The Tyrosine-225 to Phenylalanine Mutation of *Escherichia coli* Aspartate Aminotransferase Results in an Alkaline Transition in the Spectrophotometric and Kinetic pK_a Values and Reduced Values of both k_{cat} and K_m[†]

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ABSTRACT: Tyrosine-225 is hydrogen-bonded to the 3′-hydroxyl group of pyridoxal 5′-phosphate in the active site of aspartate aminotransferase. Replacement of this residue with phenylalanine (Y225F) results in a shift in the acidic limb of the pK_a of the k_{cat}/K_{Asp} vs pH profile from 7.1 (wild-type) to 8.4 (mutant). The change in the kinetic pK_a is mirrored by a similar shift in the spectrophotometrically determined pK_a of the protonated internal aldimine. Thus, a major role of tyrosine-225 is to provide a hydrogen bond that stabilizes the reactive unprotonated form of the internal aldimine in the neutral pH range. The K_m value for L-aspartate and the dissociation constant for α-methyl-DL-aspartate are respectively 20- and 37-fold *lower* in the mutant than in the wild-type enzyme, while the dissociation constant for maleate is much less perturbed. These results are interpreted in terms of competition between the Tyr225 hydroxyl group and the substrate or quasi-substrate amino group for the coenzyme. The value of k_{cat} in Y225F is 450-fold less than the corresponding rate constant in wild type. The increased affinity of the mutant enzyme for substrates, combined with the lack of discrimination against deuterium in the C_α position of L-aspartate in Y225F-catalyzed transamination [Kirsch, J. F., Toney, M. D., & Goldberg, J. M. (1990) in *Protein and Pharmaceutical Engineering* (Craik, C. S., Fletterick, R., Matthews, C. R., & Wells, J., Eds.) pp 105–118, Wiley-Liss, New York], suggests that the rate-determining step in the mutant is hydrolysis of the ketimine intermediate rather than C_α–H abstraction which is partially rate-determining in wild type.

Aspartate aminotransferase (AATase;[1] EC 2.6.1.1) is a PLP-dependent enzyme, which preferentially catalyzes the reaction

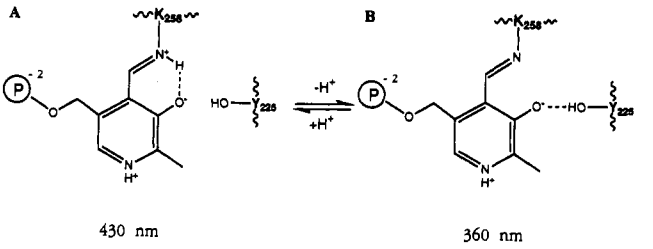
L-aspartate + α-ketoglutarate ⇌ oxalacetate + L-glutamate (1)

Over the course of the reaction, the enzyme alternates between the PLP form, in which the cofactor is linked through a Schiff's base to the ε-amino group of Lys258, and the PMP form, in which the cofactor is bound noncovalently. The reaction proceeds in two stages, in which an amino acid and water react with PLP–enzyme to form a ketoacid followed by the reaction of a second ketoacid with PMP–enzyme to give a new amino acid and water (eq 1) (Braunstein, 1973).

Crystal structures of pig cytosolic and chicken mitochondrial isozymes have been solved to high resolution (Arnone et al., 1985; Jansonius & Vincent, 1987), and the enzyme from *Escherichia coli* has been cloned and expressed (Kuramitsu et al., 1985; Malcolm & Kirsch, 1985; Fotheringham et al., 1986). An atomic resolution X-ray structure of the *E. coli* mutant K258A has been obtained (Smith et al., 1989). Nearly all of the residues lining the active site of the eukaryotic isozymes are conserved in the *E. coli* primary sequence, and the structure of the *E. coli* enzyme is virtually identical with those of the eukaryotic isozymes in its backbone tracing, and in the general disposition of the conserved active-site residues.

The protein-embedded PLP moiety makes a number of well-defined contacts with amino acid side chains, and roles for many of these residues are posited in a detailed mechanism (Kirsch et al., 1984). Several of the sites discussed in the proposal have been probed by site-directed mutagenesis experiments (Toney & Kirsch, 1987, 1989; Kirsch et al., 1987, 1990; Cronin & Kirsch, 1988; Hayashi et al., 1989; Inoue et al., 1989; Ziak et al., 1990; Kirsch & Toney, 1990; Morino et al., 1990). The role of the conserved Tyr225 is examined in this report.

Scheme I



Tyr225 donates a hydrogen bond to the O-3′ moiety of cofactor in the unprotonated enzyme form (Scheme IB) (Kirsch et al., 1984; Jansonius et al., 1985; Jansonius & Vincent, 1987). The tyrosine–cofactor interaction is disrupted upon protonation of the Schiff's base, in which the ε-nitrogen of Lys258 donates a hydrogen bond to the PLP hydroxyl group

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, *Escherichia coli* wild-type AATase; Y225F, mutant *E. coli* AATase with Tyr225 replaced by Phe; MDH, malate dehydrogenase (EC 1.1.1.37); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; L-Asp, L-aspartic acid; αKG, α-ketoglutaric acid; αMeAsp, α-methyl-DL-aspartic acid; εPLLys, N^ϵ-pyridoxyl-L-lysine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; TCA, trichloroacetic acid.

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(Scheme IA). The protonated form absorbs at 430 nm, while the unprotonated species absorbing at ~360 nm is catalytically active (Jenkins et al., 1959). It has been suggested that the hydrogen bond between Tyr225 and O-3′ decreases the pK_a of the Schiff's base nitrogen atom by about 2 units, and stabilizes the tortionally and sterically strained conformation of the internal aldimine observed in chicken mitochondrial AATase (Kirsch et al., 1984). A site-directed mutagenesis experiment in which Tyr225 is replaced by Phe (Y225F) would therefore be expected to produce an enzyme where the spectrophotometrically determined pK_a of the internal aldimine is increased relative to WT as has been recently reported (Morino et al., 1990), and this perturbation will be mirrored by a similar increase in the acidic branch of the k_{cat}/K_{Asp} vs pH profile. In order to test these predictions, Y225F was produced and characterized.

Experimental Procedures

Mutagenesis, Expression, and Purification of AATase. The subcloning, mutagenesis, and expression of the *aspC* gene of *E. coli* encoding AATase have been described previously (Malcolm & Kirsch, 1985). The mutagenesis procedure differed only in that (1) the sequence of the mutagenesis primer was 5′-AAA-ACC-CTG-GAA-AGC-GAA-GTC-3′, (2) template and primer were never exposed to temperatures below 37 °C during the mutagenesis reaction, (3) the mutant insert was retrieved from M13 replicative-form DNA, and (4) the mutant insert was subcloned into pKK 177-3 using *Eco*RI and *Hin*dIII sites. Growth and purification were performed as previously described (Smith et al., 1986) except that the cells were not induced with isopropyl β-D-thiogalactoside and were grown for 20 h. Enzyme yields were about 20–80 mg per 12 g of cell paste and were >90% pure as judged by SDS–PAGE analysis. Enzyme was stored at ~10 mg/mL in 5 or 20 mM potassium phosphate buffer at pH 7.2 with 20 μM PLP at 4 °C in the dark.

Spectrophotometric Measurements. Spectrophotometric measurements and the monitoring of kinetics were done on Cary 118C, Perkin-Elmer λ 4B, Shimadzu UV-160, or Kontron Uvikon 860 spectrophotometers. Temperature-controlled experiments were carried out at 25 °C. Circular dichroism (CD) spectroscopy was carried out on a Jasco J-600 spectropolarimeter, with [E] = 13–38 μM in 0.2 M MES (pH 6.0) or CHES (pH 10) adjusted to the appropriate pH with KOH and to I_c = 0.2 with KCl. CD spectra were corrected for the differential absorbance of buffers.

Enzyme Concentrations. Holosubunit concentrations were estimated from the absorbance at 205 nm by using the method of Scopes (1982), and by the Bradford (1976) procedure. Both methods gave values to within ±3%; molar extinction coefficients were calculated by dividing milligrams per milliliter concentrations by 43 804, the molecular weight inferred from the nucleotide sequence (Kuramitsu et al., 1985) and the formula weight of enzyme-linked PLP. The extinction coefficients at 280 nm in 5 mM KH_2PO_4/50 mM K_2SO_4 buffer (pH 7.2) are 44 000 M^{-1} cm^{-1} for WT and 40 000 M^{-1} cm^{-1} for Y225F. The WT value is 16% higher than that reported by Yagi et al. (1979), possibly due to differences in the methods of molecular weight determination.

Spectrophotometric pK_a Determination. Solutions containing 11.5 μM aliquots of the PLP forms of Y225F or WT in 5 mM CHES–KOH buffer (pH 10.5) and 0.2 M KCl were titrated by successive additions of 1–10-μL aliquots of 100 mM potassium acetate buffer (pH 3.8)/190 mM KCl, I_c = 0.2 at 25 °C in a 3-mL quartz cuvette. Enzyme solutions titrated to pH ≤7 were passed after each addition through a 0.2-μm filter to eliminate light scattering due to a small amount of precipitation below this pH. The pH was measured in the stirred cuvette before and after each absorbance measurement, and corrections were made for dilution.

Determination of Kinetic Parameters for L-Aspartate and α-Ketoglutarate. The initial steady-state rates of the reaction shown in eq 1 with substrates L-Asp and αKG were monitored at 340 nm using an MDH-coupled assay in which oxalacetate and NADH are converted to malate and NAD^+ (Kiick & Cook, 1983). Each 1-mL reaction mixture contained 0.2 M buffer, brought to the appropriate pH with KOH. The contributions of the buffer and substrates to the ionic strength were calculated, and I_c was adjusted to 0.2 by addition of KCl. Buffers were HEPES, pH 7–7.9, TAPS, pH 8–9, and CHES, pH 9.1–10. Reaction mixtures also contained 0.2–1.0 μM Y225F or 1–2 nM WT, 0.8–3.2 units of MDH, 0.2 mM NADH, 0.05–50 mM L-Asp, and 0.005–10 mM αKG. The samples (minus enzyme) were allowed to equilibrate at 25 °C for 5 min before initiation of the reaction by the addition of no more than 5% volume of buffered enzyme solution stored at 4 °C. The temperatures in positioned reaction cells were monitored over the course of an experiment, and the pH of the solutions was checked frequently. The coupling reaction was shown to be independent of MDH concentration under these conditions. The enzyme is stable over the time period of a typical experiment at the pH extremes. Two protocols were pursued: either the concentration of one substrate was varied with the concentration of the other fixed at about 10 times K_m, or both substrate concentrations were varied over an approximate range of 0.5–5 times K_m.

Spectrophotometric Determinations of the Dissociation Constants of Maleate- and α-Methyl-DL-aspartate–Enzyme Complexes. The values of the dissociation constants of maleate and αMeAsp from the PLP forms of the enzymes at various pH values were determined in MES (pH 6), I_c = 0.2 (KCl), or the buffers used for the kinetic determinations. The concentration of maleate was varied from 0.5 to 200 mM, and that of αMeAsp was from 0.05 to 30 mM. In experiments with maleate on Y225F, I_c varied from 0.2 to 0.3, and in those on WT, I_c was from 0.2 to 0.75. The extent of dissociation was determined from the dilution-corrected changes in absorbance at 430 and 355 nm (WT) or at 435 and 388 nm (Y225F).

Inhibition by Maleate. The MDH–NADH-coupled assay was used to determine the value of K_I for maleate as an inhibitor of the reaction shown in eq 1. The reaction mixtures contained 0.2 M TAPS (pH 9), 40 mM KCl, 1 nM WT or 0.12 μM Y225F, 20 units/mL MDH, 20 μM PMP, 0.2 mM NADH, and 10 mM L-Asp (WT) or 2.0 mM L-Asp (Y225F). αKG concentrations were varied from 0.075 to 2.5 mM (WT) or from 7 to 50 μM (Y225F), and the maleate concentration was between 0.5 and 24 mM. Enzyme cocktails containing AATase, MDH, NADH, and PMP were kept at 4 °C, 0.4-mL aliquots of which were transferred to cuvettes and equilibrated to 25 °C for 4 min in the cell holder. Reactions were initiated by addition of 0.4 mL of a buffered substrate and inhibitor solution equilibrated to 25 °C. A contaminating transaminase activity in commercial MDH which is activated by PMP was assayed, and its contribution to steady-state reaction rates was subtracted.

Reduction and Hydrolysis of Enzyme. Enzyme solutions (1 mg/mL) were reduced by addition of 10–50 mM sodium borohydride or reductively aminated with cysteine sulfinate. The reduced enzyme was either precipitated with 8% TCA or exchanged into 10 mM ammonium bicarbonate by ul-

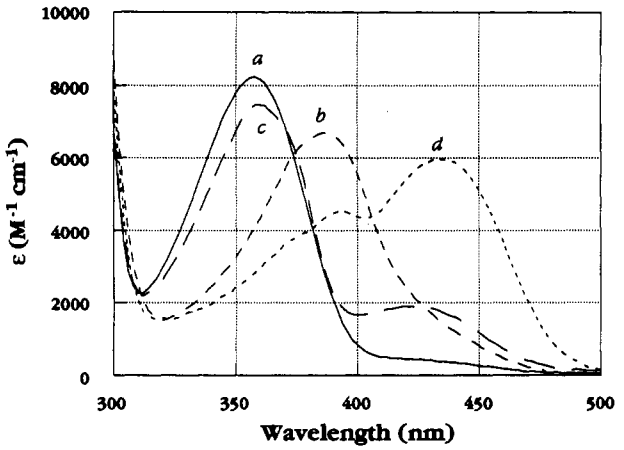


FIGURE 1: Spectra of the PLP forms of WT and Y225F aspartate aminotransferase, unliganded (a, WT; b, Y225F) and in the presence of 50 mM α-methyl-DL-aspartate (c, WT; d, Y225F). $\epsilon = A_\lambda/[E]^{A_{205}}$ where $[E]^{A_{205}}$ was determined by the method of Scopes (1982). Enzyme concentrations were 18–22 μM, buffered in 0.2 M CAPS (pH 10.4) (unliganded Y225F) or 0.2 M CHES (pH 9). The maximum absorbance wavelengths and extinction coefficients are listed in Table I.

trafiltration for subsequent acid hydrolysis. Samples of 0.25 mg were heated to 150 °C for 1 h under nitrogen in constant-boiling HCl, dried in vacuo, and resuspended in 200 μL of 100 mM sodium acetate/0.05% heptane sulfonate (pH 3.5). The hydrolysates were assayed for cofactor derivatives and tyrosine after elution from a Waters 3.9 × 300 mm C_{18} column on a Hewlett Packard 1090 liquid chromatograph. A 20-min 10–60% acetonitrile gradient in 100 mM sodium acetate/0.05% heptane sulfonate (pH 3.5) was employed with a flow rate of 1 mL/min at ambient temperatures, and eluents were monitored at 280, 290, and 335 nm, to detect tyrosine, pyridoxine, and εPLLys, respectively. In order to determine the amounts of εPLLys and tyrosine in the hydrolysates, chromatograms were compared to a standard solution containing 1 mM L-tyrosine and 45 μM synthetic εPLLys.

Synthesis of N^ϵ-Pyridoxyl-L-lysine. The synthesis of Dempsey and Christensen (1962) for the racemic compound was modified slightly in that the Schiff's base linkage between pyridoxal and N^α-acetyl-L-lysine was reduced by addition of a stoichiometric amount of sodium borohydride. The absorption spectrum of the product is identical with that published (Polyanovskii, 1963), and the concentration was determined at pH 6 by using $\epsilon_{335} = 9710\ M^{-1}\ cm^{-1}$ (Fischer et al., 1963). The product eluting with a retention time of 13 min was 90% pure as determined by HPLC analysis. An impurity with identical spectral properties eluted at 13.5 min.

Data Analysis. Unless otherwise noted, data were fitted to equations by the nonlinear regression program NLIN from the SAS Institute, Cary NC. Standard errors in k_{cat}/K_m were determined from the standard errors in k_{cat} and K_m given by NLIN and the covariances (Julin & Kirsch, 1989).

RESULTS

Visible and Near-UV Absorption Spectrophotometry. The spectra of the PLP forms of WT and Y225F in the absence and presence of αMeAsp are shown in Figure 1, and the absorbance maxima of the PLP, PMP, αMeAsp-saturated, and borohydride-reduced forms of the two enzymes are listed in Table I. Two large spectral differences between the two enzymes are manifest. First, there is a 28-nm red-shift in the absorbance maximum of the basic PLP form of Y225F relative to that of WT; second, there is a shift in the ratio of the peak heights for the two prototropic forms of the αMeAsp complexes. The more basic form predominates in the WT complex ($\epsilon_{358}/\epsilon_{422} = 3.6$), while the reverse is true for the mutant ($\epsilon_{386}/\epsilon_{434} = 0.86$).

Table I: Absorption Wavelength Maxima and Molar Extinction Coefficients (ε) for Wild-Type and Y225F Aspartate Aminotransferase[a]

		wild type		Y225F	
enzyme form	pH	absorbance maximum (nm)	ϵ^b (M^{-1} cm^{-1})	absorbance maximum (nm)	ϵ^b (M^{-1} cm^{-1})
PLP[c]	basic	358	8300	386	8500
PLP[c]	acidic	430	8200	435	8100
PMP[d]	10	335	6700	340	6700
PLP + $NaBH_4$[e]	10	332	5800	341	6500
PLP + αMeAsp[f]		358	7300	394[g]	4200
		422[g]	2000	434	5600

[a] Enzyme concentrations ranged from 10 to 22 μM. [b] Molar extinction coefficients are based on subunit concentrations determined as described under Experimental Procedures. [c] Absorbance values for determining extinction coefficients were obtained by using the A_1 from the best fit of spectrophotometric titration data (Figure 2) to eq 2. [d] PLP–enzyme in 0.2 M CHES (pH 10) was treated with 10 or 50 mM cysteine sulfinate. [e] PLP–enzyme in 0.2 M CHES (pH 10) was treated with 10 or 50 mM sodium borohydride. [f] The positions of the absorbance maxima and the extinction coefficients of the *E. coli* enzyme–α-methyl-L-aspartate complexes are independent of pH over the range 6–10.4 as has been previously observed for pig cytosolic AATase (Fasella et al., 1966). The extinction coefficients are the weighted averages of those obtained by dividing spectrophotometric determinations of A_∞ from eq 5 and 6 and enzyme absorbances in the presence of [αMeAsp] > 10 × K_D by [AATase]. [g] Shoulder.

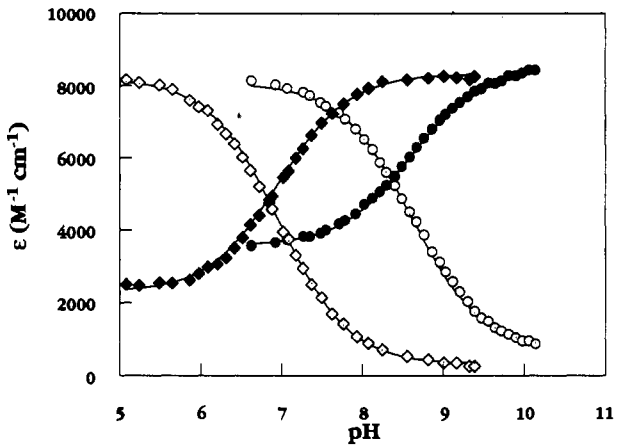


FIGURE 2: pH dependence of the extinction coefficients of WT and Y225F aspartate aminotransferase. [Enzyme] was 11.5 μM. The absorbance changes were monitored at 355 (♦) and 430 nm (◇) for WT and at 384 (●) and 430 nm (○) for Y225F. The pK_a values of WT and Y225F are shown in Table II, and the theoretical curves were calculated from eq 2 by employing the minimized parameters.

Spectrophotometric Determination of pK_a Values. The spectra of *E. coli* WT and Y225F AATase vary as a function of pH, in a manner similar to that described for the pig cytosolic isozyme (Scheme I) (Jenkins & Sizer, 1957; Jenkins et al., 1959). The absorbance values of WT and Y225F as functions of pH monitored at 355 and 430 nm (WT) or at 384 and 430 nm (Y225F) are shown in Figure 2. Data collected at 430 nm were fitted to eq 2a, and data collected at 355 or 384 nm were fitted to eq 2b, where A_1 and A_2 are the higher

$$A = \frac{A_1 - A_2}{1 + 10^{pH-pK_a}} + A_2 \quad (2a)$$

$$A = \frac{A_1 - A_2}{1 + 10^{pK_a-pH}} + A_2 \quad (2b)$$

Table II: Spectrophotometric and Kinetic Parameters of Wild-Type and Y225F Aspartate Aminotransferase

parameter	WT[a] (SE)	Y225F (SE)
Pyridoxal 5′-Phosphate Enzyme Forms		
$pK_{a,spec}$[b]	6.96 (0.02)	8.60 (0.02)
k_{cat} (s^{-1})	170	0.39[c] (0.01)
V/K_{Asp} (M^{-1} s^{-1})	1.0×10^5	5.0×10^3[d] (0.6×10^3)
pK_{a1}	7.1	8.4[d] (0.2)
pK_{a2}	9.7	9.8[d] (0.2)
Pyridoxamine 5′-Phosphate Enzyme Forms		
$V/K_{\alpha KG}$ (M^{-1} s^{-1})	2.9×10^5	5.3×10^4[d] (0.6×10^4)
pK_a	9.4	9.4[d] (0.3)

[a] WT values are from W. L. Finlayson and J. F. Kirsch (unpublished data) unless otherwise noted. [b] Weighted averages of spectrophotometrically determined pK_a values for Y225F and WT from data at two wavelengths (Figure 2) fitted to eq 2. [c] Averaged data from Figure 3B. [d] Limiting values from the data shown in Figure 3A fitted to eq 4.

and lower absorbance limits at a particular wavelength, respectively. The pK_a values of the *E. coli* enzymes are given in Table II. They are in close agreement with those reported by Cronin and Kirsch (1988), Inoue et al. (1990), and Morino et al. (1990).

pH Dependence of the Kinetic Parameters of Y225F. The steady-state values for K_m, k_{cat}, and k_{cat}/K_m for the Y225F-catalyzed reaction depicted in eq 1 were measured over a pH range of 7–10. Double-reciprocal plots of the data support a ping-pong mechanism (Velick & Vavra, 1962; Kiick & Cook, 1983). The data were fitted to eq 3 when both substrate

$$\frac{v}{[E]} = \frac{k_{cat}}{1 + K_{Asp}/[Asp] + K_{\alpha KG}/[\alpha KG]} \quad (3)$$

concentrations were varied, where v is the time-dependent change in product concentration, [E] is the concentration of Y225F, and k_{cat}, K_{Asp}, and $K_{\alpha KG}$ are the adjustable parameters. The value of k_{cat} is independent of pH in the range tested as noted earlier for pig cytosolic AATase (Velick & Vavra, 1962). The k_{cat}/K_m values were determined by fitting the data to the Michaelis–Menten equation using the program Hyper (Cleland, 1967) in experiments where one substrate concentration was varied in the presence of saturating amounts of the other.

Values of k_{cat}/K_m and k_{cat} are plotted against pH in Figure 3. The kinetic pK_a and limiting k_{cat}/K_m values were obtained from weighted nonlinear regression fitting of the data to eq 4a (k_{cat}/K_{Asp}) or 4b ($k_{cat}/K_{\alpha KG}$), where $(k_{cat}/K_{Asp})_{lim}$, $(k_{cat}/K_{\alpha KG})_{lim}$, pK_{a1}, pK_{a2}, and pK_a are the adjustable parameters. The results of the analyses are reported in Table II.

$$\frac{k_{cat}}{K_{Asp}} = \frac{(k_{cat}/K_{Asp})_{lim}}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} \quad (4a)$$

$$\frac{k_{cat}}{K_{\alpha KG}} = \frac{(k_{cat}/K_{\alpha KG})_{lim}}{1 + 10^{pH-pK_a}} \quad (4b)$$

The kinetic parameters of Y225F are markedly different from those of the WT enzyme (W. L. Finlayson and J. F. Kirsch, unpublished data), and of the pig cytosolic AATase (Velick & Vavra, 1962). k_{cat} for the reaction shown in eq 1 is reduced 450-fold in the mutant relative to the WT enzyme, and k_{cat}/K_{Asp} for Y225F is 5% that of WT. The limiting K_m values are 20 or 80 times *lower* in the mutant than they are in WT (Table III).

Association of Inhibitors with the PLP Forms of Wild Type and Y225F. Spectrophotometric titrations were carried out at several pH values in order to determine the pH dependence of the dissociation constants for the αMeAsp and maleate enzyme–PLP complexes. The association of αMeAsp or maleate with *E. coli* AATase is accompanied by spectral

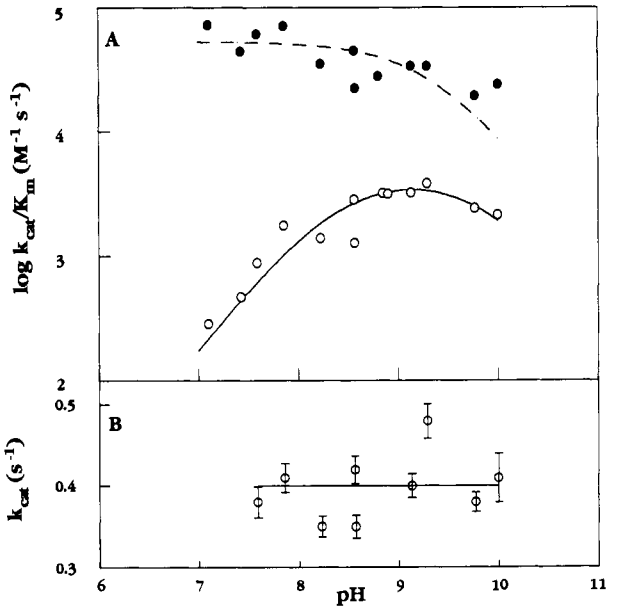


FIGURE 3: pH dependence of the kinetic parameters of Y225F aspartate aminotransferase in 0.2 M buffers adjusted with KOH and I_c = 0.2 (KCl): HEPES (pH 7–7.9); TAPS (pH 8.2–8.9); CHES (pH 9–10). (A) log (k_{cat}/K_{Asp}) (○) and log ($k_{cat}/K_{\alpha KG}$) (●). The k_{cat}/K_m values were obtained from a fit of steady-state data to eq 3 or to the Michaelis–Menten equation. The minimizing pK_a and $(k_{cat}/K_m)_{lim}$ values from the best fit of the data to eq 4 are reported in Table II. The lower point at pH 8.6 was not included in the analysis, and theoretical curves were calculated from eq 4 by using the fitted values. (B) The value of k_{cat} from eq 3. The weighted-average value represented by the line is reported in Table II.

changes from which the dissociation constants may be determined, as noted earlier for pig cytosolic AATase (Fasella et al., 1966). Absorbance data, A, from all titrations except those of Y225F at pH 8.9, were fitted to eq 5 where [L] is the maleate or αMeAsp concentration and A_0, A_∞, and K_D are the adjustable parameters.

$$A = A_0 - (A_0 - A_\infty)\frac{[L]}{K_D + [L]} \quad (5)$$

The value of the dissociation constant of the αMeAsp–Y225F complex is of the same order of magnitude as the enzyme concentration at pH 8.9, so the data were fitted to eq 6, where the parameters are as defined for eq 5 and [E] = $A = A_0 - (A_0 - A_\infty) \times$

$$\frac{(K_D + [L] + [E]) - \sqrt{(K_D + [L] + [E])^2 - 4[L][E]}}{2[E]} \quad (6)$$

[Y225F]. Weighted-average K_D values from eq 5 and 6 for data collected at two wavelengths are included in Table III. The limiting K_D values were estimated by fitting the pH-dependent K_D values to eq 7a (αMeAsp) or 7b (maleate) where the values of pK_{a1}, pK_{a2}, and pK_a are those describing the pH dependence of k_{cat}/K_{Asp}, or $k_{cat}/K_{\alpha KG}$ for Y225F and WT (Table II).

$$K_{D,lim} = \frac{K_D}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} \quad (7a)$$

$$K_{D,lim} = \frac{K_D}{1 + 10^{pH-pK_a}} \quad (7b)$$

The application of eq 7 is predicated on the assumption that the pK_a values affecting inhibitor binding are identical with those determining k_{cat}/K_{Asp}, as is the case for the pig cytosolic isozyme (Kiick & Cook, 1983). The data in Table III show that maleate has nearly equal affinity for the protonated PLP

Table III: Michaelis (K_m), Acid (K_H), Dissociation (K_D), and Inhibition (K_I) Constants for Wild-Type and Y225F Aspartate Aminotransferase

parameter	pH	WT (mM) (SE)	Y225F (mM) (SE)	WT/Y225F (SE)	$\Delta\Delta G_{app}$ (kcal mol^{-1})[f] (SE)
		Pyridoxal 5′-Phosphate Enzyme Forms			
K_H[a]		1.10×10^{-4} (0.03×10^{-4})	2.5×10^{-6} (0.1×10^{-6})	44 (2)	−2.2 (0.1)
$(K_{Asp})_{lim}$[b]		1.7	0.08 (0.01)	20	−1.8
$K_{D,Mal\text{-}PLP}$[c]	7.0	3.1 (0.1)			
	8.2		2.0 (0.8)		
	8.4	38 (3)			
	8.9	110 (10)	2.7 (0.1)		
	9.7		11 (1)		
$(K_{D,Mal\text{-}PLP})_{lim}$[d]		1.4	0.9	1.6	−0.3
$K_{D,\alpha Me\text{-}L\text{-}Asp}$[c]	6.0	8.5 (0.07)	19 (1)		
	7.0	3.6 (0.2)	1.3 (0.3)		
	8.9	3.07 (0.01)	0.12 (0.02)		
	10.0	3.8 (0.8)			
$(K_{D,\alpha Me\text{-}L\text{-}Asp})_{lim}$[d]		2.3	0.06	37	−2.1
		Pyridoxamine 5′-Phosphate Enzyme Forms			
$(K_{\alpha KG})_{lim}$[b]		0.58	0.007 (0.001)	80	[g]
$K_{I,Mal\text{-}PMP}$[e]	9.0	5.7 (0.3)	3.1 (0.4)	1.8 (0.2)	−0.3 (0.1)

[a] The spectrophotometrically determined proton dissociation constant (pK_a values are reported in Table II). [b] Values are calculated from $(V/K)_{lim}$ and k_{cat} (Table II). [c] The spectrophotometrically determined ligand dissociation constants in the buffers described under Experimental Procedures for the determination of dissociation constants. $K_{D,\alpha Me\text{-}L\text{-}Asp}$ was determined by dividing the value for the racemic compound by 2, assuming that the D-isomer does not interact with the enzyme. [d] The limiting values were estimated from eq 7a (α-methyl-L-aspartate) or eq 7b (maleate). [e] Competitive inhibition constants against the substrate α-ketoglutarate in 0.2 M TAPS, I_c = 0.2–0.3, with data fitted to eq 8. [f] $\Delta\Delta G_{app} = -RT \ln (WT/Y225F)$. [g] $\Delta\Delta G_{app}$ for $(K_{\alpha KG})_{lim}$ was not included since the value of the kinetic constant may not represent $K_{D,\alpha KG}$.

forms of WT and Y225F, while αMeAsp binds 37-fold more tightly to the mutant than to WT. The decrease in the K_D value of the αMeAsp complex parallels the 20-fold reduction in the K_m value for L-Asp (Table III).

Inhibition by Maleate. The K_I value ($K_{I,Mal\text{-}PMP}$) of maleate against the reaction depicted in eq 1 was determined by using the coupled assay at pH 9 with varying [αKG] and [L-Asp] held at $>5 \times K_m$. The data were fitted to eq 8 (Kiick & Cook, 1983), where K_{I2} is $K_{I,Mal\text{-}PMP}$, the adjustable kinetic inhibition constant; K_{Asp} and $K_{\alpha KG}$ are obtained from eq 4 and 3, and K_{I1} is $K_{I,Mal\text{-}PLP}$, the K_D value from eq 7a. It is notable that the values of $K_{I,Mal\text{-}PMP}$ for Y225F and WT are similar (Table III), while $K_{\alpha KG}$ is reduced 80-fold (see below).

$$\nu/[E] = k_{cat}[Asp][\alpha KG]/\{K_{Asp}[\alpha KG](1 + [Mal]/K_{I1}) + K_{\alpha KG}[Asp](1 + [Mal]/K_{I2}) + [Asp][\alpha KG]\} \quad (8)$$

Nature of the PLP–Enzyme Interaction in Y225F. The similarity of the absorption maxima of the unprotonated PLP form of Y225F (386 nm) (Figure 1) to free PLP (388 nm) (Harris et al., 1976) suggests that the cofactor is present as the free aldehyde rather than the imine in the active site of the mutant at pH values above the pK_a. If so, borohydride reduction of Y225F should yield pyridoxine 5′-phosphate instead of εPLLys258 5′-phosphate, which is the expected product from the Schiff's base. Sodium borohydride reduction was carried out at pH 10, and the 8% TCA supernatants of 22 μM solutions of reduced Y225F–PLP contained <5% more pyridoxine 5′-phosphate than did those from similarly treated WT as determined by using ϵ_{290} = 8400 M^{-1} cm^{-1} (Sober, 1968). The PMP form of WT was precipitated with 8% TCA in a control reaction to determine the extent of recovery of noncovalently bound cofactor. The supernatants yielded 100 ± 5% of the cofactor initially present, as determined from ϵ_{290} = 8500 M^{-1} cm^{-1} (Sober, 1968).

The quantity of εPLLys in borohydride-reduced Y225F was determined by hydrolysis of the reduced enzyme in HCl, followed by HPLC analysis. A solution containing 1 mM Tyr and 45 μM synthetic εPLLys was analyzed before and after exposure to the hydrolysis conditions in order to monitor the stability of εPLLys and to construct a standard curve. εPLLys eluted with a retention time (RT) of 13 min, but after exposure to the hydrolysis conditions, portions of this derivative were detected in two additional peaks at RT 13.5 and 16 min, the materials in which were spectroscopically indistinguishable from the parent compound. These materials were spectrally distinct from pyridoxine (RT 15.6 min) and pyridoxamine (RT 14.8 min). Although a chemical rearrangement of εPLLys may be responsible for the shifts in RT, the total derivative: Tyr ratios in four hydrolyzed vs four unhydrolyzed samples were similar (8 ± 2 and 7 ± 4, respectively). Thus, the total derivative: Tyr ratio was used to quantify the amounts of lysine-linked cofactor in protein hydrolysates.

Hydrolysis of borohydride-reduced PLP–Y225F, PLP–WT, and PMP–WT resulted in 90, 80, and 60% recoveries of hydrolyzed protein, respectively, as calculated from the tyrosine yields. In the reduced PLP forms of Y225F and WT, εPLLys yields were within ±25% of 1/1 stoichiometry with enzyme subunits, while pyridoxine was not detected, indicating that <5% of the cofactor present in the enzyme existed as the aldehyde. The PMP form of WT was hydrolyzed in a control reaction designed to detect non-active-site lysyl-Schiff's base, as well as to assess the extent of recovery of noncovalently bound cofactor. εPLLys was not found in these reactions while the ratio of pyridoxamine to protein subunits was 1:1 ± 25%. Thus, borohydride reduction traps at least 75% of the cofactor in Y225F as a Schiff's base with Lys258, in conflict with the inference from the position of the absorbance maximum.

Discussion

Spectroscopic Differences between Wild Type and Y225F. The absorbance maximum of the unprotonated PLP form of Y225F is red-shifted by 28 nm with respect to WT (358–386 nm) (Figure 1). The new maximum resembles that of free PLP at 388 nm (Harris et al., 1976) rather than that of the unprotonated Schiff's base in native AATase which absorbs maximally at 358–360 nm (Figure 1) (Jenkins & Sizer, 1957). Is this shift a consequence of internal aldimine hydrolysis above the pK_a, or of environmental factors? Borohydride reduction of the high-pH form of PLP–Y225F yielded at least 75% εPLLys after protein hydrolysis, supporting the assignment of the 386-nm species to the internal imine rather than enzyme-bound PLP aldehyde. It is possible that the equilibrium between the free aldehyde and internal aldimine is shifted toward the latter by preferential kinetic trapping of the Schiff's

base with borohydride; however, the absorbance maxima of the PMP and reduced forms of Y225F are slightly red-shifted (Table I), implicating environmental factors as responsible for the shift in the absorbance maximum.

The circular dichroism (CD) spectrum of protonated pig heart cytosolic PLP–AATase displays a positive peak at 420 nm with a broad shoulder centered at 330 nm, and a trough at 295–300 nm (Ivanov & Karpeisky, 1969). These authors suggested that the last feature is due to the presence of an active-site tyrosyl anion. The general features in the CD spectra of WT and Y225F AATase (data not shown) are similar to those described above, except that the minima at 290 nm are less pronounced in both *E. coli* enzymes than in the eukaryotic isozyme. If Tyr225 is responsible for the differential absorbance of circularly polarized light at 290 nm, then the trough at this wavelength should disappear in the mutant. The CD spectra of WT and Y225F are nearly superimposable as indicated by the ratios of the amplitudes of the deviations from the base line at 420 and 290 nm which are 7.4 (WT) and 7.7 (Y225F). Thus, the negative amplitude at 290 nm is not due to Tyr225.

Changes in the Spectrophotometrically Measured pK_a. The pK_a for the equilibrium depicted in Scheme I is increased by 1.7 units from 6.9 in WT to 8.6 in the mutant (Figure 2; Table II). Morino et al. (1990) report a similar shift for Y225F. This increase in pK_a may be ascribed to the loss of the stabilization of the 3′-O^- provided by the hydrogen bond between Tyr225 and PLP in WT.

Effects of the Mutation on the Kinetic Parameters. k_{cat} is reduced 450-fold relative to WT to 0.39 s^{-1} in Y225F (Figure 3, Table II). This large decrease in the value of k_{cat} indicates that Tyr225 is required for *efficient* catalysis of the overall transamination reaction. k_{cat} is independent of pH over the range pH 7.0–10 in common with *E. coli* WT and pig cytosolic AATase (W. L. Finlayson and J. F. Kirsch, unpublished data; Velick & Vavra, 1962). Thus, while the mutation affects the pK_a of the internal aldimine, the pK_a values of the reactant–enzyme complexes remain outside the measured range. An explanation for the decrease in k_{cat} will be suggested below. Contrasting the lowering of k_{cat} caused by the mutation Y225F are the large *decreases* in the K_m values of L-Asp and αKG (Table III), consistent with increased substrate affinity. The pK_a in the ascending limb of the pH profile for k_{cat}/K_{Asp} is increased by 1.3 units in Y225F (Figure 3) relative to WT (Table II), and the similar change in the kinetic and spectrophotometric pK_a values reinforces the interpretation that the active protonic form of the coenzyme is B in Scheme I (Jenkins et al., 1959).

*Reactions with α-Methyl-*DL*-aspartate and Maleate.* K_m values reflect a combination of microscopic rate constants and equilibria which can be partially resolved through the investigation of competitive inhibitors. αMeAsp forms a Schiff's base with the PLP form of AATase (Braunstein, 1964), but the next step in the transamination reaction, i.e., C_α proton abstraction, is not possible. αMeAsp binds to Y225F 37 times more tightly than it does to WT (Table III). The limiting values of $K_{D,\alpha MeAsp}$ and K_{Asp} are similar in both WT and Y225F, giving evidence that K_{Asp} is a measure of the dissociation constant for this substrate, i.e., that L-Asp is not a sticky substrate for *E. coli* AATase, in common with similar findings for the pig cytosolic isozyme (Kiick & Cook, 1983; Julin & Kirsch, 1989).

It is interesting to note that in the αMeAsp–enzyme complexes of WT and Y225F, the ratio of the amplitude of the absorption band occurring at 358 or 386 nm to that found at about 430 nm varies from 3.6:1.0 in WT to 0.9:1.0 in Y225F (Figure 1), while this ratio if 1:1 for pig cytosolic AATase (Fasella et al., 1966). Thus, the protonated external aldimine (430 nm) is more favored in Y225F than in WT. Fasella et al. (1966) proposed that the simultaneous existence of both forms might be due to an equilibrium proton distribution between the ε-amino group of Lys258 and the Schiff's base nitrogen, or an equilibrium between the Michaelis complex and the external aldimine. Tyr225 may influence the spectrum of the αMeAsp–enzyme complex by decreasing the basicity of the Schiff's base, thus favoring the 358-nm-absorbing species in WT.

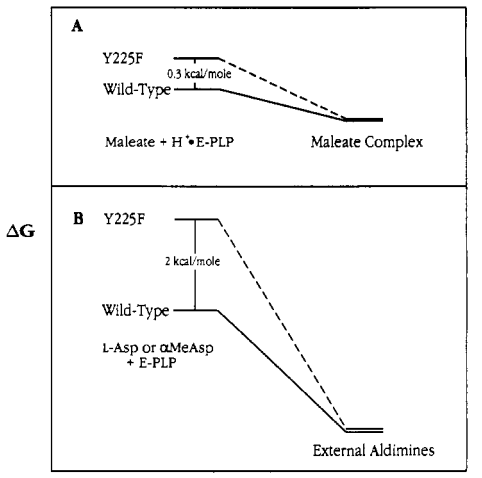
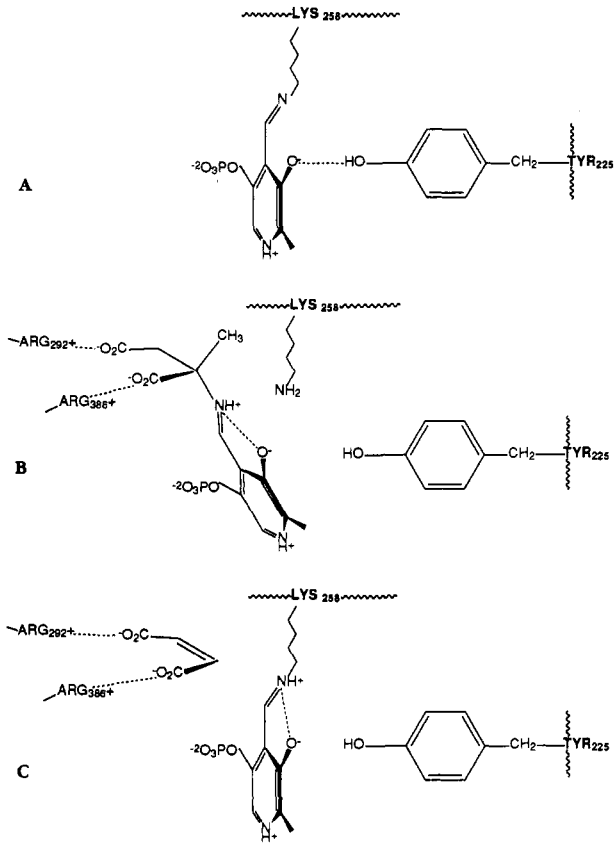


FIGURE 4: Reaction coordinate vs free energy representation of the association of maleate, αMeAsp, and L-Asp with WT and Y225F aspartate aminotransferase. The free energies of the complexed forms are arbitrarily set to equal values. The WT and mutant maleate–enzyme complexes are close to each other in relative stability (A), as is evidenced by the similarity in the K_I and limiting K_D values in Table III, indicating that the disposition of residues involved in binding this inhibitor is the same in both structures. In contrast, the free energy of formation of the external aldimine is 2 kcal/mol less in WT than in Y225F (B) (cf. the limiting values of K_{Asp} and $K_{D,\alpha MeAsp}$ in Table III), because the total free energy of complex formation in WT is reduced by that required for disruption of the cofactor–tyrosine hydrogen bond.

Maleate, a competitive inhibitor (Velick & Vavra, 1962), makes counterion interactions with the two arginines responsible for the dicarboxylic amino acid specificity (Kirsch et al., 1984). The limiting values of K_D for the maleate–PLP–enzyme complex, and the K_I for the maleate–PMP–enzyme complex at pH 9.0, are similar for WT and Y225F (Table III), indicating that the mutation does not distort the active site or alter the affinity of its protonated form for counterions. The similarity in maleate affinity for WT and mutant contrasts with the difference in αMeAsp affinity; therefore, the decrease in $K_{D,\alpha MeAsp}$ for Y225F may be cleanly assigned to the covalent transaldimination.

*Decreases in Substrate K_m and α-Methyl-*L*-aspartate K_D Values.* The above observations, vis à vis K_m values and inhibitor binding, may be explained in terms of a change in the position of the cofactor relative to Tyr225 upon transaldimination. X-ray crystallographic analyses (Kirsch et al., 1984; Arnone et al., 1985; Jansonius et al., 1985; Jansonius & Vincent, 1987) suggest that there is a strong hydrogen bond between Tyr225 and cofactor O-3′ in unliganded enzyme. The interatomic distances observed in partially refined structures (Kirsch et al., 1984) between the Tyr hydroxyl group and the cofactor O-3′ moiety in the PLP and PMP forms are 2.3 and 2.8 Å, respectively. In the complex of enzyme with *N*-(5′-phosphopyridoxyl)-L-aspartate, a stable covalent analogue of

Scheme II: Schematic Representation of (A) Selected Active-Site Residues of WT AATase and Structures of Enzyme Complexes with (B) α-Methyl-L-aspartate and (C) Maleate[a]



[a] There is a large change in cofactor tilt upon binding α-methyl-L-aspartate but not maleate (Arnone et al., 1985; Jansonius et al., 1985; Jansonius & Vincent, 1987). The absence of the Tyr-O-3′ hydrogen bond in Y225F means that less free energy is required to rotate the cofactor to the position shown in (B), accounting for the lower values of K_{Asp} and $K_{D,\alpha MeAsp}$ (Table III, Figure 4). Maleate binds to the protonated form of the internal aldimine (C) in which no or perhaps a weak hydrogen bond is formed with Tyr225; therefore, the limiting (low pH) K_D values for the maleate–PLP–enzyme complexes are similar for WT and Y225F.

the external aldimine and ketimine intermediates, a change in the cofactor tilt with respect to the protein matrix correlates with an increase of the distance between Tyr-OH and cofactor O-3′ to 3.8 Å. Thus, the crystallographic data suggest that the hydrogen bond between O-3′ and Tyr225 is broken or weakened when the coenzyme forms an external Schiff's base (Scheme II).

Removal of the tyrosyl–cofactor hydrogen bond selectively destabilizes unliganded forms of the enzyme relative to covalent enzyme complexes, because the bond is stronger in the free enzyme. The equilibrium between free enzyme and ligand vs the enzyme–ligand complex may be compared to a game of tug-of-war, where Tyr225 competes with the NH_2 moiety of αMeAsp or amino acid substrates, or the carbonyl function of α-ketoacids for binding of the cofactor. Thus, in Y225F, the absence of the Tyr-OH group allows the cofactor to adapt a position favorable for substrate binding at a free energy cost of ~2 kcal/mol less than that required for a similar reorientation in WT (Figure 4).

Reduction in k_{cat}. The k_{cat} value for WT is 450-fold greater than that for the mutant. C_α–H abstraction from L-Asp has been shown by kinetic isotope effects to be partially rate-determining for the WT *E. coli* enzyme (Kirsch et al., 1990) in common with similar findings obtained for pig cytosolic and mitochondrial isozymes (Julin & Kirsch, 1989). There is no appreciable discrimination against deuterium at the C_α position in Y225F-catalyzed transamination, showing that in this case, C_α–H abstraction is not rate-determining (Kirsch et al., 1990). This observation and the increase in affinity of Y225F for substrates suggest that ketimine hydrolysis is fully or partially rate-determining in the mutant-catalyzed reaction.

Conclusions

Tyr225 fulfills two major roles in the transamination reaction. First, it stabilizes the reactive unprotonated form of the internal aldimine at physiological pH values. Second, it increases V/K and k_{cat} for the reaction by *destabilizing* the enzyme–substrate complexes at physiological substrate concentrations. The mutation reveals that the *intrinsic* free energy of association of AATase with substrates is greater than that measured for WT enzyme, in which this excess free energy is utilized to increase the rate constants for subsequent reaction steps. Thus, these findings provide a clear example of the Circe effect (Jencks, 1975).

Acknowledgments

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Registry No. AATase, 9000-97-9; αKg, 328-50-7; PLP, 54-47-7; L-Asp, 56-84-8; L-Tyr, 60-18-4; maleic acid, 110-16-7.

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CORRECTIONS

Absorption and Fluorescence Spectroscopic Studies of the Ca^{2+}-Dependent Lipid Binding Protein p36: The Annexin Repeat as the Ca^{2+} Binding Site, by Gerard Marriott,* William R. Kirk, Nils Johnsson, and Klaus Weber, Volume 29, Number 30, July 31, 1990, pages 7004–7011.

Page 7005. In column 2, line 16 under Results, ^1L_a should read ^1L_b.

Page 7006. In column 1, line 6, and column 2, line 4, ^1L_a should read ^1L_b.

Page 7008. In the caption to Figure 7, lines 1 and 2, spectra of human and chicken p36 should read spectra of human p36.

Page 7009. In column 1, lines 26 and 30, ^1L_a should read ^1L_b.

Analysis of the Backbone Dynamics of Interleukin-1β Using Two-Dimensional Inverse Detected Heteronuclear ^{15}N–^1H NMR Spectroscopy, by G. Marius Clore,* Paul C. Driscoll, Paul T. Wingfield, and Angela M. Gronenborn*, Volume 29, Number 32, August 14, 1990, pages 7387–7401.

Page 7394. Equations 13 and 14 should read as follows:

$$J(\omega_i) = S_f^2 S_s^2 \tau_R/(1 + \omega_i^2 \tau_R^2) + (1 - S_f^2)\tau_f'/(1 + \omega_i^2 \tau_f'^2) + S_f^2(1 - S_s^2)\tau_s'/(1 + \omega_i^2 \tau_s'^2) \quad (13)$$

$$J(\omega_i) = S_f^2 S_s^2 \tau_R/(1 + \omega_i^2 \tau_R^2) + S_f^2(1 - S_s^2)\tau_s'/(1 + \omega_i^2 \tau_s'^2) \quad (14)$$

All calculations presented in the paper were carried out with the correct versions of eqs 13 and 14.