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Nuclear Relaxation Studies of the Interaction of Substrates with a Metalloaldolase from Yeast†

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ABSTRACT: The essential Zn^{2+} of yeast aldolase can be replaced by Mn^{2+} to yield an active paramagnetic holoenzyme [Kobes, R. D., Simpson, R. T., Vallee, B. L., & Rutter, W. J. (1969) *Biochemistry 8*, 585] which causes enhanced relaxation rates of substrate nuclei [Mildvan, A. S., Kobes, R. D., & Rutter, W. J. (1971) *Biochemistry 10*, 1191]. The frequency dependencies of the paramagnetic effects of aldolase–Mn^{2+} on the nuclear relaxation rates of the substrate acetol phosphate have been studied by ^1H NMR (at 100, 220, and 360 MHz), ^{31}P NMR (at 24.3, 40.5, 72.9, and 145.8 MHz), and ^{13}C NMR (at 25.1 MHz) using 90% enriched [2-^{13}C]acetol phosphate. Active-site binding of acetol phosphate in the NMR experiments was indicated by its displacement from the paramagnetic complex by the linear competitive inhibitor hexitol bisphosphate. The kinetic parameters of the exchange of acetol phosphate out of the ternary complex ($k_{off} = 1.1 \times 10^5$ s^{-1}, $E_{act} = 9.5$ kcal/mol, $k_{on} = 6.1 \times 10^7$ M^{-1} s^{-1}) determined from the temperature and frequency dependencies of $1/T_{2p}$ of ^{31}P indicated that the ternary complex is kinetically competent to be a catalytic intermediate. The correlation time for the Mn^{2+}–acetol phosphate dipolar interaction on aldolase was estimated as 2.2 ± 1.1 ns by the frequency dependence of $1/T_{1p}$ of the protons and ^{31}P of acetol phosphate, as well as that of water protons. Distances from the enzyme-bound Mn^{2+} to the methyl (9.3 Å) and methylene protons (9.5 Å) and the phosphorus (7.9 Å) of acetol phosphate, as well as to the carbonyl carbons of [2-^{13}C]acetol phosphate and [2-^{13}C]dihydroxyacetone phosphate (7.6 Å), are too great by 4.8 ± 0.6 Å for direct carbonyl coordination by the Mn^{2+}, although the conformation of bound acetol phosphate indicates that the carbonyl group points toward the Mn^{2+}. In contrast, the binary Mn–acetol phosphate complex in the absence of aldolase shows direct phosphate coordination with a Mn^{2+}–phosphorus distance of 2.9 Å. The use of Co^{2+}, a paramagnetic probe appropriate for shorter distances due to its smaller effective magnetic moment, yielded Co^{2+} to ^{31}P distances in the ternary aldolase–Co^{2+}–acetol phosphate complex consistent with that found with Mn^{2+}. While this outer sphere Co^{2+} complex was found to be kinetically competent, the existence of an inner sphere Co^{2+} complex could not be demonstrated. Hence, if the metal participates in catalysis by polarizing the carbonyl group to stabilize an enolate intermediate, it must do so through an intervening ligand. The magnitude of the Mn^{2+}–substrate distances on aldolase together with the observation of little or no change in the number of fast-exchanging water ligands on Mn^{2+} when substrates bind ($q \sim 1$) argues against an intervening water ligand. The Mn^{2+}–substrate distances are appropriate for an intervening imidazole ligand which would effectively transmit the electrophilic effect of the metal to the carbonyl group of the substrate through a hydrogen bond.

Fructose-1,6-bisphosphate aldolases (EC 4.1.2.13) from a number of sources have been divided into two mechanistic classes (Rutter, 1964). Class I aldolases, isolated from animals, higher plants, and certain bacteria, have been found to be tetrameric proteins of 150000 molecular weight (Kawahara & Tanford, 1966). Horecker and co-workers (Grazi et al., 1962; Horecker et al., 1963) showed that the reaction catalyzed by class I aldolases from muscle proceeds through a Schiff base intermediate involving an active-site lysine residue. In contrast to the class I aldolases, the class II aldolases, isolated from bacteria and fungi, apparently do not employ a Schiff base intermediate for catalysis (Horecker et al., 1963; Kobes et al., 1969). These enzymes have been found to contain one essential zinc ion per subunit (Richards & Rutter, 1961). The class II aldolases also differ in gross structure from the class I enzymes, existing as dimers with molecular weights near 80000 (Harris et al., 1969). The yeast enzyme, the prototype of class

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II aldolases, has a fourfold higher specific activity and a higher turnover number than the enzyme from rabbit muscle. It is of interest to learn the manner in which the active-site metal ion participates in catalysis in comparison with the more clearly understood mechanism of the Schiff base aldolases.

The zinc ion of yeast aldolase can be removed by treatment with chelating agents to produce an inactive apoenzyme (Kobes et al., 1969). The apoenzyme was found to be a dimer, indicating that the metal ion does not function by maintaining the dimeric structure (Harris et al., 1969). Apoaldolase tightly binds other divalent cations at the Zn^{2+} site, notably Mn^{2+} and Co^{2+}, to produce holoenzymes of lower activity (Kobes et al., 1969). Because Mn^{2+} and Co^{2+} are paramagnetic, they may be used as probes for studies of substrate or inhibitor binding to metal-substituted aldolase by magnetic resonance techniques.

In a previous study, Mildvan et al. (1971) were able to demonstrate that the Mn^{2+} bound by yeast apoaldolase was displaced by Zn^{2+}, indicating that the metals compete for the same site. It was also shown that the enzyme-bound Mn^{2+} was near the binding site of substrates and competitive inhibitors. However, the lack of a directly measured value for the correlation time for the metal–ligand dipolar interaction (τ_c) led to threefold uncertainties in the absolute values of the metal–substrate distances on the enzyme. The multifrequency–multinuclear instrumentation now available allows the measurement of correlation times and the more precise determination of distances. The present study employs ^1H, ^{31}P, and ^{13}C NMR at several field strengths to calculate the relevant metal–ligand distances and thereby the position of the substrate with respect to the bound metal as well as the conformation of the bound substrate at the active site. A major revision in our concept of the mechanistic role of the bound metal has resulted from these studies.

Materials and Methods

Enzyme Preparation and Assay. Aldolase activity was determined by the method of Rutter et al. (1966) except that 2-mercaptoethanol was excluded from the assay buffer because it has been shown to inhibit the yeast aldolase reaction (Rutter et al., 1966). Protein concentration was determined by absorbance at 280 nm [$\epsilon_{1mg/mL}$ = 1.02 (Harris et al., 1969)] or by the dye-binding method of Bradford (1976).

Aldolase was prepared from *Saccharomyces cerevisiae* according to the method described by Rutter et al. (1966) with the following modifications. The cells were broken in a colloid mill in a medium containing 0.2 mM phenylmethanesulfonyl fluoride, a serine protease inhibitor. The resulting suspension was centrifuged at 14600*g* for 20 min. The supernatant solution was then used in the ammonium sulfate fractionation described by Rutter et al. (1966). Chromatography on a DEAE-cellulose column (3 × 15 cm) was used (Richards & Rutter, 1961) to remove residual contaminating triosephosphate isomerase. Aldolase (10–50 mg) was applied to the column, and elution was carried out with a linear gradient of NaCl (10–400 mM). The aldolase activity eluted at 250 mM NaCl while the isomerase eluted at 150 mM. The purified enzyme was stored at 4 °C in an 80% saturated ammonium sulfate solution containing 50 mM β-mercaptoethanol.

The specific activity of the enzyme used for the NMR experiments (before removal of Zn^{2+}) was 100–115 units/mg, which is appropriate for the pure enzyme (Richards & Rutter, 1961). No contaminating proteins were detected by NaDodSO$_4$ gels, in which the enzyme migrated as a monomer.

All buffers used in the metal-exchange and NMR experiments were passed over Chelex 100 to remove trace metal ions. Zinc was removed from yeast aldolase by treatment of 4–70 mg/mL protein with 50 mM EDTA and 50 mM Tris-HCl, pH 7.5, for 1 h at 4 °C followed by gel filtration over Sephadex G-25 that had been treated with 10 mM EDTA, pH 7.5, and equilibrated with 50 mM Tris-HCl, pH 7.5. The apoaldolase produced by this procedure exhibited less than 1% of its original activity. Activity was restored by the addition of an equivalent of Zn^{2+}. An equivalent of Mn^{2+} yielded 15% of the activity found with Zn^{2+}, in agreement with previous observations (Kobes et al., 1969). Separation of apoaldolase from excess EDTA was monitored by measuring the longitudinal relaxation rate of water in the presence of 0.10 mM $MnCl_2$ in a 30-μL aliquot of each fraction. Mn^{2+}–aldolase is known to yield a 6-fold enhancement in relaxation rate produced by Mn^{2+} alone (Mildvan et al., 1971) while Mn–EDTA yields an enhancement of 0.3 (King & Davidson, 1958). By use of the criterion of enhancement, the apoenzyme was found to be free of EDTA.

The binding of Mn^{2+} to apoaldolase was determined by proton relaxation rate enhancement before each NMR experiment. Apoaldolase was found to bind 2 mol of Mn^{2+} per 80000 g of protein with high affinity. All of the bound Mn^{2+} could be displaced by the addition of $ZnCl_2$ except that bound to nonspecific low-affinity sites, in agreement with earlier work (Mildvan et al., 1971). Determination of free Mn^{2+} by X-band EPR (Cohn & Townsend, 1954) confirmed this result.

Substrates and Inhibitors. Acetol phosphate, a substrate analogue with low activity (Richards & Rutter, 1961; Rose & O'Connell, 1969a; Mildvan et al., 1971), was a gift of Dr. W. J. Rutter. Prior to each NMR experiment it was prepared from the cyclohexylammonium salt of its diethyl ketal by passage through Dowex 50W (H^+ form) and incubation at 40 °C for 6–12 h. The hydrolysate was neutralized by addition of 1 M potassium carbonate, which also provided potassium, an activator of class II aldolases (Kobes et al., 1969; Rutter, 1964). The sample was then lyophilized exhaustively to remove the resulting ethanol and acetic acid. Dihydroxyacetone phosphate was generated from the cyclohexylammonium salt of its dimethyl ketal prior to use by the same procedure.

Acetol phosphate concentration was measured by determination of organic phosphate according to the method described by Ames & Dubin (1960). DHAP[1] concentration was measured enzymatically by the use of α-glycerolphosphate dehydrogenase (Mildvan et al., 1971). This enzyme was purchased from Boehringer.

Hexitol bisphosphate racemic at C-2 was generated from fructose 1,6-bisphosphate by reduction with a 12-fold excess of $NaBH_4$ at pH 10 for 60 h at 4 °C (Ginsburg & Mehler, 1966). Excess reductant was destroyed by suspending Dowex 50W (H^+ form) in the solution which lowered the pH to 2. After filtration to remove the Dowex, the product was lyophilized and then dried 6 times from methanol by rotatory evaporation to remove the borate. An aqueous solution (200 mM) was neutralized with 1 M K_2CO_3. The hexitol bisphosphate produced by this procedure chromatographed as a single component on thin-layer plates (Ginsburg & Mehler, 1966) and did not contain FBP as detected by enzymatic assay (Bucher & Hohorst, 1965) using a large excess of aldolase (0.1 mg/mL).

[1] Abbreviations used: AP, acetol phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; HBP, hexitol 1,6-bisphosphate; Me_4Si, tetramethylsilane, ϵ_1* and ϵ_2* are the observed enhancements, due to the presence of the enzyme, of the longitudinal and transverse relaxation rates, respectively, of magnetic nuclei; NaDodSO$_4$, sodium dodecyl sulfate.

Preparation of ^{13}C-Enriched Acetol Phosphate. [2-^{13}C]-Acetol phosphate was synthesized from 90% enriched [1-^{13}C]acetyl chloride (12.6 mmol, Stohler Isotope Chemicals), diazomethane (Moore & Reed, 1973), and dibenzylphosphoric acid (Aldrich Chemical Co.) by a procedure similar to that reported for the synthesis of 1-halo-3-hydroxyacetone phosphates (Silverman et al., 1975). Hydrogenolysis (Brown & Brown, 1966) of the washed intermediate dibenzyl ester yielded 440 mg (23% overall yield) of crude hydroxyacetone dihydrogen phosphate, which was purified by ion-exchange chromatography on a column of Dowex-1 (Cl) resin (Pratt, 1977). The product was quantitatively recovered from the column and contained 3% inorganic phosphate, as shown by phosphate determinations before and after ashing (Ames & Dubin, 1960), using a modification (Curthoys, 1966) of the method of Fiske & Subbarow (1925). The identity and purity (≥96%) of acetol phosphate were determined by ^{1}H NMR at 60 MHz with the Hitachi Perkin-Elmer R-24B spectrometer. The ^{1}H NMR spectrum of the free-acid product at 35 °C in D_2O with external Me_4Si as reference exhibited a methyl doublet (δ 2.28 ppm, $^2J_{CH}$ = 6.0 Hz) and a methylene doublet of doublets (δ 4.74 ppm, $^2J_{CH}$ = 3.5 Hz, $^3J_{PH}$ = 8.7 Hz).

Preparation of ^{13}C-Enriched Dihydroxyacetone Phosphate. [2-^{13}C]Dihydroxyacetone phosphate was prepared from 90% enriched [2-^{13}C]glycerol by enzymatic phosphorylation and dehydrogenation, as in the corresponding synthesis of ^{14}C-labeled dihydroxyacetone phosphate (Rose & O'Connell, 1969a). Enzymatic assay showed that the phosphorylation was 92% complete under the conditions of the experiment, and *sn*-[2-^{13}C]glycerol 3-phosphate was isolated in 87% yield by chromatography on Dowex-1 acetate. The dehydrogenation step proceeded quantitatively, as shown by the absorbance of NADH in the reaction mixture. After conversion to the hydrazone, the material was purified by chromatography on a Dowex-1 formate column in hydrazinium formate buffer, pH 7.15 (Hall, 1960). The elution volume corresponded to a buffer concentration of 0.25–0.26 M. The product was liberated from the hydrazone by treatment with Dowex-50 (H) resin and then adjusted to pH 4.5 with solid $KHCO_3$ and freeze-dried. The yield of [2-^{13}C]dihydroxyacetone phosphate was 73% by enzymatic assay. The product contained 6% inorganic phosphate but was otherwise pure DHAP as determined by enzymatic assay.

Magnetic Resonance Methods. Apoaldolase to be used in ^{1}H NMR experiments was exchanged into D_2O by vacuum dialysis (Fung et al., 1974) or by lyophilization. Enzyme to be used with DHAP was treated with 10 mM glycidol phosphate (Rose & O'Connell, 1969b) for 12 h at 4 °C to inactivate any trace amounts of triosephosphate isomerase that might contaminate the aldolase preparation.

The instruments used for magnetic resonance spectroscopy at various frequencies were a Brucker WH 360/180 (360 MHz for ^{1}H, 5-mm sample tubes; 145.8-MHz for ^{31}P, 12-mm sample tubes), a Varian XL-100 (100 MHz for ^{1}H, 5-mm sample tubes; 40.5 MHz for ^{31}P, 12-mm sample tubes; 25.14 MHz for ^{13}C, 10-mm sample tubes), a Varian NV-60 (24.3 MHz for ^{31}P, 8-mm sample tubes), and a modified NMR Specialties PS 60W pulsed spectrometer for T_1 measurements of water protons at 3, 8, 15, 24.3, and 40 MHz. After the NMR experiments which lasted for as long as 36 h, at least 90% of the original activity of the enzyme was detected.

Longitudinal relaxation times (T_1) were measured by the homospoil demagnetization recovery method except for those measured on the Brucker WH 360/180 and on the PS 60W for which the inversion-recovery method was used (Mildvan & Gupta, 1978). Transverse relaxation times were estimated from the line width of resonances by using the usual relation line width (hertz) = $1/\pi T_2$.

Paramagnetic effects on relaxation rates ($1/T_{1p}$) were calculated as the difference between the relaxation rates ($1/T_1$) in the presence of Mn^{2+} and in diamagnetic controls which either lacked Mn^{2+} or contained Zn^{2+}. Both types of diamagnetic controls gave indistinguishable results.

The paramagnetic contributions to the longitudinal ($1/fT_{1p}$) and transverse relaxation rates ($1/fT_{2p}$) were used to obtain metal–substrate distances and substrate exchange rates, respectively, by using the general theory and equations reviewed elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980).

Results

Paramagnetic Effects of Mn^{2+} and Aldolase–Mn^{2+} on the Protons of Acetol Phosphate at 100 MHz. Previous binding studies using EPR and $1/T_1$ of water protons (Mildvan et al., 1971) have shown that apoaldolase has a much greater affinity for Mn^{2+} (>10^3-fold) than does acetol phosphate or dihydroxyacetone phosphate. Accordingly, the addition of these substrates to the enzyme–Mn^{2+} complex resulted in the formation of ternary aldolase–Mn^{2+}–substrate complexes with no detectable binary Mn^{2+}–substrate interaction. Hence, substrate relaxation experiments in the presence of apoaldolase were carried out under comparable conditions to ensure formation of predominantly ternary enzyme–metal–substrate complexes. Under these conditions the methyl protons of acetol phosphate yield a singlet resonance 2.17 ppm downfield from Me_4Si and the methylene protons yield a doublet ($^3J_{PH}$ = 8.2 Hz) 4.60 ppm downfield from Me_4Si. Figure 1A shows a titration measuring the paramagnetic effects of Mn^{2+} on the longitudinal relaxation rate ($1/T_{1p}$) of the methyl protons of acetol phosphate at 100 MHz in the presence and absence of apoaldolase. The presence of the apoenzyme causes a 1.9 ± 0.3-fold observed enhancement (ϵ^* = 1.9) of the effect of Mn^{2+} on $1/T_{1p}$ of the methyl protons of acetol phosphate under these conditions as reflected in the greater slope of the line with apoenzyme present (Figure 1A). This enhancement establishes the existence of a ternary complex of aldolase, Mn^{2+}, and acetol phosphate. The addition of 10 mM hexitol bisphosphate, a linear competitive inhibitor (Figure 2), decreases $1/T_{1p}$ of the methyl protons of acetol phosphate by 65% (Figure 1A). From the K_I of hexitol bisphosphate (0.22 ± 0.02 mM, Figure 2) and the previously measured dissociation constant of acetol phosphate from the active site (1.9 ± 1.0 mM; Mildvan et al., 1971), a 64 ± 16% decrease in $1/T_{1p}$ is expected under these conditions. Hence, the displacement of the expected amount of the substrate acetol phosphate by the competitive inhibitor hexitol bisphosphate is consistent with active-site binding of these compounds and with a negligible outer sphere contribution to $1/T_{1p}$ (Mildvan et al., 1980). Table I summarizes the normalized longitudinal ($1/fT_{1p}$) and transverse ($1/fT_{2p}$) relaxation rates of the methyl and methylene protons of acetol phosphate, in the presence and absence of apoenzyme, from several determinations, as exemplified in Figure 1A. Since $1/fT_{1p}$ is significantly lower than $1/fT_{2p}$ (Table I), the former is not exchange limited and may be used for distance calculations. While the enzyme significantly enhances the effects of Mn^{2+} on the $1/fT_{1p}$ (ϵ_1^* = 1.6) and $1/fT_{2p}$ (ϵ_2^* = 9.2) of the methyl protons at 100 MHz, it markedly deenhances the relaxation rates of the methylene protons (ϵ_1^* = 0.2; ϵ_2^* = 0.48), indicating a change in the relative positions of Mn^{2+} and acetol phosphate upon binding to the enzyme. Further evidence for these points will be presented in the next section.

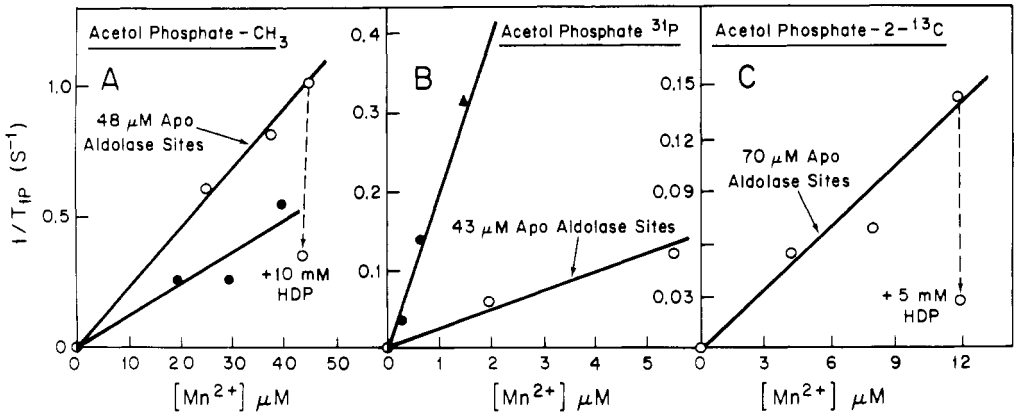

FIGURE 1: Paramagnetic effect of Mn^{2+} (●) and Mn^{2+}–aldolase (○) on longitudinal relaxation rates of acetol phosphate nuclei. Dotted line in (A) and (C) indicates displacement by the competitive inhibitor hexitol bisphosphate. Concentrations were as follows: (A) 48.2 mM acetol phosphate (●) and 50 mM acetol phosphate plus 48 μM aldolase sites (○); (B) 25.5 mM acetol phosphate (●), 22.3 mM acetol phosphate (▲), and 31.2 mM acetol phosphate plus 43 μM aldolase sites (○); (C) 48.8 mM acetol phosphate plus 70 μM aldolase sites. Each sample contained 50 mM Tris-HCl, pH 7.5, and all measurements were made at 25 °C.

Table I: Paramagnetic Effects on the Nuclei of Acetol Phosphate and DHAP[e] at 25 °C[a]

compd	metal	nucleus	frequency (MHz)	$(1/fT_{1p})_t$ (s^{-1})	$(1/fT_{2p})_t$ (s^{-1})	ϵ_1[b] (at 23.5 kG)	ϵ_2[b] (at 23.5 kG)	τ_c[c] (at 23.5 kG)(ns)	distance[d] (Å)
AP	Mn^{2+}	methyl protons	100	993 ± 305	6870 ± 1590	1.55 ± 0.55	9.19 ± 2.14	0.9 ± 0.1	9.32 ± 0.51
			220	489 ± 86					
			360	139 ± 24					
	Mn^{2+}	methylene protons	100	903 ± 530	3940 ± 740	0.19 ± 0.12	0.48 ± 0.10	1.0 ± 0.1[e]	9.47 ± 0.95
			220	1070 ± 142	8700 ± 920				
			360	508 ± 59	4040 ± 340				
	Mn^{2+}	^{31}P	24.3	1090 ± 275	(4.2 ± 2.3) × 10^4	0.13 ± 0.5	0.33 ± 0.11	2.23 ± 0.4	7.85 ± 0.52
			40.5	1010 ± 260	(1.1 ± 0.21) × 10^5				
			72.9	648 ± 250	(1.2 ± 0.16) × 10^5				
			145.8	⩽260	(1.2 ± 0.15) × 10^5				
	Co^{2+}	^{31}P	40.5	⩽5.3	202 ± 5			⩾2 × 10^{-3}	⩾6.3
	Mn^{2+}	^{13}C=O	25.14	566 ± 95					7.57 ± 0.57
DHAP	Mn^{2+}	^{13}C=O	25.14	575 ± 129	(1.17 ± 0.57) × 10^5				⩾7.55 ± 0.91[f]

compd	metal	nucleus	frequency (MHz)	$(1/fT_{1p})_b$ (s^{-1})	$(1/fT_{2p})_b$ (s^{-1})	τ_c[g] (ns)	distance (Å)
AP	Mn^{2+}	methyl protons	100	640 ± 114	748 ± 8	3.4 × 10^{-2}	5.98 ± 0.2
	Mn^{2+}	methylene protons	100	4640 ± 410	8149 ± 564	3.4 × 10^{-2}	4.30 ± 0.07
	Mn^{2+}	^{31}P	40.5	7910 ± 2260	(3.3 ± 0.9) × 10^5	3.4 × 10^{-2}	2.91 ± 0.17

[a] The subscripts t and b to $(1/fT_{1p})$ and $(1/fT_{2p})$ refer to ternary and binary complexes, respectively. All samples contained 50 mM Tris-HCl, pH 7.5, and 40–50 μM apoaldolase sites except for the ^{13}C experiments, in which the enzyme concentration was 70 μM, and the Co^{2+} experiment, in which the concentration was 180 μM. Samples for proton experiments contained 50 mM acetol phosphate and 12–44 μM $MnCl_2$ except for the sample used for the methylene protons, which contained 30 mM acetol phosphate and 14–28 μM $MnCl_2$. Samples for ^{31}P experiments contained 30 mM acetol phosphate and 0.8–5.1 μM $MnCl_2$ or 40 mM acetol phosphate and 50–170 μM $CoCl_2$. Samples for ^{13}C experiments contained 50 mM acetol phosphate and 4–12 μM $MnCl_2$ or 25 mM DHAP (keto form) and 1–3 μM $MnCl_2$. [b] Enhancements are normalized for site occupancy. No entry indicates that the corresponding binary complex was not studied. [c] Calculated from frequency dependence of $1/fT_{1p}$ of the ^{31}P and ^1H of acetol phosphate. The parameters of the Bloembergen–Morgan equation are $B = (3.5 \pm 1.4) \times 10^{20}$ s^{-2} and $\tau_v = 0.50 \times 10^{-12}$ s (Mildvan & Gupta, 1978). [d] Distances were calculated from the value of $1/fT_{1p}$ at 23.5 kG with $q = 1$ and an average τ_c of 2.2 ns. [e] Because of the larger error in $1/fT_{1p}$ at 100 MHz due to the proximity of the HDO resonance, the $1/fT_{1p}$ values at 220 and 360 MHz only were used to calculate τ_c. [f] The binding affinity of the DHAP hydrate to the aldolase-Mn^{2+} complex is unknown. By assuming it to be, at most, equal to that of DHAP, we calculate a ⩽42% decrease in the enzyme-bound DHAP and a corresponding decrease in the calculated distance from Mn^{2+} to the C-2 of DHAP by ⩽0.65 Å. This uncertainty is included in our estimate of the error in this distance. [g] This τ_c value was previously determined from the effect of the binary Mn-acetol phosphate complex on $1/T_1$ of water protons (Mildvan et al., 1971).

Paramagnetic Effects of Mn^{2+} and Aldolase–Mn^{2+} on the ^{31}P of Acetol Phosphate at 40.5 MHz. The proton-decoupled ^{31}P spectrum of acetol phosphate yields a singlet resonance 2.79 ppm downfield from 85% H_3PO_4. In contrast with the effects on the methyl protons, where the enzyme caused an enhancement (Figure 1A, Table I), in the case of phosphorus the enzyme deenhances the effect of Mn^{2+} on $1/T_{1p}$ by a factor of 8 (Figure 1B). The normalized relaxation rates of phosphorus at 40.5 MHz yields $\epsilon_1^* = 0.13$ and $\epsilon_2^* = 0.33$ (Table I). The inequality of these enhancement factors for phosphorus indicates the formation of a ternary aldolase–Mn–acetol phosphate complex rather than the simple removal of Mn^{2+} from acetol phosphate by the apoenzyme. In the binary and ternary complexes (Table I) the $1/fT_{2p}$ values of phosphorus exceed all of the $1/fT_{1p}$ values by more than an order of magnitude, indicating that none of the $1/fT_{1p}$ values are exchange limited. Hence, all are therefore suitable for distance calculations.

Paramagnetic Effects of Aldolase–Mn^{2+} on the ^{13}C Longitudinal Relaxation Rates of Acetol Phosphate and Dihydroxyacetone Phosphate at 25.14 MHz. For the purpose of obtaining the important distance from enzyme-bound Mn^{2+} to the C-2 atom of acetol phosphate, the $1/fT_{1p}$ value at this position was required. With broad-band proton decoupling, the ^{13}C resonance of C-2 of acetol phosphate, 90% enriched at this position, consisted of a doublet ($^3J_{CP} = 7.4$ Hz) 212

Table II: Determination of the Correlation Times of H_2O Protons and Calculation of the Hydration Number of Mn^{2+} in Aldolase Complexes[a]

complex	$1/fT_{1p} \times 10^{-6}$ (s^{-1}) at a frequency (MHz) of 3	8	15	24.3	40	100	$1/fT_{2p} \times 10^{-6}$ (s^{-1}) (100 MHz)	B ($s^{-2} \times 10^{-20}$)	τ_v ($s \times 10^{12}$)	τ_c at 23.5 kG ($s \times 10^9$) frequency dependence	T_{1p}/T_{2p}	q[b]
E-Mn^{2+}	2.79	2.98	3.45	3.45	2.80	0.54	7.08	0.43	2.43	5.4 ± 0.8	6.7 ± 0.3	1.0 ± 0.2
E-Mn^{2+}-DHAP	2.07	2.07	2.42	2.43	2.31	0.84	3.31	0.75	1.90	3.3 ± 0.3	3.3 ± 0.3	1.0 ± 0.2
E-Mn^{2+}-FBP	2.34	2.28	2.54	2.62	2.28	0.92	5.01	0.78	1.74	3.2 ± 0.5	4.1 ± 0.3	1.1 ± 0.2
E-Mn^{2+}-AP	1.49	1.32	1.41	1.51	1.26	0.51	2.01	1.67	2.43	2.3 ± 0.6	3.3 ± 0.3	0.5 ± 0.2

[a] Samples for water relaxation studies contained the following: 160 μM apoaldolase sites, 91.5 μM $MnCl_2$ or $ZnCl_2$, 60 mM KCl, and 40 mM Tris-HCl, pH 7.5, in a total volume of 50 μL. Substrate concentrations were as follows: DHAP, 60 mM; FBP, 20 mM; acetol phosphate, 20 mM. Measurements were made at 25 °C. [b] Calculated by fitting the $1/fT_{1p}$ measurements to the Solomon-Bloemberger-Morgan equations (Mildvan & Gupta, 1978). Errors were estimated from errors in fitting the data (6–12%), the errors in the τ_c for Mn^{2+}–H_2O interaction (9–25%), and the errors from the uncertainty in the Mn^{2+} to water proton distances of 2.87 ± 0.05 Å (Reuben & Cohn, 1970).

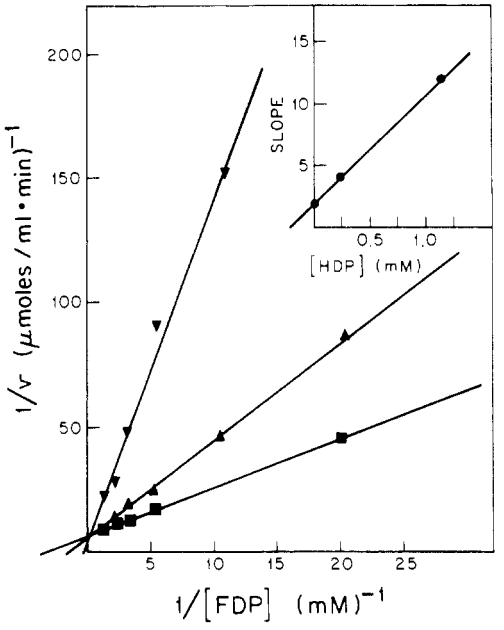

FIGURE 2: Linear competitive inhibition of fructose 1,6-bisphosphate cleavage by hexitol bisphosphate. The assay system used was the α-glycerol phosphate coupled oxidation of NADH as described under Materials and Methods. HBP concentrations were as follows: 0 (■), 0.227 (▲), and 1.14 mM (▼). Inset shows a slope replot. Enzyme concentration was 1.5 μg/mL.

ppm downfield from Me_4Si. Figure 1C shows a titration measuring the paramagnetic effect of aldolase–Mn on $1/T_1$ of C-2 of acetol phosphate, and Table I gives the $1/fT_{1p}$ value. The addition of the competitive inhibitor hexitol bisphosphate markedly decreased the paramagnetic effect of enzyme-bound Mn^{2+} on C-2 of acetol phosphate, establishing active-site binding and a negligible outer sphere contribution to $1/fT_{1p}$. The observed displacement of acetol phosphate caused by the inhibitor was 1.3–2.0-fold greater than estimated from the known dissociation constants of these compounds from the active site. This difference is probably within the experimental uncertainty of the K_D values (Mildvan et al., 1971). The $1/fT_{2p}$ values of the ^{31}P of acetol phosphate exceeded the $1/fT_{1p}$ values of all of the nuclei of acetol phosphate (Table I), indicating that the $1/fT_{1p}$ values are not exchange limited and are suitable for distance determinations.

The observed ^{13}C resonances of DHAP 90% enriched at C-2 consisted of a doublet ($^3J_{PC}$ = 6.3 Hz) at 210 ppm from Me_4Si assigned to the C-2 of DHAP and a doublet ($^3J_{PC}$ = 8.8 Hz) assigned to the C-2 of the hydrate at 72 ppm from Me_4Si. The relative amounts of DHAP and its hydrate, from the intensities of these resonances, were 1.4:1, in accord with previous proton data (Gray & Barker, 1970; Mildvan et al., 1971).

An Mn^{2+} titration (1.0–3.0 μM) of the natural and more active substrate DHAP in the presence of apoaldolase (70 μM sites) yielded a $1/fT_{1p}$ value of the carbonyl carbon indistinguishable from that found with acetol phosphate (Table I). These findings are consistent with equal distances of the carbonyl carbon atoms of both substrates from the enzyme-bound Mn^{2+}. Similar paramagnetic effects of aldolase–Mn^{2+} on the C-1 methylene protons of both DHAP and its hydrate have previously been detected (Mildvan et al., 1971). In accord with this finding, a paramagnetic effect of aldolase–Mn^{2+} on the ^{13}C-2 resonance of the hydrate of DHAP comparable in magnitude to that of DHAP was detected but was not investigated in detail. The presence of the hydrate is responsible for the greater error in the aldolase–Mn^{2+}–C-2 distance with DHAP than with acetol phosphate (Table I).

Determination of Correlation Times, Distances, and Number of Exchangeable Water Ligands in Aldolase–Mn^{2+} Complexes. The calculation of Mn^{2+} to substrate distances on enzymes from $1/fT_{1p}$ values requires a measurement of τ_c, the correlation time for the dipolar Mn^{2+}–nuclear interactions. We have previously pointed out that the most accurate method for determining τ_c is by measurement of the frequency dependence of $1/fT_{1p}$ (Mildvan et al., 1979; Mildvan & Gupta, 1978). In the present case, we have determined τ_c by this method for five separate nuclei in two ternary aldolase–Mn–substrate complexes, with similar results (Tables I and II). Thus, in the aldolase–Mn–acetol phosphate complex the τ_c values of the methyl and methylene protons of acetol phosphate are 0.9 ± 0.1 and 1.0 ± 0.3 ns, respectively (Table I), the τ_c of the phosphorus is 2.2 ± 0.4 ns (Table I), and the τ_c of the water protons is 2.3 ± 0.6 ns (Table II) at 23.5 kG. In the aldolase–Mn–DHAP complex, the τ_c of the water protons is 3.3 ± 0.3 ns (Table II). Values of 3.3–4.8 ns were estimated from the less reliable T_{1p}/T_{2p} ratio as described elsewhere (Mildvan & Engle, 1972). As with other enzyme–Mn complexes (Mildvan et al., 1979), these τ_c values are significantly lower than the tumbling time of a protein of the molecular weight of aldolase (20 ns) and are dominated by τ_s, the longitudinal electron spin relaxation time of bound Mn^{2+}. Hence, the average of the τ_c values measured by the frequency dependence of $1/fT_{1p}$ (2.2 ns) together with the extreme range (0.96–3.3 ns) was used to calculate the Mn^{2+} to substrate distances on aldolase and their respective errors (Table I). Because of the form of the relaxation equation involving the sixth power of r and the presence of τ_c both in the numerator and denominator, the errors in the calculated distances are significantly smaller than the errors in $1/fT_{1p}$ and τ_c (Table

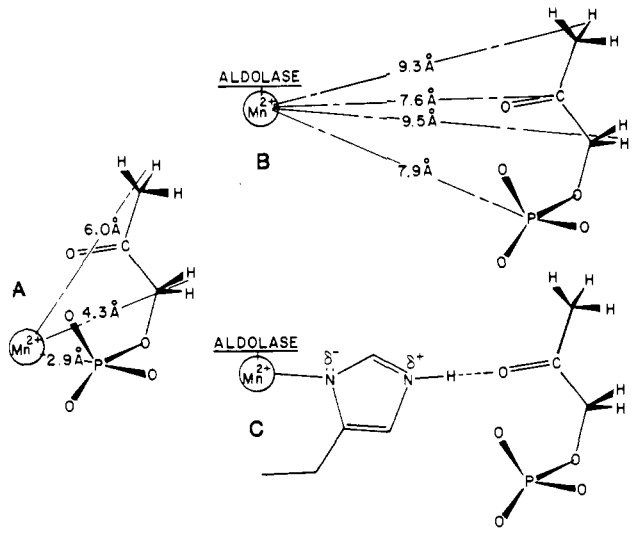

FIGURE 3: Mn^{2+}–substrate distances in (A) the Mn^{2+}–acetol phosphate complex and (B) the aldolase–Mn^{2+}–acetol phosphate complex. Part C shows an active-site structure consistent with the Mn^{2+}–substrate distances found in the aldolase–Mn^{2+}–acetol phosphate and aldolase–Mn^{2+}–DHAP complexes.

I). The Mn^{2+}–substrate distances on aldolase are much greater and more uniform than those found for the binary Mn^{2+}–acetol phosphate complex which was studied as a control (Table I, parts A and B of Figure 3). While the distances in the binary complex indicate direct and monodentate phosphoryl coordination to Mn^{2+}, those on the enzyme require an intervening ligand.

The $1/fT_{1p}$ values of H_2O were used together with the τ_c values of the Mn^{2+}–H_2O interaction and the Mn^{2+} to water proton distance of 2.87 ± 0.05 Å from X-ray data (Reuben & Cohn, 1970) to calculate q, the number of fast-exchanging water ligands on Mn^{2+}, in various aldolase complexes (Table II). The q values indicate approximately one water ligand on the enzyme-bound Mn^{2+} which exchanges with the solvent at a rate greater than 10^6 s^{-1}. The binding of substrates produces little or no change in q.

Kinetic Parameters of the Aldolase–Mn–Substrate Complexes. The $1/fT_{2p}$ value of the ^{31}P nucleus of acetol phosphate in the ternary aldolase–Mn^{2+}–substrate complex increases with frequency between 24.3 and 40.5 MHz, suggesting a shift contribution to $1/fT_{2p}$. Contact shift effects of Mn^{2+} on phosphorus have previously been found in second sphere complexes (Nowak et al., 1973). At frequencies greater than or equal to 40.5 MHz, $1/fT_{2p}$ remains constant at (1.15 ± 0.2) × 10^5 s^{-1} (Table I), suggesting that the exchange-limited ceiling has been reached; i.e., that $1/fT_{2p}$ (acetol phosphate) = the exchange rate $1/\tau_M$ (acetol phosphate). This point is established by the temperature dependence of $1/fT_{2p}$ of phosphorus (Figure 4) which shows Arrhenius behavior with a high activation energy (9.5 ± 2.4 kcal/mol). In contrast, $1/fT_{1p}$ shows essentially no temperature dependence, as expected for $1/fT_{1p}$ in the case of fast exchange (Luz & Meiboom, 1974).

Making the simplest assumption that $1/\tau_M$ (acetol phosphate) = k_{off} in the reaction

$$\text{aldolase–Mn–AP} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{AP} + \text{aldolase–Mn}$$

a value of 1.1 × 10^5 s^{-1} at 25 °C is obtained. This value greatly exceeds k_{cat} for FBP cleavage (20 s^{-1}) for the Mn^{2+}-activated enzyme (Kobes et al., 1969), indicating that the complex detected by NMR is kinetically competent to function in catalysis. The activation parameters of k_{off} are $\Delta H^{\ddagger}$ = 9

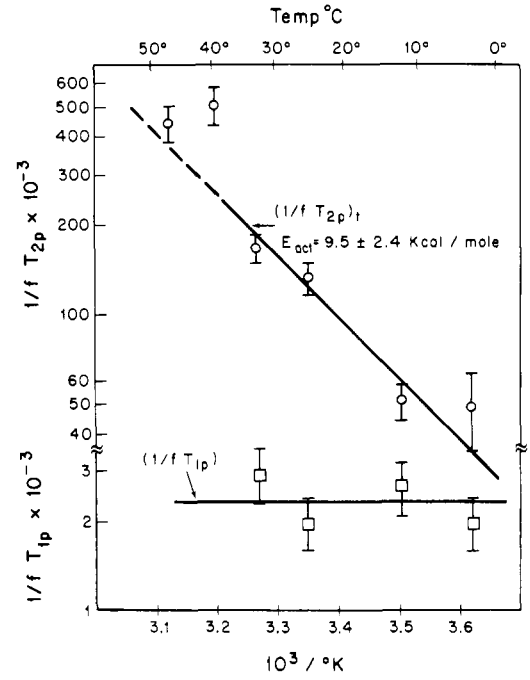

FIGURE 4: Arrhenius plot of the relaxation rates of the ^{31}P resonance of acetol phosphate. The sample contained 25.3 mM acetol phosphate, 3.0 μM $MnCl_2$ (or 3.0 μM $ZnCl_2$ in the diamagnetic control), 50 μM aldolase sites, and 50 mM Tris-HCl, pH 7.5.

kcal/mol and $-T\Delta S^{\ddagger}$ = 1.5 kcal/mol. Using the dissociation constant of acetol phosphate (1.9 mM; Mildvan et al., 1971), we calculate k_{on} to be 6.1 × 10^7 M^{-1} s^{-1}, a value lower than that expected for a diffusion-controlled reaction but larger than V_{max}/K_m for the reverse reaction (Richards & Rutter, 1961).

Although a detailed study was not made of the aldolase–Mn^{2+}–DHAP complex, the $1/fT_{2p}$ value of the ^{13}C-2 of DHAP (Table I) sets a lower limit on $k_{off} \geq (1.2 \pm 0.6) \times 10^5$ s^{-1}, which also indicates a kinetically competent complex. From this value and from the dissociation constant of DHAP (Mildvan et al., 1971), $k_{on} \geq (2.2 \pm 1.1) \times 10^7$ M^{-1} s^{-1}.

Paramagnetic Effects of Aldolase–Co^{2+} on the ^{31}P of Acetol Phosphate at 40.5 MHz. The large distances from the enzyme-bound Mn^{2+} to the nuclei of acetol phosphate leave open the possibility of a more slowly exchanging inner sphere substrate molecule which was not detected by using Mn^{2+} as the paramagnetic probe. A useful method of testing this possibility is to use a paramagnetic probe such as Co^{2+} with a smaller effective magnetic moment and hence a range of shorter measurable distances (Melamud & Mildvan, 1975). High-spin Co^{2+} is known to form a tight complex with apoaldolase, yielding an active enzyme with a k_{cat} of 130 s^{-1} (Kobes et al., 1969). The ^{31}P nucleus of acetol phosphate was studied because Mn^{2+} exerted the largest effects on this resonance (Table I). The addition of Co^{2+} (50–170 μM) to a solution containing 180 μM apoaldolase sites and 40 mM acetol phosphate resulted in a small but significant paramagnetic effect on T_2 but not on T_1 of the ^{31}P resonance at 40.5 MHz. The $1/fT_{2p}$ value (202 ± 5 s^{-1}) exceeds k_{cat} (130 s^{-1}), indicating that a kinetically competent complex was detected. Making the extreme assumptions of an undetected 30% increase in $1/T_1$ due to Co^{2+} and minimum values of g = 2.0 and τ_c = 2 × 10^{-12} s from the literature (Fung et al., 1974; Melamud & Mildvan, 1975; Sloan et al., 1975), we calculate a lower limit Co^{2+} to ^{31}P distance of ≥6.3 Å. By the use of a more appropriate g value for Co^{2+} of 4.0, a Co^{2+} to ^{31}P distance of ≥7.81 Å is calculated. These lower limit distances, which are consistent with the absolute Mn^{2+} to ^{31}P distance

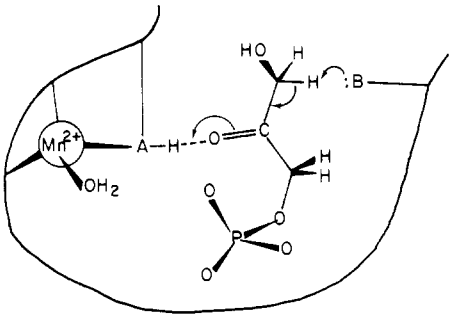

FIGURE 5: A possible mechanism for stabilization of the enolate intermediate in the aldolase reaction suggested by the Mn^{2+}–substrate distances in ternary complexes (parts B and C of Figure 3, Table I). The stereochemistry of proton abstraction is not known, although the overall reaction proceeds with retention about the C-3 atom of DHAP (Rose & Rieder, 1958).

(Table I), argue against the direct coordination of acetol phosphate by the aldolase-bound Co^{2+}.

Discussion

The presence of only one fast-exchanging water ligand on the aldolase-bound Mn^{2+} together with previous optical spectra (Simpson et al., 1971) suggests a tetrahedral geometry as is generally found with Zn^{2+}–metalloenzymes (Argos et al., 1978).

The role of the metal in the mechanism of the yeast aldolase reaction has been considered to be analogous to the role of the ϵ-amino group of lysine in muscle aldolase. The metal ion was suggested to act as an electron sink in stabilizing an enolate intermediate by direct coordination of the carbonyl oxygen of the ketone substrate (Rutter, 1964; Mildvan et al., 1971). In a previous ^1H NMR study at a single frequency (Mildvan et al., 1971), direct coordination of the carbonyl group of the substrate by the aldolase-bound Mn^{2+} was suggested despite a threefold uncertainty in the distances due to the lack of a measured τ_c value. The basis of the suggestion of direct coordination was the large T_{1p}/T_{2p} ratios of the protons of acetol phosphate and DHAP which were interpreted as being due to hyperfine contact effects on these protons. An alternative explanation for the large T_{1p}/T_{2p} ratio, established as correct in this paper, is a long τ_c value, in the nanosecond range (Table I). The measured τ_c value yields distances from aldolase-bound Mn^{2+} to the carbonyl carbon protons and phosphorus atoms of acetol phosphate and to the carbonyl carbon of DHAP too great by 4.8 ± 0.6 Å for direct carbonyl coordination (Figure 3B). A further search for a kinetically competent inner sphere substrate complex using Co^{2+} as the paramagnetic probe yielded limiting distances consistent with the absolute distances found with Mn^{2+} (Table I). Since no inner sphere complexes were detected, the active substrate complexes of yeast aldolase appear to be outer sphere (Figure 3B). Outer sphere complexes are consistent with kinetic studies which show little change in the K_m of substrates but an order of magnitude change in V_{max} on changing the metal activator (Kobes et al., 1969).

Despite its distance from the substrate, a catalytic rather than a structural role for the metal is suggested by the change in V_{max} with changing metal ions (Kobes et al., 1969) and by the orientation of the enzyme-bound acetol phosphate such that its carbonyl group points toward the metal ion. Indeed the distance from Mn^{2+} to the carbonyl carbon is the shortest of those measured (Figure 3B). It should be noted that the relative distances are more accurate than the absolute distances. Previous magnetic resonance studies of six metal-activated, carbonyl-polarizing enzymes have revealed second sphere complexes in each case (Mildvan, 1977). An intervening water ligand was suggested by a decrease of ~ 1 in q, the number of fast-exchanging water ligands, when the substrates bound to these enzymes. In the present case a smaller decrease in q with acetol phosphate and no decrease in q with DHAP are detected (Table II). This result, together with the large Mn^{2+} to substrate distances, enough for two intervening water molecules (Figure 3B), argues against an enzyme–Mn^{2+}–(H_2O)–substrate complex on yeast aldolase.

An attractive possibility is that a hydrogen-bonding group of an amino acid side chain occupies the space between the metal and the substrate. This side chain might then participate both in binding the metal and in polarizing the carbonyl group of the substrate, facilitating catalysis (Figure 5). In the mechanism of Figure 5, the metal functions to hold a general acid AH very very near the carbonyl oxygen of the substrate, stabilizing an enolate intermediate either by hydrogen bonding or by complete protonation of the enolate. This mechanism for yeast aldolase is similar in principle to that of muscle aldolase, in which covalent transformation of the carbonyl group to a Schiff base facilitates substrate protonation and carbanion formation (Riordan & Christen, 1969).

A reasonable hypothesis may be suggested for the nature of the group AH. Chemical modification studies (Ingram, 1969; Lin et al., 1972) and a requirement for exogenous thiol compounds for optimal activity (Rutter et al., 1966) suggest the presence of functional cysteine residues at the active site. However, a sulfhydryl ligand which is only 0.4 ± 0.1 Å larger than a water ligand would be too small to serve as both a metal ligand and a hydrogen-bond donor to the substrate. Moreover, sulfhydryl groups are only weak hydrogen-bond donors (Pimentel & McClellan, 1960). An imidazole of a histidine residue is the only ligand large enough to serve both purposes (Figure 3C). Furthermore, of all amino acid side chains, imidazole would most effectively transmit the electrophilic effect of the metal to the carbonyl of the substrate through a strong hydrogen bond. Indeed such a structure has been detected in several hemoproteins where an imidazole ligand of iron donates a strong hydrogen bond to a carbonyl oxygen of the peptide backbone (Valentine et al., 1979). Preliminary chemical modification and protein NMR studies at 360 MHz provide support for this hypothesis (Smith & Mildvan, 1979).

Acknowledgments

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