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Phosphorus-31 Nuclear Magnetic Resonance of Dihydroxyacetone Phosphate in the Presence of Triosephosphate Isomerase. The Question of Nonproductive Binding of the Substrate Hydrate†

Martin R. Webb,‡ David N. Standring, and Jeremy R. Knowles*

ABSTRACT: The degree of hydration of dihydroxyacetone phosphate and its binding to triosephosphate isomerase has been studied by ^{31}P NMR. The resonance of the free keto form of dihydroxyacetone phosphate, which is the form of the substrate handled by the enzyme, is broadened in the presence of enzyme, but the resonance of the hydrate is unaffected. The hydrate does not therefore bind nonproductively to the enzyme, and this fact renders further correction of the steady-state kinetic parameters unnecessary.

Many enzyme substrates exist in solution as an equilibrium mixture of two or more readily interconvertible forms of which only one is directly handled by the enzyme. For instance, substrates containing carbonyl groups may be partially hydrated (e.g., CO_2), partially enolized (e.g., acetoacetate), or exist as epimers (e.g., the α and β forms of aldoses and ketoses). While from kinetic experiments one may often determine which form of the substrate is the "active" species, it is more difficult to discover if the "inactive" form (which will be a structural analogue of the true substrate) is a competitive inhibitor. If this were true, the kinetic parameters measured on the basis of the total substrate would be in error. The purpose of this paper is to investigate the possibility of such nonpro-

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‡ Present address: Department of Biochemistry, University of Bristol, Bristol, United Kingdom.

ductive binding in the case of triosephosphate isomerase, which catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The free carbonyl form of each substrate is known to be the species directly handled by the enzyme, but the hydrate constitutes a substantial proportion of triose phosphates in aqueous solution. Thus, at 20 °C and pH 7.5 dihydroxyacetone phosphate is 45% hydrated (Reynolds et al., 1971), while only 3.3% of D-glyceraldehyde 3-phosphate is in the free aldehyde form under similar conditions (Trentham et al., 1969). The kinetic parameters for the reaction catalyzed by triosephosphate isomerase must be corrected for the presence of this inactive substrate, but they would be further changed if the hydrate were to bind nonproductively to the enzyme.

^{31}P NMR[1] allows the direct observation of the hydrate and keto forms without perturbing the system, and line broadening has been used successfully in the study of the binding of phosphoryl ligands to a number of enzymes (e.g., Feeney et al., 1975; Hull et al., 1976). The lack of sensitivity of ^{31}P NMR compared to ^{1}H NMR is more than compensated for by the simplicity of the spectra obtained and the lack of dynamic range problems associated with the water peak when observing protons.

Materials and Methods

Crystalline triosephosphate isomerase was prepared by a modification of the methods of Putman et al. (1972) and McVittie et al. (1972). Immediately before an NMR experiment the enzyme was dialyzed exhaustively against 25 mM triethanolamine hydrochloride buffer, pH 7.5, containing 10 mM EDTA, followed by concentration at 3 °C to 70–100 mg/mL using either a Sartorius membrane vacuum filtration apparatus or an Amicon CF 25 membrane cone. During the final stages of concentration, the above buffer in D_2O was added to give a solution of 33% (v/v) in D_2O. The pH was then adjusted to 6.9 (meter reading) by the careful addition of 1 N HCl, and the solution was clarified by centrifugation. All these operations were performed at 1–2 °C. The enzyme concentration was calculated using a value for $E_{280}^{0.1\%}$ of 1.21 for a 10-mm light path. The enzyme was assayed as described by Plaut and Knowles (1972).

Solutions of dihydroxyacetone phosphate in the same deuterated buffer containing 10 mM EDTA were prepared by a slight modification of the method of Ballou (1960) and assayed according to Plaut and Knowles (1972).

Broad-band proton-decoupled Fourier transform ^{31}P NMR spectra were run at 40.5 MHz on a Varian XL-100-15 instrument equipped with a variable temperature probe and a Varian 620-i 16K computer with a LINC magnetic tape unit from Computer Operations, Inc. A single side band filter was used to improve signal to noise. Spectra were obtained at 1–2 °C from 1.5–2 mL of solution in 12-mm tubes equipped with a Teflon vortex plug (Wilmad). Up to 8000 transients were accumulated within 2 h using a 1500-Hz spectral width, an acquisition time of 0.5 s, and a pulse width of 20 μs (60°). A sensitivity enhancement exponential function of time constant 0.2 s (giving a line broadening of 1.6 Hz) was applied to each free induction decay before Fourier transformation using 8K of computer memory. D_2O was used as a field-frequency lock. Chemical shifts (with reference to 85% phosphoric acid) were reproducible to ±0.05 ppm. All line widths were measured from highly expanded portions of the spectra, and were compared with that of an internal standard, triethyl phosphate.

^{1}H NMR spectra were obtained with the same instrument. For accurate integration, conditions to ensure efficient relaxation were spectral width 1000 Hz, acquisition time 4.0 s, pulse delay 16.0 s, and pulse width 10 μs (24°). Only spectra with good signal to noise were used and the integrals obtained electronically from carefully phased portions of these spectra were reproducible to within 5%.

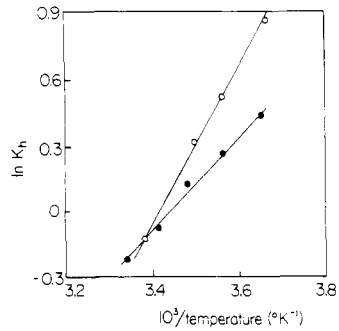

FIGURE 1: Variation of the ratio of hydrate/keto (K_h) for dihydroxyacetone phosphate, with temperature. pH 5.5 (○); pH 7.2 (●).

Results and Discussion

The overall equilibrium constant for the triose phosphate equilibrium is 22 between 22 and 37 °C (pH 7.5), and dihydroxyacetone phosphate therefore represents some 96% of the total triose phosphate in the presence of the enzyme. However, the isomerase shows a slight phosphatase activity towards its substrates, which causes some problems in the long-term observation of dihydroxyacetone phosphate resonances in the presence of high concentrations of enzyme. This hydrolytic activity is not due to extrinsic phosphatase present in the crystalline isomerase, since it is effectively eliminated by inactivation of the enzyme using the specific inactivator bromohydroxyacetone phosphate (Hartman, 1968, 1970, 1971; de la Mare et al., 1972), and such compounds as *p*-nitrophenyl phosphate are unaffected. Further, the loss of triose phosphates is not due to the uncatalyzed breakdown of D-glyceraldehyde 3-phosphate, since the rate of appearance of P_i increases with increasing isomerase levels at concentrations far above those required to maintain the triose phosphate equilibrium. Accordingly, the ^{31}P NMR spectra were obtained at 1–2 °C, at which temperature the substrate decomposition was acceptably slow. To determine the effect of temperature on the ratio of the hydrate and keto forms of dihydroxyacetone phosphate, a series of experiments was performed using ^{1}H NMR rather than ^{31}P NMR, because of its greater sensitivity and the greater reliability of intensity measurements in the absence of NOE effects. The results are summarized in Figure 1. The hydrate/keto ratio was found to be pH dependent, in addition to the known temperature dependence (Reynolds et al., 1971). The curves coincide fortuitously around room temperature. These results were qualitatively verified by ^{31}P NMR, peak assignments being in agreement with those proposed by Gray (1971). From the ^{1}H spectra, the value of K_h (= [hydrate]/[keto]) at pH 7.2 and 1 °C was found to be 1.55. The K_m for the total dihydroxyacetone phosphate was determined kinetically at pH 6.9 and 1 °C and found to be 1.34 mM, which is equivalent to a K_m^{keto} of 0.54 mM.

Proton-decoupled ^{31}P spectra of dihydroxyacetone phosphate in the absence and presence of enzyme are shown in Figure 2, together with our assignments. The keto and hydrate

[1] Abbreviations used are: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; EDTA, (ethylenedinitrilo)tetraacetic acid.

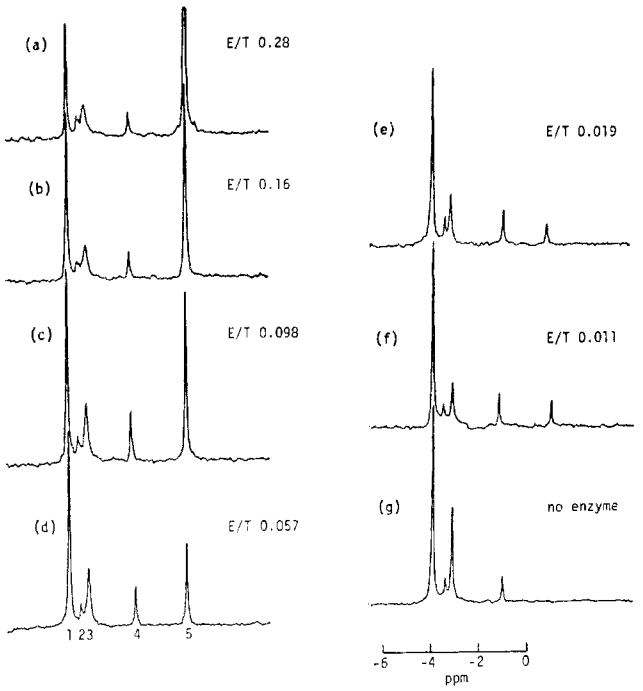

FIGURE 2: ^{31}P NMR spectra of triose phosphates in the presence of triosephosphate isomerase, pH 6.9, 1 °C. The peak assignments: peak 1, dihydroxyacetone phosphate hydrate; peak 2, glyceraldehyde 3-phosphate hydrate; peak 3, dihydroxyacetone phosphate keto form; peak 4, P_i; peak 5, triethyl phosphate (standard). The spectra are arranged in order of decreasing ratio of total enzyme to total triose phosphate (E/T): (a) 7.2 mM triose phosphate, 2.0 mN isomerase (E/T 0.28); (b) 11.6 mM triose phosphate, 1.8 mN isomerase (E/T 0.16); (c) 15.2 mM triose phosphate, 1.5 mN isomerase (E/T 0.098); (d) 21.0 mM triose phosphate, 1.2 mN isomerase (E/T 0.057); (e) 30.8 mM triose phosphate, 0.59 mN isomerase (E/T 0.019); (f) 13.1 mM triose phosphate, 0.14 mN isomerase (E/T 0.011); (g) equilibrium mixture of triose phosphates with *no* isomerase. Spectrum a is displayed with twice the vertical scale of the remaining spectra.

forms of dihydroxyacetone phosphate are in the limit of slow exchange under our conditions ($k_{hydration}$ = 0.36 s^{-1}, $k_{dehydration}$ = 0.44 s^{-1}, at 20 °C, pH 7.5; Reynolds et al., 1971). The rate constants of the individual steps of the enzyme-catalyzed reaction (Albery and Knowles, 1976) are so high that the fast-exchange condition will be met for all reasonable values of $\Delta\nu$, the chemical shift difference. (For instance, from the rate constants for the catalyzed reaction (Albery and Knowles, 1976) the lifetime of free dihydroxyacetone phosphate is between 7×10^{-5} and 1×10^{-3} s (depending upon the enzyme concentration), and for the bound substrate is $\sim 2 \times 10^{-4}$ s. For lifetimes of this size, the fast-exchange condition will be met for reasonable values (say <30 Hz) of $\Delta\nu$, the chemical shift difference, in Hz.) All enzyme-bound species and the keto forms of the two substrates therefore appear as one peak.

As the enzyme concentration is increased relative to triose phosphate, one peak, assigned to the keto form of dihydroxyacetone phosphate, is seen to broaden relative to triethyl phosphate (which binds to the enzyme with a K_i > 40 mM). Reliable figures for this broadening could be obtained only up to a point where the calculated fraction of keto form bound was 42% (though data are available for the hydrate form and P_i at much greater enzyme:substrate ratios and still show no broadening). These results are summarized in Figure 3. This figure and the preceding spectra clearly show that, whereas the addition of enzyme broadens the resonance of the keto form, *the hydrate resonance is unaffected, even at the highest*

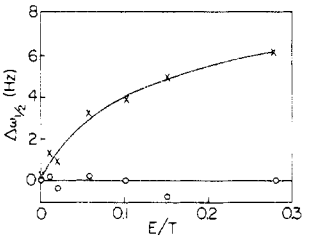

FIGURE 3: Plot of line broadening ($\Delta\omega_{1/2}$) against the ratio of total enzyme to total triose phosphate (E/T), pH 6.9, 1 °C. Dihydroxyacetone phosphate keto form (x); dihydroxyacetone phosphate hydrate (O).

enzyme:substrate ratios. The obvious interpretation of these data is that the keto form of dihydroxyacetone phosphate binds to the enzyme as expected, whereas the binding of the hydrate form is negligible by comparison. The presence of 10 mM EDTA precludes any interference from paramagnetic ions.

For the keto species, the line width at half-height, $(\omega_{1/2})_k$, will be a weighted average of the contributions of the free and bound species, assuming fast exchange obtains. Thus

$$(\omega_{1/2})_k = \frac{1-x}{\pi T_{2f}} + \frac{x}{\pi T_{2m}} + c \qquad (1)$$

where x is the fraction of ligand bound, T_{2f} and T_{2m} are the transverse relaxation times of the free and enzyme-bound species, respectively, and c is a line-width contribution deriving from instrumental filtering, sensitivity enhancements, field inhomogeneity, viscosity broadening, etc. The triethyl phosphate line width is given by

$$(\omega_{1/2})_s = \frac{1}{\pi T_{2s}} + c \qquad (2)$$

Subtracting eq 2 from eq 1 gives

$$\Delta\omega_{1/2} = x\left[\frac{1}{\pi T_{2m}} - \frac{1}{\pi T_{2f}}\right] + \left[\frac{1}{\pi T_{2f}} - \frac{1}{\pi T_{2s}}\right] \qquad (3)$$

A plot of the line broadening ($\Delta\omega_{1/2}$) vs. the fraction bound (x) should therefore be linear. The fraction of the keto form bound (x) can be calculated from the equation[2]

$$[E] = K_m\left[\frac{K'+1}{K_h+1}\right]\left[\frac{x}{1-x}\right]^2 + \left[\frac{K'+1}{K_h+1}([T]-[E]) + K_m\right]\left[\frac{x}{1-x}\right] \qquad (4)$$

K_h and K_m (for the keto form of the ligand) have the values given above, $K' = K_h K_m / K_i^{hydrate}$, and [T] and [E] are the total concentrations of triose phosphate and enzyme, respectively. K_m is a good approximation for the dissociation constant for all bound species. Since we know from Figure 3 that $K_i^{hydrate} \gg K_m$, we can use eq 4 to calculate x, and plot $\Delta\omega_{1/2}$ vs. x according to eq 3. This is shown in Figure 4 and the data are seen to be in good agreement with the theory, even when a relatively large fraction of ligand is bound. This is

[2] On the basis that enzyme-bound intermediates other than the complex with the keto form of dihydroxyacetone phosphate are present in negligible proportion (see Albery and Knowles, 1976), the scheme becomes

$$\begin{array}{ccc} \text{enzyme + keto form} & \overset{K_m}{\rightleftharpoons} & \text{(enzyme·keto form)} \\ K_h \Big\| & & \\ \text{enzyme + hydrate} & \overset{K_i^{hydrate}}{\rightleftharpoons} & \text{(enzyme·hydrate)} \end{array}$$

from which eq 4 is simply derived.

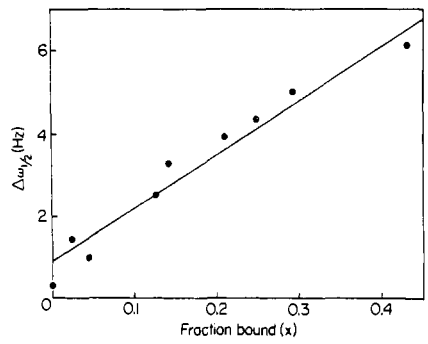

FIGURE 4: Plot of the line broadening ($\Delta\omega_{1/2}$) for the keto form of dihydroxyacetone phosphate against the fraction of this material bound to the enzyme (x), pH 6.9, 1 °C. (Data from two separate experiments.)

consistent with the conclusion that the hydrate does not bind ($K_i^{hydrate} \gg K_m$). From the slope of Figure 4, we can estimate that the line broadening observed if *all* the keto form were bound would be 14.6 Hz. This value is in good agreement with values for the total line widths (typically 15–20 Hz) of enzyme-bound phosphate in a recent study of *Escherichia coli* alkaline phosphatase by Hull et al. (1976).

Finally, chemical shift data verify the binding of the keto form but not of the hydrate of dihydroxyacetone phosphate. The latter resonance does not titrate with addition of enzyme but the former peak shows a progressive downfield shift of up to 0.3 ppm with increasing enzyme levels at pH 6.9. Similar data have been used in other studies to imply the binding of a phosphate ester as the dianion only (Feeney et al., 1975; Martinez-Carrion, 1975). At pH 6.9, the various phosphate esters present in this study already exist largely as the dianions (the pK_a values at 30 °C for dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are 6.0 and 6.3, respectively; Plaut and Knowles, 1972), but between pH 6.9 and 7.5 the resonances of both forms of dihydroxyacetone phosphate and of the hydrate of glyceraldehyde 3-phosphate all titrate smoothly downfield by about 0.4 ppm. Thus, the chemical shift observed on the binding of dihydroxyacetone phosphate (keto form) to the enzyme is consistent with its binding as the dianion. This agrees with the finding that dihydroxyacetone sulfate (an isosteric monoanion at neutral pH) does not bind to triose phosphate isomerase (J. M. Herlihy, unpublished experiments).

In summary, we conclude that the $K_i^{hydrate}$ is *at least* one order of magnitude greater than K_m^{keto}. Had the hydrate bound any more tightly than this, peak broadening would have been observed at high [E]. A K_i of this magnitude would have a negligible effect on the steady-state parameters for triosephosphate isomerase. The rate of hydrate/keto exchange for dihydroxyacetone phosphate is slow relative to the rate of the normal enzymatic reaction catalyzed by the isomerase and is also slow in an absolute sense on the NMR time scale, since there is no line broadening or chemical shift of the hydrate resonance.

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