Shift in pH-Rate Profile and Enhanced Discrimination between Dicarboxylic and Aromatic Substrates in Mitochondrial Aspartate Aminotransferase Y70H[†,‡]

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Received September 15, 1993; Revised Manuscript Received December 2, 1993[⊕]

ABSTRACT: Tyr70 of chicken mitochondrial aspartate aminotransferase was replaced with a histidine residue by oligonucleotide-directed mutagenesis. Aspartate aminotransferase Y70H retained at pH 7.5 13% of the activity toward dicarboxylic amino acids, whereas the activity toward aromatic amino acids was only 0.6% of that of the wild-type enzyme, corresponding to a 22-fold increase in the ratio of the activities toward these two types of substrates. In comparison to that of the wild-type enzyme, the low-pH limb of the pH-activity profile of the mutant enzyme was shifted to higher pH values, very likely reflecting the titration curve of the newly introduced histidine residue with a pK_a' of 6.3. Apparently, a positively charged residue at position 70 abolishes enzymic activity. The spectrophotometrically determined pK_a' value of the internal aldimine formed between pyridoxal 5′-phosphate and Lys258 in the mutant enzyme was 6.0, similar to that in the wild-type enzyme. The rate constant of the dissociation of pyridoxamine 5′-phosphate from the mutant enzyme was increased only 3 times over that of the wild-type enzyme, in contrast to the 80-fold increase in *Escherichia coli* aspartate aminotransferase Y70F [Toney, M. D., & Kirsch, J. F. (1987) *J. Biol. Chem. 262,* 12403–12405], suggesting that His70 can replace Tyr70 in forming a hydrogen bond to the coenzyme.

Aspartate aminotransferase (AspAT)[1] is a homodimeric pyridoxal 5′-phosphate-dependent (PLP-dependent) enzyme which catalyzes the reversible transfer of the amino group of aspartate or glutamate to the oxo acids 2-oxoglutarate and oxalacetate. The spatial structures have been determined for mitochondrial and cytosolic AspAT (Ford *et al.*, 1980; Borisova *et al.*, 1980; Harutyunyan *et al.*, 1982) as well as for *Escherichia coli* AspAT (Kamitori *et al.*, 1990; Danishefsky *et al.*, 1991; Jäger, 1991). On the basis of a vast amount of chemical, spectroscopic, kinetic, and crystallographic data, a detailed mechanism of enzymic transamination has been proposed (Kirsch *et al.*, 1984; Arnone *et al.*, 1985; Jansonius & Vincent, 1987). Upon formation of the Michaelis complex between the PLP form of the enzyme and the amino acid substrate, the displacement of the ϵ-amino group of Lys258 from the "internal" Schiff base by the incoming amino acid leads to the "external" PLP substrate aldimine. The crucial chemical event in enzymic transamination is the subsequent tautomerization of the external aldimine to the ketimine intermediate, which is catalyzed primarily by Lys258 (Kirsch *et al.*, 1984; Malcolm & Kirsch, 1985). Tyr70 is positioned between the 5′-phosphate group of the coenzyme and Lys258 and forms a hydrogen bond to the former (McPhalen *et al.*, 1992); in the aldimine and ketimine intermediates, another hydrogen bond appears to be formed to the ϵ-amino group of Lys258 (Kirsch *et al.*, 1984).

E. coli AspAT Y70F has been found to retain 15% of the catalytic activity of the wild-type (WT) enzyme. However, the rate constant of dissociation of PMP from the enzyme is increased 80-fold relative to WT, due to the loss of the hydrogen bond between Tyr70 and the 5′-phosphate group of the coenzyme (Toney & Kirsch, 1987, 1991). The benzene ring of Tyr70 has also been postulated to contribute to the recognition of C_5 substrates (Inoue *et al.*, 1991). In this study, we have replaced Tyr70 of chicken mitochondrial AspAT (mAspAT) with a polar histidine residue which can be protonated and deprotonated within the physiological pH range.

EXPERIMENTAL PROCEDURES

Materials. The pGEMEX expression vector was purchased from PROMEGA. Competent DH5α cells were from BRL. The expression strain *E. coli* BL21 (DE3) plysS (Grodberg & Dunn, 1988; Studier *et al.*, 1990) with the T7 RNA polymerase gene was kindly provided by Dr. F. W. Studier. Aromatic amino acids were purchased from Serva. L-Aspartic acid, pyridoxal 5′-phosphate, and pyridoxamine 5′-phosphate hydrochloride were from Merck; L-glutamic acid and L-cysteinesulfinic acid were from Sigma; 2-oxoglutaric acid and 1,4-dithio-DL-threitol were from Fluka; and isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Bachem.

Construction of the Expression System. The cDNA encoding chicken mitochondrial aspartate aminotransferase had previously been subcloned into the vector pOTS (Jaussi *et al.*, 1987). Tyr70 was replaced with a histidine residue by oligonucleotide-directed mutagenesis according to Kunkel *et al.* (1985). The pGEMEX vector which had been provided with a unique *Nde*I restriction site (Ziak *et al.*, 1993) was partially cleaved with *Xba*I and then digested with *Nde*I. The mutated cDNA was retrieved from pOTS by cleavage with

[†] This work was supported in part by the Swiss National Science Foundation Grant 31.27975.89, the Hartmann Müller-Stiftung, Zürich, and by the Italian National Research Council PF BTBS. R.J. was the recipient of a stipend from the Cloëtta Foundation, Zürich.

[‡] This study is part of an investigation of the structure and function of mitochondrial aspartate aminotransferase, a joint project with J. N. Jansonius and colleagues, University of Basel.

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[⊕] Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: AspAT, aspartate aminotransferase; mAspAT, mitochondrial AspAT; DTT, 1,4-dithio-DL-threitol; 2-OG, 2-oxoglutarate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; WT, wild type.

0006-2960/94/0433-2757$04.50/0

*Nde*I and *Xba*I, ligated into the pGEMEX vector, and amplified in competent DH5α cells using ampicillin resistance as the selection marker. *E. coli* BL21 (DE3) plysS cells were transformed with the recombinant plasmid and screened for both ampicillin and chloramphenicol resistance; positive transformants were used for expression of mAspAT Y70H.

Expression and Purification of mAspAT. The cells were grown in LB medium at 37 °C with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The same amount of ampicillin was added when the OD_{550} of the culture had reached 0.1. The culture was induced with 0.5 mM IPTG at an OD_{550} of 0.7, and the incubation was continued for a further 3 h. Typically, 10 mg of mAspAT Y70H per liter of culture was produced. The cells were harvested by centrifugation (4500*g*, 30 min, 4 °C), resuspended in lysis buffer (1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, 1 μM PMP, and 50 mM Tris chloride, pH 7.5), and disrupted by sonication (Branson Sonic Power Model 250 sonifier, 40 kHz) at 0 °C for 30 min. Cell debris was removed by ultracentrifugation (38000*g*, 30 min, 4 °C). The supernatant was dialyzed twice against 10 mM sodium phosphate and 0.1 mM PMP, pH 7.0. The dialysate was applied onto a Fractogel TSK DEAE-650 anion-exchange column equilibrated with 10 mM sodium phosphate, pH 7.0. The column was washed with two column volumes of the same buffer and subsequently eluted with a linear gradient from 0 to 500 mM NaCl in 10 mM sodium phosphate, pH 7.0. The flow-through and the immediately following gradient fractions with AspAT activity were pooled, dialyzed against 10 mM sodium phosphate, pH 6.0, and loaded onto a Fractogel TSK CM-650 cation-exchange column that had been equilibrated with 10 mM sodium phosphate, pH 6.0. After the column was washed with the same buffer, a linear gradient from 0 to 400 mM NaCl in 10 mM sodium phosphate, pH 6.0, was applied. The fractions with AspAT activity were pooled, concentrated, and chromatographed over a Sephadex G-150 column in 50 mM sodium phosphate, pH 7.5. Purified mAspAT Y70H comigrated as a single band on SDS–PAGE with the WT enzyme. WT mAspAT (210 units/mg, at 25 °C) was isolated from chicken heart as described previously (Gehring *et al.*, 1977).

Enzyme Analysis. The concentration of purified mAspAT Y70H was determined from the absorbance at 280 nm by using the molar absorption coefficient of the subunit $\epsilon = 70\,000$ M^{-1} cm^{-1} (Gehring & Christen, 1978). Absorption spectra were recorded with an 8450A UV/vis diode array spectrophotometer from Hewlett-Packard. Circular dichroism was measured with a Model J-500 spectropolarimeter from Jasco. For electrophoresis, the Phast System from Pharmacia-LKB with 10–15% SDS–polyacrylamide gradient gels was used. The gel was stained with silver (Switzer *et al.*, 1979) as described by Pharmacia. The catalytic activity of WT AspAT was measured in a coupled assay with malate dehydrogenase and 20 mM aspartate plus 20 mM 2-oxoglutarate as substrates (Birchmeier *et al.*, 1973). For measurement of the activity of mAspAT Y70H, the concentrations of both substrates were increased to 50 mM. Activity toward aromatic amino acids was determined according to Diamondstone (1966). The absorption of the aldehydes formed by decarboxylation of the oxo acid product upon addition of NaOH was measured at 330, 320, and 334 nm for tyrosine aminotransferase (TyrAT), phenylalanine aminotransferase (PheAT), and tryptophan aminotransferase (TrpAT) activity, respectively. The respective molar absorptivities were 19 900, 17 500 and 10 000 M^{-1} cm^{-1} (Mavrides & Orr, 1975).

The dissociation rate constants for the coenzyme (k_d) were determined by spectrophotometric measurement of the initial rates of coenzyme dissociation in reaction mixtures containing the enzyme plus excess PLP and aspartate in saturating concentration or excess PMP and 2-oxoglutarate (Toney & Kirsch, 1987). The reactions for the determination of $k_{d(PMP)}$ were monitored at 406 nm (ϵ_{406} = 6400 M^{-1} cm^{-1}) and 395 nm (ϵ_{395} = 6600 M^{-1} cm^{-1}) at pH 7.5 and 6.0, respectively. For calculating $k_{d(PLP)}$, the respective molar absorption coefficients ϵ_{406} = 4000 M^{-1} cm^{-1} and ϵ_{395} = 5200 M^{-1} cm^{-1} were used.

Table 1: Kinetic Parameters of mAspAT Y70H[a]

enzyme	k_{cat} (s^{-1})	$K_{m(Asp)}$ (mM)	$K_{m(2\text{-}OG)}$ (mM)	$k_{cat}/K_{m(Asp)}$ (M^{-1} s^{-1})	$k_{cat}/K_{m(2\text{-}OG)}$ (M^{-1} s^{-1})
Y70H	20.6	3.7	15.5	5.6×10^3	1.3×10^3
WT	160	0.7	1.7	2.3×10^5	9.4×10^4

[a] The assays were performed in 50 mM sodium phosphate, pH 7.5, at 25 °C. The concentrations of the substrates aspartate and 2-oxoglutarate ranged from 0.5 to 20 mM and from 0.5 to 50 mM for WT and mutant mAspAT, respectively. The kinetic parameters were determined by nonlinear regression fitting of the rates to the two-substrate ping-pong bi-bi Michaelis–Menten equation (Velick & Vavra, 1962).

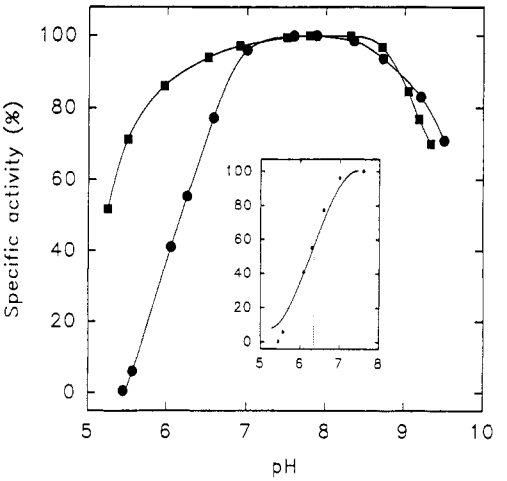

FIGURE 1: pH dependence of enzymic activity of mAspAT Y70H and the WT enzyme. Activity assays were performed in 50 mM sodium phosphate at 25 °C with 50 mM aspartate and 50 mM 2-oxoglutarate as substrates for the mutant and 20 mM of both substrates for the WT enzyme. The pH was adjusted by adding acetic acid or NaOH. Maximum specific activities were 27 and 210 units/mg for the Y70H (●) and WT (■) enzyme, respectively. In the inset, the measured values of activity of the mutant enzyme in the low-pH range were fitted to a calculated titration curve with a pK_a' value of 6.3 by a nonlinear least squares program.

RESULTS AND DISCUSSION

The pK_a value of a histidine residue which is near the physiological pH range allows one to explore the effects of both its unprotonated and protonated forms. The k_{cat} value of mAspAT Y70H at pH 7.5 was 13% of that of the WT enzyme with aspartate and 2-oxoglutarate as substrates. Compared to those of the WT enzyme, the K_m values for aspartate and 2-oxoglutarate of the mutant enzyme were found to be increased 5- and 9-fold, respectively (Table 1). Similar changes in both k_{cat} and K_m values have been reported for AspAT Y70F and AspAT Y70S (Toney & Kirsch, 1991; Inoue *et al.*, 1991). Apparently, these alterations of the catalytic parameters are due to the removal of the tyrosine residue rather than to specific effects of the substituting residues. As with the WT enzyme, maximum activity was attained between pH 7.0 and 8.5. However, if the pH was lowered to 5.5, the activity of the mutant enzyme was abolished, while the WT enzyme still retained 75% of the maximum activity (Figure 1). The low-pH limb of the pH-rate profile of mAspAT Y70H can be fitted approximately to a titration

Table 2: Catalytic Activities of mAspAT Y70H toward Aromatic Amino Acids[a]

amino acid substrate	Concn (mM)	specific activity Y70H (units/mg)	WT (units/mg)	Y70H/WT ratio (%)
aspartate	20	28	210	13.3
tyrosine	3	5.8×10^{-4}	0.085	0.68
phenylalanine	100	1.7×10^{-3}	0.314	0.54
tryptophan	40	1.5×10^{-3}	0.283	0.53

[a] The activities were measured in the presence of 1.5 mM 2-oxoglutarate and the indicated concentrations of amino acids in 50 mM sodium phosphate, pH 7.5, at 30 °C. The concentrations of the aromatic amino acids do not correspond to saturation conditions because of their limited solubility (Mavrides & Christen, 1978).

curve with $pK_a' = 6.3$ and may thus be attributed to the newly introduced histidine residue. The deviation of the low-pH data points from the calculated curve apparently is due to the protonation of residues other than His70, as indicated by the curve of the WT enzyme, and might also reflect the fact that at lower pH not only k_{cat} but also K_m is affected, i.e., that at the chosen substrate concentrations (Figure 1) k_{cat}/K_m rather than k_{cat} is measured. At pH 6.0, the k_{cat} value of the mutant enzyme was decreased to 11 s^{-1}, i.e., to half its value at pH 7.5, and K_m for aspartate was increased 6-fold to 21.3 mM; the K_m for 2-oxoglutarate (16.4 mM) was similar to that at pH 7.5. Thus, the decrease in activity of the mutant enzyme at low pH is due to both a reduced catalytic capacity and a lower affinity for aspartate. Possibly, the positively charged imidazolium group interferes with the proton acceptor–donor function of the ϵ-amino group of Lys258 in the aldimine–ketimine tautomerization. In view of the unchanged K_m value for 2-oxoglutarate at low pH, the increased K_m for aspartate might be due to electrostatic repulsion between protonated His70 and the protonated amino group of aspartate.

The catalytic activity of AspAT toward aromatic amino acids is much lower than that toward dicarboxylic substrates (Mavrides & Christen, 1978). The specific activities of the WT enzyme toward phenylalanine, tyrosine, and tryptophan range from 0.04 to 0.15% of that toward aspartate; in mAspAT Y70H these activities are further reduced to 0.002–0.006% of that toward aspartate (Table 2). Thus, mAspAT Y70H, which retains 13% of the WT activity toward aspartate, has only about 0.6% of the activity of the WT enzyme toward aromatic amino acids. This corresponds to a 22-fold increase in the ratio of the activities toward these two types of substrates. The K_m and k_{cat} values for the aromatic amino acids could not be determined because of the low solubility of the acids. Previous kinetic data have suggested that the aromatic rings of these substrates bind to a site which does not overlap with the binding site for the distal carboxylate group of dicarboxylic substrates (Mavrides & Christen, 1978; Sandmeier & Christen, 1982). A similar change in the ratio of the activities of AspAT toward aromatic and dicarboxylic substrates was also observed upon substitution of other apolar active site residues by histidine (R. A. Vacca, P. Christen, and E. Sandmeier, in preparation). The overproportionate decrease in the activity toward aromatic substrates relative to that toward dicarboxylic substrates seems to be due to a rather unspecific effect, probably to an increase in the polarity of the active site pocket interfering with the binding of aromatic substrates and their orientation required for efficient catalysis. Substitution of Arg292 by Val or Leu has been found to increase the activity toward aromatic amino acids and to decrease that toward dicarboxylic substrates, again indicating that a reduced polarity of the active site cleft is favorable for accommodating aromatic amino acid substrates (Hayashi *et al.*, 1989).

Upon addition of cysteinesulfinate, the 360-nm absorption band of the PLP form of the mutant mAspAT shifted to 330 nm, reflecting the conversion of the PLP to the PMP form; conversely, addition of 2-oxoglutarate converted the PMP form of the enzyme completely back to the PLP form, indicating that both active sites of all enzyme molecules were undergoing the transamination reaction. In the spectrophotometric pH titration of the PLP form of the mutant enzyme, the absorption maximum was shifted from 360 to 430 nm by lowering the pH value from 7.5 to 4.8, reflecting the protonation of the internal aldimine with a pK_a' value of 6.0 (Figure 2). The pK_a' value in the WT enzyme is 6.1 (Eichele *et al.*, 1978); apparently, His70 decreases it only slightly.

The CD spectra in the region of 195–250 nm and the CD bands at 360 or 330 nm of enzyme-bound PLP or PMP, respectively, were virtually identical for mAspAT Y70H and the WT enzyme (not shown). Concomitantly to the shift of maximum absorbance of PLP from 360 to 430 nm upon a

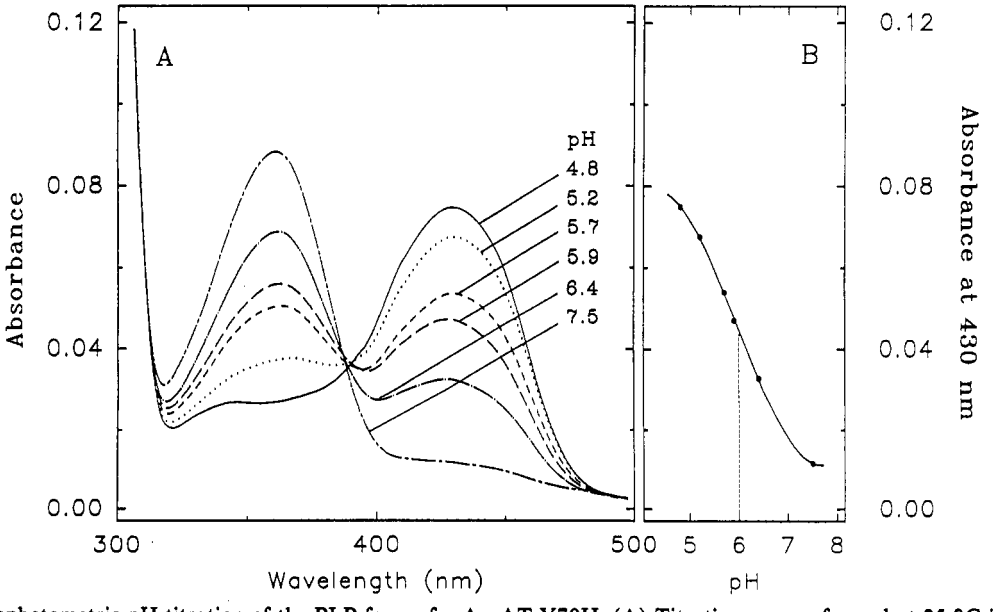

FIGURE 2: Spectrophotometric pH titration of the PLP form of mAspAT Y70H. (A) Titration was performed at 25 °C in 50 mM sodium phosphate which was adjusted to the indicated pH by adding acetic acid. (B) A theoretical dissociation curve was fitted to the experimental values.

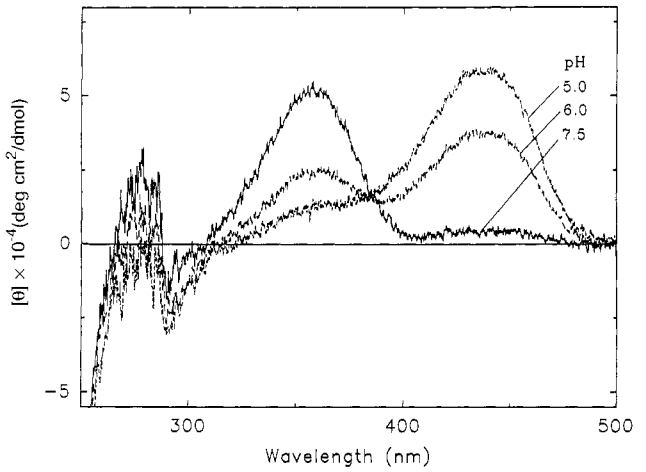

FIGURE 3: Circular dichroism spectra of the PLP form of mAspAT Y70H. The spectra were recorded at an enzyme concentration of 0.9 mg/mL in 50 mM sodium phosphate and 50 mM sodium acetate, adjusted to the indicated pH, at 25 °C.

Table 3: Rate Constants of the Dissociation of Coenzyme from mAspAT[a]

dissociating coenzyme	pH	k_d(Y70H) (s^{-1})	k_d(WT) (s^{-1})	k_d(Y70H)/k_d(WT)
PMP	7.5	7.2×10^{-4}	2.8×10^{-4}	2.6
PMP	6.0	3.8×10^{-3}	7.1×10^{-4}	5.4
PLP	7.5	5.1×10^{-5}	3.9×10^{-5}	1.3
PLP	6.0	1.0×10^{-4}	8.7×10^{-5}	1.2

[a] The measurements were performed at pH 7.5 and 6.0 at 25 °C. The reaction mixtures were composed of 1 μM mAspAT, 66 mM potassium chloride, 100 mM sodium phosphate, and either 50 mM L-aspartate plus 150 μM PLP or 30 mM 2-oxoglutarate plus 150 μM PMP.

decrease in the pH value, a small negative CD band at 295 nm was decreased further, and a positive dichroic band at 275 nm disappeared (Figure 3). These spectral changes are most likely due to protonation of the coenzyme (Ziak *et al.*, 1993) and not due to an ionized tyrosine residue, as proposed previously (Ivanov & Karpeisky, 1969).

In AspAT, the hydrogen bond between the hydroxy group of Tyr70 and the phosphate group of the coenzyme significantly contributes to the noncovalent binding of PMP, as demonstrated by *E. coli* AspAT Y70F, from which PMP dissociates 80-fold faster than from the WT enzyme (Toney & Kirsch, 1987). In contrast, mAspAT Y70H at pH 7.5 releases PMP only 3 times faster than the WT enzyme (Table 3), suggesting that a hydrogen bond might be formed between His70 and the coenzyme. The dissociation rate constants of PLP are virtually not affected by the mutation. These findings together with the largely preserved enzymic activity toward the main substrates indicate that an unprotonated histidine residue at position 70 essentially maintains the structural integrity of the active site including hydrogen bonding of the phosphate group of the coenzyme.

In the comprehensive alignment of aminotransferase sequences, Tyr70 is conserved in all enzymes of subgroup I, which includes aspartate, tyrosine, phenylalanine, alanine, and histidinol-phosphate aminotransferase. In many other aminotransferases, however, Tyr70 appears to be substituted by phenylalanine, leucine, isoleucine, or methionine, and in ornithine aminotransferase of yeast even by a histidine residue (Mehta *et al.*, 1993).

ACKNOWLEDGMENT

We thank Dr. Martin Ziak for his kind help in the construction of the expression system.

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