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Kinetics and Equilibria for the Reactions of Coenzymes with Wild Type and the Y70F Mutant of *Escherichia coli* Aspartate Aminotransferase†

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ABSTRACT: The Y70F mutant of aspartate aminotransferase has reduced affinity for coenzymes compared to the wild type. The equilibrium dissociation constants for pyridoxamine phosphate (PMP) holoenzymes, K_{diss}^{PMP}, were determined from the association and dissociation rate constants to be 1.3 nM and 30 nM for the wild type and mutant, respectively. This increase in K_{diss}^{PMP} for Y70F is due to a 27-fold increase in the dissociation rate constant. Pyridoxal phosphate (PLP) association kinetics are complex, with three kinetic processes detectable for wild type and two for Y70F. A directly determined, accurate value of K_{diss}^{PLP} for wild type enzyme has been difficult to obtain because of the low value of this constant. The values of K_{diss}^{PLP} for the holoenzymes were determined indirectly through the measured values for K_{diss}^{PMP}, glutamate–α-ketoglutarate half-reaction equilibrium constants, and the equilibrium constant for the transamination of PLP by glutamate catalyzed by Y70F. The values of K_{diss}^{PLP} obtained by this procedure are 0.4 pM for wild type and 40 pM for Y70F. The increases in K_{diss}^{PMP} and K_{diss}^{PLP} for Y70F correspond to $\Delta\Delta G$ values of 1.9 and 2.7 kcal/mol, respectively, and are directly attributed to the loss of the hydrogen bond from the phenolic hydroxyl group of Tyr70 to the coenzyme phosphate. The ΔG for association of PLP with wild type enzyme is 4.7 kcal/mol more favorable than that for PMP.

The previous paper (Toney & Kirsch, 1991) describes the properties of the aspartate aminotransferase (AATase)[1] active site mutant Y70F in its reactions with substrates. The present paper is concerned with the reactions of this mutant and the WT enzyme with coenzymes.

AATases from several species, including *Escherichia coli*, bind PLP and PMP with very high affinity such that under

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[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); wild type, wild type *E. coli* AATase; Y70F, *E. coli* AATase in which Tyr70 has been changed to phenylalanine by site-directed mutagenesis; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; E–PLP and E–PMP, PLP and PMP forms of AATase, respectively; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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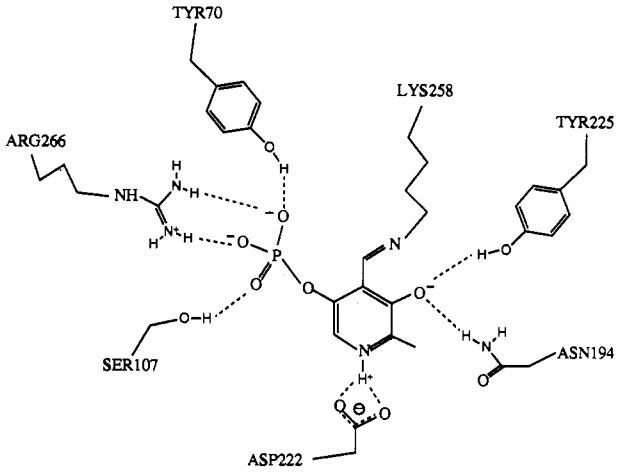
Scheme I: Some of the Specific Interactions between AATase and Coenzymes[a]



[a]Shown is PLP, which forms a covalent Schiff base with Lys258. Bound PMP makes a strong hydrogen bond to the ϵ-NH_3^+ of Lys258. A number of additional interactions have been omitted for clarity. See Jansonius and Vincent (1987) for a review of AATase structure. The role of Tyr70 is probed by the Y70F mutation.

typical assay conditions neither dissociates from the protein. This tight binding is a consequence of several specific interactions made between the coenzymes and the protein active site, some of which are shown in Scheme I. The three nonbridged oxygen atoms of the coenzyme phosphate are saturated by the protein with respect to their hydrogen-bonding capacity. The charge of the phosphate group is largely compensated by hydrogen-bonding ion pairing to Arg266 and by the presence of the N-terminus of an α-helix. The protonated pyridine nitrogen atom of the coenzyme interacts with the carboxylate of Asp222, the 3′-hydroxyl group hydrogen bonds to Tyr225 and Asn194, and the aldehyde and amino functions at C4′ interact strongly with Lys258, the former in the form of a covalent Schiff base (Kirsch et al., 1984; Jansonius & Vincent, 1987). Consistent with the stringent geometric and electronic requirements of the coenzyme binding site are the observations that modification of the coenzyme structure almost invariably decreases the affinity of the enzyme for the coenzyme (Christen & Metzler, 1985).

Among the interactions responsible for anchoring the coenzyme to the protein is the hydrogen bond donated by the Tyr70 hydroxyl group to the phosphate oxygen OP2. This bond is removed by the mutation Y70F. It was thought originally that the hydroxyl group of Tyr70 might be an important component of the catalytic apparatus, but this proposition has now been disproven (Toney & Kirsch, 1987, 1991). A preliminary report presented evidence that PMP is dissociated from the Y70F mutant more frequently than from wild type during the course of catalysis (Toney & Kirsch, 1987). Reported herein is a detailed, quantitative evaluation of the effect of this mutation on the equilibria and kinetics of coenzyme binding.

Experimental Procedures

General procedures and materials are described in Toney and Kirsch (1991). All experiments were performed in 200 mM HEPES–KOH buffer, pH 7.5/100 mM potassium chloride, unless specified otherwise. Apoenzymes were prepared by ammonium sulfate precipitation of E–PMP at pH 4.9, essentially as described by Wada and Snell (1962). Cysteinesulfinate was used to convert E–PLP to E–PMP, and a Sephadex G-25 column was used as a final step in order to remove residual ammonium sulfate.

Coenzyme Dissociation and Association Kinetics. Coenzyme dissociation rate constants were obtained by spectrophotometrically measuring the initial rates of coenzyme transamination for reactions containing only excess PLP and aspartate or PMP and α-ketoglutarate. The coenzyme dissociation step is rate-limiting under the conditions employed. See Toney & Kirsch (1987) for details of the method. The following modification was included to ensure that the measured process was enzymatic. The initial rates were measured as a function of enzyme concentration, and the dissociation rate constants were taken as the slopes of the resulting linear plots. The initial rates were independent of enzyme concentration only for the dissociation of PLP from wild type, indicating that this rate constant is inaccessible by this method.

The rate constant for the dissociation of PLP from wild type enzyme was measured directly by monitoring the release of [C4′-^{3}H]PLP from wild type holoenzyme. Apoenzyme (55 μM in 10 mM Tris-HCl, pH 8.0, 3.5 mL) was reconstituted with a 5-fold excess of [C4′-^{3}H]PMP (Julin et al., 1989). This was followed by addition of 1M HEPES–KOH, pH 7.5, and 1 M dipotassium oxalacetate to 200 mM final concentrations to convert enzyme-bound PMP to PLP. The sample was desalted on a Sephadex G-25 column, and the addition of HEPES–KOH and oxalacetate was repeated. The sample was then "washed" by repeated ultrafiltration/concentration with a Centricon 30 apparatus (Amicon) with 10 mM Tris-HCl, pH 8.0, as buffer. Washing was continued until there was less than 0.05% of the retentate ^{3}H/mL of effluent. The radioactive wild type holoenzyme was added to a reaction mixture to given final concentrations of 200 mM HEPES–KOH, pH 7.5, 100 mM potassium chloride, 1.2 mM 5′-phosphopyridoxyl-L-aspartate (included to trap nascent apoenzyme irreversibly), and 90 μM wild type in a total voluume of 1.5 mL. A small crystal of thymol was included to prevent microbial growth. The reaction mixture was maintained at 25 °C. Aliquots of 100 μL were taken at timed intervals and mixed with 900 μL of the same buffer. Following filtration through a Centricon 10 apparatus, 750 μL of the effluent was added to 10 mL of scintillant and the total ^{3}H was determined. The endpoint of the reaction was obtained by the addition of a 50-fold excess of cysteinesulfinate to a sample of the reaction, followed by KOH to 0.1 M, filtration, and counting. The reaction was followed for about 1.2 half-lives. The data were fitted to a single-exponential time course with the endpoint fixed at the measured value.

The sums of the PMP and PLP dissociation rate constants (k_{equil}) were measured for both wild type and Y70F by adding E-PMP to a reaction mixture, giving final concentrations of 200 mM HEPES–KOH, pH 7.5, 100 mM potassium chloride, 23 μM AATase, 35 μM PLP, and 35 μM PMP. The reactions were monitored at 390 nm for Y70F and at 365 nm for wild type.

Association rate constants for PMP combining with either wild type or Y70F apoenzyme were measured under pseudo-first-order conditions. Apoenzymes were present at 5 μM, and PMP was in at least 10-fold excess. The progress of the reactions was monitored by the quenching of protein fluorescence, which occurs upon PMP association (Churchich & Farrelly, 1969). The fast reaction rates were monitored with a Union Giken RA-401 stopped-flow spectrophotometer. Typically, 200 μL from each of two reservoirs was used per reaction.

The reactions between apoenzymes and PLP were monitored by the change in absorbance at 351 nm (the maximum in the

Table I: Kinetic and Equilibrium Constants for the Reactions of Wild Type and Y70F Apoenzymes with PMP and PLP[a]

	$k_{diss}^{PMP} \times 10^4$ (s^{-1})[b]	$k_{diss}^{PLP} \times 10^4$ (s^{-1})[b]	$k_{assoc}^{PMP} \times 10^{-5}$ (M^{-1} s^{-1})[c]	K_{diss}^{PMP} (nM)[d]	K_{diss}^{PLP} (pM)[e]
wild type	4.12	0.0050	3.23	1.28	0.4
	(0.06)	(0.0006)	(0.06)	(0.03)	
Y70F	111	5.9	3.66	30.3	40
	(2)	(0.3)	(0.06)	(0.7)	
Y70F/	26.9	1180	1.13	23.7	100
wild type	(0.6)	(150)	(0.03)	(0.8)	

[a] Conditions: 200 mM HEPES-KOH/100 mM potassium chloride, pH 7.5, 25 °C. Standard errors are given in parentheses. [b] All k_{diss} values except that for wild type–PLP were measured with use of a steady-state assay in which coenzyme dissociation is rate-determining. Wild type k_{diss}^{PLP} was measured by monitoring release of [^{3}H]PLP from holoenzyme. [c] Measured by monitoring the decrease in protein fluorescence on binding PMP, with use of a stopped-flow apparatus. All reaction traces were well described by single exponentials. [d] $K_{diss} = k_{diss}/k_{assoc}$. [e] These estimates have 50% errors due to the error in K_{coenz}, which was used in the calculations described in Scheme II.

difference spectrum) in the stopped-flow apparatus. Excess coenzyme and 5 μM apoenzymes were employed as for the PMP association reactions.

Half-Reaction Equilibrium Constants. The values of the equilibrium constants, K_{HR}, for the glutamate–α-ketoglutarate half-reactions (eq 1) were determined at AATase concentra-

$$\text{E–PMP} + \alpha\text{-ketoglutarate} \underset{}{\overset{K_{HR}}{\rightleftharpoons}} \text{E–PLP} + \text{glutamate} \quad (1a)$$

$$K_{HR} = \frac{[\text{E–PLP}][\text{glutamate}]}{[\text{E–PMP}][\alpha\text{-ketoglutarate}]} \quad (1b)$$

tions of 15 μM and either 0.3 mM or 1 mM initial concentrations of α-ketoglutarate. Aliquots of a 1.0 M glutamate solution were added in small volumes to 1.0-mL solutions of enzymes. The A_{360} was measured after a 1-min equilibration. Corrections were made for the effects of dilution on the concentrations of glutamate and α-ketoglutarate and the 360 nm absorbance, and the data were fitted to eq 2, where A_{360} is the

$$A_{360} = \frac{K_{HR}A_0 + A_\infty \dfrac{[\text{L-Glu}]}{[\alpha\text{KG}]}}{K_{HR} + \dfrac{[\text{L-Glu}]}{[\alpha\text{KG}]}} \quad (2)$$

360-nm absorbance. A_0, the initial absorbance, A_∞, the absorbance at infinite glutamate concentration, and K_{HR}, the equilibrium constant, are adjustable parameters.

Equilibrium Constant for the Transamination Reaction of Glutamate with Free PLP. This equilibrium constant was measured in solutions containing 200 mM HEPES-KOH, pH 7.5, 100 mM potassium chloride, 5 mM α-ketoglutarate, 5 mM glutamate, 1 μM Y70F, and either (1) 0.1 mM each PLP and PMP, (2) 0.2 mM PLP, or (3) 0.2 mM PMP. After ~15 h of reaction in the dark at 25 °C, the final concentrations of PLP and PMP were determined spectrophotometrically (Peterson & Sober, 1954) and were used in calculating the equilibrium constant. Under these conditions, the changes in α-ketoglutarate and glutamate concentrations are insignificant.

Results

Rate and Equilibrium Constants for the Reactions of Apoenzymes with Coenzymes. Table I presents a collection of rate and equilibrium constants that describe the interactions of coenzymes with wild type and Y70F apoenzymes. The dissociation rate constants for both coenzymes are greater for Y70F than for wild type: PMP is released 27-fold, and PLP

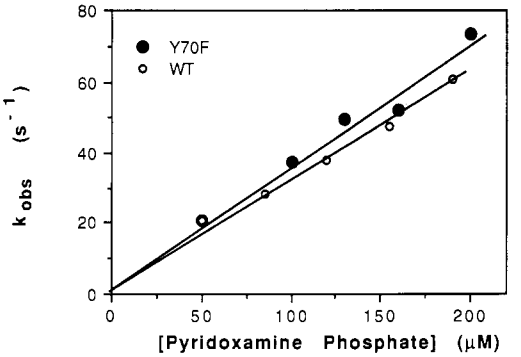


FIGURE 1: The observed first-order rate constants for the association of wild type and Y70F apoenzymes with PMP as a function of PMP concentration. Reaction traces obtained by monitoring protein fluorescence conformed to single exponentials. Apoenzyme was prepared by a procedure that avoids residual inorganic phosphate in the coenzyme site (see Experimental Procedures). Reactions were performed at 25 °C in 200 mM HEPES-KOH buffer, pH 7.5/100 mM potassium chloride.

1200-fold, faster. The discrepancy between the value of the PLP dissociation rate constant for wild type reported previously (Toney & Kirsch, 1987) and that given here is due to the very slow nature of this process and the previous measurement method. The earlier reported measurements were made at a single protein concentration, and this assay is independent of protein concentration only for the wild type–PLP complex. The rate reported earlier was due to buffer catalysis.

The sum of the PLP and PMP dissociation rate constants was measured. Enzyme containing only PMP was mixed with free PLP and free PMP and allowed to equilibrate. Under the conditions employed, the rates at which nascent apoenzymes are trapped by free coenzyme are much faster than the rates of either PMP or PLP dissociation, so that the observed k_{equil} very nearly equals the sums of the dissociation rate constants (eq 3). The observed values of k_{equil} were not

$$\text{E–PMP} + \text{PMP} + \text{PLP} \underset{\text{fast}}{\overset{k_{diss}^{PMP}}{\rightleftharpoons}} \text{E} + \text{PMP} + \text{PLP} \underset{k_{diss}^{PLP}}{\overset{\text{fast}}{\rightleftharpoons}} \text{E–PLP} + \text{PMP} + \text{PLP} \quad (3a)$$

$$k_{equil} = k_{diss}^{PLP} + k_{diss}^{PMP} \quad (3b)$$

significantly altered by increasing coenzyme concentrations to 45 μM. Because the value of k_{equil} is readily calculated from the constituent constants, its measurement provides checks on the individually measured dissociation rate constants. In the cases of wild type and Y70F, the calculated k_{equil} values are dominated by k_{diss}^{PMP} (i.e., $k_{diss}^{PLP} \ll k_{diss}^{PMP}$). Thus, k_{equil} provides an independent determination of k_{diss}^{PMP}. Indeed, k_{equil} measured for wild type [$(4.3 \pm 0.2) \times 10^{-4}$ s^{-1}] agrees well with k_{diss}^{PMP} measured by the initial rate method, and the agreement for Y70F [$k_{equil} = (8.2 \pm 0.1) \times 10^{-3}$ s^{-1}] is satisfactory (compare with column 1 of Table I).

The observed rate constants for the association of PMP with apoenzymes increase linearly with PMP concentration over the range 50–200 μM under pseudo-first-order conditions with excess coenzyme (Figure 1). The data were fitted to eq 4,

$$k_{obs} = k_{assoc}^{PMP}[\text{PMP}] + k_{diss}^{PMP} \quad (4)$$

with k_{diss}^{PMP} held constant at the independently determined value. The values of k_{assoc}^{PMP} and k_{diss}^{PMP} are reported in Table I. The calculated equilibrium dissociation constant ($K_{diss}^{PMP} = k_{diss}^{PMP}/k_{assoc}^{PMP}$) for wild type–PMP is 1.3 nM, while that for Y70F–PMP is 24-fold larger (Table I).

The kinetics describing PLP association with apoenzymes proved more complex than those of PMP. Three phases were

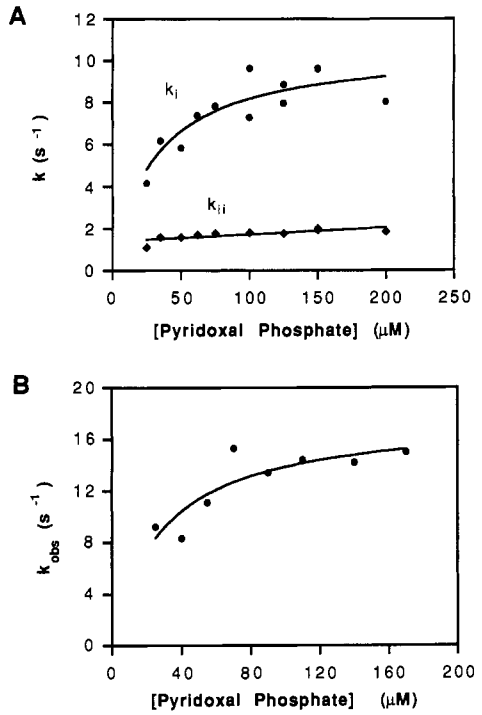


FIGURE 2: The rate constants for the association of (A) wild type and (B) Y70F apoenzymes with PLP. The nature of the reaction is complex. Three phases were detected in the wild type, and two, were detected in the Y70F traces. The rapid, first phase for both enzymes was not analyzed. Data for the second (k_i) and third (k_{ii}) phases for wild type and the second phase for Y70F are given. Apoenzyme was prepared by a procedure that avoids residual inorganic phosphate in the coenzyme site (see Experimental Procedures). Reactions were performed at 25 °C in 200 mM HEPES–KOH buffer, pH 7.5/100 mM potassium chloride.

detectable in the reaction traces for wild type, as found previously for cytosolic AATase (Vergé & Arrio-Dupont, 1981). The first phase (negative absorbance change) was over within 40 ms and was not analyzed. The data after 50 ms (positive absorbance changes) conformed well to two-exponential processes (k_i and k_{ii}, see below) and were so treated. The Y70F reactions differed in that the third (second positive) phase was not observed. The statistical significance of the two terms (a rate constant and an amplitude) added when a double-exponential vs a single-exponential equation is fit to the data was tested by using the F_χ statistic (Bevington, 1969). For tests on several representative traces, the probability that the added terms are significant (i.e., nonzero) proved greater than 99% for wild type, whereas this probability is less than 90% for Y70F. Thus, the Y70F reaction traces (in the time domain analyzed, >50 ms) conform adequately to a single-exponential model, while those for wild type require a double-exponential model to account fully for the data.

The dependence of the pseudo-first-order rate constants, for the fractions of the reactions occurring after 50 ms, on PLP concentration is shown in Figure 2. The rate constant k_i for the first process occurring with positive 351-nm absorbance change in wild type reactions shows significant dependence on PLP concentration, as does the Y70F rate constant, k_{obs}, derived from single-exponential curve fitting. The wild type k_i is generally 3-fold larger than that found for cytosolic AATase (Vergé & Arrio-Dupont, 1981), and the Y70F k_{obs} tends to be 2-fold larger than the wild type k_i. The wild type k_{ii}, corresponding to the second process with positive 351-nm absorbance change, shows only a slight dependence on PLP concentration; Vergé and Arrio-Dupont treated the corresponding cytosolic AATase process as being independent of

Scheme II: Equilibria Related to the Estimation of K_{diss}^{PLP}, the Dissociation Constant for PLP–WT and PLP–Y70F Complexes[a]

$$E + PMP + KG \underset{}{\overset{(K_{diss}^{PMP})^{-1}}{\rightleftharpoons}}$$

$$E\text{–}PMP + KG \overset{K_{HR}}{\rightleftharpoons} E\text{–}PLP + Glu \overset{K_{diss}^{PLP}}{\rightleftharpoons} E + PLP + Glu$$

$$K_{diss}^{PMP} = \frac{k_{diss}^{PMP}}{k_{assoc}^{PMP}}$$

$$K_{free} = \frac{K_{diss}^{PLP} K_{HR}}{K_{diss}^{PMP}} = \frac{[PLP][Glu]}{[PMP][KG]}$$

$$K_{diss}^{PLP} = \frac{K_{diss}^{PMP} K_{free}}{K_{HR}}$$

[a] K_{diss}^{PMP} is the equilibrium dissociation constant for the PMP holoenzyme. K_{HR} is the glutamate–α-ketoglutarate enzymatic half-reaction equilibrium constant. K_{free} is the nonenzymatic transamination half-reacton equilibrium constant. KG = α-ketoglutarate, Glu = glutamate.

Table II: Enzyme-Bound and Enzyme-Free Transamination Half-Reaction Equilibrum Constants[a]

	K_{HR}[b]		K_{free}[c]
wild type	25 (1)	pyridoxal phosphate	0.008[d] (0.004)
Y70F	6.7 (0.8)	pyridoxal	1.5[e]

[a] Conditions: 200 mM HEPES–KOH/100 mM potassium chloride, pH 7.5, 25 °C. Standard errors are given in parentheses. [b] K_{HR} is the equilibrium constant for the interconversion of enzyme-bound PLP and PMP by glutamate and α-ketoglutarate (eq 1). Measured by titration of an enzyme–α-ketoglutarate solution by glutamate, fitting the data to eq 2. [c] K_{free} (eq 7) is the equilibrium constant for the interconversion of free PL(P) and free PM(P) by glutamate and α-ketoglutarate. [d] Measured with use of the Y70F mutant as catalyst. [e] Taken from Wada and Snell (1962); measured at 37 °C in 0.1 M Tris-HCl buffer, pH 8.5.

PLP concentration. The k_i data for wild type and k_{obs} for Y70F were fitted to a rectangular hyperbola (eq 5). The

$$k_i \text{ or } k_{obs} = \frac{k_{max}[PLP]}{K_{app} + [PLP]} \quad (5)$$

resultant minimizing parameters are $k_{max} = 11 \pm 1$ s^{-1} and $K_{app} = 32 \pm 10$ μM for wild type and $k_{max} = 18 \pm 2$ s^{-1} and $K_{app} = 28 \pm 11$ μM for Y70F. The line fitted to the wild type k_{ii} data is described by slope = $(2.2 \pm 0.5) \times 10^3$ M^{-1} s^{-1} and intercept = 1.5 ± 0.1 s^{-1}. The kinetic complexity of the PLP–apoenzyme association motivated the choice of the approach shown in Scheme II (and discussed below) for the determination of K_{diss}^{PLP}.

Equilibrium Constants for the Glutamate–α-Ketoglutarate Half-Reaction. Values for K_{HR}, defined in eq 1, are collected in Table II. Two concentrations of α-ketoglutarate were used, and the same value of K_{HR} was obtained; therefore, the concentration of enzyme–substrate complexes present does not significantly affect the measured values of the equilibrium constants. The measured equilibrium constant of 25 ± 1 for wild type agrees well with that of 30 ± 3 measured independently (Inoue et al., 1989) and is similar to that of 12 measured for cytosolic AATase (Velick & Vavra, 1962). K_{HR} for Y70F is 6.7 ± 0.8, which means that the Tyr70 hydrogen bond stabilizes enzyme-bound PLP slightly more than enzyme-bound PMP.

Y70F is useful as a catalyst to achieve equilibrium between free PLP, glutamate, free PMP, and α-ketoglutarate, i.e. eqs 6 and 7, because coenzyme is dissociated from this enzyme

glutamate + PLP + apoY70F ⇌
α-ketoglutarate + PMP + apoY70F (6)

$$K_{free} = \frac{[PLP][glutamate]}{[PMP][\alpha\text{-ketoglutarate}]} \quad (7)$$

relatively rapidly. The measured value of K_{free} is 0.008 ± 0.004.

Discussion

Y70F Binds PMP Less Tightly Than Does Wild Type. A preliminary report presented evidence that PMP is dissociated faster from Y70F than from wild type (Toney & Kirsch, 1987). These initial observations were made in phosphate buffer. The more nearly complete set of measurements reported in Table I was made in HEPES–KOH buffer to circumvent any potential complications due to phosphate inhibition of coenzyme association (Arrio-Dupont, 1972).

Both PLP and PMP are dissociated significantly faster from Y70F than from wild type (Table I). These results are almost certainly a consequence of the loss of the hydrogen bond between the hydroxyl group of Tyr70 and coenzyme phosphate oxygen OP2. On the other hand, the rate constants for the association of PMP with wild type and Y70F apoenzymes are nearly the same. The transition state for the rate-determining step in the PMP binding process must therefore not involve significant interactions between the Tyr70 hydroxyl group and PMP. Thus, the difference in the equilibrium dissociation constants originates almost solely from the dissociation rate constants.

Cytosolic apoAATase dimers dissociate into monomers with an apparent dissociation constant of 0.8 μM (Cournil et al., 1975). Dissociation of apoenzyme dimers also occurs with *E. coli* AATase at similar concentrations (M.D.T. & J.F.K., unpublished results). Thus, multiple equilibria are encountered when standard equilibrium techniques are used for the direct analysis of AATase–coenzyme dissociation constants. It proved more practical to determine PMP–holoenzyme equilibrium dissociation constants from the measured coenzyme association and dissociation rate constants determined at high enzyme concentrations where the apoenzymes are fully dimerized. The calculated K_{diss}^{PMP} values given in Table I demonstrate that a major effect of Tyr70 is to stabilize the holoenzyme complex. The higher value of K_{diss}^{PMP} (30 nM) for Y70F provides an explanation for the earlier observation that the steady-state Y70F initial rates, in the absence of excess coenzyme, decrease with time (Toney & Kirsch, 1987); PMP dissociates from E–PMP under these conditions, lowering the holoenzyme concentration. The value of 1.3 nM calculated for wild type–PMP K_{diss} is in agreement with previous estimates of less than 5 nM made with cytosolic AATase (Arrio-Dupont, 1972). The calculated free energy difference for the stability of wild type– and Y70F–PMP complexes is 1.9 ± 0.1 kcal/mol.

PLP Association Kinetics and Calculation of K_{diss}^{PLP}. The association of PLP with apoAATase (*E. coli*) is rapid and occurs in a multiple-exponential process as found previously for cytosolic AATase (Vergé & Arrio-Dupont, 1981). The simplest mechanism accounting for the cytosolic AATase data is given in eq 8, where A is apoenzyme and E_1 and E_2 are

$$A + PLP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_3 \quad (8)$$

intermediates en route to the final complex, E_3; k_{-2} and k_{-3} are insignificant under the conditions of the experiment. The most likely assignments for E_1–E_3 are E_1, a noncovalent E–PLP complex; E_2, a PLP–Lys258 aldimine; and E_3, an isomerized aldimine. Fitting k_i (wild type) and k_{obs} (Y70F) vs PLP concentration to a rectangular hyperbola gives apparent dissociation constants of 30 μM, similar to previous findings (Vergé & Arrio-Dupont, 1981; Fonda & Auerbach, 1976). The major differences between the wild type and Y70F association reactions with PLP are (1) a double-exponential absorbance increase for wild type vs a single one for Y70F and (2) the 50% larger magnitudes of the Y70F rate constants compared to wild type. Within the context of the above mechanism, these data support the involvement of Tyr70 in both Schiff base formation and the subsequent isomerization. The Tyr70 hydroxyl group hydrogen bonds to the coenzyme phosphate oxygen OP2 in the holoenzyme; loss of this or some other interaction in an intermediate complex involving Tyr70 must relax the energetic requirements of these two steps in Y70F. The isomerization step might be placement of the coenzyme phosphate into the conformationally stringent binding site. If so, the inability to detect this process in the Y70F reactions indicates that it is fast compared to Schiff base formation.

A consideration of the equilibria shown in Scheme II allows the estimation of the otherwise elusive quantity K_{diss}^{PLP} from accessible equilibrium constants, namely, K_{diss}^{PMP} (Table I), K_{HR} (the glutamate–α-ketoglutarate half-reaction equilibrium constant) (Table II), and K_{free} (the equilibrium constant for the overall conversion of PLP and glutamate to PMP and α-ketoglutarate; see Results). K_{diss}^{PLP} values of 0.4 pM and 40 pM were thus calculated for wild type and Y70F, respectively. Each of these values has an associated 50% uncertainty due to the error in K_{free}. The 100-fold larger value of the calculated PLP equilibrium dissociation constant for Y70F vs wild type is less than the 1200-fold larger PLP dissociation rate constant. The remaining 12-fold difference must reside in the effective PLP association rate constants. As discussed above, the Y70F–PLP association reaction is faster and qualitatively different than that for wild type. These K_{diss}^{PLP} estimates show that, as expected, PLP is more tightly bound than PMP, due to its covalent interactions with the protein. The 2700-fold tighter binding of PLP vs PMP to wild type represents a 4.7 kcal/mol difference in free energy.

Measurements of the dissociation constants for pyridoxal and pyridoxamine binding to pyridoxamine–pyruvate transaminase gave only a 5-fold lower value of K_{diss} for pyridoxal (Ayling & Snell, 1968). Correcting for the pyridoxal aldehyde–internal hemiacetal equilibrium constant (estimated to be 80 in favor of the hemiacetal; Metzler & Snell, 1955) leads to a 400-fold lower pyridoxal–aldehyde vs pyridoxamine dissociation constant, which is still significantly smaller than the 2700-fold different observed here for the association of the phosphorylated analogues with wild type.

The value of K_{free} for the phosphorylated coenzymes can also be compared to that of 1.5 obtained for the unphosphorylated coenzymes under slightly different conditions (Wada & Snell, 1962). Correction of the latter for the aldehyde–hemiacetal equilibrium gives K_{free} = 0.02 for the aldehyde, which is close to 0.008 ± 0.004 obtained here for the phosphorylated coenzymes.

The hydrogen bond between the phenolic hydroxyl group of Tyr70 and the coenzyme phosphate clearly implicated by the X-ray structures of AATases (Jansonius & Vincent, 1987; Smith et al., 1989) thus contributes significantly to the stability of bound coenzymes: 1.9 kcal/mol for the E–PMP complex, and 2.7 kcal/mol for the E–PLP complex. The hydroxyl moiety of Tyr70 is not critical to the mechanism of transamination as demonstrated in the previous paper (Toney & Kirsch, 1991). Its main function is to stabilize the holoenzyme

complexes, which may be the reason for the strict conservation of Tyr70 in AATases (Kondo et al., 1987; Fotheringham et al., 1986).

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Thyroid Hormone Dependent Pituitary Tumor Cell Growth in Serum-Free Chemically Defined Culture. A New Regulatory Role for Apotransferrin†

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ABSTRACT: Thyroid hormone dependent GH_1 rat pituitary tumor cell growth in serum-free chemically defined medium required a serum-derived mediator (i.e., thyromedin) which was identified as transferrin [Sirbasku, D. A., Stewart, B. H., Pakala, R., Eby, J. E., Sato, H., & Roscoe, J. M. (1990) *Biochemistry 30*, 295-304]. The transferrin isolated was consistent with the equine R or D variants and was biologically active only as apotransferrin (apoTf). To determine if other variants of horse transferrin also were thyromedins, a purification was developed which yielded seven separate forms. Initially, only four of these had activity when assayed in standard "iron salts containing" medium (ED_{50} values of 290–1160 nM). To further assess activity, the iron contents of all seven were altered either by saturation with ferric ammonium citrate or by citrate/acid depletion of the metal ion. Thereafter, potencies were compared in "iron salts containing" and "iron salts reduced" media. All seven variants proved to be active as apoTf. Bioassays in which apoTf was maximized showed ED_{50} values of 2.1–3.8 nM. Conversely, assays in which thyromedins were converted to Tf·2Fe showed no activity. Previously, the only known physiological function of apoTf was that of a carrier/detoxifier of iron; this study indicates a new role in hormone-dependent pituitary cell growth.

Previous studies (Hayashi & Sato, 1976; Hayashi et al., 1978; Bottenstein et al., 1979; Hayashi, 1984) established that rat pituitary tumor cells grew in serum-free chemically defined media supplemented with several hormones including triiodothyronine (T_3),[1] Tf·2Fe, and insulin or insulin-like growth factors. More recently, we have studied T_3-dependent pituitary cell growth in serum-free medium and in cultures supplemented with hormone-depleted sera (Riss et al., 1986, 1989; Riss & Sirbasku, 1989). When we compared our results to those of others, problems were apparent.

Using the GH_4C_1 (Tashjian et al., 1970) and the GH_3 (Tashjian et al., 1968) cell lines, we found they survived

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[1] Abbreviations: apoTf, apotransferrin; CPD, cell population doublings; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; F12-DME, 1:1 (v/v) mixture of Ham's F12 nutrient medium and Dulbecco's modified Eagle's medium containing 2.2 g/L sodium bicarbonate and 15 mM HEPES, pH 7.2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; p*I*, isoelectric point; PTH, phenylthiohydantoin derivatives of amino acid residues; RP-HPLC, reverse-phase high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tf·2Fe, diferric transferrin; T_3, triiodothyronine; TFA, trifluoroacetic acid.

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