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THE FORM OF 2-PHOSPHOGLYCOLLIC ACID BOUND BY TRIOSEPHOSPHATE ISOMERASE

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SUMMARY

In NMR experiments designed to distinguish between possible enzyme-bound forms of the inhibitor 2-phosphoglycollic acid, it is found that neither of the di-anionic species, that would be consistent with the observed pH-dependence of K_i, is in fact correct. Instead, the enzyme appears to bind the tri-anionic species of this inhibitor, taking up a proton at a separate site.

The affinity of muscle triosephosphate isomerase for 2-phosphoglycollate, a potential analog of activated intermediates in substrate transformation[1], reaches a maximum at pH values where this inhibitor bears two negative charges in free solution[2]. The inhibitor might be bound in either of two di-anionic forms, I or II:

(I) (II)

We have examined NMR spectra of free and enzyme-bound 2-phosphoglycollate, making use of the pH-dependence of the resonance frequencies of the carboxyl carbon and the phosphoryl phosphorus to determine the state of ionization of these groups in the enzyme-inhibitor complex.

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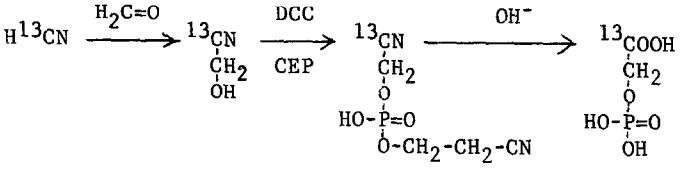
MATERIALS AND METHODS

(1-^{13}C) phosphoglycolic acid was synthesized by a procedure in which protecting groups were removed at the last stage:



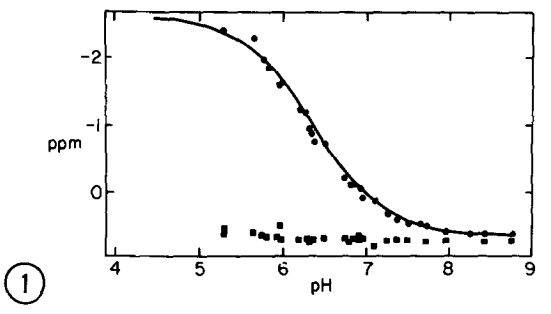
DCC = N,N'-dicyclohexylcarbodiimide
CEP = 2-cyanoethylphosphate

To K^{13}CN (1 g; 95 atom % excess, obtained from Prochem Ltd.) and HCHO (1.3 ml of 37% aqueous solution) in water (4 ml), concentrated H_2SO_4 (0.44ml) was added with stirring in an ice bath. After 1 h, the reaction was terminated by adjustment to pH 3.5 with KOH, and an oily product (0.85 g) was recovered by continuous ether extraction (14 h) and removal of ether by evaporation. Without purification, this glycolonitrile[3] (0.39 g; 6.9 mmole) was mixed with 2-cyanoethylphosphate (6.9 mmole)[4], the mixture was dried by successive evaporations with dry pyridine, and DCC (3.6 g; 17 mmole) and dry pyridine (10 ml) were added. The reaction was allowed to proceed for 18 h at room temperature, pyridine was removed and the residue was heated for 16 h under reflux in aqueous KOH (1 M; 40 ml). After adjustment to pH 9, the solution was applied to a column of Dowex 1-Cl$^-$ (50 ml bed volume), and batchwise elution was performed with water (600 ml), 0.03 N HCl (400 ml), 0.1 N HCl (400 ml) and 0.3 N HCl (400 ml) over a period of 48 h at room temperature. The desired product emerged as a sharp peak (50 ml) in 0.3 N HCl; removal of water under vacuum yielded a glass, from which traces of HCl were removed by successive evaporations with water. Potentiometric titration indicated recovery of 4 mmole of 2-phosphoglycolate, identical with the authentic compound in its mobility upon paper chromatography in butanol-propionic acid-water[5] and paper electrophoresis at pH 6.5, with detection by acid-molybdate development and irradiation.[6] Both methods indicated minor contamination with inorganic phosphate. ^{31}P-NMR showed that approximately 5% inorganic phosphate was present as a contaminant, the major product exhibiting a chemical shift identical with authentic 2-phosphoglycolate. The ^{13}C-NMR spectrum of the product exhibited only a doublet with a chemical shift identical with that of the carboxylate carbon at natural abundance in authentic 2-phosphoglycolate.

Samples of chicken muscle triosephosphate isomerase were prepared as described earlier[7]. Chemical shifts, observed at 36.4 MHz on a Bruker HFX90 for ^{31}P, are quoted downfield from trimethyl phosphate. Shifts for ^{13}C, observed at 22.6 MHz on a Bruker WH90, are quoted downfield from dioxan.

RESULTS

The proton-decoupled ^{31}P spectrum of 2-phosphoglycollate shows a single resonance that varies with pH as shown in Figure 1; the solid



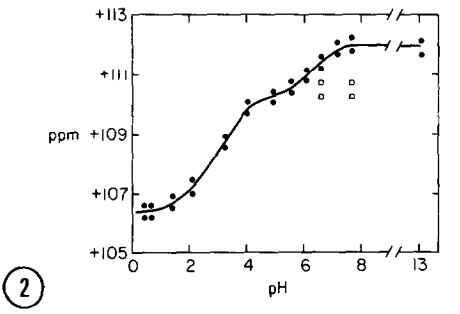


Fig. 1. ^{31}P Chemical shift of 2-phosphoglycollate, relative to trimethylphosphate, in the absence (●) and in the presence (■) of rabbit muscle triosephosphate isomerase, observed at 36.4 MHz.

Fig. 2 ^{13}C Chemical shift of 2-phosphoglycollate-1-^{13}C, relative to dioxan, in the absence (●) and in the presence (□) of chicken muscle triosephosphate isomerase, observed at 22.6 MHz.

line is a theoretical curve for titration of an acid with $pK_a = 6.4$. The proton-decoupled ^{13}C spectrum of the C(1) carbon of 2-phosphoglycollate consists of a doublet, split by the ^{31}P nucleus. The titration behavior of this doublet is shown in Figure 2. It exhibits an apparent pK_a in the neighborhood of 3.5, with a smaller deviation near pH 6.5.

In the presence of rabbit muscle triosephosphate isomerase (3 mM), the ^{31}P spectrum of 2-phosphoglycollate (4 mM) exhibits a sharp resonance at the same position as that of free phosphoglycollate, at all pH values. An additional broader peak, representing the bound inhibitor, is unaffected by changing pH as shown in Figure 1. The value of the chemical shift of the bound inhibitor (0.725 ppm) is close to the value (0.6 ppm) observed for free phosphoglycollate at high pH. The phosphate group of bound phosphoglycollate is therefore fully ionized, and this remains true over the pH range from 5.5 to 8.5. The enzyme from chicken muscle shows the same behavior.

The ^{13}C spectrum of 2-phosphoglycollate-1-^{13}C, examined under similar conditions in the presence of triosephosphate isomerase, shows two doublets. One of these is identical in position with the doublet

of the free inhibitor, and was found to increase in amplitude as the concentration of inhibitor was increase beyond one equivalent. The other doublet, representing the bound inhibitor, is centered at 110.4 ppm, and its position does not change when the pH is changed from 6.4 to 7.5. The chemical shift of the bound inhibitor is near that observed for the free inhibitor in the range where the carboxylic acid group of the free inhibitor is fully ionized (Figure 2). Thus the bound form of 2-phosphoglycollate is neither I nor II, but the fully dissociated tri-anion. The carboxylate group of this tri-anion may be involved in a hydrogen bond with a residue at the active site; this would help to explain the small downfield shift for the bound inhibitor as compared the free tri-anion (Figure 2).

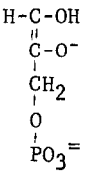
The change in ultraviolet absorption spectrum, that occurs when 2-phosphoglycollate is bound by the enzyme from chicken muscle[8], has been examined further to determine the form and stoichiometry of binding. Nonlinear regression analysis of the results indicates that 0.82 ± 0.08 moles of inhibitor are bound per mole of active site, with a Hill coefficient of 0.97 ± 0.06. The apparent dissociation constant of the enzyme-inhibitor complex is found to vary with pH in a manner consistent with that described earlier[2] for the variation of K_i with pH for the enzyme from rabbit muscle (Jones and Waley, in preparation).

2-Phosphoglycollic acid amide is found to be a very weak inhibitor of triosephosphate isomerase, with a K_i value in excess of 10^{-4} M, which may be compared with a limiting value of 4×10^{-7} M for inhibition of the rabbit muscle enzyme by 2-phosphoglycollic acid[2].

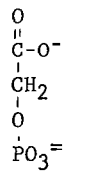
DISCUSSION

Previous studies suggest that substrates are productively bound with their phosphoryl groups fully ionized[2]. Proton abstraction from these productively bound substrates would be expected to yield tri-

anionic intermediates bearing a reasonable resemblance to the tri-anion of 2-phosphoglycollate:



intermediate



inhibitor

The stability of the inhibitory complex is thus in agreement with previous suggestions regarding the mechanism of action of this enzyme[9]. The weak binding of 2-phosphoglycollic acid amide, on other hand, appears understandable in view of its reluctance to form a comparable tri-anion.

To reconcile the apparent equilibrium binding of di-anionic forms with the present observation that the inhibitor is actually bound as the tri-anion, we are forced to conclude that the enzyme is protonated in the process of binding this inhibitor. The present experiments provide no evidence concerning the site of proton uptake. A likely candidate appears to be the active site glutamic acid residue (number 165 in the sequence of the enzyme from rabbit muscle[10]) that has been shown to be essential for catalysis[11-16] and is situated at the active site[17]. The pK_a of this group is less than 5 in the enzyme from rabbit muscle[15,2] and 3.9 in the enzyme from yeast[2]. The intrinsic affinity of the enzyme with its active site site glutamyl residue in the conjugate acid form, for the tri-anionic inhibitor, would then be several orders of magnitude more pronounced than is suggested by the observed K_i, which reaches limiting values of about 4×10^{-7} M for the enzyme from rabbit muscle[2]. This is as might be expected if the enzyme were to provide special stabilization of ene-diolate intermediates formed by proton abstraction from substrates.

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