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Role of the Histidine 176 Residue in Glyceraldehyde-3-phosphate Dehydrogenase As Probed by Site-Directed Mutagenesis†

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ABSTRACT: The catalytically essential amino acid, histidine 176, in the active site of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been replaced with an asparagine residue by site-directed mutagenesis. The role of histidine 176 as a chemical activator, enhancing the reactivity of the thiol group of cysteine 149, has been demonstrated, with iodoacetamide as a probe. The esterolytic properties of GAPDH, illustrated by the hydrolysis of *p*-nitrophenyl acetate, have been also studied. The kinetic results favor a role for histidine 176 not only as a chemical activator of cysteine 149 but also as a hydrogen donor facilitating the formation of tetrahedral intermediates. These results support the hypothesis that histidine 176 plays a similar role during the oxidative phosphorylation of glyceraldehyde 3-phosphate.

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] is a tetrameric enzyme, using NAD^+ as a cofactor. The reaction it catalyzes can be decomposed into an oxidoreduction step, with formation of an acyl-enzyme and NADH, and a phosphorylating step, including the nucleophilic attack of the acyl-enzyme by phosphate ion in the presence of NAD^+, giving rise to 1,3-diphosphoglycerate. The currently accepted mechanism (Harris & Waters, 1976), supported by extensive pre-steady-state (Trentham, 1971; Harrigan & Trentham, 1974) and steady-state kinetic experiments (Duggleby & Dennis, 1974; Meunier & Dalziel, 1978), is in favor of the formation of a thiohemiacetal intermediate involving two essential residues, Cys-149 and His-176. The His residue is postulated to act as a chemical activator by enhancing the reactivity of the thiol group of Cys-149, possibly through the formation of an ion pair with the imidazolium ring of His-176 (Polgar, 1975). The tetrahedral transition state for the hemithioacetal formation would also be stabilized by hydrogen bonding between the protonated imidazole N^ϵ of His-176 and the carbonyl oxygen atom of glyceraldehyde 3-phosphate, thus favoring a nucleophilic attack by the thiol group. A similar activation could also occur during the phosphorylating step, with the formation of a hydrogen bond between the imidazolium ring and the oxygen of the carbonyl group of the acyl-enzyme, facilitating the nucleophilic attack by the phosphate ion.

As postulated for certain dehydrogenases (Holbrook et al., 1976; Fersht, 1985), the His residue could also play a role as a base catalyst, facilitating hydride transfer, during the oxidoreduction step, in the case of GAPDH, from the thiohemiacetal intermediate toward the C4 position of the nicotinamidium ring of the coenzyme (Harris & Waters, 1976). According to the crystal structure of GAPDH from *Bacillus stearothermophilus* (Skarzynski et al., 1987; Skarzynski &

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[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); Tris, tris(hydroxymethyl)aminomethane; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; pNPA, *p*-nitrophenyl acetate; pNP, *p*-nitrophenol; IAM, iodoacetamide; G3P, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; P_i, inorganic phosphate; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms; *B. stearothermophilus*, *Bacillus stearothermophilus*; *E. coli*, *Escherichia coli*. Mutant is referred to as follows: His-176 → Asn-176 enzyme with asparagine at position 176.

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Wonacott, 1988) the positions of the thiol group of Cys-149 and of the imidazole ring of the His-176 are consistent with all these hypotheses (distance = 3.96 and 3.63 Å in the apoenzyme and holoenzyme structure, respectively).

We have recently isolated (Branlant et al., 1983) and sequenced (Branlant & Branlant, 1985) the GAPDH gene from *Escherichia coli* and have obtained a very high level of GAPDH expression in *E. coli* (80% of the soluble protein). This provided the opportunity to selectively replace amino acids postulated to be implicated in the enzymatic catalysis. In this paper, we focused our site-directed mutagenesis studies on residue His-176 in order to investigate its precise function in catalysis.

Materials and Methods

Chemicals and Reagents. All chemicals used were commercial products of reagent grade purchased from Merck. Iodoacetamide, *p*-nitrophenyl acetate, and D,L-glyceraldehyde 3-phosphate diethyl acetal were purchased from Sigma. NAD^+ was purchased from Boehringer Mannheim. Gel filtration resins ACA 34 and ACA 54 were IBF products.

Site-Directed Mutagenesis. The strategies used for construction of the recombinant phage and mutagenesis were described previously (Mougin et al., 1988). Briefly, a 20-base oligonucleotide was synthesized with a single mismatch at the first base of the His-176 codon (CAC → AAC). The method used for mutagenesis was that described by Kramer et al. (1984). Production of the mutated enzyme was from *E. coli* DF221 cells ($GAPDH^-$) transformed by the corresponding recombinant plasmids.

Enzyme Purification. After sonication of the transformed cells (grown 24 h in M63 succinate–glycerol ampicillin or LB ampicillin medium), the GAPDH enzyme was purified by ammonium sulfate fractionation (66%–88%) and chromatography on ACA 34 resin equilibrated in 20 mM potassium phosphate buffer containing 2 mM EDTA, pH 6.8. At this stage, the holoenzyme (absorbance ratio 280/260 = 1) was pure, as checked on 10% SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

Apoenzyme was prepared either by gel filtration of the holoenzyme on an ACA 54 resin equilibrated in 100 mM sodium citrate buffer containing 2 mM EDTA, pH 5.1, or passage through a minicolumn of activated charcoal (Krimsky & Racker, 1963) equilibrated in 5 mM EDTA buffer, pH 7.2. Typically, specific activities of 350 and 7 units/mg with an absorbance ratio 280/260 = 2 were attained for wild-type and mutant enzyme, respectively.

Enzymatic Activity. Enzymatic activity in the oxidative phosphorylation and K_M for NAD^+, G3P, and P_i were determined in the conditions described by Ferdinand (1964). Enzymatic activity in the reverse reaction and K_M for 1,3-DPG were determined in 10 mM Pipes buffer, pH 7.1, in the presence of NADH (0.2 mM).

(*1*) *Alkylation with Iodoacetamide (IAM).* Iodoacetamide was dissolved in water. The concentration of the stock solution was determined spectrophotometrically, according to the method of Finckle and Smith (1958) (ϵ_{375nm} = 372 $M^{-1}\cdot cm^{-1}$).

Reaction was performed in 20 mM Tris-HCl buffer, pH 7 at 37 °C, in test tubes containing wild-type apoenzyme (7.7 μN, as expressed in terms of normality N, based on a subunit molecular weight of 36 000) or His-176 → Asn-176 mutant apoenzyme (21.4 μN) and IAM (see conditions below). Aliquots were withdrawn periodically and if necessary diluted in 20 mM Tris-HCl buffer, pH 7, before determination of the residual activity. Control samples were incubated under the same conditions, except that the modifying agent was omitted.

(*a*) *Kinetics of Alkylation.* The alkylation of GAPDH by iodoacetamide is consistent with a kinetic process as shown:

$$E + nI \xrightarrow{k_2} EI_n$$

$$v = k_2(E)(I)^n$$

When $n = 1$, kinetics are of second order. In the presence of excess of alkylating agent (I), the reaction follows pseudo-first-order reaction kinetics:

$$v = k_{obsd}(E) \qquad \text{with } k_{obsd} = k_2(I)^n$$

Kinetics were measured under pseudo-first-order conditions, with a 10- to 50-fold excess of IAM over the concentration of the essential –SH groups. The pseudo-first-order constant k_{obsd} was determined for each inhibitor concentration from plots of log (A_0/A) versus time (A_0 and A correspond to the initial enzymatic activity and the activity at time t, respectively). The second-order rate constant k_2 and reaction order n with respect to IAM concentration were deduced from the equation $\log k_{obsd} = \log k_2 + n \log I$.

(*b*) *pH Dependence of the Alkylation Reaction.* Alkylation rates were determined over a wide range of pH (5.0–11.0) with different buffers (acetate, imidazole, Tris, carbonate, borate) at a constant ionic strength of 0.09. The second-order kinetic constant k_2 was calculated at each pH by dividing the first-order rate constant k_{obsd} by the concentration of IAM.

(*2*) *Hydrolysis of p-Nitrophenyl Acetate (pNPA).* pNPA was dissolved in 95% ethanol. The hydrolysis of pNPA was followed spectrophotometrically at 22 °C in 20 mM Tricine buffer, pH 7.0, containing 5% ethanol, by measuring the absorption at 400 nm of *p*-nitrophenolate ions (pNP) released during the reaction (ϵ_{400nm} = 16 500 $M^{-1}\cdot cm^{-1}$). Corrections were made for the spontaneous hydrolysis of the compound under the same experimental conditions. The presence of ethanol (5%) in the reaction medium was shown to have no effect on enzyme activity. Enzyme concentrations were 16 and 7 μN for wild-type and His-176 → Asn-176 apoenzyme, respectively, in the pre-steady-state kinetics and 16 and 8 μN for wild-type and His-176 → Asn-176 enzymes, respectively, in the steady-state kinetics. The range of substrate concentrations tested is indicated in the legends of Tables III and IV.

(*a*) *Reaction Mechanism and Kinetic Studies.* The kinetic scheme for the GAPDH-catalyzed hydrolysis of pNPA can be written in the same form as that described for α-chymotrypsin (Kézdy & Bender, 1962):

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES_1 \xrightarrow[P_1]{k_2} ES_2 \xrightarrow{k_3} E + P_2 \qquad (1)$$

with P_1 = pNP and P_2 = acetate. This well-known three-step mechanism, also observed for porcine pancreatic lipase (De Caro et al., 1986), shows two phases when substrate concentrations are in large excess over that of the enzyme ($S \simeq S_0$): a rapid pre-steady-state phase (a) indicative of acyl-enzyme formation (ES_2) and a slower phase (b) reflecting steady-state enzyme deacylation (Figure 1).

The first-order rate constant (λ) of the pre steady state is given by

$$\lambda = \frac{k_2(S_0)}{K_s + (S_0)}$$

$$\frac{1}{\lambda} = \frac{1}{k_2} + \frac{K_s}{k_2(S_0)} \qquad (2)$$

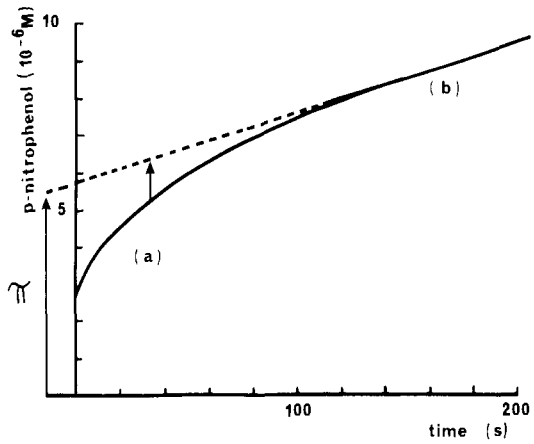


FIGURE 1: Time course of the liberation of pNP during the reaction of wild-type GAPDH with pNPA. Reaction was performed in 20 mM Tricine buffer, pH 7, containing 5% ethanol. GAPDH and pNPA concentrations were 6 μN and 8 mM, respectively. The two phases (a and b) are described under Materials and Methods.

This constant is experimentally determined by extrapolating to zero time the straight line of the steady state (π) and measuring the differences ΔA between the extrapolated line and the pre-steady-state curve (Figure 1). By plotting, for a given (S), the values log ΔA versus t, a straight line of slope $-\lambda$ is obtained. The kinetic constants K_s and k_2 can be determined from eq 2. The amount of pNP (P_1) liberated at the end of the burst is given by eq 3, which allows the determination of $K_{M(app)}$. The reaction rate at the steady state (v) is given by eq 4 and allows the determination of k_3 and $K_{M(app)}$.

$$\frac{1}{\sqrt{\pi}} = \frac{1}{\sqrt{(E_0)}} + \frac{K_{M(app)}}{(S_0)\sqrt{(E_0)}} \quad (3)$$

$$\frac{1}{v} = \frac{1}{k_3(E_0)} + \frac{K_{M(app)}}{k_3(E_0)(S_0)} \quad (4)$$

(*b*) *Buffer Concentration Dependence.* The dependence of the deacylation step on buffer concentration was followed for both enzymes at two different Tris buffer concentrations (10 mM Tris-HCl, pH 7, containing 0.45 M NaCl and 500 mM Tris-HCl, pH 7) at a constant ionic strength of 0.46 M.

(*c*) *pH Dependence of k_{cat}/K_M.* Hydrolysis of pNPA was followed for both enzymes over a wide range of pH (6.0–9.5) at a constant ionic strength of 0.09 as described in the study of alkylation by IAM. The k_{cat}/K_M value at each pH was determined from the Lineweaver–Burk plot for both apoenzymes.

Results and Discussion

Justification of the His-176 Substitution by Asn-176. Before undertaking the replacement of residue His-176 with Asn by site-directed mutagenesis, it was necessary to verify that this substitution could be accommodated in the active site. Although only the crystal structure of the enzyme from *B. stearothermophilus* has been determined at high resolution (Leslie & Wonacott, 1983; Skarzynski et al., 1987; Skarzynski & Wonacott, 1988), the active site residues of all species are almost totally conserved, so that we are confident that there can be no significant differences in structure of the *E. coli* active site region. By computer-assisted graphic simulation, His-176 can be replaced by Asn in the enzyme from *B. stearothermophilus* without perturbing the structure of the active site. The amide group can maintain a hydrogen bond with the main-chain carbonyl of serine 177, paralleling that formed with the imidazole ring of His-176 in the native enzyme.

Table I: Comparison of the Catalytic Properties of Native and Mutant His-176 → Asn-176 GAPDH

	native GAPDH	mutant GAPDH
$K_M(NAD^+)$ (10^{-3} M)	0.042	0.040
K_M(G3P) (10^{-3} M)	1.5	1.0
$K_M(P_i)$ (10^{-3} M)	22.0	22.0
k_{cat} (s^{-1})[a]	1056	18
K_M(1,3-DPG) (10^{-3} M)	0.015	0.030
k_{cat} (s^{-1})[b]	900	20

[a] GAPDH activity was determined according to the procedure of Ferdinand (1964) for the oxidative phosphorylation. [b] It was determined in Pipes buffer as described under Materials and Methods for the reverse reaction. Michaelis constants were determined by Lineweaver–Burk double-reciprocal plots.

Michaelis Constants of the His-176 → Asn-176 Mutant Enzyme. Table I shows some of the catalytic properties of the purified mutant enzyme, in comparison with those of the wild-type enzyme. No significant difference was observed for the K_M values of the substrates (G3P, P_i, and 1,3-DPG) and of the coenzyme. This result was as expected, since His-176 is not directly implicated in the binding of these ligands. It verifies that mutation does not induce local perturbation of the structure of the active site interfering with substrate binding.

However, the mutation resulted in a 60-fold reduction of k_{cat} in the oxidative phosphorylation reaction and a 45-fold reduction in the reductive dephosphorylation reaction. Such marked reductions raised the question whether the removal of the His residue might have changed the nature of the limiting step. Only the mechanism of the lobster enzyme has been studied in detail. For this enzyme, the rate-determining step in the oxidative phosphorylation process has been described to be NADH release at high pH and phosphorolysis of the acyl intermediate at low pH (Trentham, 1971). For the reverse reaction, the rate-determining step would be associated with a conformational change caused by NADH binding at high pH, and the release of G3P at low pH (Trentham, 1971).

Clearly, the catalytic mechanism is rather complex. So in order to investigate the function of His-176, we focus on two model reactions using chemical reagents as probes.

Presentation of the Two Model Reactions. A study of the kinetic behavior of the essential Cys-149 of native and mutated enzymes, in its reaction with the irreversible inhibitor iodoacetamide (MacQuarrie & Bernhard, 1971), should reveal whether His-176 affects the nucleophilic character of Cys-149. Second, the esterolytic activity of GAPDH against pNPA has been previously shown (Taylor et al., 1963). Comparison of the kinetics of pNPA hydrolysis between the mutant and the wild-type enzyme should reveal the role of His-176, acting perhaps not only as a chemical thiol activator but also as a hydrogen donor facilitating the formation of tetrahedral intermediates and as a base catalyst (Figure 2). All the studies were carried out on the apoenzyme, because of the higher reactivity of the enzyme, in the absence of NAD^+, against IAM and pNPA.

(*1*) *Alkylation with IAM.* (*a*) *Kinetics of Inactivation.* The inactivation of wild-type and mutant GAPDH by iodoacetamide follows pseudo-first-order kinetics and is concentration dependent (results not shown). The second-order rate constant and reaction order with respect to IAM can be deduced from the double logarithmic plots of the pseudo-first-order rate constant against IAM concentration (curves not shown).

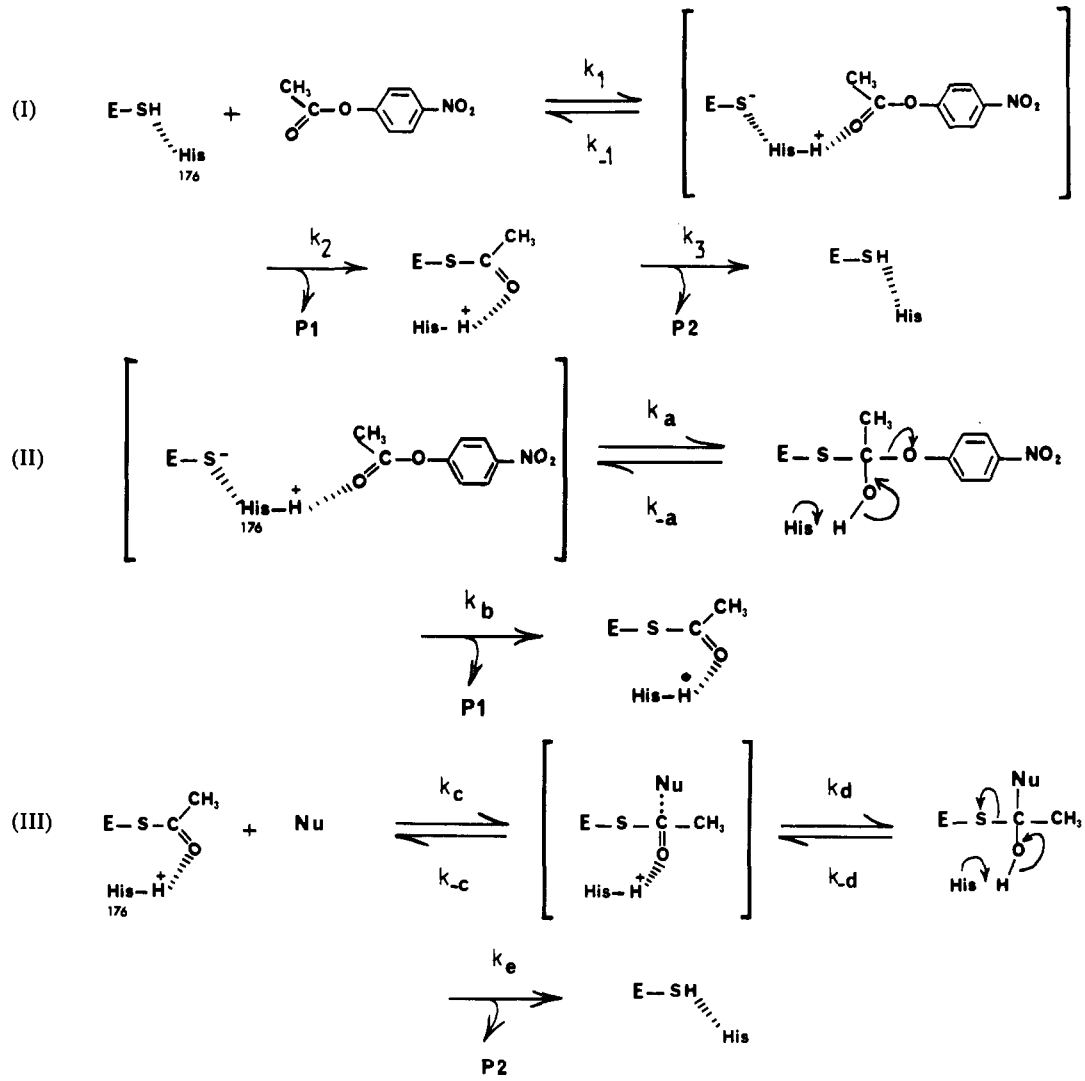


FIGURE 2: Reaction for GAPDH-catalyzed pNPA hydrolysis. P_1 and P_2 designate pNP and acetate, respectively. A three-step mechanism, as usually proposed for serine protease, was assumed (reaction scheme I). The acylating step k_2 and deacylating step k_3 can be described by a two-step and three-step mechanism, respectively (reaction schemes II and III).

Table II: Second-Order Rate Constant for the Reaction of Wild-Type and His-176 → Asn-176 Mutant Apoenzyme with IAM[a]

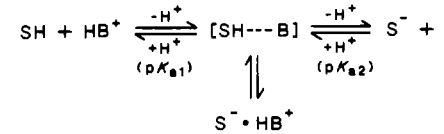
enzyme	k_2 (M^{-1} s^{-1})[b] pH 7	pH 8
wild type	30.2	84.0
mutant His-176 → Asn-176	7.2	20.0

[a] The reaction was performed at 37 °C in 20 mM Tris-HCl. Reaction order in each case is equal to 1. [b] The second-order rate constant was determined as described under Materials and Methods.

Reaction order is nearly equal to 1. This result is consistent with a loss of activity associated with the modification of a single amino acid residue, Cys-149. A 4-fold decrease in the reactivity of the Cys residue in the mutant enzyme toward IAM is observed at both pH 7 and pH 8 (Table II), reflecting the effect of the absence of His-176 on the thiol group of Cys-149.

(*b*) *pH Dependence of the Alkylation.* The pH–rate profile for the IAM inhibition of wild-type GAPDH (Figure 3A) shows a double sigmoidal behavior with two apparent pK_a's: pK_{a1} = 5.5 and pK_{a2} = 8.6. On the other hand, the profile obtained with the mutant enzyme shows a sigmoidal pattern with an apparent pK_a value of 9.3.

The characterization of two pK_a's in the wild-type enzyme suggests the presence of two reactive thiol species. As postulated by Polgar (1975), one reactive form would be an ion pair, the other one corresponding to the free mercaptide ion S^- of Cys-149:



The first pK_{a1} (5.5) could reflect the ionization of His-176. In the mutant, only the second pK_a, associated with the ionization of Cys-149, was observed with a shift of 0.7 pH unit between the wild and mutant enzyme. Moreover, at higher pH (≥10) the rates of IAM inactivation were similar for the wild and mutant enzymes. All these results confirm that His-176 interacts with Cys-149, enhancing the reactivity of the SH group, either by hydrogen-bond interaction or more probably by formation of an ion pair.

(*2*) *Esterolytic Activity.* (*a*) *Kinetics of pNPA Hydrolysis.* The esterolytic activity of GAPDH toward pNPA (Taylor et al., 1963) can be described by a mechanism involving a minimum of three steps, as indicated under Materials and Methods, and illustrated in reaction scheme I of Figure 2. Before the kinetic study was undertaken, molecular modeling, simulating the binding of tetrahedral intermediates (see reaction schemes II and III in Figure 2), was done (stereoviews not shown). Clearly, the two enantiomeric forms of both

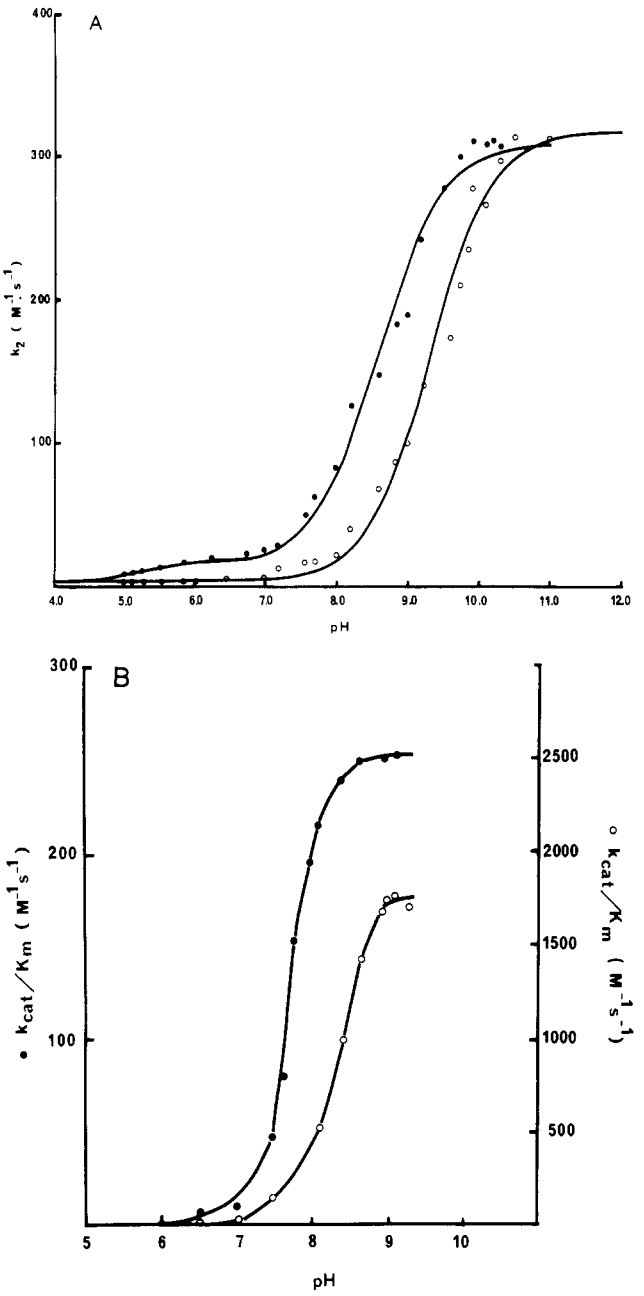


FIGURE 3: (A) pH dependence of the inactivation rate constant with IAM. Various concentrations of IAM and enzymes were used with a constant ratio IAM/GAPDH = 50. The second-order rate constants were determined at each pH as described under Materials and Methods. The solid lines are the best-fit theoretical curves calculated for ionizing groups of pK_a's = 5.5 and 8.6 for the wild-type apoenzyme (●) and a pK_a = 9.3 for mutant His-176 → Asn apoenzyme (○), respectively. Each data point represents the average of two or three measurements with less than 5% of standard error. (B) pH dependence of k_{cat}/K_M for the hydrolysis of pNPA. The k_{cat}/K_M value at each pH was determined from the Lineweaver–Burk plots for both apoenzymes as described under Materials and Methods. The solid lines are the best-fit curves drawn by eye from the experimental data, assuming graphically a pK_a = 7.7 for the wild-type apoenzyme (●) and a pK_a = 8.4 for the mutant His-176 → Asn-176 apoenzyme (○).

intermediates can be accommodated in the active site of *B. stearothermophilus* apo-GAPDH without steric hindrance. In addition, His-176 is placed so that it can act as a base catalyst on these intermediates. Under the conditions described under Materials and Methods, with pNPA concentrations in large excess over that of the enzyme, the kinetics observed with either the wild-type or mutant apoenzyme showed two phases represented in Figure 1: a rapid pre-steady-state phase, illustrated by pNP release and indicative of acyl-enzyme formation, and a slower steady-state phase associated with the deacylation step (Taylor et al., 1963; Kézdy & Bender, 1962; Hirohara et al., 1974; De Caro et al., 1986; Ascenzi et al., 1987). For both enzymes, the deacylation reaction k_3 was rate-limiting. Table III shows that, at pH 7, the acylating step k_2 was decreased 10-fold for the His-176 → Asn-176 mutant, as compared to that of the wild-type enzyme. In fact, the acylation reaction proceeds via at least two steps (see reaction scheme II of Figure 2) as already shown for α-chymotrypsin (Hirohara et al., 1974). The fact that the thiol group of Cys-149 is more basic, a stronger nucleophile, and a poorer leaving group than the pNP ion ($k_b > k_{-a}$) suggests that the nucleophilic attack of ES^- will be rate-determining (Fersht, 1985; Hupe & Jencks, 1977). Thus, k_2 is comparable to k_a. This rate-determining step of nucleophilic attack of a thiol anion on pNPA has been well illustrated by Hupe and Jencks (1977), on different chemical thiol models of varying pK_a. The 10-fold decrease of k_a observed for the mutant could be the consequence of two factors: Clearly, as shown with IAM at pH 7, the decrease of the nucleophilicity of the thiol group affects its chemical reactivity. In addition, the absence of the His residue could also decrease the electrophilic character of the ester carbon of pNPA.

Table III: Kinetic Parameters of Mutant His-176 → Asn-176 and Wild-Type ApoGAPDH in the Hydrolysis of pNPA in Pre-Steady-State Conditions[a]

	pre steady state		
enzyme[b]	K_s (10^{-5} M)	k_2 (10^{-3} s^{-1})	K_M (10^{-5} M)
wild type	380.0	240.0	3.5
mutant His-176 → Asn-176	3.3	23.0	0.4

[a] The reaction was performed at 22 °C in 20 mM Tricine buffer, pH 7, containing 5% ethanol as described under Materials and Methods. [b] Experimental conditions: GAPDH, 16 and 7 μN; pNPA, 0.2–2 and 0.02–0.2 mM; for wild-type and mutant apoenzyme, respectively. Each value represents the average of two determinations.

A 100-fold decrease of K_s is also observed for the mutant enzyme (Table III). This result is not expected and raises the question of the different binding modes of pNPA in the active site of both enzymes, i.e., His-176 → Asn-176 mutant and wild type, and thus of the significance of the k_2 values. In particular, are all binary complexes GAPDH–pNPA entirely productive in wild and mutant enzymes? By use of a computer-assisted graphic system, the binding of pNPA was simulated on both enzymes by using the model structure of the apo-GAPDH from *B. stearothermophilus*. Clearly, no structural difference could be observed for pNPA binding on both enzymes (results not shown). So the decrease of the K_s value remains to be explained and could support the idea that the kinetic scheme proposed for the acylation step would be more complicated.

The kinetic parameters of mutant His-176 → Asn-176 and wild-type apoenzyme in steady-state conditions are summarized in Table IV. An initial burst corresponding to pNP release was observed, equal in magnitude to the enzyme subunit concentration. In 20 mM Tricine buffer, pH 7, the k_3 values are similar for both enzymes, the K_M constant being better in mutant His-176 → Asn-176.

(*b*) *Variation of the Rate of Deacylation k_3 as a Function of Buffer Nucleophilicity.* To test whether the absence of His decreases the electrophilic character of the ester carbon of the thioacyl intermediate, a study of the variation of the rate-limiting deacylation step k_3 as a function of the buffer nucleophilicity was undertaken. As indicated in reaction scheme

Table IV: Kinetic Parameters of Mutant His-176 → Asn-176 and Wild-Type Apoenzyme in the Hydrolysis of pNPA in Steady-State Conditions[a]

	steady state		
enzyme[b]	K_M (10^{-5} M)	k_3 (k_{cat}) (10^{-3} s^{-1})	burst (π), pNP/E_0 (M/N)
wild type	3.20	2.20	0.95
mutant His-176 → Asn-176	0.34	1.80	0.93

[a] Reaction performed as described in Table III. [b] Experimental conditions: GAPDH, 16 and 8 μN; pNPA, 0.02–0.20 and 0.01–0.20 mM; for wild-type and mutant enzyme, respectively. Each value represents the average of two determinations.

Table V: Rate Dependence (k_{cat}) of Hydrolysis of pNPA by GAPDH as a Function of Buffer Concentration[a]

buffer	enzyme	k_{cat} (10^{-3} s^{-1})
0.01 M Tris-HCl, 0.45 M NaCl, pH 7 (I = 0.46 M)	wild type	1.00
	mutant His-176 → Asn	0.66
0.5 M Tris-HCl, pH 7 (I = 0.46 M)	wild type	6.80
	mutant His-176 → Asn	2.50

[a] The experimental conditions were as described for Table IV except that each data point represents the average of three determinations with less than 5% standard error.

III of Figure 2, the rate-limiting reaction k_3 is composed of a minimum of three steps. In this deacylation mechanism, residue His-176 could play a double role. Nucleophilic attack on the carbon of the thioacyl intermediate would be facilitated by His-176 through formation of a hydrogen bond with the oxygen of the acyl-enzyme, while also promoting the base-catalyzed release of the acetate ion. As shown in Table V, the deacylation rate constant k_3 is a function of the nucleophilicity of the buffer at a constant ionic strength. This suggests that the rate-determining step in deacylation is the nucleophilic attack, i.e., k_d.[2] This result was expected. Indeed, the release of the acetate ion, which is an efficient leaving group (pK_a = 4.8), should be much faster than the attack of the nucleophile (Hupe & Jencks, 1977).

At pH 7, increasing the Tris-HCl buffer concentration from 0.01 to 0.5 M at a constant ionic strength results in a 6.8-fold increase in k_3 for the wild type as opposed to only a 3.7-fold increase for the mutant enzyme (Table V). This difference suggests that the absence of His-176 could also decrease the electrophilic character of the carbon of the acyl intermediate in the mutant enzyme.

(*c*) *Study of the pH Dependence of* k_{cat}/K_M. In examining the pH dependence of k_{cat}/K_M, it is generally accepted (Fersht, 1985) that it provides a measure of the ionization constants of free enzyme and free substrate. In this instance, the pK_a value should reflect only the ionization of free enzyme since the substrate is un-ionized in the pH range tested.

As shown in Figure 3B, the pK_a of wild-type apoGAPDH at constant ionic strength was 7.7 while that of the His-176 → Asn-176 mutant was 8.4. This shift is in good agreement with that determined with iodoacetamide as a probe.

Conclusion

In summary, by use of iodoacetamide and *p*-nitrophenyl acetate as chemical probes, the role of His-176 has been established. It enhances the nucleophilicity of Cys-149 by lowering its pK_a as well as acting as a hydrogen donor facilitating the formation of the tetrahedral intermediates. This supports a similar role for His-176 during the oxidative phosphorylation of glyceraldehyde 3-phosphate. However, it still remains to be established whether His-176 acts as a base catalyst in the hydride transfer during the oxidative phosphorylation of glyceraldehyde 3-phosphate. The present approach does not provide an answer. In the pNPA reaction, the k_b and k_e steps seem not to be limiting, so that the participation of His-176 as a base catalyst in the decomposition of tetrahedral intermediates cannot be proven. The 60-fold reduction of k_{cat} observed with the mutant enzyme, during the oxidative phosphorylation of glyceraldehyde 3-phosphate, poses the question whether the removal of the His-176 has changed the nature of the limiting step. Preliminary steady-state experiments in which NAD^+ was added to mutant apoenzyme preincubated with glyceraldehyde 3-phosphate in the absence of phosphate show no burst of NADH production. This supports the idea that the rate-limiting step is hydride transfer and that the role of His-176 is also as a base catalyst facilitating hydride transfer in the wild-type enzyme. Stopped-flow kinetic studies are in progress to prove this point.

[2] Moreover, no effect on k_3 was observed when the ionic strength of the medium was varied, at a constant Tris concentration (results not shown). All these points strongly suggest a partition of the acyl-enzyme between water and Tris as postulated for alkaline phosphatase (Ghosh et al., 1986).

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Registry No. GAPDH, 9001-50-7; NAD, 53-84-9; G3P, 591-57-1; P_i, 14265-44-2; 1,3-DPG, 1981-49-3; pNPA, 830-03-5; IAM, 144-48-9; His, 71-00-1; Asn, 70-47-3.

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Three Residues Involved in Binding and Catalysis in the Carbamyl Phosphate Binding Site of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: Site-directed mutagenesis was used to create four mutant versions of *Escherichia coli* aspartate transcarbamylase at three positions in the catalytic chain of the enzyme. The location of all the amino acid substitutions was near the carbamyl phosphate binding site as previously determined by X-ray crystallography. Arg-54, which interacts with both the anhydride oxygen and a phosphate oxygen of carbamyl phosphate, was replaced by alanine. This mutant enzyme was approximately 17 000-fold less active than the wild type, although the binding of substrates and substrate analogues was not altered substantially. Arg-105, which interacts with both the carbonyl oxygen and a phosphate oxygen of carbamyl phosphate, was replaced by alanine. This mutant enzyme exhibited an approximate 1000-fold loss of activity, while the activity of catalytic subunit isolated from this mutant enzyme was reduced by 170-fold compared to the wild-type catalytic subunit. The K_D of carbamyl phosphate and the inhibition constants for acetyl phosphate and *N*-(phosphonoacetyl)-L-aspartate (PALA) were increased substantially by this amino acid substitution. Furthermore, this loss in substrate and substrate analogue binding can be correlated with the large increases in the aspartate and carbamyl phosphate concentrations at half of the maximum observed specific activity, $[S]_{0.5}$. Gln-137, which interacts with the amino group of carbamyl phosphate, was replaced by both asparagine and alanine. The asparagine mutant exhibited only a small reduction in activity while the alanine mutant was approximately 50-fold less active than the wild type. The catalytic subunits of both these mutant enzymes were substantially more active than the corresponding holoenzymes. Both mutant enzymes at position 137 exhibited an approximate 40-fold higher K_D for carbamyl phosphate than the wild-type catalytic subunit. The inhibition constants for acetyl phosphate were hardly altered by these mutations, while both mutations exhibited higher inhibition constants for PALA. The holoenzymes with alanine substitutions at both Arg-54 and Gln-137 exhibited loss of cooperativity, while the Arg-105 → Ala catalytic subunit exhibited cooperativity with a Hill coefficient of 2.0. The pH dependence of the reaction was unaltered by the mutations at Gln-137, although the substitutions at Arg-54 and Arg-105 both resulted in an increase in the pH optimum of the reaction. Circular dichroism (CD) spectroscopy was used to determine if the conformational change normally induced by the binding of carbamyl phosphate had been altered by any of the mutations. In every case, the magnitude of the CD difference spectrum at saturating carbamyl phosphate concentrations was reduced. The alanine substitution at Arg-105 exhibited the largest reduction, while the asparagine substitution at Gln-137 exhibited the smallest reduction. New insights into the catalytic mechanism of aspartate transcarbamylase have been obtained by combining the results of the analysis of these four mutant enzymes with previously proposed mechanisms.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the formation of *N*-carbamyl-L-aspartate from carbamyl phosphate and L-aspartate. The enzyme, used as a model system to study the molecular mechanisms of allosteric regulation [see reviews by Gerhart (1970), Jacobson and Stark (1973), Schachman (1974), Kantrowitz et al. (1980a,b), and Kantrowitz and Lipscomb (1988)], exhibits positive cooperativity for both substrates (Gerhart & Pardee, 1962; Bethell et al., 1968), and its activity is inhibited by CTP and activated by ATP, the end products of the pyrimidine and purine pathways, respectively. The enzyme is composed of three regulatory dimers (i.e., regulatory subunits) and two catalytic trimers (i.e., catalytic subunits). The regulatory subunit binds CTP and ATP but is devoid of catalytic activity while the isolated catalytic subunit exhibits no homotropic cooperativity

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