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Site-Directed Mutagenesis of *Escherichia coli* Aspartate Aminotransferase: Role of Tyr70 in the Catalytic Processes†

Katsura Inoue,‡ Seiki Kuramitsu,‡ Akihiro Okamoto,§ Ken Hirotsu,§ Taiichi Higuchi,§ and Hiroyuki Kagamiyama*,‡

Department of Medical Chemistry, Osaka Medical College, Takatsuki, Osaka 569, Japan, and Department of Chemistry, Faculty of Science, Osaka City University, Osaka-shi, Osaka 558, Japan

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ABSTRACT: Site-directed mutagenesis of Tyr70 in the active site of *Escherichia coli* aspartate aminotransferase (AspAT) followed by kinetic studies has elucidated the roles of the hydroxyl group and benzene ring of Tyr70. X-ray crystallographic analysis showed that replacement of Tyr70 by Phe did not alter the active-site conformation of the enzyme. Comparison of the kinetic parameters of the four half-transamination reactions (the pyridoxal 5′-phosphate form of the enzyme with L-aspartate or L-glutamate and the pyridoxamine 5′-phosphate form with oxalacetate or 2-oxoglutarate) between the wild-type and [Tyr70 → Phe]AspATs showed that the mutation increases the energy level of the transition state by 2 kcal·mol^{-1} for all the four substrates, suggesting some contribution of the hydroxyl group of Tyr70 to the transition state. When Phe70 was further replaced by Ser, the energy level of the transition state for L-glutamate or 2-oxoglutarate, but not for L-aspartate or oxalacetate, was further increased by 2-3 kcal·mol^{-1}, suggesting that the presence of a benzene ring at position 70 is essential for recognizing the L-glutamate–2-oxoglutarate pair as substrates.

Aspartate aminotransferase [L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1] (AspAT)[1] catalyzes the reversible transamination reaction

L-aspartate + 2-oxoglutarate ⇌ oxalacetate + L-glutamate (1)

via the "ping-pong bi-bi" mechanism (Velick & Vavra, 1962; Kiick & Cook, 1983; Jenkins & Fonda, 1985).

Recent crystallographic studies of *Escherichia coli* AspAT at 2.5-Å resolution (Kamitori et al., 1990) have revealed that the spatial structure of the *E. coli* enzyme is virtually identical with those of the animal isozymes (Ford et al., 1980; Borisov et al., 1980; Harutyunyan et al., 1982; Arnone et al., 1985a) and that most of the active-site residues are conserved and located at corresponding positions. We have examined some of the active-site residues of the *E. coli* enzyme, including Lys258,[2] Tyr225, Arg292, Arg386, and Trp140, for their

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‡ Osaka Medical College.
§ Osaka City University.

[1] Abbreviations: Asp, L-aspartate; 2-CH_3-Asp, 2-methyl-DL-aspartate; AspAT, aspartate aminotransferase; Y70X AspAT, AspAT of which Tyr70 is replaced with residue X; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EL, pyridoxal 5′-phosphate form of AspAT; EM, pyridoxamine 5′-phosphate form of AspAT; Glu, L-glutamate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OA, oxalacetate; 2OG, 2-oxoglutarate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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functional role in the catalytic process by site-directed mutagenesis.

In the present study, the contribution of Tyr70, which is conserved in every AspAT without exception from higher animals to microorganisms, to catalytic processes was examined. Crystallographic analyses (Jansonius & Vincent, 1987; Taylor et al., 1990; Kamitori et al., 1990) have shown that Tyr70 is positioned close to the 5′-phosphate group of the coenzyme [pyridoxal 5′-phosphate (PLP)] and the side chain of Lys258. A possible role of Tyr70 in proton transfer between Lys258 and the phosphate group of the coenzyme as an essential catalytic process has been postulated on the basis of crystallographic data (Kirsch et al., 1984; Arnone et al., 1985b). However, Toney and Kirsch (1987) observed that the *E. coli* AspAT mutant, in which Tyr70 was changed to phenylalanine (Y70F AspAT), retained 15% of the activity of the wild-type enzyme and dissociated the coenzyme more readily than the latter. Thus, they suggested that Tyr70 is not essential for catalytic action but functions mainly in preventing the dissociation of the coenzyme from the enzyme molecule.

In order to explore further the role of Tyr70 during catalysis, we replaced Tyr70 with phenylalanine and serine residues. On the basis of the results obtained from pre-steady-state kinetic analysis of the constructed mutants (Y70F and Y70S AspATs), we propose that, in addition to the anchoring effect of Tyr70 on the coenzyme via its phenol group, the presence of an aromatic ring at position 70 in AspAT is necessary for the recognition of L-glutamate or 2-oxoglutarate (five-carbon substrates).

Experimental Procedures

Materials

Bacterial Strains. *E. coli* TY103, a derivative of *E. coli* JM103 (Δ*lacpro*, *supE*, *thi*, *strA*, *sbcB15*, *endA*, *hsdR4*, F′-[*traD36*, *proAB*$^+$, *LacI*^{q}*ZΔM15*]) (Messing et al., 1981) bearing the genotypes *aspC*, *tyrB*, and *recA* (Yano et al., 1991), was used for the expression of mutant *aspC* genes.

Media. The YT medium (pH 7.2–7.4) used for bacterial growth and for selection of the clone carrying the mutant *aspC* gene contained 0.5% yeast extract (Nacalai Tesque, Kyoto), 0.8% Tryptone (Difco Laboratories, Detroit), and 0.25% NaCl. The *E. coli* cells that overproduce wild-type AspAT (Kamitori et al., 1987) were grown in the medium of Kuramitsu et al. (1981).

Chemicals. An "oligonucleotide-directed in vitro mutagenesis system" was obtained from Amersham Co. The other chemicals were the same as described previously (Inoue et al., 1989).

Methods

Site-Directed Mutagenesis. The synthetic oligonucleotides used for site-directed mutagenesis (Nakamaye & Eckstein, 1986) were CC-ACC(Thr67)-AAA (Lys68)-AAT(Asn69)-*NNN*(*X70*)-CTC(Leu71)-GGC(Gly72)-ATT(Ile73)-G (N indicates G, A, T, or C). The nucleotide sequence of the mutant *aspC* gene in phage M13 was determined. Then, the small restriction fragment between the *Ava*I and *Mlu*I sites of the mutated *aspC* gene incorporated into phage M13, which contained the codons for Gly36–Arg113 of AspAT, was isolated by agarose gel electrophoresis. The plasmid pKDHE19 (Kamitori et al., 1987), of which the full-length *aspC* gene had already been sequenced, was also digested with the same restriction endonucleases, and the large fragment lacking the region coding for Gly36–Arg113 was isolated by agarose gel electrophoresis. The small mutant fragment obtained from phage M13 was ligated with this large fragment from pKDHE19, to construct the plasmid pKDHE19′. The nucleotide sequence of the newly inserted fragment of pKDHE19′ was ascertained by the method of Messing (1983) with slight modification (Kuramitsu et al., 1985). The mutant AspATs were overproduced in *E. coli* TY103 harboring pKDHE19′ and purified as described previously (Inoue et al., 1989).

Crystallization and X-ray Experiments. The three-dimensional structure of the wild-type AspAT complex with 2-methyl-DL-aspartate (2-CH_3-Asp) was determined at 2.5-Å resolution as described previously (Kamitori et al., 1990). The complex of Y70F AspAT with 2-CH_3-Asp was crystallized by the hanging-drop vapor-diffusion method (McPherson, 1982) against buffered ammonium sulfate. The mutant enzyme was dialyzed against 10 mM potassium phosphate buffer at pH 7.0, containing 10 μM PLP, 1 mM EDTA, and 0.3 mM NaN_3. The reservoir ammonium sulfate solution (30 and 35% saturation) and 2-CH_3-Asp solution (0.34 M) were prepared in the same buffer. A droplet (10 μL) of the mutant enzyme solution (0.68 mM) was mixed with 10 μL of the reservoir ammonium sulfate solution and 5 μL of the 2-CH_3-Asp solution on a slide, which was then placed over a well containing 1 mL of the reservoir solution (30% saturation) and shielded with silicone grease. After one week, another 10 μL of the protein solution was added to the protein solution on the slide. After one additional week, the reservoir solution was replaced by 35%-saturated ammonium sulfate solution. Yellow prismatic crystals appeared within 7–10 days.

X-ray diffraction data to a resolution of 2.8 Å were obtained on a Rigaku rotating-anode automatic diffractometer (operated at 40 kV, 300 mA) at 11 °C. The space group was $C222_1$ with cell parameters a = 157.2, b = 85.6, and c = 79.1 Å and Z = 8. The crystal structure of the Y70F AspAT·2-CH_3-Asp complex was isomorphous with that of the wild-type AspAT-2-CH_3-Asp complex (Kamitori et al., 1990). Comparison of the observed structure factors for Y70F AspAT·2-CH_3-Asp and wild-type AspAT·2-CH_3-Asp gave R_{iso} = 0.04, which was calculated from the equation

$$R_{iso} = \sum_{hkl}|F_{Y70F} - F_{wild}| / \sum_{hkl} F_m$$

where F_{Y70F}, F_{wild}, and F_m are the observed structure factors of Y70F AspAT·2-CH_3-Asp and wild-type AspAT·2-CH_3-Asp and the mean structure factor of related reflections, respectively. Refinement was performed by use of the coordinates of the wild-type AspAT·2-CH_3-Asp structure determined at 1.8 Å (unpublished data) as a starting model, by XPLOR (Brünger et al., 1987) runs, each consisting of simulated annealing and energy/X-ray refinement with manual rebuilding using TOM (Cambillau & Horjales, 1987) between runs. The R value dropped from 0.28 to 0.20 (8743 reflections with $F > 2\sigma(F)$ out of 11 659, overall temperature factor = 13.2 Å^2, bonds root mean square deviation = 0.019 Å, water molecules not included). The overall three-dimensional structure of Y70F AspAT·2-CH_3-Asp was identical with that of wild-type AspAT·2-CH_3-Asp.

Spectrophotometric Measurements. The absorption spectra of the enzymes were recorded with a Hitachi spectrophotometer, Model 557, at protein concentrations of 10–20 μM in a 1-cm cell. The buffer solution contained 0.1 M KCl, 10 μM EDTA, and a buffer component of 50 mM MES, 50 mM HEPES, 50 mM Bicine, or 50 mM CAPS. All the spectrophotometric and pH measurements were made at 25 °C.

Determination of Protein Concentration. The concentrations of the enzymes were determined spectrophotometrically by use

[2] Amino acid residues are numbered according to the sequence of cytosolic AspAT from pig (Mehta et al., 1989).

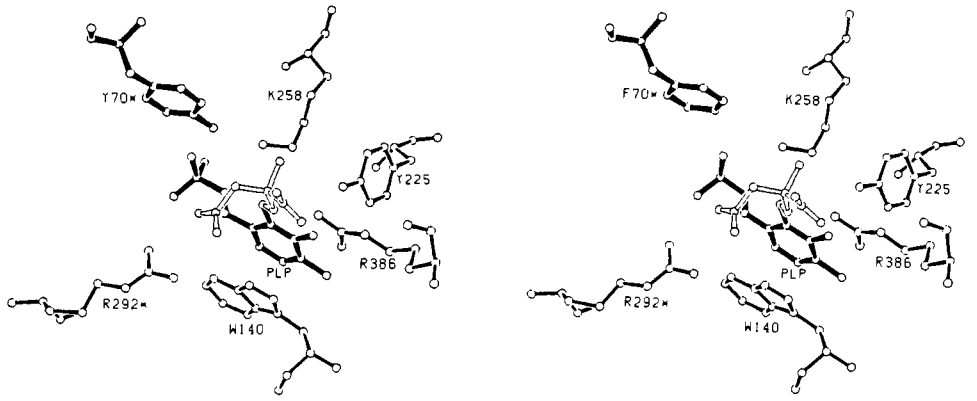

FIGURE 1: The active sites of *E. coli* wild-type AspAT·2-CH_3-Asp complex (left) and Y70F AspAT·2-CH_3-Asp complex (right) drawn by ORTEPII (Johnson, 1976). Tyr70, Phe70, and PLP are drawn as solid bonds, and 2-CH_3-Asp is drawn as clear bonds. The other residues are drawn as thin bonds.

of the following molar extinction coefficients at 280 nm: ϵ_M = 4.7 × 10^4 and 4.6 × 10^4 M^{-1} cm^{-1} for the PLP and pyridoxamine 5′-phosphate (PMP) forms, respectively, of wild-type AspAT; 4.6 × 10^4 and 4.5 × 10^4 M^{-1} cm^{-1} for the PLP and PMP forms, respectively, of Y70F AspAT and Y70S AspAT (Kuramitsu et al., 1990).

Kinetic Analysis of Enzymic Activity. The steady-state overall transamination reactions were measured spectrophotometrically at pH 8.0, 25 °C, by the method of Velick and Vavra (1962) with slight modification (Inoue et al., 1989; Kuramitsu et al., 1990). The pre-steady-state half-transamination reactions were measured spectrophotometrically at pH 8.0, 25 °C, with a stopped-flow apparatus (Applied Photophysics SF17MV or Union Giken RA-1300) as described previously (Inoue et al., 1989; Kuramitsu et al., 1990).

Reactions with Dicarboxylates. The reactions of AspATs with dicarboxylates (succinate and glutarate) were studied spectrophotometrically (Fonda & Johnson, 1970; Bonsib et al., 1975) at pH 8.0, 25 °C.

Results and Discussion

Structures of Mutant AspATs. The N-terminal amino acid sequences of the overproduced mutant AspATs were all Met-Phe-Glu-Asn-Ile-, identical with that of wild-type AspAT (Kondo et al., 1987).

Circular dichroic spectra of the mutant enzymes in the region between 200 and 250 nm showed no difference from that of the wild-type enzyme, suggesting a lack of gross structural change upon mutation (data not shown).

The structures of the wild-type AspAT·2-CH_3-Asp complex and Y70F AspAT·2-CH_3-Asp complex were compared by X-ray crystallography (Figure 1). The hydroxyl group of Tyr70 in wild-type AspAT is hydrogen-bonded to the amino group of Lys258 and to the phosphate group of PLP (Kamitori et al., 1990), as described for the animal enzymes (Jansonius & Vincent, 1987; Taylor et al., 1990). Crystallographic data for Y70F AspAT showed that the structure of the active site was virtually identical with that of the wild-type enzyme; the aromatic ring of Phe70 in Y70F AspAT occupied almost the same position as that of Tyr70 in wild-type AspAT.

The PLP form of the wild-type enzyme shows two absorption bands in the visible region at 358 and 430 nm, which have been attributed to unprotonated and protonated forms, respectively, of the Schiff base formed between PLP and the ϵ-NH_2 group of Lys258 (Kallen et al., 1985). The two mutant enzymes gave the same absorption bands as those of the wild-type enzyme. Spectrophotometric titration of these spectral bands of the mutant enzymes gave pK_a values of 6.85 (Y70F AspAT) and 6.90 (Y70S AspAT), almost identical with the corresponding value of 6.80 for the wild-type enzyme. These results showed that replacement of Tyr70 with a phenylalanine or serine residue did not affect the electronic status of the nitrogen atom of the internal aldimine.

Table I: Steady-State Kinetic Parameters[a] for Overall Transamination Reactions at pH 8.0, 25 °C

AspAT	K_m (mM) Asp	K_m (mM) 2OG	k_{cat} (s^{-1})
wild	2.0	0.60	200
Y70F	12	3.8	29
Y70S	5.0	36	7.4

[a] Abbreviations: Asp, L-aspartate; 2OG, 2-oxoglutarate.

Kinetic Analysis. The steady-state kinetic parameters of the overall transamination reactions for the wild-type and mutant AspATs are shown in Table I. Y70F AspAT retained 15% of the k_{cat} value for the wild-type AspAT. This activity agrees well with that reported by Toney and Kirsch (1987). Y70S AspAT showed 4% of the activity of the wild-type AspAT, suggesting that Tyr70 is not essential for catalytic reaction. The K_m values for aspartate and 2-oxoglutarate were increased by the mutation of Tyr70 to phenylalanine or serine; in particular, Y70S AspAT showed a 60-fold increase in the K_m value for 2-oxoglutarate. The physicochemical nature of these steady-state kinetic parameters is very complex because these parameters consist of several pre-steady-state kinetic parameters. Recent pre-steady-state kinetic analysis of the four half-transamination reactions of *E. coli* wild-type AspAT at pH 8.0 (the PLP form of AspAT with aspartate or glutamate and the PMP form of the enzyme with oxalacetate or 2-oxoglutarate) by stopped-flow spectrometry (Kuramitsu et al., 1990) has revealed that the reversible overall transamination reaction (eq 1) consists of the two half-reactions given in eq 2, as proposed previously by Fasella and Hammes

$$\mathrm{EL} + \mathrm{Asp} \overset{a}{\rightleftharpoons} \mathrm{EL{\cdot}Asp} \overset{b}{\rightleftharpoons} \mathrm{EM{\cdot}OA} \overset{c}{\rightleftharpoons} \mathrm{EM} + \mathrm{OA} \quad (2)$$

$$\mathrm{EL} + \mathrm{Glu} \overset{a}{\rightleftharpoons} \mathrm{EL{\cdot}Glu} \overset{b}{\rightleftharpoons} \mathrm{EM{\cdot}2OG} \overset{c}{\rightleftharpoons} \mathrm{EM} + \mathrm{2OG}$$

(1967), where EL, EM, Asp, OA, Glu, and 2OG represent the PLP and PMP forms of AspAT, L-aspartate, oxalacetate, L-glutamate, and 2-oxoglutarate, respectively. Each half-reaction is composed of the fast substrate-binding steps a and c in eq 2 (external aldimine formation between an amino acid substrate and the PLP form of the enzyme and ketimine formation between the corresponding keto acid and the PMP form of the enzyme) and the rate-determining interconversion between the external aldimine and ketimine complexes (step

Table II: Pre-Steady-State Kinetic Parameters[a] for Half-Transamination Reactions at pH 8.0, 25 °C

	K_d (mM)				k_{max} (s^{-1})				k_{max}/K_d (M^{-1} s^{-1})			
AspAT	Asp	Glu	OA	2OG	Asp	Glu	OA	2OG	Asp	Glu	OA	2OG
wild	4.5	38	0.035	1.3	550	700	800	600	1.2×10^5	1.8×10^4	2.3×10^7	4.6×10^5
Y70F	5.0	67	0.67	13	30	67	290	130	6.0×10^3	1.0×10^3	4.3×10^5	1.0×10^4
Y70S	17	200	1.7	40	67	6.7	250	10	3.9×10^3	34	1.5×10^5	2.5×10^2

[a] Abbreviations: Asp, L-aspartate; Glu, L-glutamate; OA, oxalacetate; 2OG, 2-oxoglutarate.

b) in eq 2. When an amino acid substrate (Asp or Glu) is added to the PLP form of AspAT (EL), an aldimine bond is formed (EL·Asp or EL·Glu) in rapid equilibrium between the substrate and PLP. The next rate-determining step is the withdrawal of the α-proton of the substrate. The next reaction is the addition of a proton to the coenzyme C4′, leading to the formation of the ketimine intermediate (EM·OA or EM·2OG). The ketimine intermediate is then hydrolyzed in rapid equilibrium to form a keto acid substrate (OA or 2OG) and the PMP form of the enzyme (EM).

In order to define the critical step affected by the mutation, pre-steady-state kinetic analysis was performed on the four sets of half-transamination reactions for these mutant enzymes according to the rate equations (Inoue et al., 1989; Kuramitsu et al., 1990)

$$E + S \underset{}{\overset{K_d}{\rightleftharpoons}} ES \xrightarrow{k_{max}} ES' \quad (3)$$

$$k_{app} = k_{max}[S]/([S] + K_d) \quad (4)$$

where K_d is the dissociation constant for the enzyme–substrate complex, ES; k_{max} is the rate constant for the rate-determining step, ES → ES′; and k_{app} is the apparent rate constant. The kinetic parameters thus obtained are summarized in Table II. For all substrates examined, the mutant enzymes showed larger K_d values than the wild-type enzyme; the increases in the K_d values of Y70F AspAT for amino acid substrates were insignificant, but those for keto acid substrates were increased by 10–20 times that of the wild-type enzyme, and the increase in K_d values was more pronounced for Y70S AspAT than for Y70F AspAT. Y70F AspAT gave smaller k_{max} values for the four substrates than the wild-type enzyme. For Y70F AspAT, the decrease in the reaction rates was more pronounced in the reactions with amino acid substrates than in those with keto acid substrates. For Y70S AspAT, the decrease in the k_{max} value for glutamate or 2-oxoglutarate (five-carbon substrate) was much more pronounced than that for aspartate or oxalacetate (four-carbon substrate). Comparison of the k_{max}/K_d values showed that the catalytic competence of Y70S AspAT was markedly decreased in the reaction with the five-carbon substrates.

Binding of Succinate and Glutarate. The PLP form of AspAT noncovalently binds succinate or glutarate (Jenkins et al., 1959). The affinity of the dicarboxylate was measured spectrophotometrically (Fonda & Johnson, 1970; Bonsib et al., 1975). Upon binding of these substrate analogues to the enzyme, the absorption spectra shift from 360 to 430 nm due to an increase in pK_a of the internal Schiff Base. As shown in Table III, the affinities of these compounds were unaltered by the mutation of position 70.

Energy Profiles for Wild-Type and Mutant AspATs. The free energy change (ΔG_s) for the fast binding step and the activation free energy change (ΔG^*) for the rate-determining step are calculated by $-RT \ln (1/K_d)$ and RT [ln (k_BT/h) − ln k_{max}], respectively (R, the gas constant; T, the absolute temperature; k_B, the Boltzmann constant; h, the Planck constant)(Fersht, 1985), to construct the free energy profiles for the half-transamination reactions of wild-type and mutant AspATs (Figure 2). The free energy difference (ΔG^*_T) between the transition state (‡) and unbound enzyme plus substrate (E + S) for each substrate was calculated from the k_{max}/K_d value according to the equation

$$\Delta G^*_T = \Delta G_s + \Delta G^* = RT\,[\ln (k_BT/h) - \ln (k_{max}/K_d)] \quad (5)$$

The ΔG^*_T values calculated from the pre-steady-state kinetic parameters (k_{max}, K_d) in Table II are shown in Figure 3.

Table III: Dissociation Constants of Dicarboxylates with *E. coli* AspATs at pH 8.0, 25 °C

	dissociation constant (mM)	
AspAT	succinate	glutarate
wild	75	150
Y70F	90	150
Y70S	100	170

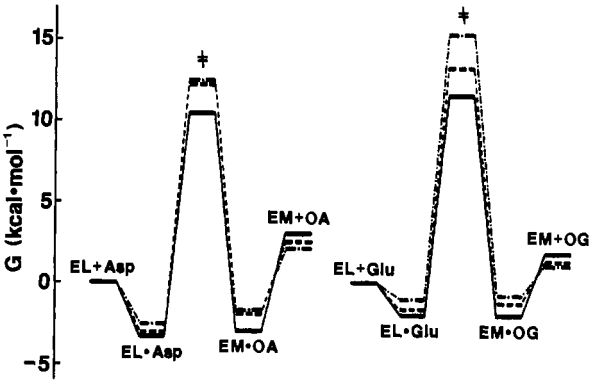

FIGURE 2: Free energy profile for the transamination reaction at pH 8.0, 25 °C. The solid line represents wild-type AspAT, the dashed line represents Y70F AspAT, and the dashed-dotted line represents Y70S AspAT. The half-transamination reaction between L-aspartate and oxalacetate (left side) or between L-glutamate and 2-oxoglutarate (right side) is shown. Abbreviations: EL, PLP form of AspAT; EM, PMP form of AspAT; Asp, L-aspartate; OA, oxalacetate; Glu, L-glutamate; OG, 2-oxoglutarate. The free energy change (ΔG_s) for the fast binding step is $-RT \ln (1/K_d)$, and the activation free energy (ΔG^*) for the rate-determining step is RT [ln (k_BT/h) − ln k_{max}]. R is the gas constant; T is the absolute temperature; k_B is the Boltzmann constant; h is the Planck constant.

The replacement of Tyr70 by phenylalanine increased ΔG^*_T of all the amino acid and keto acid substrates by 2–3 kcal·mol^{-1}, suggesting that the hydroxyl group of Tyr70 would stabilize the transition state for all the substrates, since the location of the benzene ring at position 70 is unaltered between the wild-type and Y70F AspAT, as mentioned above. The ΔG_s values for keto acid substrates were increased by about 2 kcal·mol^{-1}, whereas those for amino acid substrates were practically unaltered by the mutation (Figure 2), suggesting that the hydroxyl group of Tyr70 contributes to the binding of a keto acid substrate with the PMP form of AspAT at the ground state but not to that of an amino acid substrate with the PLP form of the enzyme. In the Michaelis complexes, Tyr70 may be hydrogen-bonded to a keto acid substrate, but not to an amino acid substrate. Then, in the succeeding step, Tyr70 would assist all of the four substrates in stabilizing the

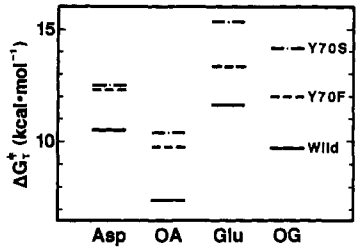

FIGURE 3: The effect of mutations on $\Delta G^{\ddagger}_{T}$ value shown as a line for each of four substrates: L-aspartate (Asp), oxalacetate (OA), L-glutamate (Glu), and 2-oxoglutarate (OG), pH 8.0, 25 °C. The solid line represents wild-type AspAT, the dashed line represents Y70F AspAT, and the dashed-dotted line represents Y70S AspAT. The free energy difference ($\Delta G^{\ddagger}_{T}$) between the transition state ($\ddagger$) and unbound enzyme plus substrate (E + S) was calculated from the k_{max}/K_d value with the equation $\Delta G^{\ddagger}_{T} = \Delta G_s + \Delta G^{\ddagger} = RT$ [ln $(k_B T/h)$ − ln (k_{max}/K_d)].

transition state. Different contributions of an amino acid residue to the transition state and to the Michaelis complex have also been observed in R386K AspAT (Inoue et al., 1989). Upon mutation of Arg386 to Lys, $\Delta G^{\ddagger}_{T}$ was increased by about 5 kcal·mol^{-1} but ΔG_s was increased by only about 2 kcal·mol^{-1}.

When Tyr70 was replaced by serine, $\Delta G^{\ddagger}_{T}$ was increased by 4–5 kcal·mol^{-1} for glutamate or 2-oxoglutarate (five-carbon substrate) but by 2–3 kcal·mol^{-1} for aspartate or oxalacetate (four-carbon substrate)(Figure 3). The profound decrease in the rate of the overall reaction for Y70S AspAT must be due to this large increase of free energy in the transition state, particularly for five-carbon substrates. It is interesting to note that the difference of $\Delta G^{\ddagger}_{T}$ between Y70F AspAT and Y70S AspAT was 2–3 kcal·mol^{-1} for five-carbon substrates, but negligible for four-carbon substrates. There is no significant difference in the dissociation constants of the four-carbon (succinate) and five-carbon (glutarate) substrate analogues among the wild-type, Y70F, and Y70S enzymes (Table III). Therefore, the presence of a benzene ring at position 70 in the AspAT structure appears to be important for correct positioning of five-carbon substrates in the active site to facilitate the transamination reaction in the transition state; the benzene ring may interact with the methylene carbon of five-carbon substrates. This newly proposed idea would be supported by the finding that Tyr70 is conserved in every already-known amino acid sequence of α-L-amino acid aminotransferases that use 2-oxoglutarate as an amino group acceptor and L-glutamate as the amino group donor to 2-oxo acids in the reverse reaction: the cytosolic and the mitochondrial isoenzymes of vertebrate AspAT, *E. coli*, *Sulfolobus solfataricus*, and *Bacillus* species AspATs, *E. coli* aromatic amino acid aminotransferase, rat tyrosine aminotransferase, and *Saccharomyces cerevisiae* and *E. coli* histidinol-phosphate aminotransferases (Christen et al., 1989; Cubellis et al., 1989; Sung, 1989; Mehta et al., 1989).

According to the mechanism previously proposed by Kirsch et al. (1984), the hydroxyl group of Tyr70 is hydrogen-bonded to the ϵ-amino group of Lys258 and to the phosphate group of the coenzyme. When the ϵ-amino group of Lys258 removes a proton from C2 of the substrate, one of the protons on the ϵ-amino group of Lys258 is transferred to the O(η) of Tyr70, and the proton bound with that oxygen is transferred to the phosphate ion of PLP. Later, however, Toney and Kirsch (1987) described that Tyr70 may function in preventing the dissociation of the coenzyme from the enzyme, but not in an essential chemical step. We also observed the easy dissociation of PMP from Y70F AspAT with the dissociation rate constants for PMP similar to those reported by Toney and Kirsch (1987) (data not shown). The present results suggest a further important functional role of Tyr70.

Conclusion. Tyr70 plays two roles in the catalytic reaction. (1) The hydroxyl group of Tyr70 stabilizes the transition state by 2 kcal·mol^{-1}. (2) The benzene ring of Tyr70 is essential for efficient use of L-glutamate and 2-oxoglutarate as substrates by the enzyme.

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Effects of Point Mutations in a Hinge Region on the Stability, Folding, and Enzymatic Activity of *Escherichia coli* Dihydrofolate Reductase†

Patricia M. Ahrweiler‡ and Carl Frieden*
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110
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ABSTRACT: The role of a hinge region in the folding, stability, and activity of *Escherichia coli* dihydrofolate reductase was investigated with three site-directed mutants at valine-88, the central residue of the hinge. The three mutants, V88A and V88I and a valine-88 deletion, were created to perturb the packing of hydrophobic residues in the interior of a loose turn formed by residues 85–91. Deleting the valine-88 residue destabilized the protein by 2.93 ± 0.6 kcal/mol as determined by equilibrium unfolding transitions in urea monitored by circular dichroism at 20 °C. Substitution of alanine for valine-88 stabilized the protein by −0.20 ± 0.02 kcal/mol, and the isoleucine substitution was mildly destabilizing by 1.73 ± 0.2 kcal/mol. Although there was no clear correlation between side-chain volume and stability, these results suggest that side-chain interactions in the interior of the turn influence the folding and stability of dihydrofolate reductase. The specific activity of the valine deletion mutant was approximately twice that of the wild-type protein while the specific activities of the V88A and V88I proteins were only slightly greater than the wild type. The full time courses of the reactions catalyzed by the mutants were almost identical with that for the wild type, indicating no major changes in the kinetic mechanism. Additionally, the rate constants associated with interconversion between various forms of the apoenzyme were identical for the mutant and wild-type enzymes. The rate constants for refolding transitions were examined by dilution of urea-inactivated protein. While the refolding properties of the V88A mutant were similar to wild type, some rate constants for phases observed in refolding of the valine deletion and the V88I mutant were decreased about 3-fold relative to the wild type. The phase most affected in both these mutants has been previously shown to be related to the formation of the binding site for dihydrofolate during refolding, indicating that the valine-88 residue may be at a region that is involved in bringing preformed elements of secondary structure together to form the dihydrofolate binding pocket.

Dihydrofolate reductase (DHFR, EC 1.5.1.3)[1] from *Escherichia coli* is an ideal candidate for studying the effects of single amino acid replacements on protein folding and stability. It is a small, monomeric protein (17 680 daltons, 159 amino acids) with no prosthetic groups or disulfide bonds. The three-dimensional structure of the binary DHFR–methotrexate complex has been determined to high resolution (1.7 Å) (Bolin et al., 1982; Filman et al., 1982), allowing some structural

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* Author to whom correspondence should be addressed.
‡ Present address: Hybritech, Inc., San Diego, CA 92126.

[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; PMSF, phenylmethanesulfonyl fluoride; H_2F, dihydrofolate; H_4F, tetrahydrofolate.

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