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Polarization of Substrate Carbonyl Groups by Yeast Aldolase: Investigation by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The infrared spectrum of the complex of D-fructose 1,6-bisphosphate bound to yeast aldolase displays three spectral features between 1700 and 1800 cm^{-1}. One of these (at 1730 cm^{-1}) corresponds to the carbonyl group of enzyme-bound D-fructose 1,6-bisphosphate and/or dihydroxyacetone phosphate. The frequency of this band, which is unaffected by the removal of the intrinsic zinc ion from the enzyme, demonstrates that this carbonyl group is not significantly polarized when the substrate binds to the enzyme. In contrast, the spectral band assigned to the carbonyl group of enzyme-bound D-glyceraldehyde 3-phosphate (at 1706 cm^{-1}) appears at a frequency 24 cm^{-1} lower than when this substrate is in aqueous solution. This shift indicates considerable polarization of the carbonyl group when D-glyceraldehyde 3-phosphate is bound at the active site. The third spectral feature (at 1748 cm^{-1}), which is observed only in the presence of potassium ion, probably corresponds to an enzymic carboxyl group in a nonpolar environment.

Enolization is a common enzyme-catalyzed reaction, yet a detailed understanding of how enzymes accelerate this process is still lacking. One means that an enzyme might employ is the polarization of the substrate's carbonyl group by a strong electrophile. Such an interaction would facilitate proton abstraction from the adjacent carbon atoms by electronic distortion of the substrate toward the structure of the transition state for enolization and by stabilization of the developing negative change on the incipient enolate oxygen in this transition state.

While attractive as a hypothesis, direct evidence for carbonyl polarization exists for only one enzyme. This evidence is based on the knowledge that the polarization of a carbonyl group weakens the carbon-oxygen double bond, resulting in a reduction in the carbonyl stretching frequency. The detection of such a change in the vibrational character of a single carbonyl group among the hundreds present in an enzyme-substrate complex requires an exceptionally sensitive spectroscopic method, such as Fourier transform infrared spectroscopy. By this means, we have earlier shown that most molecules of dihydroxyacetone phosphate experience a substantial reduction in their carbonyl stretching frequency upon binding to triosephosphate isomerase, which can be interpreted as catalytically productive polarization induced by an enzymic electrophile (Belasco & Knowles, 1980).

Fructose-1,6-bisphosphate aldolase from yeast catalyzes the abstraction of an α proton from dihydroxyacetone phosphate and the condensation of the resulting carbanionic species with D-glyceraldehyde 3-phosphate to yield D-fructose 1,6-bisphosphate. The enzyme is a dimer, and each subunit bears one tightly bound zinc ion which is absolutely required for catalytic activity (Kobes et al., 1969; Harris et al., 1969). In addition, high concentrations of potassium ion elicit a roughly 7-fold rate enhancement (Richards & Rutter, 1961). Unlike the aldolases of higher eukaryotes, the mechanism of the yeast enzyme appears not to involve an enamine intermediate (Rutter, 1964; Stribling & Perham, 1973).

The catalytic role of the essential divalent metal ion has been a matter of special interest. On the basis of NMR studies using the Mn^{2+}-substituted enzyme, Smith & Mildvan (1981) have concluded that while the carbonyl group of dihydroxyacetone phosphate bound to aldolase is oriented toward the divalent metal ion, the distance between them (>6 Å) is too great for direct, inner-sphere, coordination (Smith et al., 1980). Nevertheless, they have argued that the zinc ion might still serve as an electrophile for the carbonyl group of dihydroxyacetone phosphate (and, presumably, that of fructose 1,6-

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bisphosphate) if there is an intervening histidine residue whose imidazole group relays the metal ion's positive charge (Smith et al., 1980; Smith & Mildvan, 1981).

This paper describes experiments using Fourier transform infrared spectroscopy that were designed to examine *directly* the environments of the carbonyl groups of D-fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and D-glyceraldehyde 3-phosphate bound to yeast aldolase. We have found that whereas the carbonyl group of enzyme-bound D-glyceraldehyde 3-phosphate is considerably polarized, that of D-fructose 1,6-bisphosphate is not. Moreover, we have observed no change in the stretching frequency of the carbonyl group of D-fructose 1,6-bisphosphate upon removal of the zinc ion from the enzyme–substrate complex. Finally, we have detected a substrate-induced perturbation of the enzyme that occurs only in the presence of potassium ion.

Materials and Methods

Aldolase was prepared from fresh bakers' yeast (Federal Yeast Corp., Weymouth, MA) as described below. The enzyme was assayed by measuring the rate of enzyme-catalyzed cleavage of fructose 1,6-bisphosphate. A typical cleavage assay was initiated by the addition of yeast aldolase (approximately 0.01 unit) to a solution at 30 °C of 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] (NH_4^+ salt) buffer, pH 7.3, containing potassium acetate (100 μmol), triosephosphate isomerase (30 units), α-glycerophosphate dehydrogenase (10 units), fructose bisphosphate (2.1 μmol), and NADH (0.3 μmol), in a total volume of 1.065 mL. The rate of decrease in the absorbance at 340 nm was recorded. An enzyme unit is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of fructose bisphosphate per min under saturating conditions. The coupling enzymes, triosephosphate isomerase and α-glycerophosphate dehydrogenase, were dialyzed exhaustively against 50 mM triethanolammonium acetate buffer, pH 7.3, at 4 °C before use. A value for $A_{280nm}^{0.1\%}$ of 1.02 was assumed for yeast aldolase (Harris et al., 1969). Yeast aldolase rapidly loses activity at concentrations below 1 mg/mL. This loss is partially alleviated by dilution of the enzyme in 50 mM Hepes buffer, pH 7.3, containing 2-mercaptoethanol (10 mM) and glycerol (10% v/v) at 4 °C.

Triosephosphate isomerase (chicken muscle) was prepared according to the methods of Putman et al. (1972) and McVittie et al. (1972) and assayed by the method of Plaut & Knowles (1972). In the infrared experiments, isomerase from yeast was used, prepared according to Norton & Hartman (1972). α-Glycerophosphate dehydrogenase (yeast), hexokinase (yeast), phosphoglucose isomerase (yeast), phosphofructokinase (rabbit muscle), fructose 1,6-bisphosphatase (rabbit muscle), and glucose-6-phosphate dehydrogenase (yeast) were obtained (as crystalline suspensions in ammonium sulfate) from the Sigma Chemical Co., St. Louis, MO. ATP, NADH, $NADP^+$, dihydroxyacetone phosphate (as the dicyclohexylammonium salt of the dimethyl ketal), DL-glyceraldehyde 3-phosphate (as the barium salt of the diethyl acetal), D-fructose 1,6-bisphosphate (as the trisodium salt), Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked), and DEAE-Sephadex (A-25-120) were also obtained from Sigma. DEAE-cellulose (DE-52) was obtained from Whatman Inc., Clifton, NJ. Deuterium oxide (99.7%) was obtained from Merck & Co., Rahway, NJ. Sodium [^{13}C]cyanide (97.5%) was obtained from Prochem, Summit, NJ. D-[U-^{13}C]Glucose (83%) was obtained from Stohler Isotope Chemicals, Waltham, MA. Potassium acetate was obtained from the Fisher Scientific Co., Fair Lawn, NJ. Centriflo CF25 ultrafiltration membrane cones were obtained from the Amicon Corp., Lexington, MA. Phosphoglycolohydroxamate was obtained as a gift from Dr. D. Ponzi and Professor G. Petsko.

D-Fructose 1,6-bisphosphate was assayed by enzymic conversion either to *sn*-glycerol 3-phosphate or to 6-phosphogluconate. Dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate were assayed by enzymic reduction to *sn*-glycerol 3-phosphate. Glycolaldehyde phosphate was assayed by hydrolysis with alkaline phosphatase and reduction to ethylene glycol by yeast alcohol dehydrogenase.

Yeast aldolase was purified by a modification of the method of Jack (1973). All operations were done at 4 °C unless stated otherwise. The following buffer solutions were used. Buffer A: A solution of 0.3 M potassium hydrogen phosphate (1.5 L) was freed of heavy-metal contaminants by the addition of a saturating amount (approximately 0.1 g) of 8-hydroxyquinoline. The solution was stirred at room temperature for 30 min and then washed repeatedly (5 × 300 mL) with redistilled methylene chloride. N_2 was then bubbled through the solution to remove residual organic solvent. Immediately before use, α-toluenesulfonyl fluoride (0.1 M) in ethanol (1.5 mL) and 2-mercaptoethanol (1.1 mL) were added. Buffer B: A solution of 20 mM Tris-HCl buffer, pH 7.5, containing glycerol (5% v/v) was prepared. Before use, 2-mercaptoethanol and α-toluenesulfonyl fluoride were added to final concentrations of 10 and 0.1 mM, respectively. Buffer C: A solution of 100 mM Tris-HCl buffer, pH 7.5, containing glycerol (5% v/v) was prepared. Before use, 2-mercaptoethanol and α-toluenesulfonyl fluoride were added to final concentrations of 10 and 0.1 mM, respectively.

Bakers' yeast (1.3 kg) was immersed in liquid nitrogen for 30 min, allowed to thaw at room temperature for 2 h, and then mechanically stirred with buffer A (1.25 L) overnight. The light-brown slurry was centrifuged at 11000*g* for 1 h. To the supernatant (1460 mL), solid ammonium sulfate (613 g) was slowly added with stirring over 3 h to approximately 65% saturation. The mixture was stirred for a further 3.5 h and then centrifuged at 11000*g* for 1 h. To the supernatant (1650 mL, containing 55% of the aldolase activity) was added saturated ammonium sulfate solution (495 mL) dropwise with stirring over 30 min, and the suspension was then stirred for 20 min. The precipitate was removed by centrifugation at 11000*g* for 1 h. A further addition of saturated ammonium sulfate solution (794 mL) was then made over 1.25 h, to a final 85% saturation. After the addition of 2-mercaptoethanol (0.5 mL), the suspension was allowed to stand overnight. Centrifugation for 1 h at 11000*g* gave a tan pellet (containing 37% of the initial aldolase activity), which was dissolved in a minimal amount of buffer B (178 mL). This solution was dialyzed against three changes of buffer B (2.0 L) overnight.

The aldolase solution was diluted with 1.5 volumes of buffer B and applied to a column (5.3 cm^2 × 26 cm) of DEAE-cellulose (DE-52) previously equilibrated with buffer B. The column was washed with buffer B until protein elution (as monitored at 280 nm) had ceased. A linear salt gradient (750 + 750 mL, 50–300 mM potassium chloride in buffer B) was begun, and fractions (20 mL) were collected. Fractions containing enzyme of specific catalytic activity greater than 110 units/mg were pooled, and the solution (containing 18% of the initial aldolase activity) was concentrated to 6 mL in

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl.

an Amicon Centriflo centrifugation membrane cone.

The concentrated enzyme solution was applied to a column (5.3 cm^2 × 80 cm) of Sephadex G-150 equilibrated with buffer C, and eluted with the same buffer. Fractions containing aldolase of specific activity greater than 140 units/mg were pooled. Glycerol was added to a final concentration of 20% (v/v). The overall yield of purified aldolase was 12%. The purified enzyme (116 mg) was >95% homogeneous by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (Laemmli, 1970). The specific activity was 150 units/mg (≅100 s^{-1}). The enzyme contained less than 0.001% triosephosphate isomerase on an activity basis. The enzyme was stored frozen at −70 °C in 85 mM Tris-HCl buffer, pH 7.5, containing 2-mercaptoethanol (8 mM), α-toluenesulfonyl fluoride (0.1 mM), and glycerol (20% v/v). Under these storage conditions, the enzyme retains full activity indefinitely (>1.5 years).

D-[*U*-^{13}*C*]*Fructose 1,6-Bisphosphate.* D-[U-^{13}C]Glucose (46.6 mg, 4.1 mM) was phosphorylated at room temperature in a reaction mixture (61 mL) containing ATP (11.4 mM), magnesium chloride (4 mM), hexokinase (9 units/mL), phosphoglucose isomerase (21 units/mL), and phosphofructokinase (4 units/mL) in 57 mM ammonium bicarbonate buffer, pH 7.8. After 80 min, nucleotides were removed by six successive treatments with charcoal (1 g), followed each time by filtration. The solvent was removed by evaporation, and the residue was treated with methanol (30 mL) in order to inactivate the enzymes. After removal of the methanol by evaporation, the residue was dissolved in water (15 mL) and treated with Dowex 50 (H^+) (3.5 mL), and the solution was filtered. The filtrate was titrated to pH 7.5 with 2 N sodium hydroxide, diluted to 100 mL with water, and applied to a column (5.3 cm^2 × 11 cm) of DEAE-Sephadex (58 mL) equilibrated at 3 °C with 50 mM ammonium bicarbonate. After the column was washed with 50 mM ammonium bicarbonate (125 mL), a linear gradient of ammonium bicarbonate (750 + 750 mL, 50–800 mM) was applied, and fractions (15 mL) were collected. Fractions containing D-[U-^{13}C]fructose 1,6-bisphosphate were pooled and treated with Dowex 50 (H^+) (30 mL), and the solution was filtered. The filtrate was titrated to pH 5.7, concentrated by rotary evaporation, and stored frozen at −70 °C: yield 216 μmol (86%).

D-[*3,4*-$^{13}C_2$]*Fructose 1,6-Bisphosphate.* DL-[1-^{13}C]-Glyceraldehyde 3-phosphate was prepared by the method of Serianni et al. (1979) and purified by ion-exchange chromatography on DEAE-Sephadex (Serianni et al., 1979). Fractions containing glyceraldehyde 3-phosphate and free of glycolaldehyde phosphate were pooled, treated with excess Dowex 50 (H^+), filtered, and concentrated by evaporation. DL-[1-^{13}C]Glyceraldehyde 3-phosphate (2.63 mL, containing 0.3 mmol of the D enantiomer) was titrated to pH 7.0 with 6 N sodium hydroxide, and potassium acetate (0.32 mmol) was added. The final concentration of D-[1-^{13}C]glyceraldehyde 3-phosphate was 85 mM. Triosephosphate isomerase (200 units) and yeast aldolase (10 units) were added, and the reaction was allowed to proceed at room temperature. The progress of the reaction was monitored by assaying for D-fructose 1,6-bisphosphate. When the reaction had gone to completion (20 min), the solvent was evaporated, methanol added, and the solution again taken to dryness. The solid residue was dissolved in 0.5 M HCl (2 mL), and the solution was applied to a column of Dowex 50 (H^+) (1.2 mL) and eluted with water (5 mL). The eluate was titrated to pH 7.1 and purified by ion-exchange chromatography on DEAE-Sephadex (bicarbonate) as described above. Fractions containing [3,4-$^{13}C_2$]fructose 1,6-bisphosphate were pooled, treated with Dowex 50 (H^+) (35 mL), and filtered. The filtrate was titrated to pH 5.7, concentrated by evaporation, passed through a small column of charcoal (0.12 mL), and then stored frozen at −70 °C: yield 71 μmol (47%).

Dihydroxy[*U*-^{13}*C*]*acetone phosphate* was prepared by enzyme-catalyzed cleavage of D-[U-^{13}C]fructose 1,6-bisphosphate. To 5 mM ammonium bicarbonate buffer (250 mL) was added D-[U-^{13}C]fructose 1,6-bisphosphate (20 μmol, 80 μM), yeast aldolase (70 units), and triosephosphate isomerase (100 units). After 2 h at room temperature, the reaction was quenched with Dowex 50 (H^+) (4 g). The solution was cooled to 3 °C and passed through a column (10 mL) of Dowex 50 (H^+). The eluate was concentrated to 8 mL by evaporation, treated with methanol (120 mL), and concentrated again to 8 mL. This solution was filtered, diluted with 50 mM ammonium bicarbonate buffer, and applied to a column (1 cm^2 × 5 cm) of DEAE-Sephadex (5 mL) equilibrated at 3 °C with 50 mM ammonium bicarbonate. After the column was washed with 25 mM ammonium bicarbonate (20 mL), a linear gradient of ammonium bicarbonate (40 + 40 mL, 25–200 mM) was applied, and fractions (2 mL) were collected. Fractions containing dihydroxy[U-^{13}C]acetone phosphate were pooled, treated with Dowex 50 (H^+) (4 mL), and filtered. The filtrate was titrated to pH 4.7 and freeze-dried. The solid was redissolved in water (1 mL) and stored frozen at −70 °C: yield 26 μmol (65%). Dihydroxy[U-^{13}C]acetone phosphate prepared in this manner contained an equilibrium amount (4%) of D-[U-^{13}C]glyceraldehyde 3-phosphate.

Dihydroxy[*1*-^{13}*C*]*acetone phosphate* was prepared in a similar manner from D-[3,4-$^{13}C_2$]fructose 1,6-bisphosphate. Mass spectral analysis of the tetrakis(trimethylsilyl) derivative after reduction with sodium borohydride (Fisher et al., 1976) demonstrated that the dihydroxy[1-^{13}C]acetone phosphate thus prepared (and the D-[3,4-$^{13}C_2$]fructose 1,6-bisphosphate from which it was derived) was regiospecifically labeled with an isotopic enrichment of >95%.

Ultraviolet measurements were made with a Perkin-Elmer 554 spectrophotometer. Mass spectrometry was carried out on an AEI MS-9 instrument. 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).

Infrared measurements were made at 2-cm^{-1} resolution by 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 substrate (or inhibitor) in the presence of yeast aldolase were obtained from the ratio of a single-beam spectrum of enzyme plus substrate to a single-beam spectrum of a reference sample containing only enzyme at the same concentration. For all single-beam spectra, 500 interferograms were averaged.

A concentrated solution of native aldolase in ^2H_2O was prepared by repeatedly concentrating the enzyme solution by Centriflo ultrafiltration and rediluting with ^2H_2O containing 2-mercaptoethanol (2 mM). The protein solution was finally concentrated to about 90 mg/mL. A concentrated solution of apoaldolase in ^2H_2O was prepared from a yeast aldolase solution containing EDTA (25 mM) by repeatedly concentrating the solution by Centriflo ultrafiltration and rediluting with ^2H_2O containing EDTA (88 mM) and 2-mercaptoethanol (2 mM). The solution was finally concentrated to about 60–70

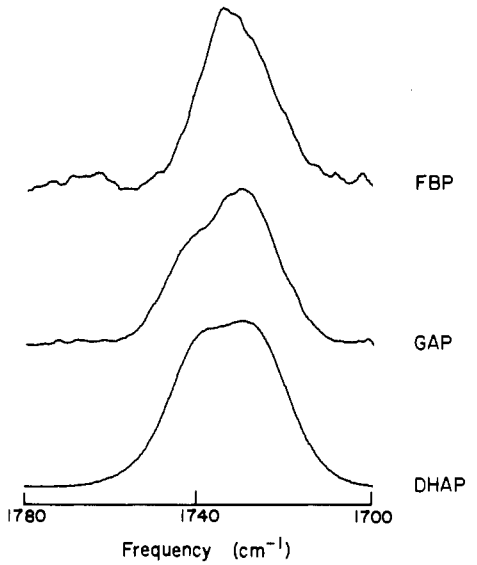

FIGURE 1: Spectra of the free substrates of aldolase. (Upper spectrum) D-Fructose 1,6-bisphosphate (tetrasodium salt, 580 mM) in ^2H_2O at 20 °C; (middle spectrum) DL-glyceraldehyde 3-phosphate (monosodium salt, 360 mM) in ^2H_2O at 25 °C; (lower spectrum) dihydroxyacetone phosphate (disodium salt, 7.4 mM) in ^2H_2O at 1 °C. (FBP, fructose bisphosphate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate.)

mg/mL. Apoenzyme prepared in this manner was less than 2% active after withdrawal from the infrared cell following an infrared measurement. Such samples could be reconstituted with zinc acetate almost to full activity (>90%). Substrates were prepared for infrared spectroscopy by titration to pH 7 (with the exception of DL-glyceraldehyde 3-phosphate, which was titrated to pH 4), freeze-drying, and redissolution in a small volume of ^2H_2O.

Results

The infrared absorption spectra of fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate are all quite similar in the carbonyl region (Figure 1). Fructose 1,6-bisphosphate, of which only 2% exists in the linear keto form at room temperature (Midelfort et al., 1976), has a carbonyl absorption at 1733 cm^{-1} ($w_{1/2}$ = 20 cm^{-1}). The carbonyl band of dihydroxyacetone phosphate [39% of which exists in the keto form at 1 °C (Webb et al., 1977)] is also centered at 1733 cm^{-1} ($w_{1/2}$ = 29 cm^{-1}), while that of glyceraldehyde 3-phosphate [only 3% aldehyde at 26 °C (Trentham et al., 1969)] appears at 1730 cm^{-1} ($w_{1/2}$ = 26 cm^{-1}). Dihydroxy[U-^{13}C]acetone phosphate has an absorption maximum at 1689 cm^{-1}. This reduction by 43 cm^{-1} of the carbonyl stretching frequency is near to the expected ~40-cm^{-1} shift for ^{13}C substitution at a carbonyl group (Pinchas & Laulicht, 1971), and similar behavior for [U-^{13}C]glyceraldehyde 3-phosphate, and [1-^{13}C]glyceraldehyde 3-phosphate is expected. On the other hand, ^{13}C substitution adjacent to the carbonyl group as in dihydroxy[1-^{13}C]acetone phosphate (and, presumably, in [3,4-$^{13}C_2$]fructose 1,6-bisphosphate) lowers the carbonyl stretching frequency by only two wavenumbers or so.

The ratio of a single-beam spectrum of a solution in 100 mM potassium acetate of D-fructose 1,6-bisphosphate (7.4 mM) and yeast aldolase (77 mg/mL, 1.9 mN in subunits) to a spectrum of a solution in potassium acetate containing only the enzyme at the same concentration gave three bands between 1700 and 1800 cm^{-1} (Figure 2). These bands were located at 1748 ($w_{1/2}$ = 12 cm^{-1}), 1730 ($w_{1/2}$ = 12 cm^{-1}), and

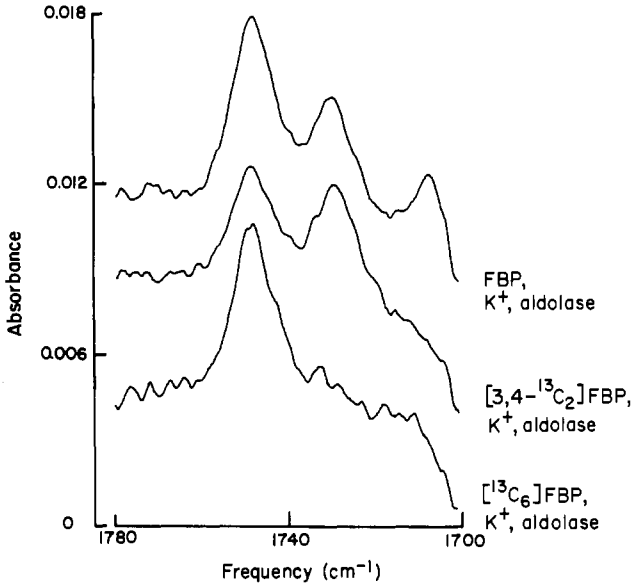

FIGURE 2: Spectra of fructose bisphosphate bound to aldolase. (Upper spectrum) Unlabeled D-fructose 1,6-bisphosphate (7.4 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.6, 1 °C; (middle spectrum) D-[3,4-$^{13}C_2$]fructose 1,6-bisphosphate (7.9 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 8.0, 1 °C; (lower spectrum) D-[U-^{13}C]fructose 1,6-bisphosphate (7.8 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.6, 1 °C. Each spectrum is a ratio against a reference spectrum of aldolase (1.93 mN) and KOAc (100 mM) in ^2H_2O at pD 7.4, 1 °C. (FBP, fructose bisphosphate.)

1706 cm^{-1} ($w_{1/2}$ = 6 cm^{-1}) and, on the basis of their frequencies and half-widths, did not correspond to any bands in the spectra of the unliganded enzyme or any of the substrates. The intensities of the three bands were independent (for at least 1 h) of the time interval between mixing the enzyme and substrate and measuring the spectrum.

The experiment was repeated with D-fructose 1,6-bisphosphate isotopically labeled at all six carbon atoms, to establish the chemical identity of the species responsible for the three absorptions shown in Figure 2. In the spectrum of D-[U-^{13}C]fructose 1,6-bisphosphate and yeast aldolase in 100 mM potassium acetate, only the band at 1748 cm^{-1} remained (Figure 2). This absorption must therefore derive from an enzyme group whose carbonyl stretching frequency is perturbed by the binding of substrate. The other two bands, which must be substrate absorptions, were presumably shifted ~40 cm^{-1} to frequencies below 1700 cm^{-1} by the isotopic substitution. However, they 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}. At the substrate concentrations used in the measurement, absorptions due to the carbonyl forms of all three substrates *not* bound to the enzyme would have been undetectable. [The overall equilibrium constant (8.1 × 10^{-5} M at 30 °C) substantially favors D-fructose 1,6-bisphosphate, but this substrate exists predominantly as a cyclic hemiketal (see above).] Consequently, the bands at 1730 and 1706 cm^{-1} must both be due to enzyme-bound substrate. This interpretation is consistent with the observed narrow half-widths, which appear to be a common feature of *enzyme-bound* species (Fisher et al., 1980). To confirm this view, the spectrum of D-fructose 1,6-bisphosphate plus aldolase in 100 mM potassium acetate was measured in the presence of the potent competitive inhibitor phosphoglycolohydroxamate (Collins, 1974; Lewis & Lowe, 1973) at a concentration adequate to displace all substrate from the active site (Figure 3). Under these conditions, both the 1730-

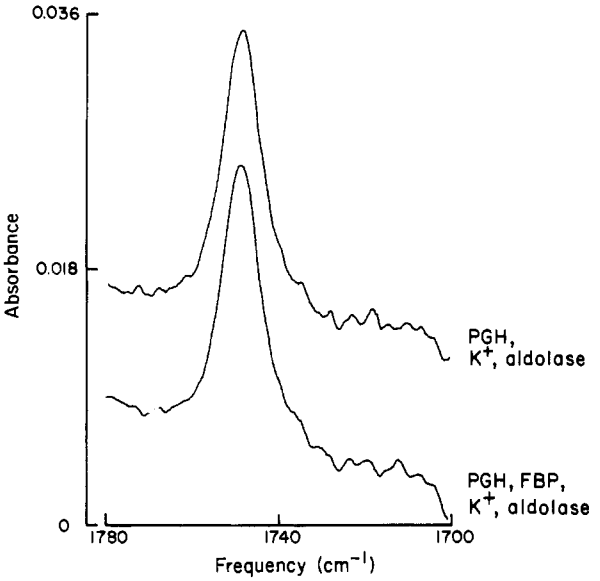

FIGURE 3: Spectra of aldolase with phosphoglycolohydroxamate, in the presence and absence of fructose bisphosphate. (Upper spectrum) Phosphoglycolohydroxamate (7.5 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.5, 1 °C; (lower spectrum) phosphoglycolohydroxamate (7.5 mM), unlabeled D-fructose 1,6-bisphosphate (7.4 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.6, 1 °C. Each spectrum is a ratio against a reference spectrum of aldolase (1.93 mN) and KOAc (100 mM) in ^2H_2O at pD 7.4, 1 °C. (PGH, phosphoglycolohydroxamate; FBP, fructose bisphosphate).

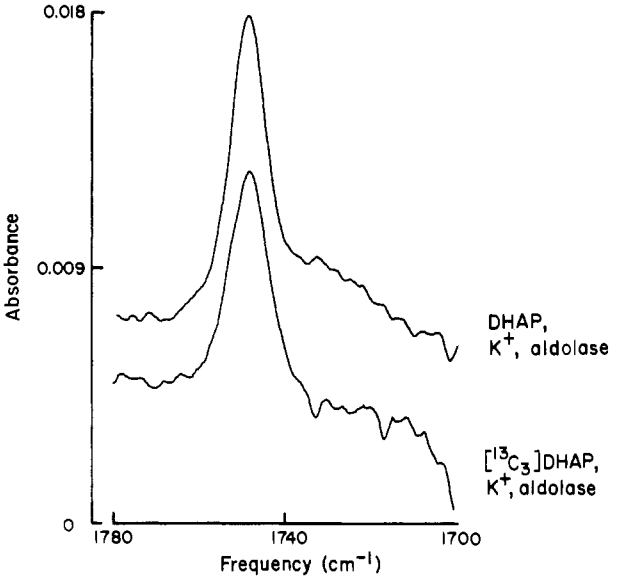

FIGURE 4: Spectra of dihydroxyacetone phosphate bound to aldolase. (Upper spectrum) Unlabeled dihydroxyacetone phosphate (4.0 mM), aldolase (1.60 mN), and KOAc (100 mM) in ^2H_2O at pD 7.1, 1 °C, as a spectral ratio against aldolase (1.60 mN) and KOAc (100 mM) in ^2H_2O at pD 7.0, 1 °C; (lower spectrum) dihydroxy[U-^{13}C]acetone phosphate (4.8 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.5, 1 °C, as a spectral ratio against aldolase (1.93 mN) and KOAc (100 mM) in ^2H_2O at pD 7.4, 1 °C. (DHAP, dihydroxyacetone phosphate.)

and 1706-cm^{-1} bands were abolished, as expected if these arise from absorptions of enzyme-bound substrate. Interestingly, the enzyme band at 1748 cm^{-1} remained. [This band also appears in the spectrum of enzyme plus phosphoglycolohydroxamate in the complete absence of substrate (Figure 3), indicating that the inhibitor perturbs the enzyme in a manner analogous to D-fructose 1,6-bisphosphate.]

The spectrum of specifically labeled D-[3,4-$^{13}C_2$]fructose 1,6-bisphosphate plus aldolase in 100 mM potassium acetate was measured (Figure 2) to identify more precisely the enzyme-bound species responsible for the bands at 1730 and 1706 cm^{-1}. In this case, the bands at 1748 and 1730 cm^{-1} were present, but the band at 1706 cm^{-1} was shifted out of view to lower frequency. Labeling of fructose 1,6-bisphosphate at C-3 and C-4 is not expected to affect its carbonyl stretching frequency significantly, nor should such labeling have much effect on the carbonyl stretching frequency of the cleavage product, dihydroxy[1-^{13}C]acetone phosphate. On the other hand, the carbonyl group of the other cleavage product, D-[1-^{13}C]-glyceraldehyde 3-phosphate, will be isotopically labeled and should therefore exhibit a substantially reduced stretching frequency. We may therefore assign the band at 1706 cm^{-1} to the carbonyl group of D-glyceraldehyde 3-phosphate bound to aldolase, while that at 1730 cm^{-1} must derive either from enzyme-bound D-fructose 1,6-bisphosphate or from bound dihydroxyacetone phosphate. For confirmation of these assignments, triosephosphate isomerase (to 7 μN) was added to a solution of fructose bisphosphate plus aldolase in ^2H_2O (under the conditions of the upper spectrum of Figure 2). Under these conditions, the substrate species are D-[1,2-$^2H_2$]glyceraldehyde 3-phosphate, [1,1-$^2H_2$]dihydroxyacetone 3-phosphate, and D-[3,4,5-$^2H_3$]fructose 1,6-bisphosphate. While deuterium substitution will shift the carbonyl absorption of the two ketonic substrates by only 1–2 cm^{-1} to lower frequencies compared to the nondeuterated analogues, deuteration of the aldehydic carbon of glyceraldehyde phosphate results in a reduction in stretching frequency of about 12 cm^{-1} (Belasco & Knowles, 1980). In accord with these expectations, the addition of a catalytic amount of isomerase to the solution that gives the upper spectrum of Figure 2 causes the disappearance of the absorption at 1706 cm^{-1} (data not shown). It is evident, then, that substitution either of ^{13}C or of ^{2}H in the aldehyde group of glyceraldehyde phosphate moves the absorption at 1706 cm^{-1} to frequencies below 1700 cm^{-1}.

In an effort to determine whether the band at 1730 cm^{-1} derived from enzyme-bound D-fructose 1,6-bisphosphate or from enzyme-bound dihydroxyacetone phosphate, the infrared spectrum of dihydroxyacetone phosphate plus aldolase was measured in the absence of D-glyceraldehyde 3-phosphate, thereby precluding the formation of D-fructose 1,6-bisphosphate (Figure 4). Only the enzyme band at 1748 cm^{-1}, superimposed on a broad, weak absorption due to free dihydroxyacetone phosphate (Figure 1), was observed. As expected, the 1748-cm^{-1} band was unperturbed when the experiment was repeated with dihydroxy[U-^{13}C]acetone phosphate (Figure 4). While we may conclude that dihydroxyacetone phosphate, like D-fructose 1,6-bisphosphate and phosphoglycolohydroxamate, induces the enzyme perturbation manifested as the absorption at 1748 cm^{-1}, the absence of a band attributable to bound dihydroxyacetone phosphate does not allow a more precise assignment of the band at 1730 cm^{-1} observed in the spectrum of D-fructose 1,6-bisphosphate plus aldolase (see below).

For examination of the roles of metal ions in yeast aldolase, the spectrum of fructose 1,6-bisphosphate plus aldolase was measured in the absence of the activating mono- and dications. When sodium ion was substituted for potassium ion, only the band at 1730 cm^{-1} was evident (Figure 5). This result demonstrates that the substrate-induced perturbation responsible for the enzyme absorption at 1748 cm^{-1} depends on the presence of potassium ion but seems to rule out a role for this ion in contributing to the environment of the carbonyl group absorbing at 1730 cm^{-1}.

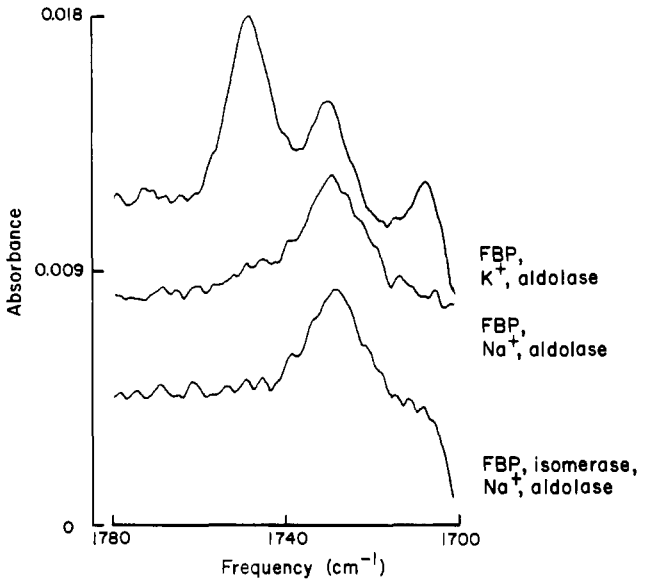

FIGURE 5: Effect of potassium ion on the spectrum of aldolase-bound fructose bisphosphate. (Upper spectrum) D-Fructose 1,6-bisphosphate (7.4 mM), aldolase (1.93 mN), and KOAc (100 mM) in ^2H_2O at pD 7.6, 1 °C, as a spectral ratio against aldolase (1.93 mN) and KOAc (100 mM) in ^2H_2O at pD 7.4, 1 °C; (middle spectrum) D-fructose 1,6-bisphosphate (6.3 mM), aldolase (1.63 mN), and NaOAc (100 mM) in ^2H_2O at pD 7.5, 2 °C, as a spectral ratio against aldolase (1.63 mN) and NaOAc (100 mM) in ^2H_2O at pD 7.4, 2 °C; (lower spectrum) D-fructose 1,6-bisphosphate (6.2 mM), aldolase (1.60 mN), triosephosphate isomerase (7.7 μN), and NaOAc (100 mM) in ^2H_2O at pD 7.5, 2 °C, as a spectral ratio against aldolase (1.63 mN) and NaOAc (100 mM) in ^2H_2O at pD 7.5, 2 °C. (FBP, fructose bisphosphate.)

The spectrum of zinc-free apoaldolase plus fructose 1,6-bisphosphate in 100 mM potassium acetate was also measured (Figure 6). The most notable feature of this spectrum was the continued presence of the substrate absorption at 1730 cm^{-1}, unperturbed by the absence of zinc in the enzyme's active site. The enzyme absorption at 1748 cm^{-1} was also retained in the spectrum of the apoenzyme–substrate complex. As expected, the band at 1730 cm^{-1} was nearly absent in a spectrum of apoaldolase plus D-[U-^{13}C]fructose 1,6-bisphosphate (83% ^{13}C enriched) but was retained in a spectrum of apoaldolase plus D-[3,4-$^{13}C_2$]fructose 1,6-bisphosphate, confirming its assignment to the carbonyl group of fructose 1,6-bisphosphate and/or dihydroxyacetone phosphate bound to the apoenzyme.

Discussion

Three conclusions can be drawn from the infrared spectra of the enzyme-bound substrates of yeast aldolase. First, the carbonyl group of D-fructose 1,6-bisphosphate and/or of dihydroxyacetone phosphate (hereafter referred to as the C-2 carbonyl group) is essentially unperturbed by binding to either the holo- or the apoenzyme. On the other hand, the carbonyl group of enzyme-bound D-glyceraldehyde 3-phosphate is substantially polarized relative to that of free glyceraldehyde 3-phosphate in aqueous solution. Third, the binding of substrate in the presence of potassium ion results in a perturbation of aldolase, which is manifested as a new absorption in the infrared spectrum of the liganded enzyme and in a change in the relative proportions of bound substrates.

C-2 Carbonyl Group. The substrate carbonyl group absorbing at 1730 cm^{-1} in the spectrum of D-fructose 1,6-bisphosphate plus yeast aldolase can be assigned to enzyme-bound D-fructose 1,6-bisphosphate and/or dihydroxyacetone phosphate. The absence of this band in the infrared spectrum of

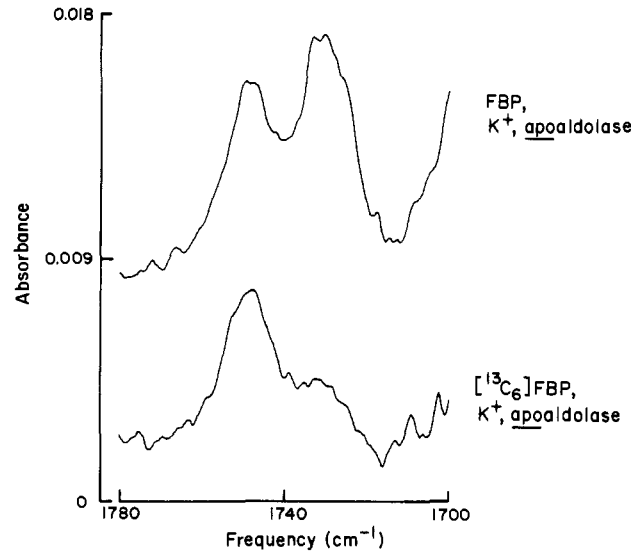

FIGURE 6: Spectra of fructose bisphosphate bound to apoaldolase. (Upper spectrum) Unlabeled D-fructose 1,6-bisphosphate (7.8 mM), aldolase (1.30 mN), KOAc (100 mM), and EDTA (74 mM) in ^2H_2O, pD 7.85, 1 °C; (lower spectrum) D-[U-^{13}C]fructose 1,6-bisphosphate (8.6 mM), aldolase (1.30 mN), KOAc (100 mM), and EDTA (74 mM) in ^2H_2O, pD 7.85, 1 °C. Each spectrum is a ratio against a reference spectrum of aldolase (1.30 mN), KOAc (100 mM), and EDTA (74 mM) in ^2H_2O, pD 7.85, 1 °C. (FBP, fructose bisphosphate.)

dihydroxyacetone phosphate plus aldolase (see below) eliminates the possibility that it is an absorption of dihydroxyacetone phosphate in a *binary* complex with aldolase. However, because the same carbon atom contributes to the carbonyl groups of both D-fructose 1,6-bisphosphate and its cleavage product, dihydroxyacetone phosphate, isotopic labeling cannot distinguish the carbonyl group of the former bound to aldolase in a binary complex from that of the latter bound to the enzyme in a ternary complex including D-glyceraldehyde 3-phosphate. It is, nevertheless, reasonable to suppose that the enzymic environments of these two carbonyl groups are much the same due to the similarity of dihydroxyacetone phosphate and the carbonyl-containing half of D-fructose 1,6-bisphosphate in terms of structure, binding site, and mechanism (i.e., aldol cleavage resembles enolization). Consequently, whatever can be ascertained about the environment of the carbonyl group of either one of these enzyme-bound substrates is likely to pertain to both.

The most striking feature of the enzyme-bound C-2 carbonyl group is that its stretching frequency (1730 cm^{-1}) is virtually the same as those of the carbonyl groups of free D-fructose 1,6-bisphosphate and free dihydroxyacetone phosphate in aqueous solution (1733 cm^{-1}). Clearly, if there is an enzymic electrophile interacting with the C-2 carbonyl group, it is no more effective than water in its ability to perturb the electron distribution of that carbonyl group.

This result is in sharp contrast to that of a similar study of the environment of the carbonyl group of dihydroxyacetone phosphate bound to triosephosphate isomerase. In that case, the predominant infrared band for the enzyme-bound substrate appeared at 1713 cm^{-1}, indicating substantial polarization of the carbonyl group by a potent enzymic electrophile[2] (Belasco & Knowles, 1980). Why should two enzymes, both of which catalyze the enolization of dihydroxyacetone phosphate, behave

[2] We have recently confirmed our infrared band assignments in the experiments with triosephosphate isomerase by using dihydroxyacetone phosphate specifically labeled at C-1 with ^{13}C. These assignments had previously been made by utilizing [1(*S*)-^{2}H]dihydroxyacetone phosphate.

so differently? One important difference between the mechanisms of these enzymes is that the conversion of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate catalyzed by triosephosphate isomerase must proceed via a protonated enediol intermediate, whose formation requires the interaction of a general acid catalyst with the carbonyl group of the substrate. Aldolase, in contrast, need not provide a proton donor for the carbonyl group of dihydroxyacetone phosphate because an anionic enediol*ate* intermediate is all that is required (and may, in fact, be preferable) for condensation with D-glyceraldehyde 3-phosphate to yield D-fructose 1,6-bisphosphate. Nevertheless, it remains unclear whether the catalytic efficiency of yeast aldolase is enhanced or diminished by its failure to polarize the C-2 carbonyl group. Additional infrared investigations of other isomerases and aldolases will be necessary in order to determine whether a pattern exists for these two mechanistically related classes of enzymes.

Smith and Mildvan have proposed that the zinc ion of yeast aldolase serves as an electrophile for the carbonyl group of dihydroxyacetone phosphate by polarizing an intervening histidine residue (Smith et al., 1980; Smith & Mildvan, 1981). However, we have observed *no change* in the carbonyl stretching frequency of the enzyme-bound C-2 carbonyl group when either the zinc ion is removed or potassium ion is replaced by sodium ion. Since one would have expected this carbonyl stretching frequency to increase in response to the loss of the enzymic electrophile, our data would appear to argue against such a role for either the zinc or the potassium ion. One way these results might be reconciled with the proposal that zinc acts indirectly as an electrophile for the C-2 carbonyl group would be to postulate that the loss of the zinc ion is compensated for by the protonation of the putative intervening histidine residue, whose pK_a might be higher in the absence of the metal ion. If an imidazolium ion and a zinc-coordinated imidazole group were equally effective electrophiles, the carbonyl stretching frequency of the bound substrate would be unaffected by the removal of the divalent metal ion. Even if this suggestion is true, it leaves unresolved the reason for the nearly complete lack of catalytic activity exhibited by the apoenzyme (Kobes et al., 1969).

The curious absence of a detectable substrate carbonyl absorption in the infrared spectrum of the binary complex of aldolase and dihydroxyacetone phosphate is most likely due to the fact that only a small fraction of enzyme-bound substrate is in the keto form. Aldolase may be able to bind one or both of the cyclic hemiketal anomers of D-fructose 1,6-bisphosphate and to catalyze their ring opening, yielding the reactive linear keto form (Schray et al., 1975; Midelfort et al., 1976). It is therefore reasonable to suppose, by structural and mechanistic analogy, that aldolase can also bind the hydrated form of dihydroxyacetone phosphate (there is evidence for this: Mildvan et al., 1971) and catalyze its dehydration, producing the keto form. If, in a binary complex with aldolase, the equilibrium between the hydrate and keto forms of dihydroxyacetone phosphate substantially favors the former, it would be difficult to detect any carbonyl band in the infrared spectrum of enzyme-bound dihydroxyacetone phosphate. Similarly, the substrate carbonyl absorption would be quite weak if, on the surface of the enzyme, the enediol(ate) form of dihydroxyacetone phosphate is significantly more stable than the keto form.

Carbonyl Group of D-*Glyceraldehyde 3-Phosphate.* The carbonyl group of D-glyceraldehyde 3-phosphate in a ternary complex with yeast aldolase and dihydroxyacetone phosphate exhibits a stretching frequency of 1706 cm^{-1}, a value substantially below that of free D-glyceraldehyde 3-phosphate in aqueous solution (1730 cm^{-1}). This shift of 24 cm^{-1} is somewhat larger than that exhibited by dihydroxyacetone phosphate bound to triosephosphate isomerase (19 cm^{-1}; Belasco & Knowles, 1980).

Clearly, the carbonyl group of D-glyceraldehyde 3-phosphate undergoes substantial polarization upon binding to aldolase. The most appealing explanation for this polarization is to postulate the existence of an enzymic electrophile in the vicinity of the carbonyl oxygen. By polarizing the carbonyl group, this electrophile would reduce the electron density at the carbonyl carbon and facilitate nucleophilic addition by the enolate form of dihydroxyacetone phosphate, thereby increasing the catalytic efficiency of the enzyme. In fact, this electrophile could well be the Brønsted acid that protonates the alkoxide ion transiently generated by nucleophilic addition. The deprotonated form of this electrophile would then be the base responsible for initiating aldol cleavage in the reverse reaction by abstracting the hydroxyl proton at C-4 of D-fructose 1,6-bisphosphate.

For the sake of comparison, it is worth considering an extreme case of carbonyl polarization, the protonation of the carbonyl oxygen of acetone. The reduction in the carbonyl stretching frequency is here 99 cm^{-1} (Clemett, 1970), and it is accompanied by an increase of some 10^{18}-fold in the relative thermodynamic stability of the adduct deriving from nucleophilic addition of hydroxide ion to the carbonyl group. The 24-cm^{-1} shift observed for D-glyceraldehyde 3-phosphate bound to aldolase might therefore be expected to have a proportionate effect (smaller, but still dramatic in catalytic terms) on its reactivity.

Another contribution to the electric field experienced by the carbonyl group of D-glyceraldehyde 3-phosphate might derive from the other substrate present at the enzyme's active site in the ternary complex. Rose and co-workers have suggested that D-glyceraldehyde 3-phosphate does not bind to yeast aldolase until after the enolization of enzyme-bound dihydroxyacetone phosphate (Rose et al., 1965). Consequently, dihydroxyacetone phosphate in the ternary complex may exist in the anionic enolate form, with a partial negative change on C-1.[3] Since this carbon atom lies adjacent to the carbonyl carbon of D-glyceraldehyde 3-phosphate (to which it adds nucleophilically), its negative charge would repel electrons away from the carbonyl carbon and toward the carbonyl oxygen. Once again, the effect would be to weaken the carbonyl double bond and to lower the observed carbonyl stretching frequency of the bound D-glyceraldehyde 3-phosphate. So that the magnitude of such a perturbation by the enolate anion of dihydroxyacetone phosphate could be assessed, an attempt was made to determine the carbonyl stretching frequency of enzyme-bound D-glyceraldehyde 3-phosphate in the absence of dihydroxyacetone phosphate. Unfortunately, as was the case with the binary complex of aldolase and dihydroxyacetone phosphate, no substrate carbonyl absorption was visible above 1700 cm^{-1}.[4] One must conclude either that D-glyceraldehyde 3-phosphate does not bind in the absence of dihydroxyacetone phosphate or that, if bound, only a small fraction of it exists in the aldehyde form. For example, in a binary complex, D-glyceraldehyde 3-phosphate might bind as the hydrate, a form structurally analogous to the lower half of D-fructose

[3] If dihydroxyacetone phosphate in the ternary complex exists only as the enediol(ate), the substrate absorption at 1730 cm^{-1} would then be due to enzyme-bound D-fructose 1,6-bisphosphate.

[4] Interestingly, D-glyceraldehyde 3-phosphate alone did not induce the enzyme's spectral feature at 1748 cm^{-1}.

1,6-bisphosphate. Such behavior would then preclude infrared detection of the aldehyde carbonyl group.

Finally, it is noteworthy that no band appears at 1706 cm^{-1} in the absence of potassium ion. Either the carbonyl group of D-glyceraldehyde 3-phosphate assumes a normal stretching frequency of about 1730 cm^{-1} when potassium ion is replaced by sodium ion, or the internal equilibrium between enzyme-bound D-fructose 1,6-bisphosphate and its enzyme-bound cleavage products is shifted in favor of the former. A catalytic amount of triosephosphate isomerase was added to a solution of fructose bisphosphate plus aldolase containing sodium ion in place of potassium to distinguish between these possibilities. If the absence of potassium had caused the carbonyl group of glyceraldehyde phosphate to assume its unperturbed frequency of 1730 cm^{-1}, deuteration of the aldehydic carbon would have produced a peak at 1718 cm^{-1}, which is the stretching frequency for the unperturbed 1-^{2}H-labeled material (Belasco & Knowles, 1980). No peak or shoulder at 1718 cm^{-1} was observed (Figure 5), from which we may conclude that removal of potassium ion changes the relative proportion of enzyme-bound hexose bisphosphate and triose phosphates in favor of the hexose bisphosphate. This conclusion is consonant with the observed kinetic effects of potassium ion, which are discussed in the following paper (Kadonaga & Knowles, 1983).

Enzymic Carbonyl Group. The binding of D-fructose 1,6-bisphosphate, of dihydroxyacetone phosphate, or of phosphoglycolohydroxamate to yeast aldolase induces the appearance of a new infrared spectral feature at 1748 cm^{-1}, which has been assigned to the liganded enzyme. The only type of enzymic carbonyl group likely to absorb at such a high frequency is a protonated carboxyl group in a nonpolar environment. Such an environment would be required to explain both the high pK_a of this carboxyl group (>7) and its high carbonyl stretching frequency. [Monomeric aliphatic carboxylic acids in carbon tetrachloride exhibit carbonyl stretching frequencies near 1760 cm^{-1}, which are shifted to ~1720 cm^{-1} in polar alcohol solvents (Avram & Mateescu, 1972).] Whether, in the unliganded enzyme, the enzymic carboxyl group is deprotonated, or protonated but in a somewhat more polar environment, is not certain.

Potassium ion induces a 7-fold enhancement in the rate of enzyme-catalyzed aldol cleavage (Richards & Rutter, 1961), though its mechanistic or structural function remains to be established. Interestingly, potassium (but not zinc) is also required for the appearance of the enzymic carbonyl band at 1748 cm^{-1}. The determination of the mechanistic role (if any) of this unusual carboxyl group and the manner of its perturbation by potassium and substrate must presumably await X-ray crystallographic analysis of the enzyme and its complexes.

Registry No. D-Fructose 1,6-bisphosphate, 488-69-7; aldolase, 9024-52-6; D-glyceraldehyde 3-phosphate, 591-57-1; potassium, 7440-09-7; dihydroxyacetone phosphate, 57-04-5; phosphoglycolohydroxamate, 51528-59-7.

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