Articles

Contribution to Catalysis and Stability of the Five Cysteines in *Escherichia coli* Aspartate Aminotransferase. Preparation and Properties of a Cysteine-Free Enzyme†

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ABSTRACT: The five cysteines, at positions 82, 191, 192, 270, and 401, of *Escherichia coli* aspartate aminotransferase (AATase) were, individually and in some combinations, converted to alanine by site-directed mutagenesis (C82A, C191A, C192A, C270A, C401A). Cys-191, which is conserved in all AATase isozymes, was mutated to serine as well (C191S). A quintuple mutant, with all cysteines converted to alanines (Quint), was also constructed. The effects of these single and multiple mutations were examined by steady-state kinetics and urea denaturation. The thermal stabilities of Quint and of the wild-type enzyme (WT) were determined by differential scanning calorimetry. The mutants had k_{cat} values up to 50% greater than that of WT and K_M^{Asp} and $K_M^{\alpha\text{-KG}}$ values up to 1.5- and 3.3-fold higher than that of WT. The mutants C82A and C191A exhibit nearly the same C_M in urea denaturation experiments as WT, while the other single mutants and Quint are less stable, with C_M differences of up to 0.7 M urea. Quint is also less thermostable than WT, with a ΔT_M of 3.3–4.4 °C. Thus the five cysteine replacements yield small, but significant, changes in catalytic and denaturation parameters, but none of the cysteines was found to be essential. The changes manifested in the mutation of the conserved Cys-191 to alanine are no greater than those observed with the four nonconserved cysteines. We consider the evolutionary implications of these findings.

Aspartate aminotransferase (AATase;[1] EC 2.6.1.1) is a pyridoxal phosphate containing (PLP) enzyme, which catalyzes the transfer of the α-amino group of L-aspartate to α-ketoglutarate, producing oxaloacetate and L-glutamate

$$\text{Enz-PLP} + \text{L-aspartate} \rightleftharpoons \text{Enz-PMP} + \text{oxaloacetate}$$

$$\text{Enz-PMP} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{Enz-PLP} + \text{L-glutamate} \quad (1)$$

The AATase of *Escherichia coli* has been cloned and overexpressed (Malcolm & Kirsch, 1985; Kuramitsu et al., 1985), and high-resolution X-ray structures have been determined (Smith et al., 1989; Kamitori et al., 1990). The roles of many of the active-site residues in catalysis have been examined by site-directed mutagenesis (Malcolm & Kirsch, 1985; Toney & Kirsch, 1987, 1989; Cronin & Kirsch, 1988; Hayashi et al., 1989; Inoue et al., 1989; Goldberg et al., 1991).

The role of cysteines in AATase has been of historic interest. Chemical modification studies on pig and chicken mitochondrial AATases showed that Cys-166 is susceptible to "syncatalytic modification"; that is, in the presence of substrates, the reactivity of this thiol toward DTNB and *N*-ethylmaleimide increases by 10-fold (Gehring & Christen, 1975, 1978). This cysteine is in the domain interface and becomes exposed in the transition from the open to closed form of the enzyme upon the binding of substrate. This residue is not conserved in the *E. coli* isozyme, which has five other cysteines (Table I). Alkylation of one or more of the five cysteines of *E. coli* AATase, in the presence of urea, prevented refolding while nonalkylated enzyme does refold with high yield (Planas & Kirsch, 1990, 1991). This earlier investigation left open the question of whether one or more cysteines is essential for refolding or if the introduction of a bulky, charged substituent, e.g., (carboxymethyl)- or *S*-(aminoethyl)cysteine, prevented refolding. Site-directed mutagenesis permits evaluation of the contributions to catalysis and stability of each of the cysteine residues of *E. coli* AATase.

The role of cysteines in unrelated transaminases has been assessed previously. Merola et al. (1989) reported that single cysteine to glycine mutations of the three cysteines in the D-amino acid transaminase of a thermophilic *Bacillus* strain YM-1 had little effect on activity or thermal stability. The consequences of multiple Cys to Gly mutations were not determined by these workers. This enzyme has little homology

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, wild-type *Escherichia coli* AATase; Quint, *E. coli* AATase in which Cys-82, Cys-191, Cys-192, Cys-270, and Cys-401 have been mutated to Ala; C82A, C191A, C192A, C270A, and C401A, *E. coli* AATases in which the residues of Cys-82, Cys-191, Cys-192, Cys-270, and Cys-401, respectively, have been changed to Ala; C191S, *E. coli* AATase in which the residue Cys-191 has been changed to Ser; MDH, *E. coli* malate dehydrogenase; α-KG, α-ketoglutarate; DSC, differential scanning calorimetry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid; GuHCl, guanidine hydrochloride.

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Table I: Cysteine Residues in Aspartate Aminotransferase Isozymes

isozyme	residue number[a]														
	12	52	80	**82**	166	**191**	**192**	216	251	253	**270**	275	361	390	**401**
m chicken[b]	G	C	T	A	C	C	A	N	V	S	F	C	C	A	A
m turkey	G	C	T	A	C	C	A		V	S	F	C	C		A
m pig	G	S	C	A	C	C	A	N	C	C	F	C	C	A	A
m rat	G	S	C	A	C	C	A	N	C	C	F	C	C	A	A
m human	C	S	C	A	C	C	A	N	C	C	F	C	C	A	A
c chicken[b]	A	V	R	N	R	C	A	C	F	A	L	G	S	C	A
c pig	A	V	R	C	R	C	A	F	F	A	L	A	S	C	A
E.Coli	A	S	G	C	H	C	C	G	I	A	C	A	S	A	C

[a] From Mehta et al. (1989). The cysteine sites of *E. coli* AATase are underlined. [b] m denotes the mitochondrial isozymes; c denotes the cytosolic isozymes.

with the *E. coli* AATase (Mehta et al., 1989). Two thermophilic aspartate aminotransferases which lack cysteine have been cloned and sequenced. The AATase gene from the thermoacidophilic archaebacterium, *Sulfolobus solfataricus*, has no codons for cysteine in the open reading frame (Cubellis et al., 1989). Recent amino acid analysis of the purified protein confirms the absence of cysteine (G. Marino, personal communication). Sequence analysis of this enzyme shows a very low degree of similarity to the AATase isozymes listed in Table I. A cysteine-less AATase was also cloned from the thermophilic *Bacillus* strain YM-2 (Sung et al., 1991). Alignment of the sequence of this enzyme with other AATases shows only 13–14% identity to *E. coli* and mammalian AATases, but 34% identity with the *S. solfataricus* enzyme. Those residues that are invariant among other transaminases and to which essential catalytic and structural roles have been ascribed (Mehta et al., 1989) are also conserved in these thermophilic AATases (Sung et al., 1991).

The three-dimensional crystal structure of *E. coli* AATase, shown in Figure 1, shows that the five natural cysteines exist as free thiols (Smith et al., 1989). Two of these, Cys-82 and Cys-401, are near the surface of the molecule. None of the cysteines is in the active-site cleft of AATase. Cys-191, however, is conserved among all of the isozymes (Table I). Cysteine is also present in this position in the closely related *E. coli* enzyme, tyrosine aminotransferase (Mehta et al., 1989). If a residue is retained over the course of evolution from *E. coli* to mammals and birds, it is likely that it has an important function, in either catalysis, protein folding, or stability. The function of the conserved Cys-191 is not known. The importance of this cysteine, and that of the four nonconserved cysteines, was examined by single and combined replacement.

Materials and Methods

Materials

L-Asp, α-KG, NADH, PLP, and DTNB were purchased from Sigma. Urea (crystalline, enzyme grade) and DTT were from BRL. CHES, HEPES, MES, and TAPS were obtained from Aldrich. Centricon 30 microconcentrators were Amicon products. MDH was purified from an AATase-deficient *E. coli* strain and was a generous gift of Mr. Edward Neymark. Oligonucleotides for site-directed mutagenesis were obtained from Mr. James Onuffer of the DNA Synthesis Facility, University of California, Berkeley.

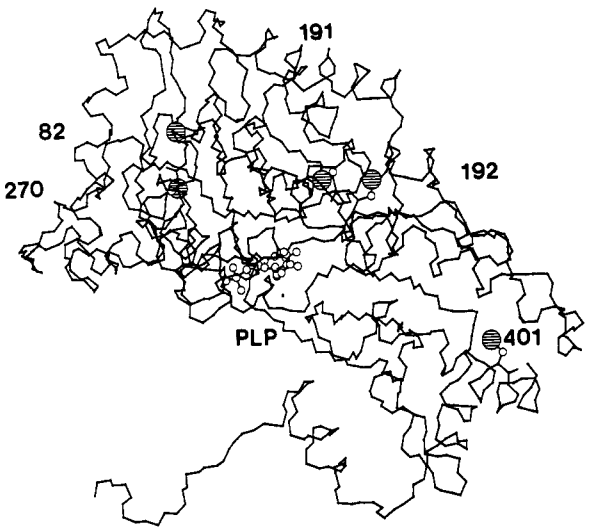

FIGURE 1: α-Carbon structure of one monomer of *E. coli* aspartate aminotransferase. The five cysteines of the wild-type enzyme are highlighted, with shaded sulfur atoms. The active-site Lys-258-PLP aldimine is included for reference (Smith et al., 1989).

Methods

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed by the Kunkel method (Kunkel, 1985), as described previously (Danishefsky et al., 1991). The presence of the desired mutation(s) and the lack of second-site mutations were confirmed by dideoxy sequencing (Sanger et al., 1977) of the entire gene.

Enzyme Purification and Assays. WT and mutant AATases were expressed as previously described (Danishefsky et al., 1991). The enzymes were purified by the method of Cronin and Kirsch (1988), with the following modifications: (1) DTT replaced 2-mercaptoethanol in the chromatography buffers. (2) A pH precipitation was performed before the ammonium sulfate precipitation, in the following manner: Sodium acetate was added to the crude extract to 20 mM, and the pH was adjusted to 5.0 with acetic acid, while stirring briskly. The precipitated material was removed by centrifugation at 13000*g* for 25 min. The pH of the supernatant was readjusted to 7.0 before the addition of solid ammonium sulfate. (3) The gel filtration resin was Sephadex G-100 instead of Ultrogel AcA 34. (4) The column profiles and

Table II: Steady-State Kinetic Parameters for Wild-Type and Mutant Aspartate Aminotransferases[a]

enzyme	no. of detns	k_{cat} (s^{-1})	K_M^{Asp} [b] (mM)	$K_M^{\alpha\text{-KG}}$ [b] (mM)	k_{cat}/K_M^{Asp} (M^{-1} s^{-1} × 10^4)	$k_{cat}/K_M^{\alpha\text{-KG}}$ (M^{-1} s^{-1} × 10^5)
WT	1	159 ± 2	1.75 ± 0.04	0.48 ± 0.01	9.08 ± 0.13	3.32 ± 0.05
Quint	3	238 ± 8	2.71 ± 0.22	1.59 ± 0.14	9.04 ± 0.53	1.56 ± 0.07
C82A	1	195 ± 5	1.70 ± 0.11	0.55 ± 0.04	11.4 ± 0.54	3.52 ± 0.19
C191A	3	175 ± 8	2.73 ± 0.17	0.54 ± 0.06	6.44 ± 0.51	3.31 ± 0.22
C191S	2	181 ± 4	1.70 ± 0.09	1.56 ± 0.08	10.7 ± 0.20	1.16 ± 0.02
C192A	2	152 ± 7	1.44 ± 0.06	0.61 ± 0.04	10.6 ± 0.30	2.53 ± 0.04
C270A	3	186 ± 9	2.24 ± 0.28	0.65 ± 0.03	8.35 ± 0.61	2.87 ± 0.04
C401A	1	177 ± 4	2.24 ± 0.12	0.57 ± 0.03	7.92 ± 0.31	3.11 ± 0.14
triple mutants						
C82A/C270A/C401A	1	201 ± 3	2.18 ± 0.08	1.04 ± 0.04	9.19 ± 0.26	1.93 ± 0.05
C191A/C192A/C270A	1	227 ± 3	2.72 ± 0.08	1.14 ± 0.03	8.34 ± 0.16	1.99 ± 0.04
C191S/C192A/C270A	1	177 ± 3	1.52 ± 0.07	1.81 ± 0.07	11.7 ± 0.40	0.978 ± 0.023

[a] The assays were performed in 200 mM potassium HEPES, at 25 °C. The values of k_{cat}, K_M, and k_{cat}/K_M were determined as described under Materials and Methods. [b] Aspartate concentrations were from 0.75 to 15 mM, and α-KG concentrations were from 0.3 to 6.0 mM.

elution position of AATase were determined by the absorbance at 280, 360, and 430 nm, rather than enzyme activity. The purity of the enzymes was estimated visually from sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be 95–99%.

Enzyme concentration was determined by absorbance at 205 nm (Scopes, 1974), using A(1 mg/mL) = 31 absorbance units. Spectrophotometric and kinetic measurements were performed on Perkin Elmer Lambda 4B and Kontron Uvikon 860 spectrophotometers with constant-temperature baths set at 25 °C.

Steady-State Kinetics. Steady-state activity of AATase, using the L-Asp/α-KG substrate pair, was measured by coupling the production of oxaloacetate to MDH and monitoring the decrease in absorbance at 340 nm at 25 °C (Cronin & Kirsch, 1988). The assay conditions were 200 mM HEPES, pH 7.5, 100 mM KCl, 150 μM NADH, 10 units/mL MDH, 0.75–15 mM Asp, and 0.3–6 mM α-KG. The k_{cat} and K_M values for the Asp/α-KG reaction were determined from a matrix of initial rates using five aspartate and five α-KG concentrations. These rates were fit to the equation describing a ping-pong mechanism (Velick & Vavra, 1962)

$$\frac{v}{E_t} = \frac{k_{cat}[\text{Asp}][\alpha\text{-KG}]}{K_M^{Asp}[\alpha\text{-KG}] + K_M^{\alpha\text{-KG}}[\text{Asp}] + [\text{Asp}][\alpha\text{-KG}]} \quad (2)$$

by nonlinear regression, using the program of R. Viola (personal communication). Errors in k_{cat}/K_M were determined as described by Julin and Kirsch (1989).

Spectrophotometric Titrations. The pK_a of the internal aldimine between Lys-258 and the bound PLP was determined as previously described (Goldberg et al., 1991). The surface thiols of WT and mutant AATases were quantitated with DTNB. DTT was removed with Centricon centrifugal microconcentrators, prior to the reaction with DTNB. Enzymes were diluted to 6–7 μM (monomer) in 50 mM TAPS, pH 8.0, and 2 μM PLP and incubated with 500 μM DTNB at 25 °C. The increase in absorbance at 412 nm was monitored for 1 h, by which time the reaction of the accessible cysteines was complete.

Urea Denaturation. WT and mutant AATase stocks of 30 μg/mL in 200 mM HEPES, pH 7.5, 1 mM DTT, 20 μM PLP, and 0–7 M urea were incubated for 12–20 h at room temperature. An aliquot of the stock was diluted into an assay buffer, containing 200 mM HEPES, pH 7.5, 100 mM KCl, 10 mM Asp, 3 mM α-KG, 150 μM NADH, and 10 units/mL MDH, and the AATase activity was measured by the coupled MDH assay. The final concentration of urea in the assay mixture was between 0 and 70 mM. Controls showed that concentrations up to 80 mM urea had no effect on the MDH activity in the reaction cuvette.

The dependence of AATase activity on urea concentration was determined from the initial linear rates of reaction. These data were fit to the following equation, which describes a two-state model of protein unfolding, by nonlinear regression:

$$K_{obs} = \exp\left\{\frac{-(\Delta G_u^{H_2O} + m[\text{urea}])}{RT}\right\} \quad (3)$$

where

$$K_{obs} = \frac{A_0 - A_i}{A_i} \quad (4)$$

where R is the gas constant, T is the absolute temperature (298 K), $\Delta G_u^{H_2O}$ is the free energy of unfolding in 0 M urea, and m is an adjustable parameter related to the cooperativity of unfolding. A_0 is the activity observed at 0 M urea, and A_i is that measured at a given, nonzero concentration of urea, i M (such that percent activity, at i M urea, is $A_i/A_0 \times 100$). This analysis is essentially that of Pace (1986).

Differential Scanning Calorimetry. The instrumentation employed was a Microcal MC2 differential scanning calorimeter, interfaced with an IBM-XT computer for data collection and analysis. The scan rate was 49–55 °C/h, over a temperature range of 30–80 °C. The buffers were either 40 mM potassium HEPES, pH 7.5, 40 mM potassium MES, pH 6.2, or 40 mM potassium TAPS, pH 8.4. The pH was chosen such that the ionic strength remained constant with the buffers employed. Protein concentrations were 2.0–4.9 mg/mL. The data were analyzed with the DA-2 software package provided by Microcal. The curves were normalized by the empirical selection of a base line, and the calorimetric enthalpy was calculated from the integrated area of the endotherm(s). The peak of the endotherm gave the T_M value.

Results

Kinetic Analysis. The steady-state kinetics of WT and mutant AATases were examined, with the L-Asp/α-KG substrate pair. The Michaelis–Menten parameters are presented in Table II. All single mutations of Cys to Ala (or Ser in the case of C191S), as well as the quintuple mutation of the five cysteines to alanines, yield active enzymes.

Each of the single mutations, however, does result in some slight to moderate change in the catalytic parameters of the enzyme. All of the single mutants, except C192A which is essentially unchanged, have a slightly increased k_{cat} relative to the WT enzyme. Larger changes are observed in the K_M values. All of the mutations give increased K_M values for α-KG, with C191S having the most pronounced effect, a 3.2-fold increase. The effect of the single mutations on K_M

Table III: Parameters Describing the Urea-Induced Denaturation of Wild-Type and Mutant Aspartate Aminotransferases[a]

enzyme	C_M (M urea)	ΔG^{H_2O} (kcal/mol)	$-m$ (kcal mol^{-1} M^{-1})	$\Delta\Delta G^{H_2O}$ [b] (kcal/mol)	$\Delta\Delta G^{3.9M}$ [c] (kcal/mol)
WT	3.89 ± 0.26	9.96 ± 0.46	2.56 ± 0.12		
Quint	3.16 ± 0.15	8.92 ± 0.30	2.83 ± 0.10	1.0 ± 0.5	2.1 ± 0.6
C82A	4.06 ± 0.15	8.67 ± 0.22	2.13 ± 0.06	1.3 ± 0.5	−0.4 ± 0.5
C191A	3.87 ± 0.17	7.74 ± 0.24	2.00 ± 0.06	2.2 ± 0.5	0.05 ± 0.5
C191S	3.41 ± 0.18	8.12 ± 0.30	2.38 ± 0.09	1.8 ± 0.5	1.1 ± 0.6
C192A	3.53 ± 0.13	8.32 ± 0.21	2.35 ± 0.06	1.6 ± 0.5	0.8 ± 0.5
C270A	3.51 ± 0.19	7.50 ± 0.29	2.14 ± 0.08	2.5 ± 0.5	0.8 ± 0.6
C401A	3.41 ± 0.26	13.2 ± 0.73	3.87 ± 0.21	−3.2 ± 0.9	1.9 ± 0.9

[a] The data were treated as described under Materials and Methods. Two complete urea denaturation curves were determined for each variant, with duplicate measurements of the activity made for each urea concentration. The thermodynamic values were calculated for a 0.7 μM standard state, which was the monomer concentration used in the experiments. [b] $\Delta\Delta G^{H_2O} = \Delta G^{H_2O}$(WT) − ΔG^{H_2O}(mutant). [c] $\Delta\Delta G^{3.9M} = \Delta G$(WT at 3.9 M urea) − ΔG(mutant at 3.9 M urea). 3.9 M urea is the C_M for WT AATase.

for L-Asp is either no significant change (C82A and C191S), a minor decrease of 1.2-fold (C192A), or an increase of 1.3- to 1.6-fold (C270A, C401A, and C191A). Only one of the mutants, C82A, has a higher $k_{cat}/K_M^{\alpha\text{-KG}}$ value (352 000 M^{-1} s^{-1}) than does WT (332 000 M^{-1} s^{-1}), although C191A is nearly the same (331 000 M^{-1} s^{-1}). The largest change among the single mutants is seen with C191S, which due to the large increase in $K_M^{\alpha\text{-KG}}$ has a $k_{cat}/K_M^{\alpha\text{-KG}}$ value which is 2.9-fold less than that of WT. The k_{cat}/K_M^{Asp} values are either increased by up to 1.3-fold or decreased by no more than 1.4-fold.

The two triple mutants C82A/C270A/C401A and C191A/C192A/C270A exhibit kinetic parameters that are intermediate between those of WT and the quintuple mutant (Quint). Both k_{cat} and K_M values are increased. The triple mutant, in which three of the buried cysteines, Cys-191, Cys-192, and Cys-270, have been changed to Ala, is quite similar to Quint, except that the $K_M^{\alpha\text{-KG}}$ value is not as high (1.14 and 1.59 mM, respectively). The triple mutant C191S/C192A/C270A is notable in that it is the only mutant enzyme where the $K_M^{\alpha\text{-KG}}$ is greater than the K_M^{Asp} value, and thus the $k_{cat}/K_M^{\alpha\text{-KG}}$ value is less than the k_{cat}/K_M^{Asp} value.

Quint exhibits a k_{cat} value 1.5-fold higher than that of WT. The K_M values for L-Asp and α-KG are increased 1.5- and 3.3-fold, respectively. This results in a k_{cat}/K_M^{Asp} that is virtually the same as that of WT (90 400 and 90 800, respectively) and a $k_{cat}/K_M^{\alpha\text{-KG}}$ that is 2.1-fold lower than that characterizing WT.

Spectrophotometric Titration. The protonation state of the internal aldimine formed from the ε-amino group of Lys-258 and the bound PLP can be determined spectrophotometrically as the protonated form absorbs maximally at 430 nm while the free base's maximum is at 360 nm (Kallen et al., 1985). The pK_a so obtained is 7.11 ± 0.01 for Quint (data not shown), a value virtually identical to that of WT, 7.16 ± 0.05 (Cronin & Kirsch, 1988).

Surface Thiol Accessibility. Previous work from this laboratory (Planas & Kirsch, 1991) had shown that one cysteine of *E. coli* AATase is accessible to DTNB in the native conformation. The X-ray structure suggests that this thiol might be either Cys-82 or Cys-401. The single Cys to Ala mutants, C82A and C401A, allow a direct determination of the identity of the DTNB-accessible thiol. WT, C82A, C401A, and Quint were reacted with excess DTNB (Figure 2). Both WT and C401A have a cysteine that reacts with DTNB in the native conformation of AATase, while C82A does not. This result shows that it is Cys-82 which is the reagent-accessible thiol. The time course of the surface thiol reaction with DTNB fits a first-order rate equation. The rate constant (and amplitude) for WT and C401A are 0.13 min^{-1} (0.70 SH/subunit) and 0.14 min^{-1} (0.65 SH/subunit), respectively. C82A and Quint have amplitudes of 0.09 and 0.06 surface cysteines per subunit, respectively, after a 60-min reaction.

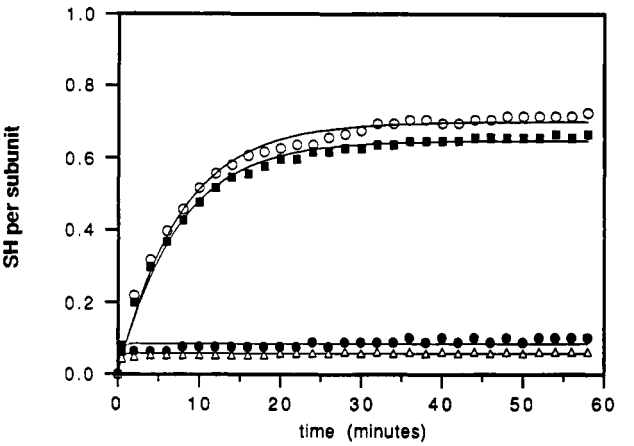

FIGURE 2: Time course of the reactions of wild-type and mutant aspartate aminotransferases with DTNB. The reaction conditions were 50 mM TAPS, pH 8.0, 500 μM DTNB, and 6–7 μM subunit. The reactions were monitored by change in absorbance at 412 nm at 25 °C. WT, ○; C401A, ■; C82A, ●; and Quint (cysteine-free quintuple mutant), △.

Urea Denaturation. The stability of the WT and mutant AATases was examined by measuring the dependence of enzyme activity on urea concentration. It was established that the exponential loss in activity is complete at urea concentrations equal to C_M, 5 and 6 M urea, in less than 12 h for both Quint and WT. Urea denaturation is largely reversible. After incubation in 6 M urea (200 mM HEPES, pH 7.5, 1 mM DTT, 20 μM PLP, and 0.3 mg/mL enzyme) at 25 °C for 24 h, Quint recovered 78% activity when diluted 10-fold with 200 mM HEPES, pH 7.5, 1 mM DTT, and 20 μM PLP. WT recovered 77% activity after 16 h, but only 60% activity after 24 h in 6 M urea. Thus the urea unfolding curves and parameters presented in Table III and Figure 3 represent near-equilibrium transitions.

The midpoint of the transition, C_M, is that concentration of urea which results in a 50% loss in activity. The C_M determined by activity for the WT enzyme (C_M = 3.9 M) is similar to that found by fluorescence monitoring, 3.7 M urea (A. Planas, unpublished results). The ideal standard parameter for comparison of the free energies of unfolding of mutant proteins is $\Delta G_u^{H_2O}$. Its evaluation, however, requires a long extrapolation to 0 M urea from ΔG_u values measured at a series of specific urea concentrations. Thus, the errors in $\Delta G_u^{H_2O}$ may be larger than the differences among the variant proteins. To overcome this problem, the relative ΔG_u values are evaluated at 3.9 M urea, the C_M of wild type. Kellis et al. (1989) have discussed the advantages and disadvantages of this approach.

The single mutation C82A which lacks the DTNB-accessible thiol is the only variant which is more stable than WT, as indicated by the C_M and $\Delta\Delta G^{3.9M}$ values. The other single

Table IV: Thermal Denaturation Parameters for Wild-Type and Cysteine-Free (Quint) Aspartate Aminotransferases[a]

enzyme	buffer, pH	no. of runs	minor T_M (°C)	major T_M (°C)	calorimetric ΔH (kcal/mol)
WT	TAPS, 8.4	1	61.8	64.7	341
WT	HEPES, 7.5	3	61.6 ± 1.3	65.5 ± 0.3	336 ± 14
Quint	HEPES, 7.5	2	57.6 ± 0.0[b]	61.1 ± 0.1	336 ± 6
WT	MES, 6.2	2		62.2 ± 0.7	381 ± 10
Quint	MES, 6.2	1		58.9	383

[a] The experiments were performed as described under Materials and Methods. Buffer concentrations were 40 mM, and the protein concentration was 2.0–4.9 mg/mL. The data were fitted with the DA-2 software package of Microcal. [b] The same value was obtained in both experiments.

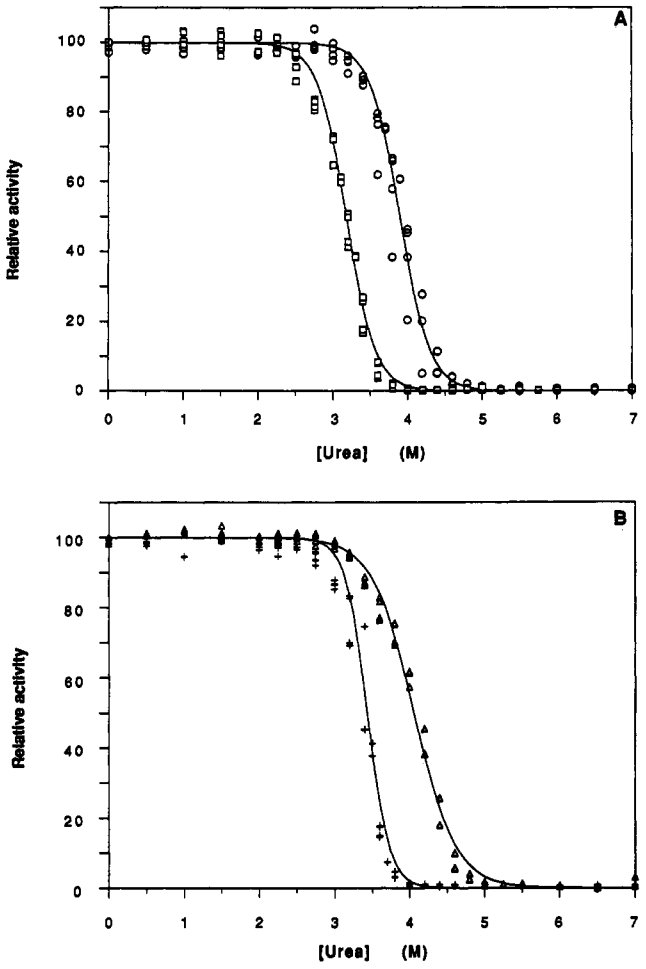

FIGURE 3: Dependence of enzyme activity on urea concentration for WT and mutant aspartate aminotransferases. Samples containing 30 μg/mL enzyme were incubated at 25 °C, in 0–7 M urea, 200 mM HEPES, pH 7.5, 1 mM DTT, and 20 μM PLP, for 12–20 h. The lines were fit to a two-state model as described under Materials and Methods. (Panel A) WT, ○; and Quint (cysteine-free quintuple mutant), □. (Panel B) C82A, △; and C401A, +.

mutants have C_M and $\Delta\Delta G^{3.9M}$ values which are intermediate between those of WT and Quint. C401A is more stable than WT as determined from the $\Delta G_u^{H_2O}$ values and exhibits a much more cooperative transition (Figure 3B). However, the C_M for C401A is 3.4 M urea, significantly lower than that of WT (3.9 M urea). Quint, with a C_M = 3.2 M urea, is the least stable mutant.

Differential Scanning Calorimetry. The relative thermal stabilities of WT and Quint were examined by differential scanning calorimetry. Plots of ΔC_p versus temperature for WT and Quint are shown in Figure 4. The midpoints of the thermal denaturation transitions, T_M, correspond to the peaks of the profiles in this figure. The T_M and calorimetric ΔH values for WT and Quint are presented in Table IV.

Attempts were made to characterize qualitatively the two endotherms observed in the scans conducted at pH 7.5. The estimated purity of the enzymes (>95%) makes it unlikely that the minor endotherm represents the denaturation transition of a contaminating protein. A control experiment was done with WT enzyme which had been treated with 3 mM α-KG to convert any trace PMP bound to the enzyme to PLP. The excess substrate and any product formed were removed by Centricon centrifugal microconcentration, and the sample, with 2 μM PLP added, was melted by DSC. The scan still showed two endotherms with the same T_M values as given in Table IV (data not shown). Thus the peaks are not due to AATase containing two different forms of the cofactor, or to the presence of apoenzyme. The area of the minor endotherm decreased relative to that of the major endotherm as protein concentration was increased from 2.0 to 7.6 mg/mL (data not shown).

Quint is less stable to thermal denaturation than WT, both at pH 6.2 and at pH 7.5. The ΔT_M between Quint and WT, for the HEPES endotherms, is 4.0 and 4.4 °C for the minor and major endotherms, respectively. This difference is less at the lower pH of the MES scans, ΔT_M = 3.3 °C.

Discussion

Catalysis. The mutation of each of the cysteine residues of *E. coli* AATase, individually, yielded AATase variants with k_{cat} values 0–18% higher than those of WT. The K_M^{Asp} and $K_M^{\alpha\text{-KG}}$ values of the variants were equal to or within a factor of 1.6- and 3.3-fold, respectively, of those exhibited by the WT enzyme. The amino acid at position 192 is alanine in all other known AATase sequences (Table I); therefore, the observation that C192A has kinetic parameters very similar to those of WT is not unexpected. The single mutations C82A, C270A, and C401A show less than a 1.6-fold increase in k_{cat} and K_M values relative to WT. The substitution of alanine for the conserved Cys-191 produces an enzyme whose major catalytic difference from WT is a modest 1.6-fold increase in K_M^{Asp}. Replacement of Cys-191 by serine resulted in an enzyme with a WT-like K_M^{Asp}, but a 3.2-fold higher $K_M^{\alpha\text{-KG}}$. It seems that the less conservative Cys to Ala mutation, C191A, results in a better enzyme than the C191S variant. Thus, the quintuple mutant, Quint, was constructed with all five cysteines mutated to alanine.

The k_{cat} value of Quint is 50% greater than that of WT, but the K_M values are increased by 1.5- and 3.3-fold for L-Asp and α-KG, respectively. The steady-state parameters of the single Cys to Ala mutants are generally intermediate between those of Quint and WT. The question of the additivity of the effects of multiple mutations in proteins has been reviewed recently (Wells, 1990). He finds generally that, if the mutated residues do not interact with each other directly, or indirectly through electrostatic or structural perturbations, the sum of the free energy changes for the individual mutations is nearly equal to that of the multiple mutant. This additivity principle is applicable to catalysis (k_{cat} and k_{cat}/K_M values) as well as stability. Evaluations of the additivity of the changes in k_{cat}, k_{cat}/K_M^{Asp}, and $k_{cat}/K_M^{\alpha\text{-KG}}$ values for Quint and the three triple mutants were made. Perturbations on the steady-state pa-

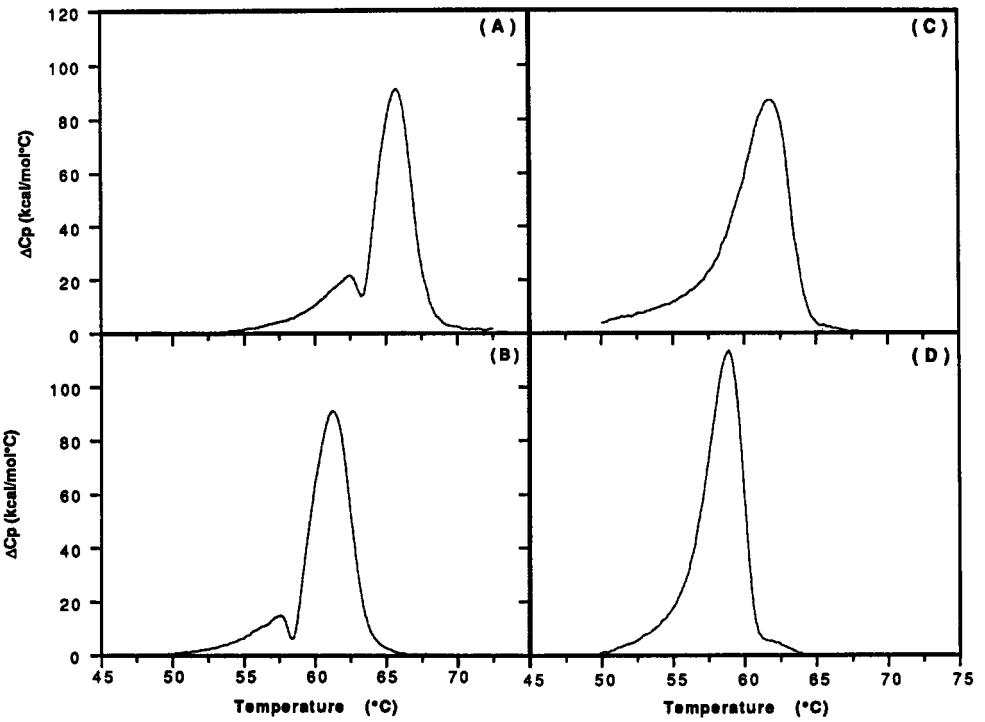

FIGURE 4: Differential scanning calorimetry profiles of WT and Quint aspartate aminotransferases. The scans have been corrected for protein concentration so that ΔC_p is in units of kcal·mol^{-1}·°C^{-1}. (Panel A) WT in 40 mM HEPES, pH 7.5. (Panel B) Quint (cysteine-free quintuple mutant) in 40 mM HEPES, pH 7.5. (Panel C) WT in 40 mM MES, pH 6.2. (Panel D) Quint in 40 mM MES, pH 6.2.

rameters resulting from individual mutations are too small to permit quantitative conclusions to be drawn.

Stability. None of the mutations of the five cysteines, either independently or in concert, severely destabilized the protein. Changing the surface cysteine, Cys-82, to alanine yielded an enzyme slightly more stable to urea denaturation than WT. As with the kinetics, it was found that the C191A mutation had less effect on the properties of the enzyme than the seemingly more conservative C191S mutation. C191A has the same C_M as WT, while that of C191S is decreased by nearly 0.5 M urea.

With the exception of C401A, all of the variants showed similar cooperativity in unfolding in urea. This can be seen by the very similar values of m in Table III, and by the parallel slopes of the denaturation curves in the transition region in Figure 3. It is interesting that the single mutant C401A unfolds more cooperatively (Figure 3B), with a value of m that is higher than for the other enzymes. Residue 401 is in the interface between two α-helices which compose part of the large and small domains of AATase (Smith et al., 1989). Cys-401 is part of helix 16 of the small domain and contacts Thr-340 and Leu-341 which are near the C-terminus of the long helix 13 of the large domain. This contact is near the surface of AATase. Cys-401 of the WT enzyme is DTNB-accessible in solutions of 1 M urea (A. Planas, unpublished observations). The contacts of these three residues may be important to the stability of the domain interface, and hence by lessening the contact by replacing Cys-401 with Ala, the enzyme unfolds more cooperatively. As seen in Table I, only the *E. coli* AATase has a cysteine at position 401; the other isozymes have Ala-401. Leu-341 is conserved among all of the isozymes, but the residue at position 340 is not (Mehta et al., 1989). *E. coli* AATase has Thr-340, while the other isozymes have either Arg or Asn, which are bulkier than threonine and may compensate for the decrease in size of Ala relative to Cys at position 401.

The combination of the five individual mutations to yield Quint results in an enzyme that is significantly less stable than WT, as judged by chemical and thermal denaturation. The urea denaturation parameters of the single Cys to Ala mutants are generally intermediate between those of WT and Quint, and the individual free energy changes are approximately additive. Summation of the $\Delta\Delta G^{H_2O}$ and $\Delta\Delta G^{3.9M}$ values for the five Cys to Ala mutants gives +4.4 ± 1.3 and +3.2 ± 1.4 kcal/mol, respectively. The $\Delta\Delta G^{H_2O}$ and $\Delta\Delta G^{3.9M}$ values of Quint are +1.0 ± 0.5 and +2.1 ± 0.6 kcal/mol, respectively (Table III). The propagated errors are large relative to the subtle changes between the variant enzymes. However, the effects of the single mutations on the parameter $\Delta\Delta G^{3.9M}$ appear to be additive.

The DSC experiments demonstrate that Quint is less thermostable than WT, as judged from its lower T_M, at both pH 7.5 and pH 6.2. The calorimetric enthalpy of unfolding, ΔH_{cal}, is the same for both enzymes. Unfortunately, it was not possible to determine accurately a heat capacity difference, ΔC_p, between the native and denatured forms of either protein as the C_p base line of the native enzyme was apparently higher than that of the denatured enzyme due to aggregation. Thus, neither the ΔG of unfolding nor the $\Delta\Delta G$ of Quint relative to WT could be determined.

Only one endotherm was seen in earlier DSC experiments on pig heart cytosolic AATase (Relimpio et al., 1981). These studies were done in 50 mM Tris, pH 8.2, and the enzyme exhibits a higher T_M, 79.5 °C, than that of *E. Coli* WT AATase. This suggests that the pig heart enzyme melts in one cooperative transition with no populated intermediates. Chemical denaturation of the *E. coli* enzyme by GuHCl does show two transitions however. The unfolding intermediate can be stabilized in 1 M GuHCl and isolated. It has been identified as a monomeric species (Herold & Kirschner, 1990). The urea-induced denaturation reported here was monitored by loss of enzyme activity. A monomeric intermediate would have escaped detection as the active site is located at the dimer interface (Ford et al., 1980). Two transitions are observed in the DSC scans at pH 7.5 and 8.4. The enthalpy of the initial endotherm is dependent on protein concentration, suggesting

that this transition may represent the dissociation of the dimer to a monomeric intermediate.

Evolutionary Considerations. The results presented here demonstrate that none of the cysteines of *E. coli* AATase is essential for catalytic activity or stability. It is not clear why Cys-191 is conserved among the AATase isozymes, from such evolutionarily distant sources as pig, chicken, and *E. coli*. This residue is buried and is not accessible to small molecules or proteins in the cell. Therefore, it seems unlikely that Cys-191 plays an in vivo role in protein–protein interactions, protein localization, or turnover. There is a difference in the kinetic parameters between C191A and WT AATase, a 1.6-fold increase in K_M^{Asp} and a 1.4-fold decrease in k_{cat}/K_M^{Asp}. However, this catalytic difference is not substantially greater than the effects seen in any of the other Cys to Ala mutants at nonconserved positions. Whether these minor catalytic effects of C191A are evolutionarily significant is not known. It is noteworthy that the K_M and V_{max}/K_M of rat heart cytosolic AATase and pig heart cytosolic AATase, when assayed in a similar manner, show differences of 1.9-fold for K_M^{Asp}, 3.4-fold for $K_M^{\alpha\text{-KG}}$, and 1.4- and 2.4-fold for V_{max}/K_M^{Asp} and $V_{max}/K_M^{\alpha\text{-KG}}$, respectively (Mavrides & Nadeau, 1987; Schlegel & Christen, 1978). The magnitudes of these differences are greater than those seen by the mutation to alanine of the conserved Cys-191 in the *E. coli* enzyme. Mutation of the conserved Cys-191 (to Ala) has no effect on the stability of the enzyme to urea denaturation, while mutations of the nonconserved cysteines 192, 270, and 401 do.

Sequence alignment of 12 AATase isozymes shows that Cys-191 is in a region that is highly conserved (Mehta et al., 1989). This includes three invariant residues in all aminotransferases, Asn-194, Pro-195, and Gly-197. The global integrity of the sequence of this region may be essential for stability or catalysis and thus has been largely maintained through the course of evolution, although the identity of individual sites, such as Cys-191, is not so critical as long as the mutation is conservative.

Why then is Cys-191 conserved in AATase when the catalytic and stability consequences of the C191A mutation are no greater than those observed for mutation of the nonconserved cysteines? The properties of the C191S enzyme and consideration of the genetic code suggest a possible explanation. The codons for Cys are UGC and UGU. Base changes in the third position result in termination or a Cys to Trp mutation. Changes in the second position yield Phe, Ser, or Tyr, while those in the first position gave Arg, Ser, or Gly. Of these possible single base pair mutations, only Cys to Ser would be considered conservative. The C191S mutant, unlike C191A, as described above, differs significantly from WT AATase, in both kinetic and stability properties. Thus, the only one-base, conservative mutation available to Cys-191 has *large*, measurable phenotypic consequences. The Cys to Ala mutation requires two base changes. If Cys-191 were present in the progenitor gene of AATase, we speculate that it could have been effectively frozen by the fact that any accessible (i.e., one-base) change results in a detrimental alteration of the protein's behavior.

Since none of cysteines is essential individually, it was possible to construct a quintuple mutant which contains no cysteines. The generation of this cysteine-free AATase, Quint, provides a platform onto which uniquely placed cysteine residues can be engineered. This may allow the introduction of unnatural amino acids into proteins by combining the techniques of site-directed mutagenesis and chemical modification to take advantage of the unique chemistry of cysteine.

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Registry No. AATase, 9000-97-9; Cys, 52-90-4; Ala, 56-41-7; Ser, 56-45-1; Asp, 56-84-8; α-KG, 328-50-7.

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The Rate of Formation of Transition-State Analogues in the Active Site of Adenosine Deaminase Is Encounter-Controlled: Implications for the Mechanism†

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ABSTRACT: We have previously shown that purine riboside, when bound to adenosine deaminase, forms a complex in which C-6 of the purine is tetrahedral [Kurz, L. C., & Frieden, C. (1987) *Biochemistry 26*, 8450]. We now report the rates of formation of enzyme–inhibitor complexes of two types, those which do and those which do not form such tetrahedral intermediates. In both cases, the rates are encounter-controlled since the progress curves for formation of the complexes are well-described by a simple second-order approach to equilibrium and the rate constants show an inverse solvent viscosity dependence. Assuming that the formation of the intermediate–analogue complex is preceded by an initial ground-state analogue complex, the lifetime of that ground-state complex must be less than ~20 μs. All of the enzyme–inhibitor complexes studied share three characteristics: (1) the complexes generate large UV-difference spectra; (2) a substantial solvent isotope effect is found on the enzyme's affinity for the inhibitors; and (3) a new signal appears in the CD spectra of the complexes. Two of the nucleosides studied, 1-deazapurine riboside and 1-deazaadenosine, form complexes which appear to mimic a ground-state rather than a reactive intermediate when bound to adenosine deaminase. We find that the values for the association rate constants for those inhibitors which form intermediate analogues are very similar to that for adenosine. The presence of a significant solvent isotope effect on the affinity of all inhibitors is attributable in part to a large transfer isotope effect on the free ligand and in part to an effect on the bound ligand. This complicates use of the solvent isotope effect in applications of the multiple isotope method for estimating intrinsic isotope effects and commitment factors.

Adenosine deaminase (EC 3.5.4.4) catalyzes the hydrolysis of (deoxy)adenosine to (deoxy)inosine (Figure 1). Reaction pathways have been proposed (Figure 1) with several discrete chemical intermediates (Wolfenden et al., 1969; Frick et al., 1987; Weiss et al., 1987) or an S_N2 mechanism lacking any stable species on the path from (enzyme-bound) reactants to products (Wilson et al., 1991). Support for a hydrate tetrahedral intermediate has come from studies of alternative substrates (Evans & Wolfenden, 1973), transition-state analogues (Wolfenden et al., 1969), UV-difference spectra (Kurz & Frieden, 1983), and solvent isotope studies (Kurz & Frieden, 1983). ^{13}C NMR studies of the enzyme complex with purine riboside (Kurz & Frieden, 1987; Jones et al., 1989) gave evidence for the formation of an analogue of a tetrahedral intermediate, and that conclusion is now confirmed by the crystal structure of the purine riboside–enzyme complex (Wilson et al., 1991). Thus, with purine riboside, the enzyme catalyzes formation of the tetrahedral intermediate, the first half of an addition–elimination sequence, but is unable to complete turnover owing to its lack of a leaving group at C-6.

In this report, our approach is to use inhibitors that are able to participate in only part of the catalytic cycle in order to isolate and study part of the mechanism without the complication of enzyme turnover. We seek evidence for each of the intermediate species shown in Figure 1. We believe that

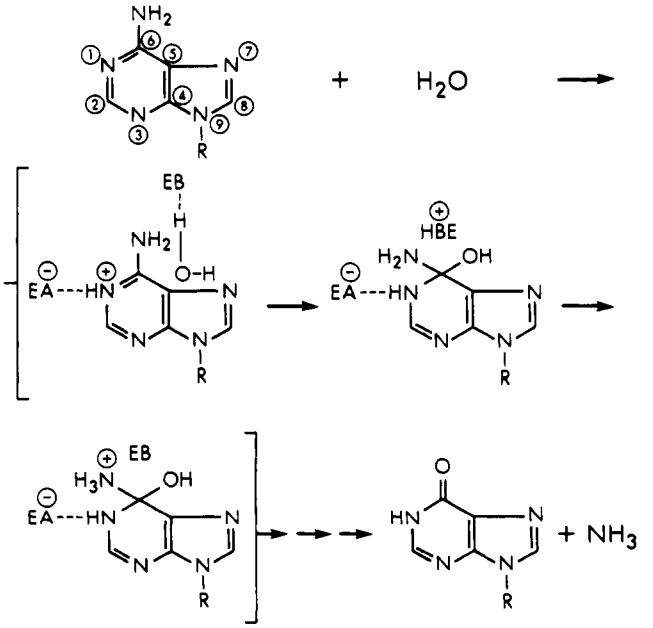

FIGURE 1: Reaction catalyzed by adenosine deaminase including the structures of possible intermediates.

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