The K258R Mutant of Aspartate Aminotransferase Stabilizes the Quinonoid Intermediate*

Michael D. Toney and Jack F. Kirsch
From the Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720

Lys-258 of aspartate aminotransferase forms a Schiff base with pyridoxal phosphate and is responsible for catalysis of the 1,3-prototropic shift central to the transamination reaction sequence. Substitution of arginine for Lys-258 stabilizes the otherwise elusive quinonoid intermediate, as assessed by the long wavelength absorption bands observed in the reactions of this mutant with several amino acid substrates. The external aldimine intermediate is not detectable during reactions of this mutant with amino acids, although the inhibitor α-methylaspartate does slowly and stably form this species. These results suggest that external aldimine formation is one of the rate-determining steps of the reaction. The pyridoxamine-5'-phosphate-like enzyme form (330-nm absorption maximum) is unreactive toward keto acid substrates, and the coenzyme bound to this species is not dissociable from the protein.

Aspartate aminotransferase is a PLP-dependent enzyme which preferentially catalyzes the reversible interconversion of aspartate and α-ketoglutarate to oxalacetate and glutamate. The ready availability and ease of studying this enzyme have instigated a large body of research, making it the best understood PLP-dependent enzyme involved in amino acid metabolism (Christen and Metzler, 1985; Jansonius and Vincent, 1987).

The central intermediate proposed for all PLP-catalyzed reactions (Braunstein and Shemyakin, 1953; Metzler et al., 1954) is the resonance stabilized carbanion formed on cleavage of a bond to Cα of the external aldimine intermediate (Scheme 1). This delocalized carbanion is called the "quinonoid" intermediate because of its resemblance to para-quinone. The kinetic competence of this species in enzymatic reactions has most often been demonstrated in the reactions of noncognate substrates or coenzymes where the quinonoid is either a dead-end product (Ulevitch and Kallen, 1977), or in part by the payment of reaction energetics have been greatly perturbed (Chen et al., 1987). For example, the reaction of erythro-β-hydroxynorvaline with aspartate aminotransferase produces a strong, long-lived quinonoid absorption band (Jenkins, 1964; Toney and Kirsch, 1991). Aspartate aminotransferase does not display a significant build up of the quinonoid intermediate in reactions with aspartate or glutamate (Farrell and Hammes, 1967; Gehring, 1986; Gehring et al., 1987) but does with the reactive substrate cysteine sulfinate. It is possible for the central 1,3-prototropic shift to proceed without the formation of a quinonoid intermediate. Double kinetic isotope effect studies suggest that this occurs in the reaction of cytosolic aspartate aminotransferase with aspartate (Julin and Kirsch, 1989). The suggested mechanism is that Lys-258 donates a proton to C4' simultaneously with Cα proton abstraction.

Previous site-directed mutagenesis studies have conclusively demonstrated that the Schiff base-forming Lys-258 provides general base catalysis of the 1,3-prototropic shift central to the transamination reaction (Toney and Kirsch, 1989; Ziuk et al., 1990; Toney and Kirsch, 1992). The substitution of arginine for Lys-258 produces a catalytically inactive enzyme (Morino et al., 1990) which, as demonstrated herein, undergoes half-reactions with amino acids in which the quinonoid intermediate is readily observed.

EXPERIMENTAL PROCEDURES

General—K258R was constructed as described previously for K258M; Toney and Kirsch, 1992. The protein was purified according to Cronin and Kirsch (1988). L-Cysteine sulfinic acid was purchased from Aldrich. All other chemicals were from Sigma.

Absorbance spectra and kinetics were measured with a Unikov 860 spectrophotometer (Kontron Instruments) interfaced to an IBM-compatible personal computer. Curve fitting was performed with the nonlinear regression program ENZFITTER (R. J. Leatherbarrow, Biosoft Publishing Co.). All experiments were conducted at 25 °C in 0.1 M HEPES-KOH, pH 7.5, except as noted.

Kinetic Analysis of K258R Reactions—The spectral changes in the coenzyme were monitored in single turnover transamination half-reactions under pseudo-first order conditions with ~10 μM K258R subunits and amino acid concentrations in large excess. Ionic strength varied from 0.05 to 0.07 M.

The reactions of keto acid substrates were monitored by periodic spectrophotometric scans of the coenzyme region over the course of the experiments, which lasted for 2 days (oxalacetate) to 2.5 months (α-ketoglutarate).

α-Methylaspartate Aldimine Formation—The spectrum of the K258R-α-methylaspartate aldime was obtained after 10 min of reaction with 0.1 M α-methyl-DL-aspartate. The kinetics of aldime formation with 50 μM α-methyl-DL-aspartate were monitored at 460 nm.

Resolution of K258R—Dissociation of 330 nm absorbing coenzyme from K258R was attempted by three methods: 1) precipitation with ammonium sulfate, pH 2, and allowing the precipitate to stand for 20 h, as described for K258A (Toney and Kirsch, 1992), 2) partial unfolding in 1 or 2 M guanidine hydrochloride (Herold and Kirschner, 1967). 3) Dissociation of 330 nm absorbing coenzyme from K258R was attempted by three methods: 1) precipitation with ammonium sulfate, pH 2, and allowing the precipitate to stand for 20 h, as described for K258A (Toney and Kirsch, 1992), 2) partial unfolding in 1 or 2 M guanidine hydrochloride (Herold and Kirschner, 1967).
The only absorption bands observed during the course of the reaction are the same as those found in wild type reactions (Jenkins, 1990). After 2 h of reaction, the protein was dialyzed against 6 liters of 2 mM HEPES-KOH, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA, 50 μM PLP, and 0.5 mg/ml K258R in a total volume of 10 ml. After 2 h of reaction, the protein was dialyzed against 6 liters of 2 mM HEPES-KOH, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA at 4°C to refold the protein, and 3) dialysis (2 ml of 1 mg/ml K258R) against 2 liters of 0.1 M sodium hydroxide at 4°C for 15 h.

RESULTS

K258R Reactions with Amino Acids Exhibit a Quinonoid Absorption Band—Fig. 1 shows the coenzyme absorption spectra of K258R before and approximately 30 s after cysteine sulfinate was added to a final concentration of 5 mM. The addition of the amino acid yields a transient increase in absorbance centered at 525 nm, consistent with a quinonoid intermediate (Kallen et al., 1988; Metzler et al., 1988), which decays to give a 330-nm band as the final product of the reaction. The K258R spectrum in the absence of substrate agrees with that previously reported (Kuramitsu et al., 1990) with excess PLP (the unfolding buffer consisted of 0.1 M guanidine hydrochloride, 1 mM dichothreitol, 0.5 mM EDTA, 50 μM PLP, and 0.5 mg/ml K258R in a total volume of 10 ml. After 2 h of reaction, the protein was dialyzed against 6 liters of 2 mM HEPES-KOH, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA at 4°C to refold the protein, and 3) dialysis (2 ml of 1 mg/ml K258R) against 2 liters of 0.1 M sodium hydroxide at 4°C for 15 h.

Kinetics of the Reaction of K258R with Cysteine Sulfinate—The only absorption bands observed during the course of the reaction are at 525, 405, and 330 nm. The time courses for these three wavelengths, at 5 mM cysteine sulfinate, are shown in Fig. 2. The decrease in absorbance at 405 nm follows a single exponential decay (Equation 1).

\[ A_{405} = (A_0 - A_s)e^{-kt} + A_s \]  

The curve shown fitted to the 525-nm absorbance data is characteristic of that of an intermediate, B, in a three component, serial mechanism (Moore and Pearson, 1981).

\[ A_{525} = \frac{k_1}{k_2 - k_1} (e^{-kt} - e^{-k'_t}) + A_{525}^{\infty} \]  

The time course for the appearance of the final product (i.e. C in Equation 2), which absorbs maximally at 330 nm, is described by Equation 4.

\[ A_{330} = (A_{330}^{\infty} - A_{330}^{\infty}) \frac{k_1}{k_1 - k_2} (e^{-kt} - e^{-k'_t}) + A_{330}^{\infty} \]  

The adjustable parameters in each of the above regression analyses were the absorbance \(A_{330}, A_{525}, A_{405}\), and \(A_{330}^{\infty}\), as appropriate) and rate constant values. The values of the rate constants obtained from the curve fitting are: \(k_1 = (1.61 \pm 0.02) \times 10^{-2} \text{ s}^{-1} \) (405 nm), \(k_2 = (2.65 \pm 0.05) \times 10^{-2} \text{ s}^{-1}\), and \(k_3 = (1.67 \pm 0.04) \times 10^{-2} \text{ s}^{-1}\) (525 nm), and \(k_4 = (1.00 \pm 0.04) \times 10^{-2} \text{ s}^{-1}\) and \(k_5 = (1.38 \pm 0.02) \times 10^{-2} \text{ s}^{-1}\) (330 nm). The three kinetic traces were obtained from separate reactions, thus the variation in the parameters. Conditions: 10 μM K258R, 0.1 mM HEPES-KOH, pH 7.5, 25°C.

FIG. 2. Kinetic traces for the reaction of K258R with 5 mM cysteine sulfinate. The wavelengths at which the data were collected are indicated. The curves drawn through the 405-, 525-, and 330-nm data represent the best fits to Equations 1, 3, and 4, respectively, which describe the kinetics of the three component, serial mechanism given in Equation 2. The values of the rate constants obtained from the curve fitting are: \(k_1 = (1.61 \pm 0.02) \times 10^{-2} \text{ s}^{-1}\) (405 nm), \(k_2 = (2.65 \pm 0.05) \times 10^{-2} \text{ s}^{-1}\), and \(k_3 = (1.67 \pm 0.04) \times 10^{-2} \text{ s}^{-1}\) (525 nm), and \(k_4 = (1.00 \pm 0.04) \times 10^{-2} \text{ s}^{-1}\) and \(k_5 = (1.38 \pm 0.02) \times 10^{-2} \text{ s}^{-1}\) (330 nm). The three kinetic traces were obtained from separate reactions, thus the variation in the parameters. Conditions: 10 μM K258R, 0.1 mM HEPES-KOH, pH 7.5, 25°C.

The adjustable parameters in each of the above regression analyses were the absorbance \(A_{330}, A_{525}, A_{405}\), and \(A_{330}^{\infty}\), as appropriate) and rate constant values. The values of the rate constants obtained from the curve fitting are given in Fig. 2.
reaction with 100 mM α-methyl-DL-aspartate. Reaction with this inhibitor converts the initial 405-nm absorbance band to one of 430 nm, characteristic of an external aldimine. The observed pseudo-first order rate constant for formation of this external aldimine is (1.8 ± 0.4) × 10^{-1} s^{-1}. This is 1100-fold less than the value of 2.3 ± 0.15 s^{-1} observed with K258A and α-methyl-DL-aspartate under the same conditions (Toney and Kirsch, 1992).

**K258R Does Not React with α-Keto Acids**—No changes in the coenzyme absorption spectrum of K258R-PMP were observed with 100 mM oxalacetate or α-ketoglutarate and incubation for 2 days or 2.5 months, respectively.

The Coenzyme Form Absorbing at 330 nm Cannot Be Dissociated from the Enzyme—Three methods previously proven successful in removing coenzyme from wild type and other aspartate aminotransferase mutants

The kinetic traces shown in Fig. 2 demonstrate that the quinonoid species formed from K258R and cysteine sulfinate is kinetically competent (i.e., it is formed and decays with rates that are no slower than the overall rate of conversion of aldimine to ketimine at any time point). This suggests, but does not prove, that the quinonoid is directly on the reaction pathway. An alternative kinetic mechanism is one where the quinonoid is formed off the reaction pathway in equilibrium with an intermediate on the pathway (Julin and Kirsch, 1989). The on-the-pathway intermediate in equilibrium with the quinonoid would most likely be the external aldimine since the ketimine absorbs near 330 nm (Toney and Kirsch, 1992) where a lag phase is observed. The equilibrium constant would, in this case, greatly favor the quinonoid over the external aldimine since no absorption for the latter (430 nm) is observed during the reactions.

**The Reaction of K258R with α-Methyl-DL-aspartic Acid**—The reactions of K258R with amino acid substrates do not generate detectable transient absorbance at 430 nm characteristic of external aldimines. Rather, the 405-nm absorbance of K258R-PLP converts directly to the quinonoid (525 nm) absorption band, although the chemical mechanism dictates an external aldimine precursor. The spectra of Figs. 1 and 3 demonstrate that the inhibitor α-methyl-DL-aspartic acid does form a stable external aldimine with the characteristic 430-nm absorption band (Kallen et al., 1985). The observed rate constant for this reaction is 1100-fold smaller than that measured for K258A, which is also unable to form an internal Schiff base linkage (“internal aldimine”) with PLP, and is thus likewise incapable of employing the facile transamination mechanism utilized by wild type enzyme. The x-ray structure of K258R (Kamitori et al., 1990) suggests that this is due to a mispositioning of the PLP aldehyde function due to steric interactions between PLP and the arginine side chain. Thus the rate of decrease of A_{430} probably reflects rate-determining external aldimine formation and not Ca proton abstraction.

Two mechanisms for quinonoid formation need consideration. The close proximity of the positively charged guanidino group of Arg-258 to Ca of the external aldimine intermediate might facilitate proton abstraction by solvent (which must occur in the reactions of K258A and K258M with amino acids in the absence of exogenous amines (Toney and Kirsch, 1989, 1992). Alternatively, the free base form of Arg-258 might directly abstract the Ca proton. The independence of the observed pseudo-first order rate constant for wild type on pH between 5 and 10 indicates that the pK_a of the Arg-258 ε-amino group in the external aldimine complex is less than 5 (Kiick and Cook, 1983), although the ε-amino group of free lysine has a pK_a value of 10.5. The Arg-258 guanidino group (pK_a = 12.5 for free arginine) might have a similar environmentally induced ~6 unit decrease in pK_a value. Thus, at pH 7.5 (the pH employed) a significant fraction of the Arg-258 guanidino group might be in the reactive free base form. This latter mechanism of direct proton abstraction by Arg-258 is favored due to the similarity to the wild type reaction.

The higher stability of the quinonoid formed with K258R compared to wild type is explained by the greater basicity of the arginine versus the lysine side chain functional group. The quinonoid intermediate is an ion pair constituted by the positively charged functional group at position 258 and the carbanionic pyridoxyl moiety. Considered simply, the 100-fold greater basicity of the Arg-258 guanidino group, compared to the Lys-258 ε-amino group, will enhance the stability of this ion pair.
Aspartate Aminotransferase Quinonoid Intermediate

Scheme 2. Mechanistic proposal for the reaction of K258R with amino acid substrates. K258R-PLP (I) binds amino acid to form the Michaelis complex (II) which slowly reacts to form the external aldimine intermediate (III). Fast deprotonation of the external aldimine by the free base form of Arg-258 (or by solvent) leads to an inactive enzyme form in the mispositioned form of the Arg-258 guanidino group (or possibly by solvent) leads to the ketimine intermediate (IV). The ketimine (V) produced by reprotonation of the quinonoid at C4' subsequently reacts via undefined steps to produce an enzyme with covalently bound PMP.

Quinonoid formation with concomitant increase in $A_{280}$ would follow rapidly.

K258R-PMP Is a Covalently Inactivated Enzyme—The inability to dissociate 330 nm absorbing coenzyme (PMP or a derivative thereof) from K258R by three methods previously proven successful with other aspartate aminotransferase variants indicates that the coenzyme is unusually strongly bound to the protein. The stability of this species to 0.1 M sodium hydroxide raises the possibility of covalent attachment, which is, to the authors' knowledge, unprecedented for vitamin B$_{6}$-dependent enzymes. These results explain the lack of reactivity of this enzyme form toward α-keto acids since the coenzyme would not be free to interact with these substrates.

Mechanistic Proposal for the Reaction of K258R with Amino Acids—The results discussed above can be accommodated in the mechanistic proposal of Scheme 2. The amino acid substrate initially binds noncovalently to K258R-PLP (I) to form the Michaelis complex (II). External aldimine (III) formation then occurs slowly via attack of the substrate amino group on the mispositioned PLP aldehyde function. The external aldimine is comparatively rapidly deprotonated by the free base form of the Arg-258 guanidino group (or possibly by solvent) to give the quinonoid intermediate (IV), which slowly reprotonates at C4' to give the ketimine intermediate (V) as inferred from the increase in $A_{280}$. The exact fate of this latter intermediate is undetermined, but a subsequent step(s) leads, via an unknown mechanism, to an inactive enzyme form in which the coenzyme is covalently bound.

REFERENCES
Abbott, E. H. & Bobrik, A. (1973) Biochemistry 12, 846
Braunstein, A. E. & Shemyakin, M. M. (1953) Biochemistry (Moscow) 18, 393
Chen, V. J., Metzler, D. E. & Jenkins, W. T. (1987) J. Biol. Chem. 262, 1442
Christen, P. & Metzler, D. E. (eds) (1985) Transaminases, John Wiley & Sons, New York
Cronin, C. N. & Kirsch, J. F. (1988) Biochemistry 27, 4572
Dunathan, H. C. (1971) Adv. Enzymol. 35, 79
Gehring, H. (1986) Eur. J. Biochem. 159, 291
Gehring, H., Tobler, H. P., Kocher, S., Christen, P. (1987) in Biochemistry of Vitamin B$_{6}$ (Korpela, T. & Christen, P., eds) p. 115, Birkhauser Verlag, Basel
Herold, M. & Kirschner, K. (1990) Biochemistry 29, 1907
Jansonius, J. N. & Vincent, M. G. (1987) in Biological Macromolecules and Assemblies (Jurnak, F. & McPherson, A., eds) Vol. 3, pp. 187-285, John Wiley & Sons, New York
Jenkins, W. T. (1964) J. Biol. Chem. 239, 1742
Julin, D. A. & Kirsch, J. F., (1989) Biochemistry 28, 3825
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Rakost, I. M., Savin, F. A., Torchinsky, Y. M. & Ueno, H. (1985) in Transaminases (Christen, P. & Metzler, D. E., eds) pp. 37-100, John Wiley & Sons, New York
Kamitori, S., Okamoto, A., Hirotsu, K., Higuchi, T., Kuramitsu, S., Kagamiyama, H., Matsuura, Y. & Katsube, Y. (1990) J. Biochem. (Tokyo) 108, 175
Kicik, D. M. & Cook, P. F. (1983) Biochemistry 22, 375
Kuramitsu, S., Inoue, Y., Tanase, S., Morino, Y. & Kagamiyama, H. (1987) Biochem. Biophys. Res. Commun. 146, 416
Metzler, D. E., Ikawa, M. & Snell, E. E. (1984) J. Am. Chem. Soc. 76, 648
Metzler, C. M., Harris, A. G. & Metzler, D. E. (1988) Biochemistry 27, 4923
Moore, J. W. & Jenkins, W. G. (1981) Kinetics and Mechanism, 2nd ed., pp. 290-296, John Wiley & Sons, New York
Morino, Y., Shimada, K. & Kagamiyama, H. (1990) Ann. N. Y. Acad. Sci. 585, 32
Toney, M. D. & Kirsch, J. F. (1989) Science 243, 1485
Toney, M. D. & Kirsch, J. F. (1991) Biochemistry, 30, 7466
Toney, M. D., and Kirsch, J. F. (1992) Protein Sci., in press
Ulevitch, R. J. & Kallen, R. G. (1977) Biochemistry 16, 5350
Ziai, M., Jaassi, K., Gehring, H. & Christen, P. (1990) Eur. J. Biochem. 187, 329