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Nuclear Magnetic Resonance and Chemical Modification Studies of the Role of the Metal in Yeast Aldolase†

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ABSTRACT: The C-2 proton resonances of approximately six of the ten histidine residues per monomer of yeast aldolase are detected at 360 MHz. In the metal-free apoenzyme, all of these detected resonances shift upfield with increasing pH* (uncorrected pH meter reading in ^2H_2O solutions) between 6.5 and 8, in a manner typical for histidines. In the Zn^{2+}–metalloenzyme, 3 ± 1 of these proton signals remain downfield as a single resonance over a wide range of pH* values, indicating metal coordination of these imidazoles. This downfield signal is not detected in the paramagnetic Co^{2+}–aldolase complex. Chemical modification of the Zn^{2+}–enzyme by diethyl pyrocarbonate results in acylation of histidines as detected by an increase in absorbancy at 240 nm and loss of enzymatic activity, both occurring with the same second-order rate constant (70 M^{-1} min^{-1} at pH 6.0, 20 °C). Titrations with diethyl pyrocarbonate monitoring enzymatic activity, optical absorbancy at 240 nm, and 360-MHz NMR of the histidine C-2 protons indicate that all ten histidines react in parallel. Modification of Mn^{2+}-substituted aldolase with diethyl pyrocarbonate, monitoring the paramagnetic effect of the bound Mn^{2+} on $1/T_1$ of water protons, reveals a transient increase in $1/T_1$ which occurs during enzyme inactivation, presumably due to an increased accessibility of the bound Mn^{2+} to water. This is followed by a decrease in $1/T_1$ due to the dissociation of Mn^{2+} from the enzyme, as detected by EPR. These results provide independent evidence for binding of imidazole ligands to the metal. Thiomethylation with methyl methanethiosulfonate of approximately one of the five cysteine residues per monomer of Zn^{2+}–aldolase, Mn^{2+}–aldolase, or apoaldolase (followed by reconstitution with Zn^{2+}) results in loss of activity. Such modification of Mn^{2+}–aldolase does not alter the metal site as measured by $1/T_1$, nor does it cause the dissociation of Mn^{2+}, but it does prevent the binding of the substrate fructose diphosphate. These findings indicate that the essential thiol functions at the active site, not as a metal ligand but possibly as a general base. The present results are consistent with the coordination of the enzyme-bound metal by three imidazole ligands, and previous data indicate one rapidly exchanging water ligand [Smith, G. M., Mildvan, A. S., & Harper, E. T. (1980) *Biochemistry 19*, 1248]. If the coordination geometry is tetrahedral [Simpson, R. T., Kobes, R. D., Erbe, R. W., Rutter, W. J., & Vallee, B. L. (1971) *Biochemistry 10*, 2466], then all four of the metal ligands are identified. The results are also consistent with a metal-bound imidazole functioning to polarize the carbonyl group of dihydroxyacetone phosphate by hydrogen bonding.

Fructose-1,6-diphosphate aldolases catalyze the reversible condensation of dihydroxyacetone phosphate (DHAP)[1] and glyceraldehyde 3-phosphate to form fructose 1,6-diphosphate, an essential reaction in the pathways for fermentation and synthesis of hexoses. The enzyme is therefore widely distributed in nature. The fructose-1,6-diphosphate aldolases from lower organisms (class II aldolases) differ from those of higher plants and animals (class I aldolases) in that they do not employ a Schiff base intermediate (Rutter, 1964). The class II aldolases contain a metal ion, usually Zn^{2+}, at the active site (Warburg & Christian, 1943; Rutter, 1964). Yeast aldolase is a dimer of molecular weight 75 000–80 000 (Harris et al., 1969) and contains one essential metal site per subunit (Harris et al., 1969; Richards & Rutter, 1961; Mildvan et al., 1971). The zinc ion of yeast aldolase can be removed by high concentrations of EDTA to yield an inactive apoenzyme (Kobes et al., 1969). Activity can be recovered by the addition to the apoenzyme of stoichiometric amounts of Zn^{2+}, Co^{2+}, Mn^{2+}, or Fe^{2+}, though all ions other than Zn^{2+} yield a holoenzyme of lower activity (Kobes et al., 1969; Rutter, 1964).

Inhibition by low concentrations of chelating agents (Warburg & Christian, 1943; Rutter, 1964), optical spectroscopy with the Co^{2+}–enzyme (Simpson et al., 1971), and early distance measurements with the Mn^{2+}–enzyme (Mildvan et al., 1971) implicated the metal ion as a direct participant in catalysis although there was a 3-fold uncertainty in the metal–substrate distances (Mildvan et al., 1971). A mechanism based on this early evidence proposed that the metal ion catalyzed the condensation by polarizing the carbonyl group of DHAP via direct coordination to facilitate deprotonation

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[1] Abbreviations used: DEP, diethyl pyrocarbonate; MMTS, methyl methanethiosulfonate; FDP, fructose diphosphate; DHAP, dihydroxyacetone phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; Tris, tris(hydroxymethyl)aminomethane; pH*, uncorrected pH meter reading in ^2H_2O solutions; $NaDodSO_4$, sodium dodecyl sulfate.

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at C-3 by a nearby general base (Rutter, 1964; Mildvan et al., 1971). It has since been shown that the distance from the metal to the substrate carbonyl carbon (7.5 Å) measured in the Mn^{2+}- and Co^{2+}-enzyme complexes is too far for direct coordination (Smith et al., 1980). The orientation of the substrate carbonyl group toward the metal suggests that the metal might still polarize the carbonyl group through an intervening ligand. The metal–substrate distances are appropriate for the imidazole moiety of histidine as an intervening ligand (Smith et al., 1980), and theoretical calculations (Valentine et al., 1979) indicate that a metal-coordinated imidazole is a strong hydrogen-bond donor.

In this work, we present data which establish the presence of several histidine ligands to the metal in yeast aldolase. Further experiments demonstrate the requirement for one or more histidines both for the binding of the metal activator and for catalytic activity. Preliminary reports of this work have been published (Smith & Mildvan, 1979; Smith, 1980).

Materials and Methods

Enzyme Preparation and Assay. Yeast aldolase was prepared from *Saccharomyces cerivisiae* and stored as previously described (Smith et al., 1980). The protein was found to be essentially homogeneous by $NaDodSO_4$ gel electrophoresis and by specific activity (115 units/mg). The enzyme was found to lose activity slowly during storage, but only batches of enzyme having a specific activity of >100 units/mg were used for experiments.

Enzymatic activity (cleavage of FDP) was determined by the α-glycerolphosphate dehydrogenase coupled system described by Rutter et al. (1966) except that β-mercaptoethanol was omitted from the assay. Protein concentration was measured by UV absorbance [$\epsilon_{280nm}^{1mg/mL} = 1.02$ (Harris et al., 1969)] or by the method of Bradford (1976).

The zinc ion of yeast aldolase was removed by 50 mM EDTA as previously described (Smith et al., 1980), after which the enzyme was inactive. Addition of 2 equiv of Zn^{2+} per 80 000 daltons restored activity to 100–110% of that of the holoenzyme before removal of zinc. Addition of 2 equiv of Mn^{2+} produced a holoenzyme that was 15% as active as the Zn^{2+} holoenzyme, in accord with previous results (Kobes et al., 1969; Mildvan et al., 1971; Smith et al., 1980).

Modification of Histidine Residues. Diethyl pyrocarbonate (DEP) was obtained from Sigma. It was dissolved in 0.1 M potassium phosphate or potassium carbonate buffer, pH 6.0. The extinction coefficient for (ethoxycarbonyl)histidine determined at 240 nm obtained by reacting 5 mM *N*-acetyl-L-histidine with a 4-fold excess of DEP at pH 6.0 ($\epsilon_{240nm} = 3500$) was within the range reported in the literature (Choong et al., 1977; Melchior & Fahrney, 1970; Miles, 1977). Conversely, the concentration of DEP was determined by reacting limiting amounts of DEP with 10 mM *N*-acetyl-L-histidine at pH 6.0, using this extinction coefficient. The half-life of a 50 mM stock solution of DEP in potassium phosphate buffer at pH 6.0 was found to be ~25 min at 50 °C and ~75 min at 10 °C.

Modification of histidine residues of yeast aldolase was monitored by the change in absorbance at 240 nm after initiation with DEP. Ammonium sulfate and β-mercaptoethanol, which would interfere with the reaction, were removed from protein samples by gel filtration on Sephadex G-25 prior to each series of experiments. Assays of the apoenzyme or Mn^{2+}–enzyme that was modified were performed in the usual assay mixture that was supplemented with 2 equiv of $ZnCl_2$ per active site to ensure saturation of the enzyme with the best metal activator (Mildvan et al., 1971).

Analysis of the kinetics of the second-order reaction of DEP with the His residues of aldolase was done by computer simulation, since this reaction is complicated by the pseudo-first-order hydrolysis of the (ethoxycarbonyl)histidine product (EOC-His) and of the reagent DEP itself. The rate equations for the relevant species can be written as follows:

$$\frac{d[His]}{dt} = -k_2[His][DEP] \quad (1)$$

$$\frac{d[DEP]}{dt} = -k_1[DEP] - k_2[His][DEP] \quad (2)$$

$$\frac{d[EOC\text{-}His]}{dt} = k_2[His][DEP] - k_3[EOC\text{-}His] \quad (3)$$

in which k_2 is the second-order rate constant for the reaction of DEP with histidine and k_1 and k_3 are pseudo-first-order rate constants for hydrolysis of DEP and (ethoxycarbonyl)histidine, respectively. These equations cannot be solved explicitly except in special cases. Therefore, Euler's approximation (eq 4) was

$$C_{t+\Delta t} = C_t + \left(\frac{dC}{dt}\right)_t \Delta t \quad (4)$$

used to calculate the concentration of each species at suitably small increments of time over the entire course of the reaction from trial rate constants, where C_t represents the concentration of a given species at time t. The rate constants k_1, k_2, and k_3 were simultaneously adjusted, using a modified Levenberg–Marquardt algorithm (Brown & Dennis, 1972), until the fit of the experimental data to eq 1–3 could not be perceptibly improved by further iteration. The total concentration of imidazole moieties [i.e., ten per aldolase active site (Harris et al., 1969)] was used in the kinetic equations because all ten residues appeared to react at the same rate (see Results). For kinetic analysis involving monitoring the loss of enzymatic activity, the residual activity was assumed to be proportional to the concentration of unmodified His residues, an assumption that was justified by preliminary experiments (see Results).

Modification of Cysteine Residues. MMTS, obtained from Aldrich, was dissolved in 0.1 M Tris-HCl, pH 7.5, and added in 15–40-μL aliquots to a 1-mL solution containing 2–5 mg/mL aldolase. After a 20-min incubation period at 4 °C to allow all of the MMTS to react with protein thiol groups (Smith et al., 1975), the total number of unreacted thiol groups was determined by adding a 40-μL aliquot of the reaction mixture to an assay solution containing 6 M urea, 150 μM Ellman's reagent, and 0.1 M Tris-HCl, pH 7.5, in a total volume of 0.35 mL. The total thiol concentration was then determined from the absorbance at 412 nm, using a molar extinction coefficient of 13.6×10^3. Unreacted MMTS can potentially react with the 5-thio-2-nitrobenzoate produced by the cleavage of Ellman's reagent, giving the appearance that more than the actual number of protein SH groups had reacted. This phenomenon appears to have been more of a problem at high concentrations of MMTS relative to enzyme–SH groups (i.e., toward the end of a titration) under the conditions used.

Magnetic Resonance Measurements. ^1H NMR spectra of aldolase were recorded on a Bruker WH 180/360 spectrometer operating at 360 MHz or a Bruker WM 250 spectrometer operating at 250 MHz. For such experiments, the enzyme was exchanged into D_2O by lyophilization and dissolution in D_2O, followed by a second lyophilization and dissolution or by repeated vacuum filtration (Fung et al., 1974).

The measurement of solvent water proton relaxation rates in chemical modification experiments was performed on a modified NMR Specialties pulsed spectrometer at 24.3 MHz

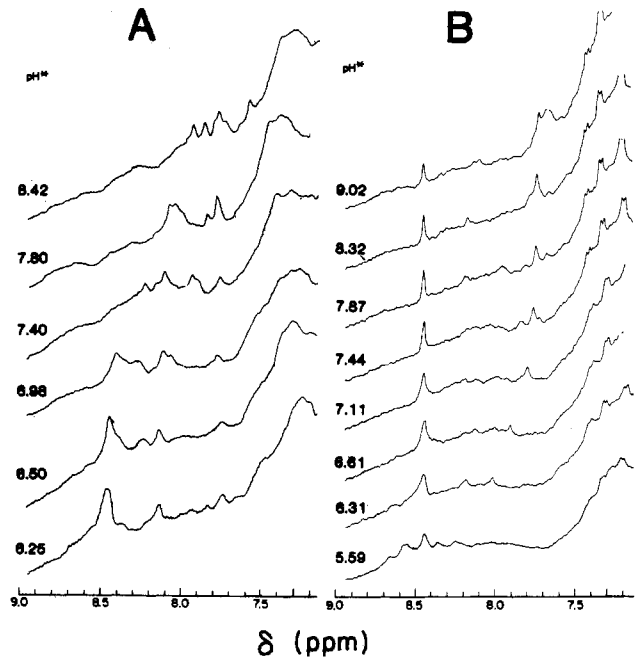

FIGURE 1: Effect of pH* on the chemical shifts of the histidine resonances of apoaldolase (A) and of Zn^{2+}–aldolase (B) at 360 MHz. The enzyme concentration was 0.6 mM subunits in D_2O. Each spectrum required 1024 acquisitions using 16K data points, a sweep width of 5 kHz, a 3-Hz line broadening, and a 90° pulse repetition rate of 4.0 s. The probe temperature was 20 °C.

(Mildvan & Engle, 1972). The measurement of free Mn^{2+} was carried out on a Varian E-4 EPR spectrometer. For these experiments, the modification reaction was initiated by addition of DEP at a controlled temperature (21 °C). An aliquot of the sample was immediately drawn into a quartz capillary EPR tube and placed in the microwave cavity, which was held at the same temperature as that of the pulsed NMR spectrometer probe (21 °C), and the remainder of the sample was placed in the spectrometer for water relaxation measurements. EPR measurements were made by scanning the Mn^{2+} spectrum and recording the time at which each resonance line appeared. The time of measurement was taken to be the midpoint between the times of the maximum and subsequent minimum in the derivative spectrum. The Mn^{2+} concentration was calculated by using separate standard curves for each line because of the well-known nonequivalence of the six EPR lines.

Results

^1H NMR of His C-2 Protons of Yeast Aldolase. The effects of pH* on the aromatic region of the 360-MHz ^1H NMR spectrum of yeast apoaldolase are shown in Figure 1A. The entire proton spectrum was found to be relatively constant over the pH range of 4–9 except for the resonances of the histidine C-2 protons which shift upfield with increasing pH by ~1 ppm in response to deprotonation (Markley, 1975). Yeast aldolase contains ten histidine residues per monomer (Harris et al., 1969). From the appearance of the spectrum of the apoenzyme at pH* 8.42 (Figure 1A), which reveals a total of six peaks or shoulders, and from the ratio of the integrated intensity of the entire histidine C-2 region to the average intensity of those individual peaks which titrate, it is estimated that approximately six of the ten histidine residues are observed. The corresponding His C-4 H resonances were not visible due to overlap with the bulk of the aromatic resonances. Because of the similar line shape of the individual histidine resonances, even when they are resolved, detailed titration curves cannot as yet be drawn without an extensive study involving selective

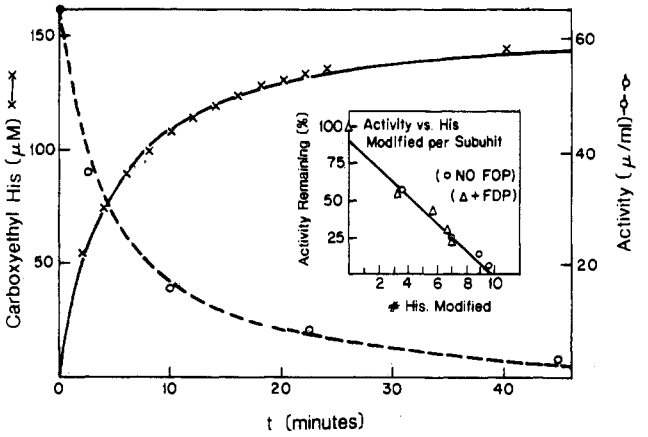

FIGURE 2: Time course of the reaction of diethyl pyrocarbonate with yeast aldolase at 20 °C as monitored by loss of activity and acylation of histidine. The enzyme concentration was 15.5 μM subunits in 0.1 M potassium phosphate, pH 6.0. The reaction was initiated by addition of 2.84 mM DEP. The points represent the measured activity or the measured extent of acylation, and the curves are calculated from the rate eq 1–3, assuming $k_1 = 0.11$ min^{-1}, $k_2 = 70$ M^{-1} min^{-1}, and $k_3 = 0$. The average deviation of the theoretical curves from the experimental data is 1.4% for the inactivation and 2.2% for the acylation. Inset: Correlation of loss of activity with the number of histidine residues modified.

deuteration of the C-2 positions (Markley, 1972). Nonetheless, all of the observed His C-2 H resonances behave typically, moving ~1 ppm upfield in response to deprotonation.

A similar titration of the Zn^{2+}–enzyme yielded the data shown in Figure 1B. Again, from the appearance of the spectrum at pH* 5.59 and from an analysis of intensity ratios, approximately six of the ten histidine residues are also resolved in the Zn^{2+}–enzyme. Although most of the His C-2 proton resonances move upfield with increasing pH, one resonance remains in the downfield position at 8.45 ppm. This resonance corresponds to 3 ± 1 protons as judged by comparing its integrated intensity to the average intensity of those individual peaks which titrate. His C-2 proton resonances that do not respond to increasing pH have been observed in systems in which one of the imidazole nitrogen atoms is coordinated to a metal ion (Cohen & Hayes, 1974). In such cases, the pK value for dissociation of the remaining nitrogen-bound proton is greater than 10, much higher than that in diprotonated imidazoles (Hanania et al., 1966; Hanania & Irvine, 1964).

If an equivalent amount of Co^{2+} rather than Zn^{2+} is added to the apoenzyme, the resulting proton spectrum either at 360 MHz or at 250 MHz is very similar to that of the Zn^{2+}–enzyme, except that the signal at 8.45 ppm is not detected, presumably due to paramagnetic broadening. This provides further evidence for direct imidazole coordination by the activating divalent cation.

Modification of Histidine Residues by Diethyl Pyrocarbonate. The results of a typical modification experiment are shown in Figure 2. To ensure that turbidity did not contribute to the increase in optical density at 240 nm, it was necessary to follow the reactions of DEP with aldolase and with *N*-acetyl-L-histidine by measuring the UV spectra at various times after initiation with DEP. The optical density at wavelengths ≥275 nm remained unchanged, indicating that turbidity did not contribute to the measured absorbance. The total number of histidine residues modified was ten per subunit (Figure 2 inset), in agreement with the number of histidine residues per monomer determined by amino acid analysis (Harris et al., 1969), which demonstrates that the reaction was essentially complete. The enzymatic activity decreased progressively as the extent of modification increased. The results

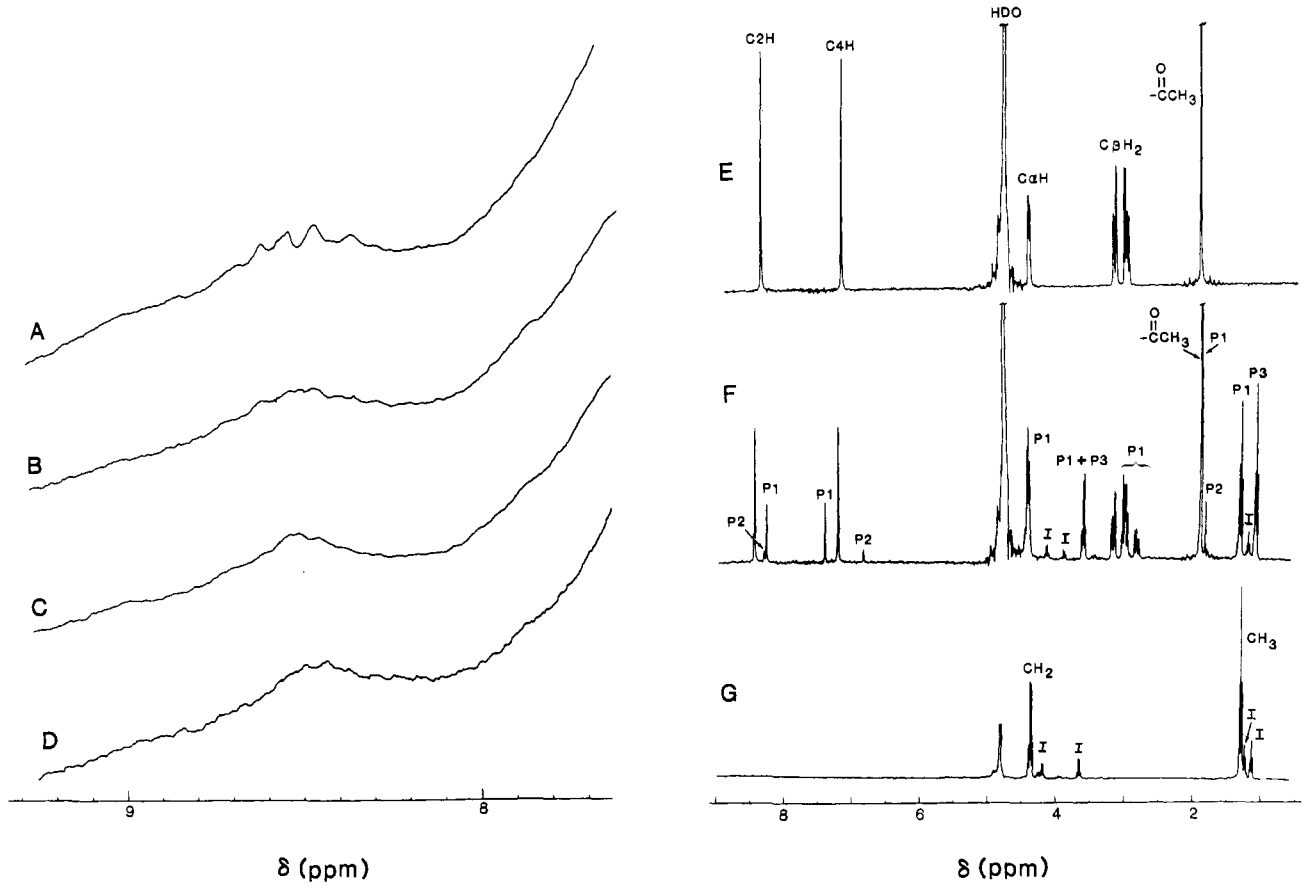

FIGURE 3: Effect of diethyl pyrocarbonate on the 360-MHz proton NMR spectrum of yeast aldolase and of N^{α}-acetyl-L-histidine. The samples contained 0.1 M potassium phosphate buffer, pH* 6.0, in D_2O and the following components: (A) 1.0 mM Zn–aldolase sites; (B) 2.0 mM DEP and 0.95 mM Zn–aldolase sites; (C) 4.0 mM DEP and 0.91 mM Zn–aldolase sites; (D) 8.0 mM DEP and 0.83 mM aldolase sites; (E) 10.0 mM N^{α}-acetyl-L-histidine; (F) 2.6 mM DEP and 8.7 mM N^{α}-acetyl-L-histidine; (G) 5.0 mM DEP. NMR parameters are as in Figure 1 except that 512 acquisitions were used for the protein and 100 acquisitions and a line broadening of 0.1 Hz were used for the small molecules. The assignments of the resonances of the small molecules are as indicated. The resonances labeled I result from minor impurities or breakdown products of DEP. The resonances of the reaction products (P) are assigned as follows: P1, imidazole–*N*-(carboxyethyl)-N^{α}-acetyl-L-histidine; P2, imidazole–*N*,*N*-bis(carboxyethyl)-N^{α}-acetyl-L-histidine; P3, ethyl carbonate. The shifts (δ) and coupling constants (J) for DEP are $\delta(CH_3) = 1.33$, $\delta(CH_2) = 4.33$ ($^3J = 7.5$ Hz). Those for P-1 are $\delta(CH_3) = 1.41$, $\delta(CH_2) = 3.66$ ($^3J = 8$ Hz), $\delta(CH_3CO) = 1.97$, $\delta(C_{\beta}H_A) = 2.88$ ($^2J = 9$ Hz), $\delta(C_{\beta}H_B) = 3.04$, $\delta(C_{\alpha}H_x) = 4.45$ ($^3J_A = 9$ Hz, $^3J_B = 6$ Hz), δ(C-4 H) = 7.36, δ(C-2 H) = 8.20. Those for P-2 are $\delta(CH_3CO) = 1.92$, δ(C-4 H) = 6.81, δ(C-2 H) = 8.24. Those for P-3 are $\delta(CH_3) = 1.19$, $\delta(CH_2) = 3.66$ ($^3J = 8$ Hz).

indicate that DEP was not selective for particular His residues, reacting with all at approximately the same rate. Thus, the assumption that enzymatic activity is proportional to the concentration of unmodified histidine used in the kinetic analysis is justified. The reason for the approximately equal reaction rates of all of the histidines, despite their differing environments, is not clear, but similar behavior has been described in other enzymes (Miles, 1977). The second-order rate constant (k_2) for both modification of histidine and loss of activity was found to be 70 M^{-1} min^{-1}. The pseudo-first-order rate constant (k_1) for the spontaneous hydrolysis of DEP in the presence of aldolase under the conditions of Figure 2 was 0.11 min^{-1}, corresponding to a half-life of 6.3 min rather than 25 min as found in the absence of aldolase. Thus, in the experiment shown in Figure 2, the concentration of DEP would be decreased to a negligible level after ~45 min, and the reaction should cease at that time. The observed decrease in the half-life of DEP in the presence of aldolase was presumably due to catalysis of its hydrolysis by nucleophilic groups of the protein (Dann & Britton, 1974). The pseudo-first-order rate constant (k_3) for hydrolysis of (ethoxycarbonyl)histidine was found to be negligible. The values of k_1, k_2, and k_3 were used in the rate equations (eq 1–3) to generate the theoretical curves which provided a good fit to the kinetic data (Figure 2).

The presence of saturating levels of FDP did not protect any histidine residues against modification nor did it decrease the rate of inactivation. In preliminary experiments, the lifetime of DEP was decreased by the presence of a nucleophilic contaminant in our tetracyclohexylammonium fructose 1,6-diphosphate, leading to an apparent decrease in the rate of inactivation that mimicked protection by the substrate (Smith & Mildvan, 1979). The lack of appreciable protection is not surprising, however, because all His residues reacted with DEP at about the same rate despite the fact that approximately four of the ten histidines are not detected by NMR and may be buried inside the globular protein.

The reaction of the His residues with DEP can be monitored by 360-MHz ^1H NMR, as shown in Figure 3A–D. The resonances assigned to His C-2 protons broaden and may also shift upfield. In a model reaction using *N*-acetyl-L-histidine, the resonance of the C-2 proton is found to shift upfield by 0.17 ppm (Figure 3F).

Attempts to reverse the DEP modification of yeast aldolase with hydroxylamine (Miles, 1977) were not successful since the unmodified and modified enzymes were found to precipitate, and the unmodified enzyme lost substantial activity in the presence of 50 mM hydroxylamine, reflecting irreversible denaturation. The precipitation of the protein also precluded absorbancy studies at 240 nm. Amino acid analyses of 6 N HCl hydrolysates of native, partially (50%) modified, and completely modified aldolase as well as of completely modified aldolase which had been treated with 50 mM hydroxylamine

Table I: Modification of Histidine and Cysteine by Diethyl Pyrocarbonate[a]

activity remaining (%)	unreacted His[b] per monomer	total His[c] per monomer	free SH[d] per monomer	Δ(SH)/Δ(activity)
100	10.21	10.12	4.66	
80	8.21		4.54	0.60
60	6.30		4.45	0.53
	5.44	9.59		
29	3.15		4.27	0.55
17	2.00		4.06	0.72
8	1.05	10.15	3.73	1.01
after treatment[e] with H_2NOH		10.13		

[a] Aldolase (29.9 μM subunits) was reacted with 2.13 mM DEP in 0.1 M potassium phosphate, pH 6. Aliquots were withdrawn at various times for the indicated measurements. [b] (Carboxyethyl)-histidine was determined by A_{240}; unreacted histidine was calculated by subtraction. [c] Determined by amino acid analysis by comparing the amount of histidine to the amount of lysine, of which there are 25.3 residues per 40 000 daltons. [d] Determined by Ellman's reagent. [e] Aldolase (27.2 μM subunits) modified to 8% residual activity was incubated in 180 mM H_2NOH for 24 h.

for 24 h all yielded 10 histidine residues per monomer within experimental error (10.2 ± 0.3). These observations rule out irreversible cleavage of the imidazole ring (Melchior & Fahrney, 1970) following modification or NH_2OH treatment.

Despite reports that DEP is specific for histidine at pH 6 (Muhlrad et al., 1967; Dann & Britton, 1974), other nucleophilic residues, notably cysteine, tyrosine, and lysine, have been found to be modified at pH values as low as 4 (Melchior & Fahrney, 1970; Miles, 1977; Burstein et al., 1974). The absence of change in the UV spectrum of aldolase at 278 nm indicates that tyrosine was not modified significantly during inactivation. The concentration of cysteine thiol groups was monitored by using Ellman's reagent during the reaction of aldolase with DEP, and the data are shown in Table I. It appears that some cysteine was acylated by DEP. However, the ratio of modified cysteines to the fractional loss of activity is significantly less than 1 (0.56 ± 0.04) over the first two-thirds of the titration. This observation, together with the finding that 1.4 ± 0.3 cysteines per subunit must be modified to inactivate the enzyme (vide infra), and the agreement of the rate constants for histidine modification and loss of activity argue against cysteine modification as being primarily responsible for the inactivation of aldolase by DEP. Similarly, the agreement in rate constants for histidine modification and enzyme inactivation by DEP argues against lysine modification as being responsible for the inactivation.

For further characterization of the role of histidines in maintaining the structure and catalytic activity of aldolase, the reaction of DEP with Mn^{2+}–aldolase was monitored by water relaxation rate measurements and by EPR. A plot of the time course of the reaction is shown in Figure 4. For these measurements, high concentrations of Mn^{2+}–aldolase and proportionally higher DEP concentrations were required in order to observe changes in the parameters with the required accuracy. At the concentrations used in these experiments, the loss of activity and increase in absorbance at 240 nm are rapid. Occurring over the time span of this rapid loss in activity is a small but highly reproducible increase in the observed enhancement (ϵ^*) of the effect of aldolase-bound Mn^{2+} on the solvent water proton relaxation rate, indicating a change in the environment of the bound Mn^{2+}. This rapid increase in ϵ^* is followed by a slower decrease in ϵ^* which is paralleled by the appearance of free Mn^{2+} in the reaction

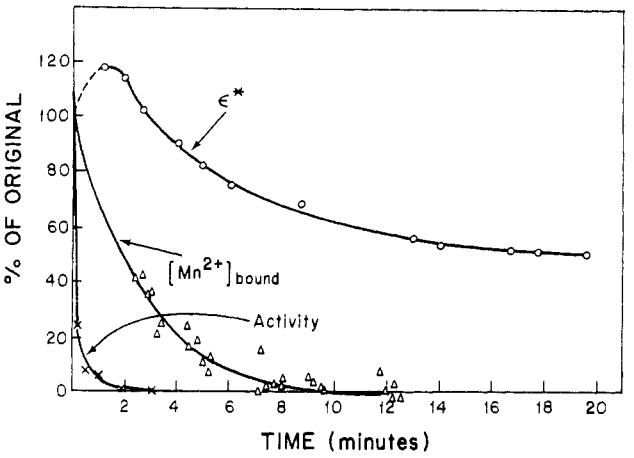

FIGURE 4: Time course of inactivation of Mn^{2+}–aldolase, monitoring the release of bound Mn^{2+} by EPR and the enhancement (ϵ^*) of $1/T_1$ of water protons. The sample contained 61.3 μM apoaldolase sites, 45 μM $MnCl_2$, and 9.7 mM DEP in 50 mM potassium bicarbonate, pH 6.0, at 21 °C. Water proton $1/T_1$ rates and EPR measurements of free Mn^{2+} were made on the same sample, while activity was measured on a duplicate sample as described under Materials and Methods.

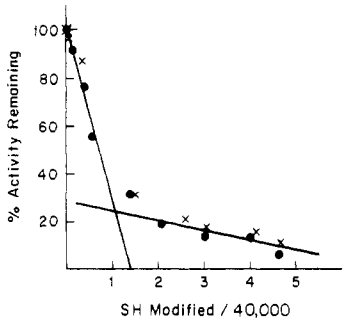

FIGURE 5: Titration of Zn^{2+}–aldolase and apoaldolase, with MMTS measuring inactivation. The solution contained either 100 μM Zn^{2+}–aldolase (●) or 100 μM apoaldolase (×) and 0.1 M Tris-HCl, pH 7.5 (total volume 1 mL). After addition of 10–40-μL aliquots of 0.1 mM MMTS and incubation at 4 °C for 20 min, activity and unreacted thiol concentration were determined as described under Materials and Methods.

mixture. The observation that the final enhancement approached 50% of its initial value rather than 16% (Figure 4) suggests that some of the Mn^{2+} is bound to lower affinity sites that have a higher enhancement factor. Two such sites per 80 000 daltons are known to exist on the native enzyme (Mildvan et al., 1971).

Modification of Thiol Groups by MMTS. The results of a titration of yeast aldolase and the apoenzyme with MMTS are shown in Figure 5. Before modification, the number of reactive thiol groups was found to be 4.6/40 000 daltons. Extrapolation of the extensive initial phase of the titration curve shows that modification of 1.4 ± 0.3 thiol groups per subunit renders the enzyme inactive. The value slightly exceeding unity and the extrapolated intercept of the latter phase of the plot greater than the expected upper limit value of five cysteines per subunit probably result from the reaction of residual MMTS with the 5-thio-2-nitrobenzoate produced by the cleavage of Ellman's reagent, as discussed under Materials and Methods. The presence of saturating concentrations of FDP or the removal of metal by EDTA did not affect either of the extrapolated intercepts in such titrations. Inactivation of the enzyme by modification of the cysteine residues of Mn^{2+}–aldolase did not significantly change the observed enhancement (ϵ^*) of $1/T_1$ of water protons, nor did it cause the enzyme to lose bound Mn^{2+}.

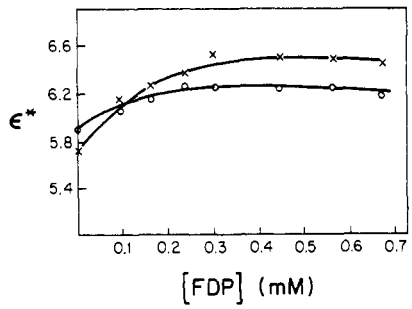

FIGURE 6: Effect of thiomethylation of Mn^{2+}–aldolase on FDP binding. Mn^{2+}–aldolase (×) and thiomethylated Mn^{2+}–aldolase (○) were titrated, with FDP measuring $1/T_1$ of water protons. Both solutions contained 48.5 μM aldolase sites, 30.7 μM $MnCl_2$, 0.1 M KCl, and 0.1 M Tris-HCl, pH 7.5. The modified Mn^{2+}–enzyme which retained 39% of its activity was prepared by preincubation with 0.8 equiv of MMTS for 20 min. Mn^{2+}–enzyme solutions were titrated with identical solutions which also contained 2.0 mM FDP.

As a test for the interaction of thiol-modified aldolase with its substrates, Mn^{2+} was bound to partially modified apoaldolase, yielding a complex with an ϵ^* value indistinguishable from that of unmodified Mn^{2+}–aldolase. Titrations with FDP were performed while monitoring the solvent water proton relaxation rate. The partially modified enzyme, which retained 39 ± 5% of its activity, showed 45 ± 10% of the expected change in ϵ^* upon binding of FDP while the K_D of FDP (0.08 mM) was unaltered (Figure 6). Hence, within the errors of the measurements, the effects of FDP can be explained by the interaction of the substrate only with the residual unmodified enzyme.

Discussion

Three independent pieces of evidence indicate imidazole ligands to the essential metal on yeast aldolase: the failure of 3 ± 1 histidine residues of the metalloenzyme to titrate between pH* 6 and 9, the absence of this signal in the presence of the paramagnetic Co^{2+} ion, and the exposure and release of the metal upon chemical modification of histidine residues.

Acid–base titrations of histidine residues of proteins have yielded valuable information on several enzymes (Markley, 1975). Of particular relevance to this study are systems in which the C-2 H resonance of a His residue does not shift over the pH range 5–9. In such cases, the resonance is assigned to a residue that is not available for titration such as the ligand His-18 of cytochrome *c* (Cohen & Hayes, 1974). In the aromatic region of the ^1H NMR spectrum of yeast aldolase, the proton resonance at 8.45 ppm is firmly assigned to the C-2 protons of 3 ± 1 His residues by virtue of its chemical shift and its intensity. The absence of these overlapping resonances in the spectrum of the apoenzyme together with the titration data strongly suggests that 3 ± 1 His residues coordinate to the zinc ion via their ring nitrogen atoms. The p*K*s of the remaining ring NH groups are expected to be high for imidazole ligands, generally greater than 10 (Hanania et al., 1966; Hanania & Irvine, 1964). These coordinated imidazole ligands would be protonated on both nitrogens only at low pH (<5) when the metal ion would dissociate.

Independent evidence for the role of histidine in metal binding was obtained by the magnetic resonance study of aldolase modification with DEP. Whereas the inactivation of Mn^{2+}–aldolase by the modification of cysteine had no significant effect on the water proton relaxation rate enhancement, modification of histidine caused a biphasic change in the enhancement. The first phase, an increase in ϵ^*, which occurred during the loss of enzymatic activity, is consistent with an increased exposure of the bound Mn^{2+} to water, possibly due to the loss of an imidazole ligand (Mildvan & Engle, 1972). The second phase, a decrease in ϵ^*, corresponds to the dissociation of Mn^{2+} from the active site.

The finding of histidine ligands for Zn^{2+} on aldolase is not surprising since one or more imidazole ligands are usually found on Zn^{2+}–metalloenzymes by X-ray diffraction (Argos et al., 1978; Lipscomb, 1980). Moreover, as in many other Zn^{2+}–metalloenzymes (Lipscomb, 1980), the coordination geometry about the metal on aldolase appears to be tetrahedral as suggested by the optical and CD spectra of Co^{2+}–aldolase (Kobes et al., 1969). If so, then all of the four ligands to the metal may now be known since we have previously detected a rapidly exchanging water ligand on the Mn^{2+}–enzyme (Smith et al., 1980), and we now detect approximately three histidine ligands on the Zn^{2+}–enzyme.[2]

The observation that the loss of activity upon modification of histidine is much faster than the loss of the metal from the active site suggests a catalytic role for at least one of the histidine ligands, in addition to the complexation of the metal. Our previous study (Smith et al., 1980) indicated that the escape rate of the water ligand of the enzyme-bound Mn^{2+} is not significantly decreased in the presence of substrates and that the metal–substrate distances are appropriate for an intervening imidazole group but are too large for an intervening water ligand. It is therefore reasonable to conclude that the keto substrate (FDP or DHAP) interacts indirectly with the metal such that its carbonyl oxygen accepts a hydrogen bond from the imidazole NH group of a histidine ligand. A theoretical treatment of metal-coordinated imidazole in such hydrogen-bonded systems (Valentine et al., 1979) indicates that the strength of such a hydrogen bond would be enhanced by the presence of the metal and would be sufficient to polarize the carbonyl group to stabilize an enolate intermediate.

The importance of the thiol groups of yeast aldolase has been examined by other workers (Ingram, 1969; Lin et al., 1972). Titration with either Ellman's reagent or *p*-hydroxymercuribenzoate revealed 4.5 sulfhydryl groups per 40 000 daltons, one of which was necessary for activity. With these bulky reagents, the nonessential thiol groups were modified preferentially over the essential group, resulting in no loss of activity until the last thiol group was titrated. With the smaller reagent, MMTS, used in the present studies, the essential thiol is modified first, indicating that it may actually be more reactive but somewhat less accessible to large reagents than are the nonessential thiol groups. The resulting modified enzyme has an intact metal site but can no longer bind FDP, indicating that the essential thiol is not a metal ligand but that it interacts with the substrate at the active site. The results with MMTS are consistent with those of affinity labeling of yeast aldolase with haloacetol phosphates (Lin et al., 1971), *N*-(bromoacetyl)ethanolamine phosphate,[3] and with DL-2-chloro-2-deoxyglyceraldehyde 3-phosphate,[3] all of which inactivate the enzyme by modifying a single thiol group. This group has been suggested to function as a general base to deprotonate C-3 of DHAP (Lin et al., 1972). Our observation that the active-site cysteine is more reactive toward MMTS at pH 7.5 than the nonessential cysteine residues supports the idea that the active-site thiol is at least partially deprotonated at this pH value and could therefore act as a general base. The functioning

[2] Three histidine ligands and one water ligand on Zn^{2+} are also found on carbonic anhydrase by X-ray analysis (Lindskog et al., 1971). Unlike carbonic anhydrase, however, yeast aldolase does not catalyze the hydrolysis of nitrophenyl acetate using the assay of Armstrong et al. (1966) (G. M. Smith, unpublished experiments).

[3] G. M. Smith and F. C. Hartman, unpublished experiments.

of the essential thiol of muscle aldolase as a general base to deprotonate the nearby C-4 hydroxyl group of FDP has been proposed by Horecker et al. (1972).

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