J. Microbiol. Biotechnol. (2001), **11**(5), 838–844

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Fuculose-1-Phosphate Aldolase of *Methanococcus jannaschii*: Reaction of Histidine Residues Connected with Catalytic Activities

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Received: May 8, 2001
Accepted: August 30, 2001

Abstract The enzyme Fuc aldolase from *Methanococcus jannaschii* that catalyzes the aldol condensation of DHAP and L-lactaldehyde to give fuculose-1-phosphate was inactivated by DEP. The inactivation was pseudo first-order in the enzyme and DEP, which was biphasic. A pseudo second-order rate constant of 120 $M^{-1}min^{-1}$ was obtained at pH 6.0 and 25°C. Quantifying the increase in absorbance at 240 nm showed that four histidine residues per subunit were modified during the nearly complete inactivation. The statistical analysis and the time course of the modification suggested that two or three histidine residues were essential for activity. The rate of inactivation was dependent on the pH, and the pH inactivation data implied the involvement of an amino acid residue with a pK_a value of 5.7. Fuc aldolase was protected against DEP inactivation by DHAP, indicating that the histidine residues were located at the active site of Fuc aldolase. DL-Glyceraldehyde, as an alternative substrate to L-lactaldehyde, showed no specific protection for Fuc aldolase.

Key words: *Methanococcus jannaschii*, hyperthermophile, fuculose-1-phosphate aldolase, chemical modification, diethylpyrocarbonate

L-Fuculose-1-phosphate aldolase [EC 4.1.2.17] catalyzes an aldol-type condensation of dihydroxyacetone phosphate (DHAP) with L-lactaldehyde to yield fuculose-1-phosphate. The fuculose-1-phosphate aldolase enzyme studied so far exhibits a high specificity for the DHAP nucleophile, yet accepts a wide variety of different aldehyde electrophiles as well [8]. The enzyme has been identified and cloned in *E. coli* and is assumed to be involved in the dissimilation of fuculose [2]. Fuculose-1-phosphate aldolase belongs to the type II aldolases, which require metal ions such as Zn^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}, as cofactors. The metal ion in type II aldolases serves as the Lewis acid in the active site.

The structure of fuculose-1-phosphate aldolase from *E. coli* has been determined at a 2.4 Å resolution [6, 10]. The structure showed that dihydroxyacetone phosphate ligates a zinc ion with its hydroxyl and keto oxygen atoms, shifting E73 away from the zinc coordination sphere to a nonpolar environment. The aldehyde oxygen participates in Zn^{2+} coordination, facilitating the reaction by rendering the aldehyde carbon more positive. The enzyme complex with a Zn^{2+} ion shows three His residues as metal binding sites among four His residues. H92, H94, and H155 coordinate with Zn^{2+} in order to facilitate the proper catalysis. The amino acid sequence around these three histidines is well conserved among type II aldolase genes as well as the *M. jannaschii* aldolase gene.

Although the DNA coding sequence for fuculose-1-phosphate aldolase [4], a number of mechanistic studies [6, 10, 26], and the thermostability of a variety of enzymes in thermophilic bacteria [3, 13, 24] have already been reported, little information concerning the identity of the active site amino acids involved in substrate binding and/or catalysis is available. Studies on the imidazole modification of 4-hydroxy-4-methyl-2-oxoglutarate aldolase from *Pseudomonas ochraceae* and fructose-1,6-diphosphate aldolase from rabbit muscle tissue have previously been reported [5, 16]. Yet, beyond these observations on the potential role of histidine residues, studies to determine the role of the active-site amino acid of fuculose-1-phosphate aldolase are lacking.

Accordingly, in this report, the chemical modification of fuculose-1-phosphate aldolase by diethylpyrocarbonate (DEP) was employed in an attempt to show the importance of histidine residues in the catalytic function. As a result, chemical and kinetic evidence of the involvement of histidine residues in fuculose-1-phosphate aldolase catalysis is provided based on the pH dependence of inactivation,

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substrate protection studies, CD spectral studies, and detection of N-carbethoxyhistidine by UV difference spectra.

MATERIALS AND METHODS

Chemicals

The *Methanococcus jannaschii* was obtained from Deutsch Sammlung von Mikroorganismen (DSM #2661). The diethylpyrocarbonate (ethoxyformic anhydride), Tris (hydroxymethyl) aminomethane, DHAP, DL-glyceraldehyde, β-nicotinamide adenine dinucleotide in a reduced form, α-glycerophosphate dehydrogenase, zinc chloride, zinc sulfate, and potassium chloride were all purchased from Sigma Chemical Co. (St. Louis, U.S.A.). The ethylenediaminetetraacetic acid was purchased from Shinyo Pure Chemical Co. (Osaka, Japan). The trimethylphosphonoacetate was purchased from Aldrich Chemical Co. (Milwaukee, U.S.A.). The hydroxylamine hydrochloride was purchased from Showa Chemicals Inc. (Dsaka, Japan). The imidazole was purchased from Fluka (Switzerland). The acetonitrile was purchased from Merck. All other chemicals were of pure or extra pure grade and commercially available.

Enzyme Purification

Fuculose-1-phosphate aldolase was purified from *Methanococcus jannaschii* as described previously [4]. Briefly, the cording region of the aldolase gene was expressed in *E. coli* using a pET system to a level of 30% of the total cellular proteins. The aldolase was purified to more than 95% homogeneity by heat treatment and ion-exchange chromatography. The enzyme preparations used in this investigation were homogeneous, as judged by polyacrylamide gel electrophoresis.

Enzyme Assay

The aldolase activity was measured by determining the concentration of the remaining DHAP, as described by Ghalambor and Heath [8]. The reduced amount of NADH was measured by α-glycerophosphate dehydrogenase absorbed at 340 nm (ε_{340}=6,220 $M^{-1}cm^{-1}$) at 25°C, using a Hewlett Packard 8452 Diode Array spectrophotometer equipped with a constant temperature cell housing and Shimadzu UV. All reactions were carried out in a 1-ml cuvette with a 1-cm light path length and incubated for at least 5 min in the cell. The standard assay reaction mixture contained a 100 mM Tris-HCl buffer (pH 8.0), 1.0 mM DHAP, 15.0 mM DL-glyceraldehyde, and 0.2 mM $ZnCl_2$ in a total of 1.0 ml. The catalytic reaction was initiated by adding 13 mM of aldolase to the reaction mixture at 37°C. After 10 min of incubation, 200 ml of the sample was transferred to 1 ml of the secondary reaction solution containing a 100 mM Tris-HCl buffer (pH 7.5), 0.3 mM NADH, 50 mM EDTA, and 2.0 units of α-GDH. The amount of DHAP was calculated from the reduced amount of NADH using α-glycerophosphate dehydrogenase. By comparing the amount of DHAP in the absence (OD_i) and presence (OD_f) of fuculose-1-phosphate aldolase in the aldol condensation reaction, the specific activity of the enzyme was calculated. The enzyme activity was defined as the difference between OD_i and OD_f. One unit of enzyme activity was defined as the quantity that catalyzed the decomposition of one mmole of DHAP per minute under the standard conditions of the above two-step assay.

Chemical Modification with Diethylpyrocarbonate

Stock solutions of diethylpyrocarbonate were freshly prepared in acetonitrile immediately before use. The molar concentration of DEP was determined spectrophotometrically by its reaction with 10 mM imidazole in a 100 mM potassium phosphate buffer, pH 6.0. The increase in absorbance at 240 nm was measured and the concentration was estimated using a value of 3.0×10^3 $M^{-1}cm^{-1}$ [19, 22]. Inactivation by DEP was carried out at 25°C in a reaction mixture containing a 100 mM potassium phosphate buffer (pH 6.0), the enzyme (1.0 mM), and different concentrations of the reagent, as indicated in the figure legends. The reaction was started by the addition of DEP, and a 400 μl aliquot of the modified enzyme was assayed for the remaining activity at the indicated time intervals. The concentration of acetonitrile was kept below 5% (v/v) throughout the experiment.

Absorption Spectra Measurement

The ultraviolet spectra were measured using a Shimadzu UV with a 1-cm path length cell at 25°C. The absorbance difference was measured at various time periods. The fluorescence spectra were measured using an ISS spectrophotometer at 25°C and an excitation wavelength of 260 nm. Potassium phosphate buffers, pH 6.0, containing the respective modifiers served as controls.

Circular Dichroism

The CD spectra were recorded on a Jasco J-715CD spectropolarimeter with a cell length of 0.1 cm at 25°C. One-hundred mg aliquots of each native and modified protein in a potassium phosphate buffer at pH 8.0 were used for the CD analysis. The CD data were the average of five spectra. The CD spectra were analyzed using a program based on J-700 for Windows Secondary Structure Estimation.

pH Studies

The pH dependence of the inactivation rate was obtained using the above procedure with 100 mM MES (pH 5.0–6.0), 100 mM MOPS (pH 6.0–7.5), and 100 mM TAPS (pH 7.5–9.0). In all cases, the stability of the enzyme was determined by incubating the enzyme at the pH of interest and assaying aliquots at several time intervals at pH 9.0. Since DEP is known to be unstable at higher pH values, it

Table 1. Substrate effects of various compounds relative to DEP modification of aldolase[a].

Substrate	Relative activity (%)
Control	100
DEP	42
DEP+DHAP (0.1 mM)	81
DEP+DL-Glyceraldehyde (0.75 mM)	49
DEP+Zn^{2+} (0.2 mM)	51
DEP+KCl (5 mM)	54

[a]Aldolase activity was assayed as described in Materials and Methods. The relative activity refers to the percentage of activity obtained by comparison with the nonactivation control.

was necessary to determine the half-life of the reagent as a function of pH. The half-life of DEP under various conditions employed for the modification reaction was determined using the method of Berger [1]. In all cases, the degradation of DEP was not a significant problem over the course of the reaction.

Aldolase Protection from DEP Inactivation by Substrates

Aldolase (1.67 μM) was incubated with 5 mM DEP in a 100 mM phosphate buffer pH 6.0 with the substrates listed in Table 1, at the indicated concentrations. The substrate concentrations were used to their K_m values. After incubating for 6 min at 25°C, the levels of enzyme activity were determined. The remaining activity refers to the percent of activity obtained by comparison with the nonactivation control.

RESULTS

Inactivation of Aldolase by DEP

Fuc aldolase was treated with increasing concentrations of DEP, and the time course of the loss of enzymatic activity of the DEP-treated enzyme was followed. Since DEP undergoes hydrolysis in aqueous solutions, the inactivation process was corrected for the spontaneous hydrolysis of DEP as described by

$$\ln(A/A_o)=-(k/k')I_o(1-e^{-k't}) \quad (1)$$

where A/A_o is the fractional activity remaining at time t, I_o is the initial concentration of DEP, k' is the pseudo first-order rate constant for the inactivation of Fuc aldolase by DEP, and k' is the constant for the spontaneous hydrolysis of DEP [9]. In order to estimate the value of k', DEP was incubated in 100 mM potassium phosphate buffer (pH 6.0) at 25°C. At various time intervals, aliquots were removed and the amount of DEP remaining was determined with excess imidazole and measuring the absorbance increase at 240 nm, as described under Materials and Methods. The value of k' was estimated to be 0.015 min^{-1}. Plots of $\ln A/A_o$ versus $(1-e^{-k't})/k'$ at various DEP concentrations were not linear, as shown in Fig. 1A, indicating that the inactivation

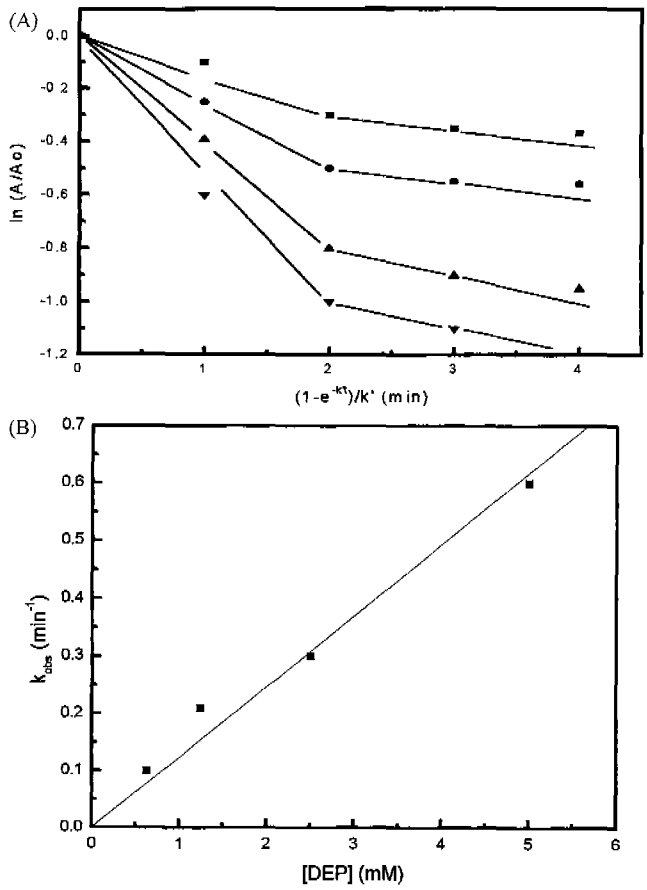

Fig. 1. Inactivation of fuculose-1-phosphate aldolase by DEP. (A) The enzyme (1 μM) was incubated in a solution of a 250 mM potassium phosphate buffer (pH 6.0). The reaction was started by the addition of indicated concentrations of DEP in acetonitrile, and then aliquots of the modified enzyme were assayed for the remaining activity at incubated time intervals. The concentrations of DEP were as follows: (■), 0.625 mM; (●), 1.25 mM; (▲), 2.5 mM; (▼), 5 mM. (B) Second-order rate constant.

process appeared to be a fast phase and followed by a slow phase. A second-order rate constant (k_2) of 120 $min^{-1}M^{-1}$ was obtained from the slope of the plot of k_{obs} versus the DEP concentration (Fig. 1B). This result indicated that no reversible complex was formed prior to the inactivation process. The values for k_{obs} were obtained from the slopes of the linear plots in Fig. 1A.

Correlation between Aldolase Activity and Histidine Residues Modified by DEP

The modification of the histidine residues in aldolase was monitored by the absorbance changes in the UV region. The difference in the absorption spectrum of the DEP-treated enzyme versus the unmodified enzyme at pH 6.0 revealed only a single peak at 240 nm, which is characteristic of an N-carbethoxyimidazole derivative (Fig. 2). Modification of either a cysteine or tyrosine residue by DEP was not detected since no change was observed in the difference

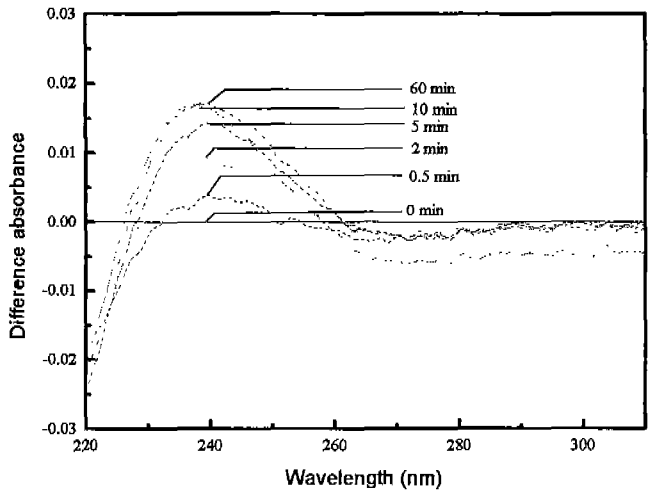

Fig. 2. Difference absorbance of fuculose-1-phosphate aldolase by DEP.

Difference spectra of DEP-modified fuculose-1-phosphate aldolase. Aldolase (5 μM) in a 100 mM potassium phosphate buffer (pH 6.0) was incubated with 2 mM DEP at 25°C. At the indicated times, the spectra were acquired versus an identical reference sample consisting of only the enzyme and buffer.

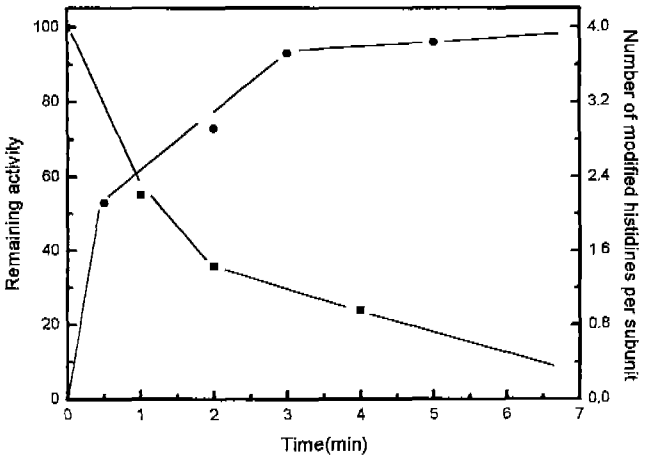

Fig. 3. Number of histidine residues modified and remaining activity.

Relationship between the extent of histidine modification and the loss of catalytic activity of fuculose-1-phosphate with 3 mM DEP. The number of modified histidine residues was determined by measuring the absorbance increase of the sample at l=240 (●). Samples were assayed for remaining enzyme activity (■) at each time.

spectrum at 230 nm for cysteine modification and/or 278 nm for tyrosine modification [11, 12, 18, 20, 25].

This increase in absorbance at 240 nm upon treatment with DEP was used to estimate the number of histidine residues modified within the protein. Fuc aldolase contains four histidine residues at positions 72, 87, 89, and 147 in its primary structure, as predicted from its DNA sequence [17] and determined from a total amino acid analysis performed on recombinant Fuc aldolase. The number of histidine residues modified by DEP, as determined by the difference spectra at 240 nm, correlated with the loss of enzyme activity (Fig. 3). Sequential modification of the histidine residues was achieved by the addition of increasing amounts of DEP. A plot of the residual enzyme activity versus the number of histidine residues modified by DEP showed that all four histidine residues in the subunit were modified to inactivate the enzyme completely. To calculate the number of essential histidine residues in Fuc aldolase, the statistical method of Tsou [9] was utilized:

$$x=(p-m)/p=(A/A_0)^{1/\mu} \quad (2)$$

This equation relates the remaining activity A/A_0 when m groups have reacted with the number of essential residues (μ) and fraction of residues remaining (x), on the assumption that all of the modifiable residue (p) is equally reactive towards the modifying reagent (DEP) and that the modification of any of the essential residues results in complete inactivation. In this plot, the whole number μ is varied in value from μ=1 to n, where n equals the possible number of modifiable groups. The value of μ that produces the highest correlation coefficient when the data are fit to a straight line provides an estimate of the essential number of modified residues. Over the course of the modification of Fuc aldolase, the number of modified histidine residues and the remaining activity at a given time were simultaneously determined. A plot of x vs. $(A/A_0)^{1/i}$ gave a curvature regardless of the i number (data not shown). The curvature relationship between the residual activity and the amount of the modified residues implied that more than two of the four reactive residues were catalytically important for the enzyme activity. When Fuc aldolase was incubated with 5 mM DEP, a fast phase based on a time dependent inactivation resulted in only 50% activity within one minute, indicating that two or three of the four histidine residues appeared to be essential for the enzyme activity.

Substrate Protection of Aldolase Against Inactivation by DEP

The effect of the substrate on inactivation was examined by incubating aldolase with 5 mM DEP in the presence of DHAP, DL-glyceraldehyde, Zn^{2+}, and KCl. The ability of the substrate, DHAP, and DL-glyceraldehyde to protect aldolase against inactivation by DEP was evaluated and the results are shown in Table 1. DHAP as a carboxyl donor provided a high level (81%) of protection for aldolase against DEP inactivation. These results suggest that DHAP interacted with a histidine(s) at or near the active site of aldolase or that the DHAP binding induced a conformational change that prevented any histidine modification by DEP. In contrast, DL-glyceraldehyde as a carbonyl acceptor provided less than 49% protection, whereas Zn^{2+} as a cofactor and KCl as a salt provided less than 51% and 54% protection, respectively.

pH Dependence of Inactivation

The pK value of a modified residue can be estimated from the pH dependency of the inactivation rate. The rate of inactivation of Fuc aldolase by DEP was determined using 6 mM DEP as the function of the pH. The inactivation of Fuc aldolase by DEP, was examined over the pH range of 5.5–8.5. At all pH values, the process was first order with respect to DEP, as shown at pH 7.0. Thus, the 6 mM concentration of DEP was used at all pH values. A plot of the experimentally determined k_{obs} values as a function of pH indicated that DEP inactivation of Fuc aldolase was pH dependent (Fig. 4). The dependence of inactivation on the pH was evaluated in greater detail according to:

$$k_{obs}=k_{obs(max)}/(1+[H^+]/K_a) \quad (3)$$

Equation (3) can be rearranged to the following linear form:

$$k_{obs}[H^+]=K_a k_{obs(max)}-K_a k_{obs} \quad (4)$$

where K_a is the acidic dissociation constant of the reacting group and $k_{obs(max)}$ is the second-order rate constant of the unprotonated reacting group [23]. When the dependency of k_{obs} over a pH range of 5.5–8.5 was analyzed according to Eq. (4) (from a plot of $k_{obs}[H^+]$ versus k_{obs}), a pK_a value of 5.7±0.2 and $k_{obs(max)}$ value of 0.25 min^{-1} were obtained. As discussed previously, the UV difference spectrum of DEP-treated Fuc aldolase versus the untreated enzyme at pH 6.0 and λ=240 nm was indicative of a protein-bound N-carbethoxyhistidine. Accordingly, these results suggested that the DEP inactivation of Fuc aldolase between pH 5.5–8.5 resulted from the modification of a histidine residue with a pK_a of 5.7 or from the modification of one or more histidine residues with closely related pK_as.

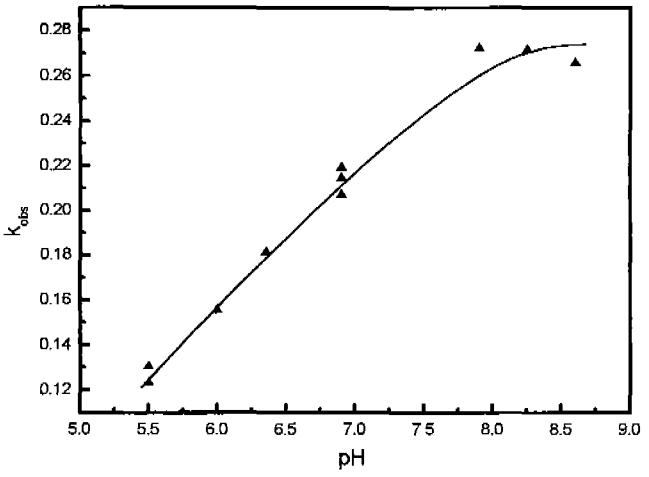

Fig. 4. Effect of pH on DEP fuculose-1-phosphate aldolase inactivation.

The aldolase (1.67 μM) was incubated with 3 mM DEP at various pH values. Aliquots were taken at various time intervals for determination of the residual activity. The rate constant (k_{obs}) is the second-order rate constant of the unprotonated reacting group.

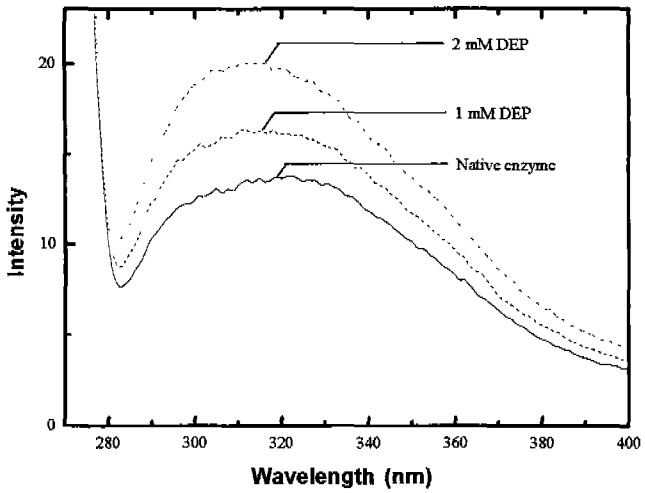

Fig. 5. Emission spectra of DEP-modified fuculose-1-phosphate aldolase.

Aldolase (1.67 μM) in a 100 mM potassium phosphate buffer (pH 6.0) was incubated with various concentrations for 10 min. The reaction mixture was dialyzed against the same buffer, and the emission spectra were determined as described in Materials and Methods.

Structural Analysis

A pronounced effect was observed on the emission spectrum of intrinsic fluorescence, which was characterized by a large increase in intensity at 320 nm (Fig. 5). The intensity of the maximum emission wavelength apparently changed within 10 nm. The CD spectrum of the native Fuc aldolase and DEP-treated Fuc aldolase is shown in Fig. 6. Both the native and DEP-treated aldolases exhibited negative cotton effects through 206 nm and 224 nm, respectively. The structural analysis revealed a 30% α-helix, 36% β-sheet,

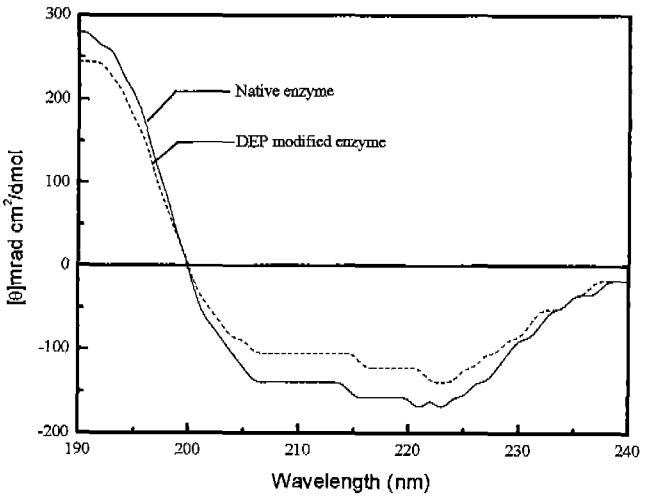

Fig. 6. Effect of DEP modification on the secondary structure of fuculose-1-phosphate aldolase.

Each protein was present at a concentration of 0.1 mg/ml in 25 mM potassium phosphate buffer (pH 7.5). The data were the average of five spectra. Native enzyme, solid line; DEP modified enzyme, dashed line.

and 34% random-coil structure for the native aldolase. An analysis of the DEP-treated aldolase indicated certain changes in the secondary structure, as revealed by a 42% α-helix, 20% β-sheet, and 38% random-coil structure.

DISCUSSION

Chemical modification studies have been utilized to identify functionally important histidine residues in *Methanococcus jannaschii* Fuc aldolase. Several reports describe the utilization of DEP to explore the role of histidine residues in a number of enzymes [7, 8, 15 19]. Chemical modification studies have shown that Fuc aldolase is inactivated by DEP in a time-dependent manner. The current kinetic analysis of the inactivation revealed that the reaction between DEP and Fuc aldolase appeared to occur in two phases; a fast one followed by a slower one. There are several possible explanations for the biphasic curves in the first-order plots. First, since the reagent itself is unstable, the biphasicity could have resulted from a decrease in the reagent concentration over time. Although this can be a factor in long kinetic studies, it was not a major problem in this case. The rates of the fast and slow phases of the reaction are roughly so, and 10 times faster than that of the reagent loss. Second, there were two or more different histidines with different rate constants for modification which affected the activity to differing extents (some directly, others through conformational changes). Third, the modification of histidines that did not affect the activity directly may have induced structural changes in the proteins influencing the histidine(s) that did affect the activity.

The second and third explanations differ according to their direct or indirect action on the activity or binding. In the third case, protein rearrangement based on DEP modification should occur at a rate approximating the slow phase, while this is not necessarily true for the second case. A plot of the pseudo-first-order rate constant extracted from the initial linear region indicated that the reaction between the enzyme and the reagent was a simple bimolecular reaction. A second-order rate constant of 120 $M^{-1}min^{-1}$ was determined for the inactivation of Fuc aldolase by DEP. The values for k_{obs} were obtained from the slopes of the linear plots in Fig. 1A. These values fell within the range of the rate constants obtained for the inactivation of other proteins containing important histidine residues, for which values between 0.67 and 368 $M^{-1}min^{-1}$ have been reported [7, 15, 17].

Although DEP is generally considered to selectively modify histidine residues in a pH range of 5.5–8.5 [17], the functional groups of other amino acids may also react with DEP. The UV difference spectrum of the DEP-treated Fuc aldolase versus the untreated Fuc aldolase in a 100 mM potassium phosphate buffer (pH 6.0) only showed an increase in absorbance at 240 nm, which is characteristic of a protein bound N-carbethoxyhistidine [20, 21]. The possible modification of either a tyrosine or cysteine residue by DEP was not detected in the spectrum. Activity restoration by hydroxylamine was not attempted because of the reaction with DHAP. However, the UV difference spectrum of the hydroxylamine-treated Fuc aldolase only showed a decrease in absorbance at 240 nm. The rates of inactivation of Fuc aldolase by DEP in different buffers were dependent on the pH, and a value of 5.7 was calculated for the pK_a of the modified residue. Except for the pH dependency studies, the modification studies of Fuc aldolase were all performed at pH 6.0. At this pH, the evidence only suggests the modification of histidine residues.

The complete modification of all four histidines in Fuc aldolase by DEP was achieved after prolonged reactions in the presence of an excess of DEP. The correlation between the number of modified residues and the enzyme activity revealed four histidine residues. Although four histidine residues appeared to lead to a complete loss of aldolase activity, according to Tsou's statistical analysis [9] and the time course of the modification (Fig. 3), two or three of the four reactive residues were revealed as essential for the enzyme activity. The rate of inactivation of Fuc aldolase was dependent on the pH, and pK_a 5.7 was calculated for the modified residue. It was observed that the DEP inactivation of Fuc aldolase occurred more significantly at the slower rates. This observation suggests that the protonation process was slower.

DHAP protected Fuc aldolase from inactivation by DEP, suggesting that the DHAP binding site was located near essential histidine residues. The observed protection could also indicate that, upon substrate binding, conformational changes occurred within Fuc aldolase that limited the accessibility of DEP to these residues. The protection afforded by DL-glyceraldehyde was significantly smaller than that provided by DHAP. This result could reflect that the binding site of DL-glyceraldehyde was away from the histidine sites or that the DL-glyceraldehyde binding induced the conformational change of the enzyme. In all likelihood, the enzyme conformation adopted in the presence of the individual substrates differed from that in the ternary complex, and the substrate binding pockets in the former were not fully defined.

The structure of L-fuculose-1-phosphate aldolase from *E. coli* shows that dihydroxyacetone phosphate ligates a zinc ion with its hydroxyl and keto oxygen atoms, shifting Glu73 away from the zinc coordination sphere to a nonpolar environment [6]. The activation of DHAP for a nucleophilic attack is achieved by deprotonating its C-3 atom. This proton is accepted by the carboxylated Glu73 that positions appropriately in a nonpolar environment between the side chains of Phe76, Phe131, Leu136, and His155, after it has left the Zn^{2+} coordination sphere. In contrast, the structural change in the DEP-treated aldolase

was attributed to the nonpolar environment in the Zn^{2+} coordination sphere.

Chemical modification studies on Fuc aldolase have revealed that two or three histidines play an important role in the aldol condensation catalyzed by Fuc aldolase. All four histidine residues were found to be in conducive in inactivating the enzyme. Further research is now focusing on site-directed mutagenesis in order to elucidate the catalytic function and role of histidine residues.

Acknowledgment

A Korea Research Foundation Grant supported this work (KRF-1999-D00320).

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