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Direct Observation of Substrate Distortion by Triosephosphate Isomerase Using Fourier Transform Infrared Spectroscopy†

Joel G. Belasco and Jeremy R. Knowles*

ABSTRACT: The infrared spectrum of dihydroxyacetone phosphate bound to triosephosphate isomerase has been measured. There are two carbonyl bands corresponding to the bound substrate, with an intensity ratio of about 3:1. Relative to the carbonyl absorption of dihydroxyacetone phosphate in free solution, the major band is shifted by 19 cm^{-1} to 1713 cm^{-1}, providing direct evidence of enzyme-induced distortion of the substrate. This strain is probably attributable to an enzymic electrophile that polarizes the carbonyl group of the substrate and thereby promotes catalysis.

One of the devices that have been proposed to account for the catalytic effectiveness of enzymes is ground-state destabilization. The theoretical basis for the catalytic advantage thus achieved is well established. As formulated by Jencks (1975), the geometric and electronic distortion of the enzyme and its substrate in the enzyme–substrate complex toward the transition-state structure reduces the activation free energy required for reaction and thereby results in a rate acceleration.

Although the logic of this concept has held a good deal of appeal for enzymologists, it has been a considerable challenge to establish conclusively the involvement of ground-state destabilization in real enzyme systems. Various methods have been utilized toward this end, including X-ray crystallography (Phillips, 1967; Smith et al., 1978), ultraviolet and electron spin resonance spectroscopy (Vallee & Williams, 1968), binding isotope effects (Thomson et al., 1964; Bush et al., 1971; de Juan & Taylor, 1976), NMR spectroscopy (Sykes et al., 1971), extended X-ray absorption fine structure (Eisenberger et al., 1978; Tullius et al., 1978), and comparative studies of substrate and inhibitor dissociation constants (Thompson, 1974).

Vibrational spectroscopy has seen rather limited use in establishing ground-state strain. Although this method can, in principle, provide a very direct measure of bond strength on a time scale (10^{-14} s) that essentially freezes molecular motion, the lack of sensitivity has until recently limited its usefulness in biochemistry, with the notable exception of resonance Raman studies of chromophoric protein ligands (Carey, 1978). The only example of the use of vibrational spectroscopy in an attempt to detect strain in a nonchromophoric enzyme–substrate complex was by Riepe & Wang (1968), who used infrared spectroscopy to conclude that carbon dioxide bound at the active site of carbonic anhydrase is *not* under appreciable strain [see also the study of carboxyhemoglobin (Alben & Caughey, 1968)]. However, doubts have since been raised as to whether the carbon dioxide whose

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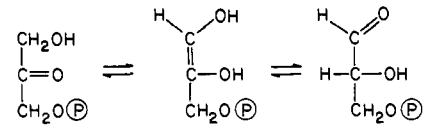
0006-2960/80/0419-0472$01.00/0

infrared spectrum was being measured was bound productively (Khalifah, 1971).

Triosephosphate isomerase (EC 5.3.1.1) catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate by an enolization mechanism involving



a *cis*-enediol intermediate (Rieder & Rose, 1959). The enzyme is a symmetrical dimer requiring no metal ion for activity. On the basis of their observation that dihydroxyacetone phosphate is reduced by sodium borohydride 8 times faster when bound to the enzyme than when in free solution, Webb & Knowles (1974) proposed the existence on the enzyme of an electrophilic group more effective than water at polarizing the substrate carbonyl. While such a polarization was an attractive proposal in that it would also increase the acidity of the α protons and thereby promote enolization, an approximation effect arising from the binding of borohydride at the active site prior to its reaction with dihydroxyacetone phosphate could also have produced the observed rate acceleration. Moreover, this approach could not distinguish between polarization in the ground state and stabilization of the incipient alkoxide ion in the transition state.

The recent development of Fourier transform infrared spectrometers with on-line data processing capabilities has allowed a quantum leap in the ease with which the vibrational frequencies of individual functional groups of enzyme-bound substrates can be examined, and we decided to employ this technique to determine directly whether the carbonyl group of dihydroxyacetone phosphate is polarized by triosephosphate isomerase. Surprisingly, we have observed two carbonyl bands for enzyme-bound dihydroxyacetone phosphate. The larger of these absorbs at a frequency 19 cm^{-1} below that of free dihydroxyacetone phosphate and provides convincing evidence for catalytically productive ground-state strain induced in the substrate by triosephosphate isomerase.

Materials and Methods

Crystalline triosephosphate isomerase was prepared from chicken muscle by a modification of the methods of Putman et al. (1972) and McVittie et al. (1972) and from yeast by the method of Norton & Hartman (1972). Specifically alkylated triosephosphate isomerase was prepared by treating the chicken muscle enzyme with bromohydroxyacetone phosphate and then with sodium borohydride (de la Mare et al., 1972). Aldolase (from rabbit muscle, as a crystalline suspension in ammonium sulfate), α-glycerophosphate dehydrogenase (from yeast, as a crystalline suspension in ammonium sulfate), dihydroxyacetone phosphate (as the dicyclohexylammonium salt of the dimethyl ketal), and Dowex 50 W (H^+ form; 100–200 mesh; 4% cross-linked) were obtained from the Sigma Chemical Co., St. Louis, MO. $D_2{}^{16}O$ (99.8% D) and NaOD (99% D) were obtained from the Aldrich Chemical Co., Milwaukee, WI. $D_2{}^{18}O$ (99.9% D; 99.5% ^{18}O) was obtained from Prochem, Summit, NJ. Sodium borodeuteride was obtained from Alfa-Ventron, Danvers, MA. Centriflo CF25 ultrafiltration membrane cones were obtained from Amicon Corp., Lexington, MA. DEAE-cellulose (DE-52) was obtained from Whatman, Ltd., Maidstone, U.K.

Ultraviolet measurements were made with a Perkin-Elmer 575 spectrophotometer. Values of $A_{280}{}^{0.1\%}$ of 1.21 and 1.00 were assumed for triosephosphate isomerase from chicken muscle (de la Mare et al., 1972) and yeast (Norton & Hartman, 1972), respectively. Aldolase and α-glycerophosphate dehydrogenase were in all instances treated with bromohydroxyacetone phosphate to inactivate traces of triosephosphate isomerase (de la Mare et al., 1972) and then dialyzed exhaustively. Measurements of pH were made at room temperature with a Radiometer PHM64 pH meter and a Microelectrodes, Inc., MI-410 combination microelectrode. Reported pD values are 0.4 unit higher than those actually read on the pH meter (Glasoe & Long, 1960). Mass spectrometry was carried out on an AEI MS-9 instrument.

[1(S)-^{2}H]Dihydroxyacetone phosphate was prepared by aldolase-catalyzed exchange of the *pro-S* hydrogen of dihydroxyacetone phosphate (Bloom & Topper, 1958). Aldolase (1 mg, 14 units) was combined with 0.20 mL of 1.0 M triethanolamine hydrochloride buffer, pH 7.7, containing EDTA (10 mM), and this mixture was freeze-dried, redissolved in 2 mL of D_2O, and filtered. Dihydroxyacetone phosphate (21.5 μmol) was added, and the isotopic exchange was allowed to proceed for 2 h at room temperature. The reaction mixture was passed through a small column (2 mL) of Dowex 50 (H^+) and washed through with water (2 mL). The eluate was titrated to pH 4 with 0.1 M NaOH, freeze-dried, and redissolved in D_2O (100 μL). This solution was titrated to pD 7.3 with 1 M NaOD just prior to use. A portion of this material was reduced with sodium borohydride and then trimethylsilylated to yield *rac*-[1(*S*)-^{2}H]tetrakis(trimethylsilyl)glycerol 3-phosphate (Fischer et al., 1976). Mass spectrometric analysis (Duncan et al., 1971; Curstedt, 1974) revealed that the material was >97% deuterated at C-1 and also 40% deuterated at C-3 (Lowe & Pratt, 1976).

[1,2-$^2H_2$]-D-Glyceraldehyde 3-Phosphate. Dihydroxyacetone phosphate monosodium salt (65.5 μmol) that had been freeze-dried and redissolved in 400 mM triethylammonium bicarbonate in D_2O (1.0 mL) was added to freeze-dried aldolase (1 mg, 14 units). After 80 min at 25 °C, triosephosphate isomerase (0.04 mg) in D_2O (0.5 μL) was added. After a further 110 min the reaction mixture was freeze-dried, treated with methanol (2 mL) to denature the enzymes, and evaporated to dryness. The residue was dissolved in D_2O (1 mL), and the solution was applied to a small column of Dowex 50 (D^+) (1.25 mL), which was then eluted with water (3.5 mL). The eluate was titrated to pH 4.9 with 0.1 M NaOH and freeze-dried.

The residue was dissolved in 20 mM ammonium bicarbonate (32 mL) containing NADH (1.34 mM), aldolase (1 mg, 14 units), and α-glycerophosphate dehydrogenase (1 mg, 165 units). The reaction was allowed to proceed at 30 °C while the absorbance at 340 nm of a small portion was monitored. After 47 min the reaction had reached completion. Dowex 50 (H^+) (4 g) was added, and the reaction mixture was filtered. The filtrate was treated with prewashed charcoal (0.2 g), filtered, treated with more charcoal (0.1 g), and filtered again, to give a solution with an absorbance at 259 nm of less than 0.01. This solution was concentrated to a volume of 5 mL, titrated to pH 3.9 with 0.1 M NaOH, and freeze-dried. The residue was treated with methanol (2 mL), evaporated to dryness, and redissolved in water (1 mL). This solution was applied to a small column of Dowex 50 (H^+) (0.5 mL) and eluted with water (3 mL). The eluate was titrated to pH 4.7 with 0.1 M NaOH. The yield of D-glyceraldehyde 3-phosphate was 24.8 μmol (38%, based on starting dihydroxyacetone phosphate). The product was free of dihydroxyacetone phosphate and contained L-glycerol 3-phosphate (27.6 μmol). The infrared spectrum in D_2O at 25 °C had a single carbonyl band at 1718 cm^{-1}, compared to 1730 cm^{-1} for the mono-

sodium salt of undeuterated glyceraldehyde 3-phosphate. All but ~1 cm^{-1} of this difference can be attributed to the effect of deuterium substitution at C-1. As a check on the extent of deuterium substitution, a portion was reduced with sodium borohydride to yield glycerol 3-phosphate, which on mass spectrometric analysis (as described above) revealed ≥98% deuteration at one site on C-1.

Phosphoglycolohydroxamate was prepared by condensation of methyl glycolate with dibenzyl phosphorochloridate (Atherton, 1957), followed by hydrogenolysis over palladium on charcoal, and then hydroxylaminolysis. The product was purified by ion-exchange chromatography on DEAE-cellulose, converted to the dicyclohexylammonium salt, and recrystallized from acetone–water [see also Collins (1974) and Lewis & Lowe (1977)].

Infrared measurements were made at 2-cm^{-1} resolution using a Nicolet 7199 Fourier transform infrared spectrometer purged with nitrogen. The sample cell had CaF_2 windows and a 0.108-mm Teflon spacer and was mounted on a water-jacketed cell holder cooled to 1 °C. In all measurements the solvent was deuterium oxide. "Double-beam" infrared spectra of dihydroxyacetone phosphate in the presence of triosephosphate isomerase were obtained from the ratio of a single-beam spectrum of 65 or 70 μL of a concentrated enzyme solution (67–105 mg/mL) plus 5 μL of concentrated dihydroxyacetone phosphate (108–126 mM) to a single-beam spectrum of a reference sample containing an equal volume of the same enzyme solution diluted with 5 μL of $D_2{}^{16}O$ or $D_2{}^{18}O$. For the single-beam spectra, 500 interferograms were averaged, requiring an acquisition time of ~8.5 min.

Concentrated solutions of native and alkylated triosephosphate isomerase in $D_2{}^{16}O$ were prepared by dialyzing the enzyme against 25 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (0.25 mM) and then exhaustively concentrating the enzyme solution by Centriflo ultrafiltration and rediluting with $D_2{}^{16}O$, before finally concentrating it to about 100 mg/mL. A concentrated solution of triosephosphate isomerase in $D_2{}^{18}O$ was prepared by diluting 225 μL of a 99 mg/mL solution of the enzyme in $D_2{}^{16}O$ with 125 μL of $D_2{}^{18}O$, reconcentrating by Centriflo centrifugation to a volume of about 225 μL, and then repeating this process 3 times. This procedure gave a solution of 89 mg/mL triosephosphate isomerase in ≈85% $D_2{}^{18}O$. The pD of the concentrated enzyme samples thus prepared was 7.4–7.7 without adjustment.

Concentrated solutions of dihydroxyacetone phosphate were prepared by freeze-drying a solution of the monosodium salt of dihydroxyacetone phosphate, redissolving in $D_2{}^{16}O$ to a concentration of 150 mM, and titrating to pD 7.4 with 1 M NaOD (except in the preparation of dihydroxyacetone phosphate in $D_2{}^{18}O$, in which case the monosodium salt of dihydroxyacetone phosphate was titrated to pH 7.0 with 0.1 M NaOH, freeze-dried, and redissolved in $D_2{}^{18}O$ to a concentration of 108 mM).

Determination of the Sites of Deuterium Incorporation into Dihydroxyacetone Phosphate by Concentrated Triosephosphate Isomerase. Solutions in $D_2{}^{16}O$ (75 μL; pD 7.5) of dihydroxyacetone phosphate (8.0 mM; ditriethylammonium salt) with and without chicken muscle triosephosphate isomerase (76 mg/mL) were prepared and left for 35 min at 3 °C. A solution of 400 mM triethylammonium bicarbonate in $D_2{}^{16}O$ (50 μL) was added, followed immediately by sodium borodeuteride (1.0 mg, 24 μmol). After 10 min, methanol (2.0 mL) was added, and the solutions were left for 1 h at room temperature. The solution containing precipitated protein was centrifuged and decanted. After removal of borate as the trimethyl ester [by repeated evaporation of methanol from the solution made acidic with Dowex 50 (H^+)], the samples were analyzed by mass spectrometry as described above. This analysis revealed that the glycerol 3-phosphate derived from dihydroxyacetone phosphate not treated with enzyme was 90% deuterated at C-2 by sodium borodeuteride. The glycerol 3-phosphate from dihydroxyacetone phosphate that had been incubated with concentrated triosephosphate isomerase, in addition to being 90% deuterated at C-2, was completely deuterated at only one site on C-1 and also 8% deuterated at C-3.

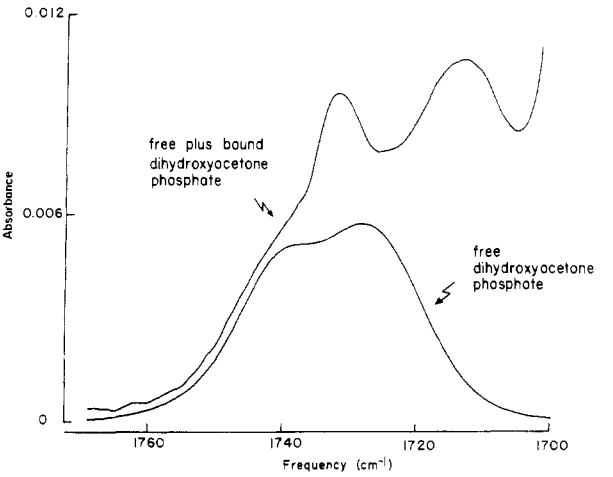


FIGURE 1: Infrared spectra of free [1(*R*)-^{2}H]dihydroxyacetone phosphate (lower trace) and of free plus enzyme-bound [1(*R*)-^{2}H]-dihydroxyacetone phosphate (upper trace). The ratio of bound to free dihydroxyacetone phosphate in the keto form is estimated to be about 0.7:1 [see Albery & Knowles (1976a) and Webb et al. (1977)].

Results

The infrared absorption spectrum of the dianionic form of dihydroxyacetone phosphate has a carbonyl band centered at 1733 cm^{-1} ($w_{1/2}$ = 29 cm^{-1}) which consists of two component peaks with absorption maxima at about 1738 and 1730 cm^{-1} (Figure 1). The observed band shape, which was only slightly affected by changes in ionic strength (0.01–1.0), cannot be ascribed to Fermi resonance since it was unaltered when the carbonyl band was shifted to 1697 cm^{-1} by isotopic substitution with ^{18}O. An alternative explanation is that the component peaks arise from cisoid and transoid conformations of the doubly charged phosphate group relative to the carbonyl group, inasmuch as the ion–dipole interaction will strengthen the C=O double bond (and raise its stretching frequency by a few wavenumbers) in the cisoid conformer and weaken the C=O bond (thereby lowering its stretching frequency) in the transoid conformer. Reducing the charge on the phosphate group would then be expected to lessen the difference between the effects of these two phosphate orientations on the carbonyl stretching frequency. This prediction was confirmed by the infrared spectra of the monoanionic ($\bar{\nu}_{CO}$ = 1736 cm^{-1}) and free acid ($\bar{\nu}_{CO}$ = 1738 cm^{-1}) forms of dihydroxyacetone phosphate, both of which had a single, symmetrical, and somewhat narrower ($w_{1/2}$ = 26 cm^{-1}) carbonyl absorption.[1]

The ratio of a single-beam spectrum of a solution in D_2O of dihydroxyacetone phosphate (8.4 mM) and triosephosphate isomerase (63 mg/mL, 2.4 mN in subunits) (which catalyzes the production of [1(*R*)-^{2}H]dihydroxyacetone phosphate) to

[1] The small increase in carbonyl stretching frequency observed for the monoanionic and free acid forms may be attributed to the increased electron-withdrawing character of the less highly charged phosphate group.

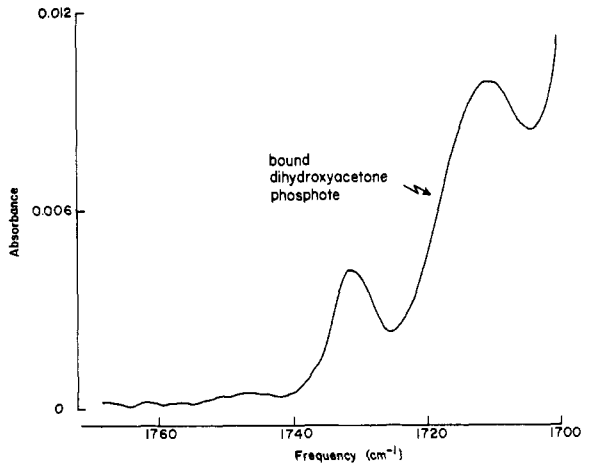


FIGURE 2: Infrared spectrum of enzyme-bound [1(*R*)-^{2}H]dihydroxyacetone phosphate, obtained by spectral subtraction to give a flat base line above 1740 cm^{-1}.

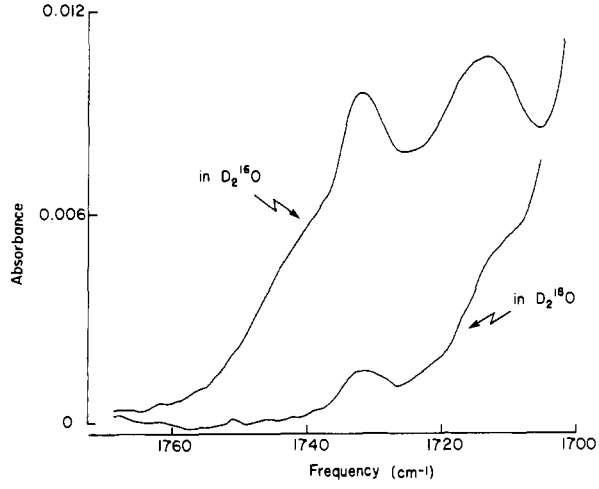


FIGURE 3: Infrared spectra of free plus enzyme-bound [1(*R*)-^{2}H;-1,2-$^{18}O_2$]dihydroxyacetone phosphate (lower trace) and of free plus enzyme-bound [1(*R*)-^{2}H;1,2-$^{16}O_2$]dihydroxyacetone phosphate (upper trace).

a spectrum of a solution containing only the enzyme at the same concentration gave two new bands between 1700 and 1800 cm^{-1} superposed on the absorption of unbound [1(*R*)-^{2}H]dihydroxyacetone phosphate (Figure 1). The two new bands were narrow and centered at 1713 cm^{-1} ($w_{1/2}$ = 13 cm^{-1}) and 1732 cm^{-1} ($w_{1/2}$ = 8 cm^{-1}) with an area ratio of about 3:1 (Figure 2). These two bands did not correspond to any bands in the spectrum of the unliganded enzyme or that of the free substrate. Their intensity was dependent on the enzyme concentration but was independent (for at least 1 h) of the time interval between mixing the enzyme and substrate and measuring the spectrum. Precisely the same bands were observed with the enzymes from chicken muscle and yeast. [All subsequent experiments were performed with the chicken muscle enzyme.]

The bands at 1713 and 1732 cm^{-1} could not be due to the carbonyl absorption of unbound glyceraldehyde 3-phosphate, whose keto form would be present at only 0.3% of the concentration of the keto form of unbound dihydroxyacetone phosphate. Moreover, they do not correspond to any of the likely hydrolysis products of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Both in the presence and in the absence of triosephosphate isomerase, dihydroxyacetone absorbs at 1737 cm^{-1} ($w_{1/2}$ = 19 cm^{-1}), glyceraldehyde absorbs at 1733 cm^{-1} ($w_{1/2}$ = 25 cm^{-1}), and methylglyoxal absorbs at 1726 cm^{-1} ($w_{1/2}$ = 21 cm^{-1}). Nor could either of these bands have arisen from fructose 1,6-bisphosphate (which could, in principle, be formed by an aldolase contaminant) since only 2% of fructose 1,6-bisphosphate exists as the keto form (Midelfort et al., 1976).

The new bands evidently arise from absorptions unique to the enzyme–substrate complex. Several experiments were performed to determine their origin more precisely.

To eliminate the possibility that either of the new bands corresponded to substrate bound outside the active site, two experiments were done. First, when the potent inhibitor phosphoglycolohydroxamate, a transition-state analogue (Collins, 1974), was included at a saturating concentration (6.5 mM) along with the enzyme and substrate in the infrared sample, the narrow bands at 1713 and 1732 cm^{-1} were found to be absent. Furthermore, when the active site of the enzyme was blocked by specific alkylation of the active-site residue Glu-165 by reaction with bromohydroxyacetone phosphate (de la Mare et al., 1972), the two new bands were again missing.

Another possibility was that one or both of the bands might be due to the vibration of a functional group of the enzyme. In order to rule this out, we obtained the spectrum of the enzyme-bound substrate in $D_2{}^{18}O$. Even at neutral pH, the carbonyl oxygen of dihydroxyacetone phosphate undergoes rapid isotopic exchange with the solvent, yielding an isotopically labeled species that absorbs at 1697 cm^{-1} (in the presence of triosephosphate isomerase, both the C-1 and C-2 oxygens are exchanged). In contrast, none of the oxygens of the enzyme (amide, carboxylate, and hydroxyl oxygens) should exchange under these conditions. The infrared spectrum obtained in $D_2{}^{18}O$ showed only weak absorptions at 1732 and 1713 cm^{-1} due to the residual ^{16}O content (≈15%) of the sample (Figure 3). Presumably, there were corresponding bands shifted 30–40 cm^{-1} to lower frequencies by isotopic substitution, but these were obscured by the intense amide I band of the protein, whose precise cancellation in the sample and reference is unreliable below 1700 cm^{-1}. Since the bands at 1713 and 1732 cm^{-1} were shifted by specific isotopic labeling of the substrate, they could not arise from vibrations of enzyme functional groups.

To determine whether the new bands were absorptions of bound dihydroxyacetone phosphate, bound D-glyceraldehyde 3-phosphate, or the bound enediol intermediate [dihydroxyacetone phosphate comprises ≥70% of the enzyme-bound species at equilibrium (Albery & Knowles, 1976a)], the original experiment was repeated with [1(*S*)-^{2}H]dihydroxyacetone phosphate, deuterated at the site *not* labelized by triosephosphate isomerase (Rieder & Rose, 1959). Because the C=C stretch of 1,2-*cis*-difluoro[1-^{2}H]ethylene absorbs at a frequency 20 cm^{-1} below that of the undeuterated compound (Craig & Overend, 1969), it is reasonable to expect that the corresponding absorption of the *cis*-enediol intermediate deuterated at C-1 would be shifted by a comparable amount.[2] Similarly, the carbonyl band of [1,2-$^2H_2$]-D-glyceraldehyde 3-phosphate appears 11–12 cm^{-1} below that of [2-^{2}H]-glyceraldehyde 3-phosphate. In contrast to these large effects, the carbonyl of [1-^{2}H]dihydroxyacetone phosphate absorbs at 1732 cm^{-1}, only 1 cm^{-1} lower than that of the unlabeled compound. When [1(*S*)-^{2}H]dihydroxyacetone phosphate was combined with the enzyme (giving [1,1-$^2H_2$]dihydroxyacetone phosphate), the narrow bands previously at 1713 and 1732 cm^{-1} were shifted only slightly to 1712 and 1730 cm^{-1}, respectively. In a control experiment, dihydroxyacetone phos-

[2] In general, deuteration of an olefinic carbon atom reduces the double bond stretching frequency by 10–20 cm^{-1} (Pinchas & Laulicht, 1971).

phate incubated with triosephosphate isomerase in D_2O under the conditions of the infrared spectral measurements was shown to incorporate only one deuterium at C-1. These results demonstrate that both of the two new absorptions derive from dihydroxyacetone phosphate bound at the active site of the enzyme.

Discussion

The 19-cm^{-1} shift observed for the predominant band in the infrared spectrum of dihydroxyacetone phosphate bound to triosephosphate isomerase is quite substantial, and it is worth inquiring as to its origin.[3] The simplest and most appealing explanation is that it is due to the polarization of the substrate's carbonyl group by an electrophile on the enzyme, which is consistent with the substrate's ready reduction by borohydride (Webb & Knowles, 1974) and rapid enolization (Hall & Knowles, 1975) when bound to the enzyme. Supporting this view are preliminary X-ray crystallographic studies on the enzyme–substrate complex, which reveal that Lys-13 and His-95 lie very close to the carbonyl oxygen of dihydroxyacetone phosphate (Phillips et al., 1977; D. C. Phillips and I. A. Wilson, personal communication).

The hydroxyl group of enzyme-bound dihydroxyacetone phosphate is probably cisoid with respect to the carbonyl group, as this conformation should allow facile formation of the *cis*-enediol intermediate (Rose, 1962). Nevertheless, it seems unlikely that the substrate itself, using its α-hydroxyl group, could alone act as a more effective electrophile than water. The carbonyl stretching frequency in carbon tetrachloride of 5β-cholestan-5β-ol-6-one, in which the α-hydroxy ketone is locked in a cisoid conformation and forms an intramolecular hydrogen bond, is 14 cm^{-1} below that of its epimer, 5α-cholestan-5α-ol-6-one, in which an intramolecular hydrogen bond is geometrically precluded (Oki et al., 1968). The effect of the intramolecular hydrogen bond is reduced to 10 cm^{-1} when the small difference in the carbonyl frequencies of the parent ketones (5β- and 5α-cholestan-6-one) is taken into account (Jones & Kime, 1966). This difference is less than half of the shift observed when acetone is removed from carbon tetrachloride and allowed to hydrogen bond to water. It therefore seems unlikely that the effect on the carbonyl group of dihydroxyacetone phosphate when it binds to the enzyme is due to a frozen cisoid conformation, because replacement of hydrogen bonding to water with intramolecular hydrogen bonding should *raise* the carbonyl stretching frequency of the bound substrate.

An increase of about 9° in the C–C–C angle of the enzyme-bound substrate could also, in principle, lower its carbonyl stretching frequency by 19 cm^{-1} (Halford, 1956). Such an increase would, however, strain the substrate away from the ideal geometry of the transition state for hydride addition and would therefore be incompatible with the increased rate of borohydride reduction observed for dihydroxyacetone phosphate when bound to the enzyme (Webb & Knowles, 1974; Brown & Ichikawa, 1957). The same conclusion can be drawn by examining the π^* orbital (LUMO) of a similarly strained acetone molecule, which is expected to interact less favorably with an incoming hydride ion because of the LUMO's higher energy and smaller carbon p-orbital coefficient.

Alternatively, the carbonyl stretching frequency of the bound substrate might be influenced by the orientation or the ionization state of its phosphate group. While the spectrum of free dihydroxyacetone phosphate suggests that a transoid conformation of the phosphate group relative to the carbonyl group does not normally reduce the carbonyl stretching frequency by more than a few wavenumbers, a highly strained conformation with the phosphate group of the bound substrate in very close proximity to the carbonyl carbon might lower the stretching frequency by the requisite amount. However, without the participation of an electrophile, the introduction of a negative charge so near to the carbonyl carbon would be expected to impede, rather than enhance, the addition of a nucleophile such as borohydride.[4] The carbonyl stretching frequency is also relatively insensitive to the state of ionization of the phosphate group (see above) and would, if anything, be expected to *increase* somewhat under the influence of the phosphate binding site, which is likely to consist of a set of good hydrogen-bond donors (Belasco et al., 1978; D. C. Phillips and I. A. Wilson, personal communication).

Finally, there is the possibility that the observed shift in frequency results from partial C–H bond cleavage in the ground state of the enzyme–substrate complex which adds enolate character to the bound dihydroxyacetone phosphate. While this explanation is rather easily reduced to a philosophical question of causality resembling that of the chicken and the egg, it may also be disputed on the more empirical basis that in the absence of an enzymic electrophile, it once again is difficult to reconcile additional enolate character with a greater susceptibility to borohydride reduction.

It seems, therefore, that the strain observed for the predominant form of the complex of dihydroxyacetone phosphate with triosephosphate isomerase is primarily the result of the polarization of the substrate's carbonyl group by an enzymic electrophile more effective than water. The decrease in stretching frequency (19 cm^{-1}) is quite large, roughly 20% of that observed for acetone when it is fully protonated in concentrated sulfuric acid (Clemett, 1970). Not only will the strain induced by the electrophile be relieved in the transition state, but the electrophile should also serve to stabilize the negative charge that accumulates on the incipient enolate oxygen in the transition state and thereby further augment the enzyme's catalytic power.

There remains to be considered the origin of the second, minor carbonyl band for the bound substrate, which is hardly shifted relative to that of the substrate in free solution. While Fermi resonance unique to enzyme-bound dihydroxyacetone phosphate cannot be ruled out, the most likely explanation for the existence of two infrared bands for the bound substrate between 1700 and 1750 cm^{-1} is that there are two structurally distinct forms of the enzyme–substrate complex. For instance, the enzymic electrophile could, in principle, interact either with the substrate carbonyl, thereby polarizing it, or with the C-1 hydroxyl group, which would have a negligible effect on the carbonyl stretching frequency. Such a flip-flopping would not be unexpected since the electrophile may also be involved in polarizing the carbonyl group of the other substrate, glyceraldehyde 3-phosphate, in the reverse reaction. Alternatively, the two bands might arise from productively and nonproductively bound substrate rotamers that differ in the environment experienced by the carbonyl group. A nonproductive

[3] Because the conformation of enzyme-bound dihydroxyacetone phosphate is not yet established, the center of the carbonyl band for the free dianionic substrate is used as the point of reference for comparisons of stretching frequencies, with the caveat that doing so introduces an uncertainty in these comparisons of ±5 cm^{-1}.

[4] For a similar reason, while the negative charge of the active site base Glu-165 might contribute to the electric field that induces the polarization of the substrate's carbonyl group, it is unlikely to be solely responsible.

conformation of the enzyme–substrate complex, while unappealing to the biochemist accustomed to attributing infallibility to enzymes, might not be as deleterious as at first anticipated. Triosephosphate isomerase has evidently evolved to a state of virtual perfection as a catalyst (Albery & Knowles, 1976b), and a less than maximal rate of enolization can be accommodated without a significant reduction in the efficiency of catalysis of the overall reaction.

Acknowledgments

We are grateful to Dr. Mark Fisher for providing the yeast triosephosphate isomerase used in these experiments, to Professor Alex Nickon for stimulating discussions, and to Glen Graham for helping with the PRDDO calculations. The Fourier transform infrared spectrometer was obtained with funds from the National Science Foundation and the Materials Research Laboratory, Cambridge, MA.

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