected in the absence of enzyme in any of the conditions used. With different enzyme concentrations, essentially the same amounts of pyrophosphate were found after long incubation intervals at 30 °C in the presence of either ethylene glycol or PEG 8000. For instance, in presence of 57% (w/v) ethylene glycol and 0.2, 1, or 5 μg/mL inorganic pyrophosphatase, the concentrations of pyrophosphate accumulated in a medium containing 50 mM MOPS buffer (pH 7.2), 1 mM P_i, and 8 mM Mg after 8 h of incubation at 30 °C were 0.09, 0.07, and 0.09 μM, respectively. Under the same conditions and in the presence of

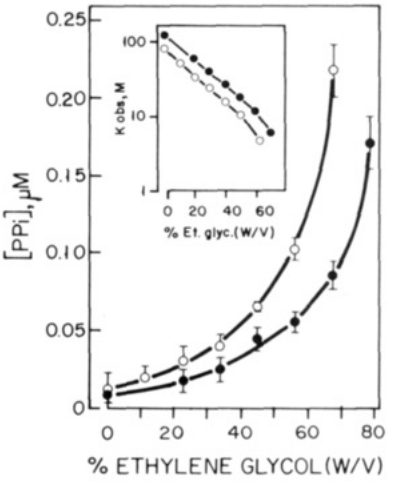

FIGURE 3: Effect of ethylene glycol. The assay medium consisted of 10 mM $MgCl_2$, 1 mM [^{32}P]P_i, 1 μg/mL inorganic pyrophosphatase, and either (●) 50 mM MOPS buffer (pH 7.2) or (○) 50 mM Tris-HCl buffer (pH 8.0). Essentially the same results were obtained after 3 and 6 h of incubation at 30 °C. The values are averages (±SE) of four experiments. The inset shows the values of K_{obsd} calculated from the equilibrium concentrations of pyrophosphate attained in presence of different concentrations of ethylene glycol.

50% (w/v) PEG 8000 and 0.5, 1, or 5 μg/mL inorganic pyrophosphatase, the concentrations of pyrophosphate were 1.49, 1.58, and 1.43 μM, respectively. In all subsequent experiments, it was assumed that equilibrium had been attained if the concentration of radioactive pyrophosphate in solution was the same in the presence of two enzyme concentrations differing by a factor of 5 after 2 and 4 h of incubation in the presence of ethylene glycol or after 4 and 8 h in the presence of PEG.

Using high enzyme concentrations (0.5–1.0 mg of protein/mL), Janson et al. (1979) and Springs et al. (1981) have shown the formation of enzyme-bound pyrophosphate. The amount of bound pyrophosphate formed was proportional to the amount of enzyme present and on a molar basis corresponded to about 10% of the total enzyme used. The concentrations of enzyme used in this paper are much smaller than those required to measure enzyme-bound pyrophosphate. In the presence of 1 μg of enzyme protein/mL, the concentration of bound pyrophosphate present in the mixture should be about 0.003 μM [molecular weight of pyrophosphatase is 63 000–71 000 (Cooperman, 1982)], i.e., much smaller than those measured in Figures 2–5. Thus, the measured pyrophosphate corresponds to the equilibrium concentration in solution, and the error derived from enzyme-bound pyrophosphate is not significant.

Effect of Cosolvents. In a previous paper (de Meis, 1984) we observed a decrease of K_{obsd} for pyrophosphate hydrolysis when ethylene glycol (66% w/v) was added to the assay medium. These experiments were performed at pH 7.8 in only one organic solvent concentration. We now show that increasing concentrations of ethylene glycol lead to a progressive increase in the equilibrium concentration of pyrophosphate (Figure 3). This effect was observed both at pH 7.2 and at pH 7.8. The inset to Figure 3 shows that at these two pH values there was a linear decrease of K_{obsd} as the concentration of ethylene glycol in the medium was raised. This linear relationship was not observed with the use of PEG 8000 (Figure 4). There was a large increase of the equilibrium level of pyrophosphate both at pH 7.2 and at pH 7.8 when the PEG concentration was raised above 40% (Figure 4). At present we do not know the reason for the different profiles attained in Figures 3 and 4. Polymers of ethylene glycol were more effective than either ethylene glycol or dimethyl sulfoxide in

Table I: Effects of Dimethyl Sulfoxide, Ethylene Glycol, and Polymers of Ethylene Glycol on K_{obsd} of PP_i Hydrolysis[a]

additions	X_{sol}	pH 7.2 [PP_i] (nM)	pH 7.2 K_{obsd} (M)	pH 7.2 $\Delta G°_{obsd}$ (kcal mol^{-1})	pH 8.0 [PP_i] (nM)	pH 8.0 K_{obsd} (M)	pH 8.0 $\Delta G°_{obsd}$ (kcal mol^{-1})
none	0	12 ± 2 (4)	85.5	−2.7	3 ± 1 (8)	345.6	−3.5
dimethyl sulfoxide, 50% (w/v)	0.210	35 ± 7 (4)	28.9	−2.0			
ethylene glycol, 50% (w/v)	0.172	48 ± 8 (4)	20.8	−1.8	32 ± 2 (4)	31.3	−2.1
PEG 400, 50% (w/v)	0.039	88 ± 18 (6)	11.3	−1.5	81 ± 17 (6)	12.3	−1.5
PEG 1450, 50% (w/v)	0.011	166 ± 23 (4)	6.0	−1.1	75 ± 31 (4)	13.3	−1.6
PEG 3500, 50% (w/v)	0.005	368 ± 34 (6)	2.7	−0.6	261 ± 15 (6)	3.8	−0.8
PEG 8000, 50% (w/v)	0.002	1393 ± 85 (11)	0.7	0.2	8305 ± 136 (8)	0.1	1.3

[a] The assay media contained 50 mM MOPS buffer, pH 7.2, 1 mM [^{32}P]P_i, and 8 mM $MgCl_2$ or 50 mM Tris-HCl buffer, pH 8.0, 1 mM [^{32}P]P_i, and 0.9 mM $MgCl_2$. The enzyme concentrations were 0.01 and 0.05 μg/mL in totally aqueous media, 0.1 and 0.5 μg/mL in the presence of ethylene glycol and dimethyl sulfoxide and 1 and 5 μg/mL in the presence of different PEG polymers. Essentially the same results were obtained in each case with both enzyme concentrations after 4 or 7 h of incubation at 30 °C. The pyrophosphate concentrations are the average ±SE of the number of experiments shown in parentheses. X_{sol} is the mole fraction of organic solvent.

Table II: Association Constants[a]

concn of ethylene glycol (% w/v)	PO_4^{3-} K_2 (M^{-1})	PO_4^{3-} β (M^{-1})	$P_2O_7^{4-}$ K_1 (M^{-1})	$P_2O_7^{4-}$ K_2 (M^{-1})	$P_2O_7^{4-}$ β_1 (M^{-1})	$P_2O_7^{4-}$ β_2 (M^{-1})	$P_2O_7^{4-}$ β_3 (M^{-1})
0	5.5×10^6	67	2.0×10^8	1.2×10^6	1156	2.0×10^5	55
11			3.2×10^8	1.7×10^6	1367	1.2×10^5	50
22			3.4×10^8	2.2×10^6	2082	2.1×10^5	
33	13.5×10^6	274	3.4×10^8	2.2×10^6	2695	2.4×10^5	136
44			3.9×10^8	2.5×10^6			
55			3.4×10^8	2.9×10^6			
66	20.9×10^6	327	3.4×10^8	3.0×10^6			

[a] For PO_4^{3-}, K_2 refers to the association constant of the reaction $H^+ + HPO_4^{2-} \rightleftharpoons H_2PO_4^-$ and β to the association constant of the reaction $Mg^{2+} + HPO_4^{2-} \rightleftharpoons MgHPO_4$. For $P_2O_7^{4-}$, K_1 refers to the association constant of the reaction $H^+ + P_2O_7^{4-} \rightleftharpoons HP_2O_7^{3-}$, K_2 to $H^+ + HP_2O_7^{3-} \rightleftharpoons H_2P_2O_7^{2-}$, β_1 to $Mg^{2+} + HP_2O_7^{3-} \rightleftharpoons MgHP_2O_7^-$, β_2 to $Mg^{2+} + P_2O_7^{4-} \rightleftharpoons MgP_2O_7^{2-}$, and β_3 to $Mg^{2+} + MgP_2O_7^{2-} \rightleftharpoons Mg_2P_2O_7$. Essentially the same results were obtained in at least two independent experiments for each condition. For pyrophosphate, $K_1' = K_1 (1 + \beta_2[Mg^{2+}] + \beta_3[Mg^{2+}]^2)/(1 + \beta_1[Mg^{2+}])$ and $K_2' = K_2 + \beta_1 K_2[Mg^{2+}]$. For phosphate, $K_2' = K_2 + \beta K_2[Mg^{2+}]$. K_1' and K_2' are the apparent acidity constants in the presence of $MgCl_2$ (Lambert & Watters, 1957). The values were corrected for the free Mg^{2+} concentration with the aid of a computer.

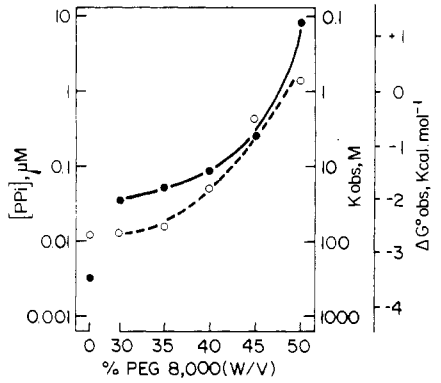

FIGURE 4: Effect of PEG 8000 on K_{obsd}. The assay medium consisted of (○) 50 mM MOPS buffer (pH 7.2), 1 mM [^{32}P]P_i, and 8 mM $MgCl_2$ or (●) 50 mM Tris-HCl buffer (pH 8.0), 1 mM [^{32}P]P_i, and 0.9 mM $MgCl_2$. Essentially the same results were obtained after 4 and 8 h of incubation at 30 °C in the presence of either 0.05 or 2.5 μg of inorganic pyrophosphatase/mL.

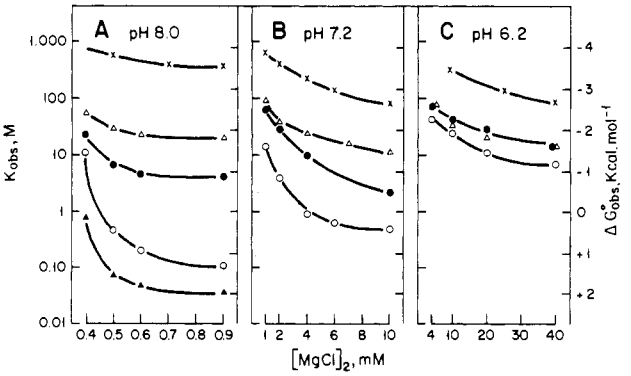

FIGURE 5: Effects of pH, Mg^{2+} and polymers of ethylene glycol on K_{obsd}. In (▲) the [^{32}P]P_i concentration was 0.1 mM (A). In all other conditions, it was 1 mM. The buffers used were (A) 50 mM Tris-HCl (pH 8.0), (B) 50 mM MOPS (pH 7.2), and (C) 50 mM MES (pH 6.2). The enzyme concentration was either 0.01 μg/mL (×), 0.1 mg/mL (△), or 1.0 μg/mL (●, ○, ▲). Essentially the same results were obtained after 3 and 6 h of incubation at 30 °C. (×) Totally aqueous medium, (△) 66% (w/v) ethylene glycol, (●) 50% (w/v) PEG 3500 and (○, ▲) 50% (w/v) PEG 8000.

decreasing the K_{obsd} for pyrophosphate hydrolysis. This effect is more pronounced the higher the molecular weight of the polymers (Table I and Figure 5). The pyrophosphate synthesized in the presence of 50% (w/v) PEG is readily hydrolyzed when the concentration of the polymer in the medium is suddenly decreased (Figure 2B).

Effects of pH and $MgCl_2$. It has been shown in previous papers (Flodgaard & Fleron, 1974; de Meis, 1984) that for a given P_i concentration the K_{obsd} of pyrophosphate hydrolysis in totally aqueous medium becomes smaller with increasing $MgCl_2$ concentrations in the medium. This effect depends on the pH value. The lower the pH, the more $MgCl_2$ is needed to decrease K_{obsd}. Figure 5 shows that $MgCl_2$ and pH had the same effects on K_{obsd} when measured in presence of organic solvents. An interesting finding is that the ability of PEG to decrease K_{obsd} is attenuated as the pH of the medium is decreased from 8.0 to 6.2 (Figure 5). At pH 8.0, PEG 8000 was much more effective than ethylene glycol in decreasing K_{obsd} of pyrophosphate hydrolysis (Figure 5A). However, at pH 6.2 this difference is practically abolished.

K_{eq} of Ionic Reactions. In order to analyze the effect of organic solvents on the K_{eq} of hydrolysis of the different ionic forms of pyrophosphate, the association constants of Mg^{2+} and H^+ with P_i and pyrophosphate in the presence of organic solvents are needed. As far as we know, there are no values reported in the literature for the Mg^{2+} association constants in presence of ethylene glycol. Therefore, we measured the association constants in totally aqueous medium and in the

Table III: Thermodynamic Parameters for Ionic Reactions[a]

reaction	H_2O		ethylene glycol 33% (w/v)		ethylene glycol 66% (w/v)	
	K_{eq} (M)	$\Delta G°$ (kcal/mol)	K_{eq} (M)	$\Delta G°$ (kcal/mol)	K_{eq} (M)	$\Delta G°$ (kcal/mol)
pyrophosphate hydrolysis						
$HP_2O_7^{3-} + HOH \rightleftharpoons H_2PO_4^- + HPO_4^{2-}$	7452 ± 923	−5.4	366 ± 28	−3.5	97 ± 5 (161 ± 25)	−2.8 (−3.1)
$H_2P_2O_7^{2-} + HOH \rightleftharpoons 2H_2PO_4^-$	34155 ± 4230	−6.3	2207 ± 171	−4.6	672 ± 36 (1112 ± 172)	−3.9 (−4.2)
$MgP_2O_7^{2-} + HOH \rightleftharpoons MgHPO_4 + HPO_4^{2-}$	88 ± 11	−2.7	11 ± 1	−1.4	2.2 ± 0.1 (3.2 ± 0.5)	−0.5 (−0.7)
$MgHP_2O_7^- + HOH \rightleftharpoons MgHPO_4 + H_2PO_4^-$	431 ± 53	−3.6	37 ± 3	−2.2	11.8 ± 0.6 (12.4 ± 1.9)	−1.5 (−1.5)
$Mg_2P_2O_7 + HOH \rightleftharpoons 2MgHPO_4$	107 ± 13	−2.8	21 ± 2	−1.8	5.2 ± 0.3 (4.8 ± 0.7)	−1.0 (−0.9)
contribution of H^+ or Mg^{2+}						
$H^+ + HPO_4^{2-} \rightleftharpoons H_2PO_4^-$	5.5×10^6	−9.3	13.5×10^6	−9.8	20.9×10^6	−10.1
$Mg^{2+} + HPO_4^{2-} \rightleftharpoons MgHPO_4$	67	−2.5	274	−3.4	327	−3.5

[a] In presence of 66% ethylene glycol the values were calculated by using two different sets of values for β_1, β_2, and β_3. In the first set it was assumed that in the presence of 66% ethylene glycol the values of the three association constants were the same as in the presence of 33% ethylene glycol. For the second set (in parentheses), it was assumed that raising the concentration of ethylene glycol from 33% to 66% would promote an increase in the association constants equal to that promoted by the addition of 33% ethylene glycol to a totally aqueous medium. In this case the values of β_1, β_2, and β_3 used were 4.24×10^3, 2.70×10^5, and 217 M^{-1}, respectively. Notice that K_{eq} of the ionic reaction calculated with the two sets of association constants were practically the same. In the pH range used (Figures 2A, 3, and 5 and Table I), the concentrations of the ionic species $H_3P_2O_7^-$ and $H_4P_2O_7$ are much smaller than the total concentration of pyrophosphate in solution. Therefore, reactions involving these ionic species were ignored. For calculation of K_{eq} for ionic reactions in totally aqueous medium we used experimental values presented in previous paper (de Meis, 1984), where the effects of Mg^{2+} and pH on K_{obsd} in totally aqueous medium were explored in more detail.

presence of different ethylene glycol concentrations (Figure 6 and Table II). The constants could not be measured in the presence of PEG due to the low solubility of the Mg^{2+} complexes of P_i and pyrophosphate. Apparent association constants of H^+ with orthophosphate and pyrophosphate ions were calculated from the apparent pK determined by titration with standardized solutions of NaOH or HCl (Smith & Alberty, 1956; Lambert & Watters, 1957; Grace & Dunaway-Mariano, 1983). The apparent stability constants of Mg^{2+} with orthophosphate and pyrophosphate ions were calculated from the decrease in pH at the apparent pK point caused by the addition of Mg^{2+} during the titration measurement as described by Smith & Alberty (1956) and by Lambert & Watters (1957). The titrations were performed with a microburet in a volume of 3 mL under an atmosphere of nitrogen at 30 °C and with constant stirring. Potassium chloride (0.2 M) was present in order to minimize changes in ionic strength. In the presence of ethylene glycol, the pH readings were corrected as described by Bates et al. (1963) and by Grace & Dunway-Mariano (1983). The association constant value found in totally aqueous medium (Table II) is in general agreement with those reported in the literature. For PO_4^{3-}, the reported values for K_2 and β vary from 5.3×10^6 to 8.1×10^6 and from 60 to 76, respectively (Peacock & Nickles, 1969; Grace & Dunaway-Mariano, 1983; Frey & Stuehr, 1972; Smith & Alberty, 1956). For $P_2O_7^{4-}$, the values for K_1, K_2, β_1, β_2, and β_3 vary from 1.6×10^8 to 2.3×10^8, 10^6 to 1.2×10^6, 1148 to 1513, 2.57×10^5 to 2.63×10^5, and 214 to 219, respectively (Grace & Dunaway-Mariano, 1983; Frey & Stuehr, 1972; Hogfeldt, 1982). In the presence of ethylene glycol we observed a small but consistent increase for six of the seven association constants measured (Figure 6 and Table II). An increment from 8.1×10^6 to 1.7×10^7 has been reported for the association constants of PO_4^{3-} (K_2) and from 1.6×10^8 to 2.8×10^8 for $P_2O_7^{4-}$ (K_1) in the presence of 25% (v/v) ethylene glycol (Grace & Dunaway-Mariano, 1983). These values correspond fairly well with those calculated by interpolating our data to 25% ethylene glycol. The association constant for pyrophosphate with Mg^{2+} (β_1, β_2, and β_3) could only be measured in the presence of 11–33% (w/v) ethylene glycol (Table II). At higher concentrations of this solvent there was precipitation of magnesium pyrophosphate complexes.

The values of K_{obsd} measured in Table I include contributions by the K_{eq} values for different ionic species of P_i and pyrophosphate free and in complex form with magnesium. With the use of the association constants shown in Table II, the K_{eq} of the different ionic reactions were calculated from the experimental values attained in totally aqueous medium and in presence of 33 or 66% (w/v) ethylene glycol (Table III). In each of the reactions shown in Table III one of the five forms of pyrophosphate is a reactant, either free or in complex form with magnesium. The data of Table III show that ethylene glycol promotes a decrease of the K_{eq} of the five ionic reactions calculated. The $\Delta G°$ for pyrophosphate hydrolysis may vary depending on whether Mg^{2+} and H^+ participates in the reaction, either as a reactant (consumed) or as a product (produced). The contribution of H^+ and Mg^{2+} to the values of $\Delta G°$ of pyrophosphate hydrolysis correspond to the $\Delta G°$ values for the association or dissociation of HPO_4^{2-} and H^+

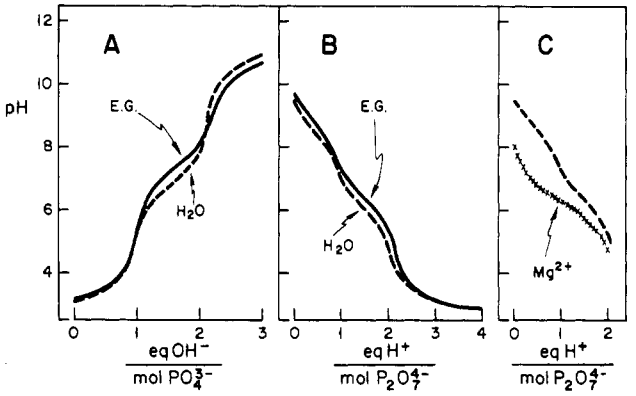

FIGURE 6: Association constant measurements. (A) Titration of 1 mM phosphoric acid with 0.1 N NaOH in presence of 0.2 M KCl in totally aqueous medium (dashed line) or in the presence of 66% (w/v) ethylene glycol. (B) Titration of 1 mM $Na_4P_2O_7$ with 0.1 HCl in presence of 0.2 M KCl in totally aqueous medium (dashed line) or in the presence of 66% (w/v) ethylene glycol. (C) Titration of 1 mM $Na_4P_2O_7$ with 0.1 N HCl in the presence of 0.2 M KCl and 33% (w/v) ethylene glycol either with (crosses) or without (dashed line) 1.5 mM $MgCl_2$. The apparent pK_1 and pK_2 values were determined as the pH values at which the ratio of equivalents of OH^- per mole of PO_4^{3-} or equivalents of H^+ per mole of $P_2O_7^{4-}$ was 0.5 or 1.5, respectively, where PO_4^{3-} and $P_2O_7^{4-}$ refer to the total concentrations of orthophosphate and pyrophosphate present in any form (Lambert & Watters, 1957). In (C), β_1, β_2, and β_3 for pyrophosphate were calculated from the pH values at the apparent pK in the absence and presence of Mg^{2+} (Table II). Only the pK values in the pH range of 5.0–9.0 were considered.

or Mg^{2+} (de Meis, 1984). Table III shows that ethylene glycol promotes a small change in the contribution of each H^+ or Mg^{2+} consumed or produced. Taken together, the data of Table III indicate that the decrease of K_{obsd} observed in the presence of ethylene glycol is not related to a decrease of the K_{eq} of a particular ionic reaction, but it is probably due to a general decrease of the K_{eq} of all ionic reactions. Due to the experimental difficulties in measuring the association constant of Table II in the presence of PEG 8000, the effect of this cosolvent on the K_{eq} of the different ionic reactions of pyrophosphate hydrolysis could not be estimated. It might be possible that the effect of PEG 8000 in decreasing the K_{obsd} of pyrophosphate hydrolysis observed when the pH is decreased from 8.0 to 6.2 (Figure 5) is related to a large change on the association constant of P_i and pyrophosphate with H^+ and Mg^{2+}.

Discussion

Water on the Enzyme Surface. The water molecules that organize around a protein in solution have been found to have properties that are different from those of the medium "bulk water"; e.g., they exhibit a lower vapor pressure, lower mobility, and greatly reduced freezing point [for review, see Cook & Kuntz (1974)]. Similar changes on the properties of water are observed in mixtures of organic solvents and water (Dobson, 1925; Malcolm, 1957; Amis & Hinton, 1973; Cooke & Kuntz, 1974; Chan & Van Hook, 1976). The data presented in this paper show that the K_{eq} for hydrolysis of pyrophosphate in aqueous solution varies significantly following the addition of organic solvents (Figure 5 and Table I). This finding suggests that the difference in energies of hydrolysis between phosphate compounds in solution and those on the enzyme surface could be ascribed to the different properties of bulk water and water on the enzyme surface.

Yeast inorganic pyrophosphatase retains a tightly bound pyrophosphate (Janson et al., 1979; Springs et al. 1981; Cooperman, 1982) that has a K_{obsd} for hydrolysis of 4.8 (Springs et al., 1981). Table I shows that the addition of organic solvents promotes a decrease of K_{obsd} to values similar or significantly lower than those determined for the tightly bound pyrophosphate.

Water Activity. The thermodynamic equilibrium constant for pyrophosphate hydrolysis is calculated by taking into consideration the activities of pyrophosphate, P_i, and H_2O according to

$$K_{eq} = [P_i]^2/([PP_i][H_2O])$$

The different organic solvents used promote a small decrease of water activity as calculated from partial vapor pressure data (Dobson, 1925; Malcolm & Rowlinson, 1957; Chan & Van Hook, 1976). The addition of 50% (w/v) dimethyl sulfoxide promotes a decrease of water activity from 1.00 (pure water) to 0.74 at 25 °C and to 0.77 at 70 °C. In the presence of 50% PEG 300 the water activity is 0.90 at 30 °C and 0.93 at 65 °C. For PEG of molecular weight higher than 300, vapor pressures were measured in temperatures varying between 50 and 65 °C. At 65 °C the water activity of mixtures containing 49–50% (w/v) of either PEG 3000 or PEG 5000 is 0.97 (Malcolm & Rowlinson, 1957). These values indicate that the decrease of K_{obsd} observed in Figure 5 and Table I is not related to a change of water activity but rather to changes on the activities of P_i and (or) pyrophosphate. There is a larger decrease of water activity in the presence of dimethyl sulfoxide than in the presence of polymers of ethylene glycol. However, the polymers of ethylene glycol are more effective than dimethyl sulfoxide in decreasing K_{obsd} of pyrophosphate hydrolysis; water activity varies little when the molecular weight of PEG increases, but the decrease of K_{obsd} is more pronounced the higher the molecular weight of the polymer. Finally, were the decrease of K_{obsd} related solely to a change of water activity of the medium and not to a change of the activities of P_i and pyrophosphate, then the different cosolvents used should promote a discrete increase of K_{obsd}, varying from 3 to 26%, and not a decrease of K_{obsd} of several orders of magnitude (Table I).

There is a qualitative correlation between the ability of the organic solvent to break the structure of bulk water and to decrease the energy of hydrolysis of pyrophosphate. Viscosity measurements of water–organic solvent mixtures in a temperature range of 20–60 °C (Ueberreiter, 1980a,b, 1982) revealed that at the concentrations used in Table I polymers of ethylene glycol are more effective than ethylene glycol and dimethyl sulfoxide in breaking the structure of water. This effect increases with increasing molecular weight of the polymer. The similarity between the two effects of the organic solvents raises the possibility that the activities of P_i and (or) pyrophosphate may vary when the structure of water is changed.

Solvation Energy. The concept of high-energy compounds has been analyzed primarily from a theoretical viewpoint. Until 1969, water was ignored in the different theoretical formulations reported (Kalckar, 1941; Hill & Morales, 1951; Boyd & Lipscomb, 1960; Pullman & Pullman, 1963). In 1970 George et al. and later Haynes et al. (1978) proposed that the energy of hydrolysis of pyrophosphate and other phosphate compounds might be determined by the difference in solvation energies of reactants and products. One of the implications of this formulation is that the activities of P_i and (or) pyrophosphate may vary depending on their degree of interaction with the solvent. The data presented in this paper can be interpreted according to the concept of solvation energy. It may be that the different cosolvents used promote a discrete change of solvation of P_i and (or) pyrophosphate. The solvation energies of the different ionic forms of P_i vary between 76 and 637 kcal mol^{-1} and those of pyrophosphate between 84 and 584 kcal mol^{-1} (George et al., 1970). These high values indicate that a small change of P_i and (or) pyrophosphate solvation would be sufficient to account for the decrease of the energy of pyrophosphate hydrolysis observed in Table I. In support of this proposal is the finding that the solvation of ions such as NO_3^-, SO_4^{2-}, Mg^{2+}, Cu^{2+}, and Na^+ is significantly altered in presence of organic solvents [for review, see Amis & Hinton (1973)].

Registry No. PEG, 25322-68-3; P_i, 14265-44-2; Me_2SO, 67-68-5; $HO(CH_2)_2OH$, 107-21-1; H^+, 12408-02-5; Mg, 7439-95-4; pyrophosphate, 14000-31-8; inorganic pyrophosphatase, 9024-82-2.

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