The role of the conserved Lys68*:Glu265 intersubunit salt bridge in aspartate aminotransferase kinetics: Multiple forced covariant amino acid substitutions in natural variants

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Abstract

The role of the Lys68*:Glu265 intersubunit salt bridge that is conserved (Csb) in all known aspartate aminotransferases (AATases), except those of animal cytosolic, Ac (His68*:Glu265), and plant mitochondrial, Pm (Met68*:Gln265), origins, was evaluated in the *Escherichia coli* AATase. Two double-mutant cycles, to K68M/E265Q and the charge reversed K68E/E265K, were characterized with the context dependence (*C*) and impact (*I*) formalism, previously defined for functional chimeric analysis. Mutations of Lys68* with Glu265 fixed are generally more deleterious than the converse mutations of Glu265 with Lys68* fixed, showing that buried negative charges have greater effects than buried positive charges in this context. Replacement of the charged Lys68*:Glu265 with the K68M/E265Q neutral pair introduces relatively small effects on the kinetic parameters. The differential sensitivity of $k_{\text{cat}}/K_{\text{M, L-Asp}}$ and $k_{\text{cat}}/K_{\text{M, } \alpha\text{-KG}}$ to salt bridge mutagenic replacements is shown by a linear-free energy relationship, in which the logarithms of the latter second order rate constants are generally decreased by a factor of two more than are those of the former. Thus, $k_{\text{cat}}/K_{\text{M, L-Asp}}$ and $k_{\text{cat}}/K_{\text{M, } \alpha\text{-KG}}$ are 133 and 442 $\text{mM}^{-1}\text{s}^{-1}$ for the wild-type (WT) enzyme, respectively, but their relative order is reversed in the more severely compromised mutants (14.8 and 5.3 $\text{mM}^{-1}\text{s}^{-1}$ for K68E). A Venn diagram illustrates apparent forced covariances of groups of amino acids that accompany the naturally occurring salt bridge replacements in the Pm and Ac classes. The more deeply rooted tree indicates that the Csb variant was the ancestral specie.

Keywords: Aspartate aminotranferase; context dependence; covariance; double-mutant cycles; evolution; impact; kinetics; salt bridge; Venn diagram

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Abbreviations: AATase, aspartate aminotransferase; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; OAA, oxaloacetate; α -KG, α -ketoglutarate; eAATase, *E. coli* AATase; *I*, impact; *C*, context dependence; WT, wild type; MDH, malate dehydrogenase; Csb, conserved salt bridge; Pm, plant mitochondrial; Ac, animal cytosolic; PID, protein identification number; $\cap$, intersection of two sets; His₆-tag; 6-histidine tag.

*Residues pertaining to the opposite subunit.

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Aspartate aminotransferase (AATase) is a pyridoxal phosphate- (PLP) dependent enzyme that belongs to the subfamily I α of aminotransferases (Jensen and Gu 1996). This enzyme catalyzes the reversible transfer of the α -amino group from four- and five-carbon dicarboxylic amino acids to the corresponding α -keto-acids by a ping-pong bi bi mechanism. The transamination reaction is described by the two half reactions shown in equations 1 and 2.

X-ray structures of AATases have been determined for *Escherichia coli* (Jager et al. 1994; Kamitori et al. 1990; Okamoto et al. 1994; Smith et al. 1989), yeast cytosolic (Jeffery et al. 1998), pig heart cytosolic (Arnone et al. 1985), and mitochondrial and cytosolic chicken (Ford et al. 1980; Malashkevich et al. 1993; McPhalen et al. 1992) AATases. The enzyme is a homodimer with the two active sites situated at the interface. Each subunit contains a PLP molecule anchored to its large domain (residues 46–329). Three residues belonging to the opposite subunit, Tyr70* (Toney and Kirsch 1991a,b), Arg292* (Cronin and Kirsch 1988), and Asn297*, (Luong and Kirsch 2001) have been shown to have a direct role in the catalysis of the reaction, thus explaining the obligate homodimeric active form. Substrate association induces a change from the “open” to the “closed” conformation of the enzyme by rotation of the small domain (residues 16–45 and 330–409) into the active site (Fukumoto et al. 1991; Jager et al. 1994; Picot et al. 1991; Sandmeier and Christen 1980). Some small domain residues such as Arg386 (Danishefsky et al. 1991; Matharu et al. 2001), which interacts with the α -carboxylate of the substrate, play a key role in the activity of the enzyme.

A schematic view of the *E. coli* aspartate aminotransferase (eAATase) active site is shown in Scheme 1. The direct roles in catalysis or PLP binding of nearly all active-site residues have been evaluated by site-directed mutagenesis. First shell active site residues are defined as those that participate in catalysis through direct interactions with the substrate(s) or cofactor. Accordingly, the second shell designation applies to those amino acids that interact directly with, or are spatially close to, first shell residues. Generally, first shell residues play essential roles in enzyme activity. The AATase second shell residues that are important for activity include His143 (Yano et al. 1991) and Cys191 (Gloss et al. 1996; Jeffery et al. 2000). The side chains of these two amino acids interact directly with first shell residues Tyr225 and Asp222, respectively.

The side chains of Lys68* and Glu265 form the only intersubunit salt bridge situated in the second shell of eAATase, which is highly conserved among AATases. Adjacent to Lys68*:Glu265 are first shell active-site residues Arg266 and Tyr70*, both of which make important contacts with the phosphate moiety of PLP (Scheme 1). The Pm and Ac AATases are the only isoforms that have different pairs of amino acids at positions 68* and 265. These are Met68*:Gln265 and His68*:Glu265, respectively. Evolutionary analysis points to Lys68*:Glu265 as the extant pair in the ancestral AATase. Other second shell residues vary in an exclusive manner when the Lys68*:Glu265 pair is replaced in natural variants.

The importance of the Lys68*:Glu265 salt bridge on eAATase kinetics was explored by mutagenic replacements via two double-mutant cycles leading ultimately to the charge-inverted configuration K68E/E265K and to the neu-

Scheme 1. Partial active site of eAATase. The first shell active site residues Tyr70*, Thr109, Asp222, Tyr225, Lys258, and Arg266 make important interactions with the bound PLP while two of the second shell residues, Lys68* and Glu265, form an intersubunit salt bridge. Residues from the opposite subunit are separated by the arc.

tral pair K68M/E265Q. It is shown that the information gleaned from double-mutant cycles can be significantly enhanced by incorporating the impact (*I*) and context dependence (*C*) parameters, recently introduced for the quantitative analysis of functional chimeras (Luong and Kirsch 2001).

Results and Discussion

Theory

Chimeric constructs have proven to be powerful instruments for understanding component contributions to higher order assemblies in biology (Henzl et al. 1998; Smith et al. 1998). A chimera is constructed by judicious substitution within homologous biological macromolecules or assemblies. Scheme 2 (left) illustrates the construction of two functional chimeras. The changes in any addressable parameter caused by transforming the specie *X* into *Y* ($X \rightarrow Y$) is reflected in the values of $\Delta\Delta G$ defined by equation 3.

$$\Delta\Delta G_{X \rightarrow Y}^{param.} = -RT \ln \left(\frac{param.^Y}{param.^X} \right) \quad (3)$$

It was shown recently that functional chimeras may be analyzed quantitatively with the parameters of context depen-

Scheme 2. Chimeras and double-mutant cycles. Chimeras (*left*): A and B represent homologous biological assemblies, for example, proteins, protein/protein, or protein/nucleic acid complexes. The white areas represent fields of identity, whereas the dotted and solid gray squares show regions that differ. A chimera is constructed by substitution of a piece of A (S or R) with the corresponding part of B (upper arrows), whereas the retro-chimera is formed by the replacement of the indicated section of B with that of A (lower arrows). The $\Delta\Delta G$ values reflect the change imposed on any addressable parameter, such as ligand affinity or a kinetic constant (Luong and Kirsch 2001). The illustrated substitutions yield the four hybrid species, shown in the middle of the left side. It is not possible to convert A to B without additional substitution, for example, substructure T. Double-mutant cycles (*right*) are limiting examples of chimeras in the sense that there are only two amino acid differences between the WT and the double mutant DM. The DM, in general, would not be a naturally occurring protein. WT is converted to DM with two mutations. Note that the directions of the lower sets of arrows are reversed in the left- and right-hand figures, reflecting the historical treatment of double-mutant cycles.

dence (C) and impact (I) defined in equations 4 and 5 (Luong and Kirsch 2001).

$$C = \Delta\Delta G_{S_{A \rightarrow B}} + \Delta\Delta G_{S_{B \rightarrow A}} \quad (4)$$

$$I = \Delta\Delta G_{S_{A \rightarrow B}} - \Delta\Delta G_{S_{B \rightarrow A}} \quad (5)$$

C is a measure of the interaction of the replaced region with its surroundings. Its value approaches zero where the transformations are context independent. I is a quantitative measure of the importance of this region in the probed parameter. Qualitatively, four combinations of I and C are possible (large I , small C ; large I , large C ; small I , small C , and small I , large C). The interpretation for each possibility has been described previously (Luong and Kirsch 2001). For example, large I and small C values show that the probed position is quantitatively important for the addressed function and that that substitution is context independent.

The I values (equation 5) evaluate the absolute free energy differences elicited by chimeric replacements by subtracting $\Delta\Delta G$ for the effect of a forward and reverse replacement set on a given parameter. It is, however, sometimes useful to compare I values from different chimeras. This can be accomplished by normalization according to equation 6.

$$I_N = I_i / I_T \quad (6)$$

where I_N is the normalized impact value, I_i is the impact value for any given replacement and I_T would express the total $\Delta\Delta G$ differentiating the two natural forms, A versus B in Scheme 2 (left). Although I_i values will generally be smaller than I_T , they may approach or even exceed the latter value if the context dependence C_i for that replacement set is large.

In the specific case where A and B differ by only two amino acids, the construct of chimeras is reduced to a

double-mutant cycle (Scheme 2, right). The traditional analysis of double-mutant cycles defines the mutations as proceeding vectorially from the wild type (WT) to the double mutant. To apply the parameters of context dependence and impact it is therefore necessary to reverse the signs of $\Delta\Delta G_{\text{RS} \rightarrow \text{RS}}$ and $\Delta\Delta G_{\text{RS} \rightarrow \text{RS}}$. Thus, the expressions for C and I as applied to a double-mutant cycle becomes

$$C_{(\text{S} \rightarrow \text{S})} = \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} + (-\Delta\Delta G_{\text{RS} \rightarrow \text{RS}}) \quad (7)$$

$$I_{(\text{S} \rightarrow \text{S})} = \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} - (-\Delta\Delta G_{\text{RS} \rightarrow \text{RS}}) \quad (8)$$

Double-mutant cycle analysis is used to measure the effect of the interaction between two residues on a given parameter. A quantitative value of these interactions is given in terms of variation in free energy, $\Delta\Delta G_{\text{int}}$ (Ackers and Smith 1985; Horovitz 1987, 1996; Pons et al. 1999). The constraints of the thermodynamic cycles (Scheme 2) lead to two equivalent expressions for $\Delta\Delta G_{\text{int}}$, both of which are found in the literature.

$$\Delta\Delta G_{\text{int}} = \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} + \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} - \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} \quad (9)$$

$$\begin{aligned} \Delta\Delta G_{\text{int}} &= \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} - \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} \\ &= \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} - \Delta\Delta G_{\text{RS} \rightarrow \text{RS}} \end{aligned} \quad (10)$$

Equation 9 defines $\Delta\Delta G_{\text{int}}$ as the difference between the sum of the $\Delta\Delta G$ values for the single mutations and the double mutation. It follows from equations 7 and 10 that $\Delta\Delta G_{\text{int}} = C$ and that $C_{(\text{S} \rightarrow \text{S})} = C_{(\text{R} \rightarrow \text{R})}$, but $I_{(\text{S} \rightarrow \text{S})}$ will generally not be equal to $I_{(\text{R} \rightarrow \text{R})}$. The signs of the quantities C and I provide additional information. For example, where C and I have opposite signs the second mutation added to the first has a larger effect than when it is introduced in the WT framework (equations 7 and 8).

Steady-state kinetics

The six constructed eAATase mutants were designed to probe the catalytic importance of the intersubunit salt bridge that exists between Glu265 and Lys68* in the second shell of most AATase active sites (Scheme 1). The distance between the ϵ -NH₂ group of Lys68* and the carboxylate side chain of Glu265 is 2.9 Å in the unliganded eAATase structure (PDB Id: 1ASN), otherwise known as the open conformation. This distance is not appreciably different from that seen in the liganded (PDB Id: 1ASM), or closed conformation, indicating that this salt bridge is not one of the amino acid side-chain interactions that is rearranged as a consequence of ligand association.

The steady-state kinetic parameters for each of the six eAATase salt bridge mutations are shown in Table 1. The $\Delta\Delta G^\ddagger$ values represented in Figure 1 reflect the changes in $k_{\text{cat}}/K_{\text{M}, \alpha\text{-KG}}$, $k_{\text{cat}}/K_{\text{M}, \text{L-Asp}}$, and k_{cat} between the mutants and the WT. The bar graph shows that although the mutations cause differential effects on each of the kinetic parameters, they generally follow the same trend. Mutations at position 68 (K68M and K68E) substantially decrease the values of $k_{\text{cat}}/K_{\text{M}, \alpha\text{-KG}}$, $k_{\text{cat}}/K_{\text{M}, \text{L-Asp}}$, and k_{cat} , whereas the mutations E265Q and K68M/E265Q perturb the kinetic parameters to a much lesser extent. The inset of Figure 1 shows that the logarithms of $k_{\text{cat}}/K_{\text{M}, \alpha\text{-KG}}$ versus $k_{\text{cat}}/K_{\text{M}, \text{L-Asp}}$, normalized by the WT values, yield a line of slope 1.8 ± 0.2 . The linearity of the plot is artificially enhanced by the autocorrelation of $k_{\text{cat}}/K_{\text{M}, \alpha\text{-KG}}$ with $k_{\text{cat}}/K_{\text{M}, \text{L-Asp}}$ because all of the kinetic constants were obtained from the same set of data (Estell 1987). Nonetheless, it is clear that the $\Delta\Delta G^\ddagger$ for the keto-acid half reaction is 1.8 times more sensitive to the salt-bridge disruption than is the amino acid half reaction. The value of K_{half} for equation 2 is given by

$$K_{\text{half}} = \frac{[\text{L} - \text{Glu}] \cdot [\text{Enz} - \text{PLP}]}{[\alpha - \text{KG}] \cdot [\text{Enz} - \text{PMP}]} \quad (11)$$

Table 1. Steady-state kinetic parameters for WT and eAATases with the L-Asp/ α -KG substrate pair

Enzyme	k_{cat} (s ⁻¹)	$k_{\text{cat}}/K_{\text{M}, \text{L-Asp}}$ (M ⁻¹ s ⁻¹ × 10 ³)	$k_{\text{cat}}/K_{\text{M}, \alpha\text{-KG}}$ (M ⁻¹ s ⁻¹ × 10 ³)	$K_{\text{M}, \text{L-Asp}}$ (mM)	$K_{\text{M}, \alpha\text{-KG}}$ (mM)
WT	259 (8)	133 (8)	442 (31)	1.9 (0.2)	0.59 (0.05)
K68E	19.9 (0.6)	14.8 (0.5)	5.3 (0.2)	1.34 (0.06)	3.7 (0.2)
E265K	11.2 (0.5)	48 (4)	50 (4)	0.24 (0.03)	0.23 (0.03)
K68E/E265K	19.9 (0.5)	89 (4)	82 (9)	0.22 (0.02)	0.24 (0.03)
K68M	18.4 (0.6)	32 (2)	18 (1)	0.58 (0.04)	1.05 (0.07)
E265Q	81 (2)	153 (10)	405 (31)	0.53 (0.02)	0.20 (0.01)
K68M/E265Q	75 (5)	102 (6)	240 (14)	0.73 (0.08)	0.31 (0.03)

Conditions: 200 mM TAPS-KOH (pH 8.4), 100 mM KCl, 20 μ M PLP, 150 μ M NADH, 8 units/mL MDH, 25°C. The AATase activity was measured by coupling OAA formation to MDH. Standard errors are given in parentheses. (WT) wild type; (eAATase) *E. coli* aspartate aminotransferase.

Fig. 1. Effect of single and double mutations in the Lys68*:Glu265 salt bridge on the kinetic parameters of *E. coli* aspartate aminotransferase (eAATase). The values are represented in terms of $\Delta\Delta G_{WT \rightarrow mutant}^\ddagger$ (equation 3). $k_{cat}/K_{M, \alpha-KG}$ (solid white), $k_{cat}/K_{M, L-Asp}$ (solid black), and k_{cat} (dotted). The numerical values are given in Figure 2. The standard errors for each determination are ≤ 0.08 kcal/mole. The inset shows the correlation of the effects on the normalized values of $\log k_{cat}/K_{M, L-Asp}$ with those on $\log k_{cat}/K_{M, \alpha-KG}$ for the six intersubunit salt-bridge mutations. The slope of the line is 1.8 ± 0.2 ($r = 0.95$).

This quantity is decreased by salt-bridge mutations, therefore they stabilize the pyridoxamine phosphate (PMP) relative to the PLP forms of the enzyme. The reasons for this are unknown.

Kinetic analysis of salt-bridge mutations that result in buried-single or double-like charges

Generally, mutations that reverse one or both charges of the intersubunit salt bridge elicit larger effects on the kinetic parameters, $k_{cat}/K_{M, \alpha-KG}$, $k_{cat}/K_{M, L-Asp}$, and k_{cat} , than those at the same locus that neutralize the salt-bridge charges (K68E > K68M, E265K > E265Q, and K68E/E265K > K68M/E265Q). For example, the values of $k_{cat}/K_{M, \alpha-KG}$ for the mutants K68E and E265K are 3.4- and 8.1-fold lower than those shown by K68M and E265Q, respectively, showing that charge reversal for this parameter is more deleterious than a single-charge burial. The value of k_{cat} for the E265K mutant is similarly 7.2-fold lower than it is for E265Q. However, the k_{cat} for K68M and K68E are the same within experimental error. Mutations that result in the burial of one or two negative charges, K68M and K68E, generally reduce the values of the kinetic parameters by larger factors

than do mutations that sequester single or double positive charges, E265Q and E265K (Fig. 1; Table 1).

Double-mutant cycles

Double-mutant cycles are used to extract the specific free energy of interaction between two residues of a protein or protein/protein interaction (Theory section). The two double-mutant cycles evaluated here are presented in Figure 2. The changes in $k_{cat}/K_{M, \alpha-KG}$, $k_{cat}/K_{M, L-Asp}$, and k_{cat} resulting from the mutations at position 68 and/or 265 are expressed in terms of $\Delta\Delta G^\ddagger$ according to equation 3. Decreases and increases in the parameter values are indicated by positive and negative values of $\Delta\Delta G^\ddagger$, respectively. Charge reversal or neutralization of the negative charge present at position 265 in the single mutants K68E or K68M generally leads to a 53% to 81% recovery of the $\Delta\Delta G^\ddagger$ values associated with the kinetic parameters. Conversely, the addition of K68M to the E265Q mutant does not rescue activity, showing further that in this system a buried positive charge is less deleterious than a buried negative charge.

These data show that the intersubunit salt bridge Lys68*:Glu265 can be substituted with the two uncharged residues present in plant mitochondrial AATases at these

Fig. 2. Double-mutant cycle analysis of the Lys68*:Glu265 salt bridge on the kinetic parameters $k_{\text{cat}}/K_{\text{M, L-Asp}}$ (dotted arrows), $k_{\text{cat}}/K_{\text{M, } \alpha\text{-KG}}$ (solid arrows), and k_{cat} (dashed arrows). Charge-to-charge and charge-to-neutral replacement mutations are in the upper and lower sections of the figure, respectively. The numbers and their associated standard errors (in parentheses) represent the changes in free energies of activation ($\Delta\Delta G^\ddagger$) for the specified kinetic parameters.

positions (Met68*:Glu265) (Fig. 3) with relatively minor consequences ($\Delta\Delta G^\ddagger$ 0.74 kcal/mole). Moreover, K68M/E265Q has less of an effect on the kinetic parameters than K68E/E265K, which restores the interactions between these two residues. This set of observations shows qualitatively that the residues at positions 68 and 265 make important interactions with their surroundings in the WT enzyme. Evaluation of C and I values quantifies these interactions.

The high values of C (>1 kcal/mole) of the two double-mutant cycles studied (Fig. 4) reflect the importance of the salt-bridge Lys68*:Glu265 interactions in eAATase catalytic activity. The consequences of introducing oppositely charged mutations at these positions are on average 0.9 kcal/mole greater than those affected by charge-neutralization mutations.

Impact

The data reported here show that the integrity of the Lys68*:Glu265 salt bridge, which is in the second shell of the active site (Scheme 1), is an important determinant of catalytic activity. The I values (equation 8) for $k_{\text{cat}}/K_{\text{M, } \alpha\text{-KG}}$,

$k_{\text{cat}}/K_{\text{M, L-Asp}}$, and k_{cat} provide a quantitative measure of the importance of each mutation on the individual kinetic parameters. The signs of the I values (Fig. 4, bottom) for the mutations at position 68 and 265 show that burying negative charges (I_{K68E} and I_{K68M}) decreases the activity of the enzyme ($I > 0$), while shielding those negative charges (I_{E265K} and I_{E265Q}) rescues $k_{\text{cat}}/K_{\text{M}}$ for both substrates ($I < 0$). Therefore, Lys68* is a more important determinant for $k_{\text{cat}}/K_{\text{M, } \alpha\text{-KG}}$ and $k_{\text{cat}}/K_{\text{M, L-Asp}}$ than is Glu265. The value of I_{E265K} for k_{cat} clearly indicates that formation of an oppositely charged salt bridge is insufficient to rescue the catalytic activity of the K68E mutation for this kinetic parameter. The large difference in the values of I_{E265K} versus I_{E265Q} for k_{cat} shows that this parameter is relatively insensitive to the neutralization of the Glu265 negative charge but is very sensitive to the presence of a positive charge at position 265.

Are there forced covariances caused by replacements in the 68:265 pair?*

A multiple sequence alignment of 32 AATases from different organisms (Fig. 3) shows that the intersubunit salt bridge

Fig. 3. Partial primary sequence alignment of aspartate aminotransferases (AATases) from different organisms. Lys68* region (left) and Glu265 region (right). Cytosolic (c), mitochondrial (m), and chloroplast (cl). The arrows show the positions of the intersubunit salt-bridge residues Lys68* and Glu265 as well as first shell active site residues Tyr70*, Lys258, and Arg266. The alignment was produced with PileUp using the default settings within GCG. The protein identification numbers (PubMed PID) for each sequence are given in Figure 5.

Lys68*:Glu265 is conserved in 70% of the sequences. The phylogram constructed from this alignment (Fig. 5) groups AATases into five main branches: bacterial, animal cytosolic (Ac), plant cytosolic, plant mitochondrial (Pm), and animal mitochondrial. Only the Pm (Met68*:Gln265) and Ac (His68*:Glu265) groups do not conserve the Lys68*:Glu265 salt bridge. Although the tree does not differentiate between His and Lys as the amino acid present at position 68 in the ancestral AATase, it eliminates Met68* as the progenitor, because (Lys, His)68* has deeper roots. Glu is

the ancestral residue at position 265 for the same reason. The experiments described here show that the Lys68*:Glu265 ion pair can be replaced with Met68*:Gln265 with relatively small (two- to threefold) effects on the principal kinetic parameters associated with the AATase reaction. It is not known if these in vitro differences (Table 1) are of functional consequence in vivo. In other words, did the evolutionary changes that occurred at positions 68* and 265 occur as independent events or were additional mutations required to maintain the fitness of AATase in vivo? This

Fig. 4. Context dependence (C , top) (equation 7) and impact (I , bottom) (equation 8) of substitutions in the intersubunit salt-bridge Lys68*:Glu265 of eAATase. The standard errors for each determination are ≤ 0.1 kcal/mole. The left- and right-hand sides of the figure correspond to the upper and lower sections of Figure 2, respectively.

question can be addressed by examining if the natural variants occur as similarly isolated pair replacements or as coordinated changes with other amino-acid substitutions. The evolutionary relationships between Csb, Pm, and Ac AATases are conveniently visualized in the Venn diagram of Figure 6. A total of 74 residues are invariant among them; thus, their function is independent of the nature of the 68*:265 interface. However, six residues are conserved only in the Csb and Ac class (Leu35 through Val273) and another eight are constant in the Csb and Pm group (Asp15 through Glu322). Finally, a total of 20 amino acids shown in the white areas of Figure 6 are unique to a single classification. Because there are no common covariant residues that are unique to the Pm and Ac class (Fig. 6), it is likely that Lys68* rather than His68* was present in the ancestral AATase.

Replacement of the Lys68*:Glu265 pair with either of the natural substitutions is accompanied by a total of 13 covariant changes (Val15 through Asn322 in the Ac Group and Val35 through Leu273 in the Pm Group). A 14th residue Ile291 (Csb) changes to Ala291 (Pm) or Val291 (Ac). Twelve of these covariant amino acids are located in regions of the AATase three-dimensional structure that might be expected to influence the catalytic properties of the enzyme. Three of these, at positions 56, 147, and 295, are in the subunit interface; seven, at positions 15, 17, 18, 73, 112, 291, and 295, are within 4 Å of a first shell active site residue; and five, at positions 15, 17, 18, 19, and 35, are part of the N-terminal portion of the mobile small domain. Additionally, Asp15 and residues adjacent to position 322 (323–325) are thought to fulfill key functions in the rotation of the small domain that occurs on substrate binding (Jager et al. 1994; Jensen and Gu 1996; Mizuguchi et al. 2001; Picot et al. 1991).

It is important to emphasize that neither of the two Csb replacements, Pm or Ac, creates a buried charge (His68* potentially forms a salt bridge with Glu265). These observations are in accord with the mutagenesis studies that show clearly that buried charges in the 68*:265 locus are deleterious to the kinetic parameters (see above). Mutations that are singly detrimental but restore normal fitness in combination have been referred to as compensatory neutral mutations (Kimura 1985). At least two base changes are required to convert Lys68*:Glu265 to Met68*:Gln265. Given that none of the possible single substitution evolutionary intermediates between Lys68*:Glu265 and Met68*:Gln265 are found in nature; the substitutions at the 68*:265 pair appear to have arisen from compensatory neutral mutations. Malcolm et al. 1990 described a similar set of observations for a triplet of amino acids in game-bird lysozymes that lie together in the three-dimensional structure just beneath the active site cleft. Only two triplets are found in nature (Thr40, Ile55, Ser91 and Ser40, Val55, Thr91). Constructed proteins with all six possible intermediates in the three-step evolutionary pathway between these two triplets differed substantially from either WT enzyme in their thermostabilities, indicating that the triplet residues also represent compensatory neutral mutations and are thus forced covariants.

Although it is shown here that the mutation of Lys68*:Glu265 to Met68*:Gln265 has a small effect on the kinetics, the covariance analysis from Figure 6, which shows a number of additional accompanying substitutions and indicates strongly that these changes occurring naturally in isolation would result in an enzyme that is in some additional way suboptimal (e.g., lowered affinity for PLP or decreased stability). The nature of the selective pressure that forced these covariances and the order by which the successive mutations took place can not presently be decided. Suppose for example that Met68*:Gln265 arose in the Pm AATases in

Fig. 5. Phylogram of AATases. Cytosolic (c), mitochondrial (m), chloroplast (cl). The tree was built from the multiple sequence alignment of Figure 3 with the application PAUP using a branch-and-bound tree search method, parsimony optimality criterion, and midpoint method for rooting. PubMed PID numbers are given in parentheses. Residues in proteins that replace one or both components of the generally conserved Lys68*:Glu265 salt bridge are in bold type.

advance of the other covariant changes. This may have provided some selective overall advantage, but as judged from the extant evolutionary record, this would have been overall suboptimal. Therefore, further compensatory amino-acid replacements would have been forced. They would not occur at residues that interact directly with substrates or PLP (first shell) because these were already optimized. The rescuing substitutions would be expected at second shell residues if improvements in catalytic activity were required or in less well-defined regions to improve stability.

Materials and methods

Site-directed mutagenesis

Mutations were introduced by recombinant polymerase chain reaction (PCR) (Higuchi 1990). The entire eAATase open reading frame and 151 bp of the eAATase promoter region were amplified in the final PCR reaction, which introduces the coding sequences for a 6-histidine tag (His₆-tag) at the carboxy-terminus of eAATase followed by a primer-derived *Bam*HI restriction site. The 5' primer used in the final PCR reaction spans the endogenous

Fig. 6. Venn diagram demonstrating the covariant residue changes accompanying natural substitutions in the Lys68*:Glu265 salt bridge. The solid, dashed, and dotted ellipses describe, respectively, those amino acids that covary with AATases having (a) a conserved salt bridge (Csb, Lys68*:Glu265), (b) the animal cytosolic enzymes (Ac, His68*:Glu265), and (c) the plant mitochondrial AATases (Pm, Met68*:Gln265). The phylogram of Figure 5 identifies the individual AATase isoforms that were compared. The amino acids indicated in the white areas for each AATase group are those that covary with the residues at positions 68 and 265 and are not shared by the other two groups. For example, Val35 and Met73 are found only in the Pm group and thus covary with Met68*:Gln265. The lightly shaded areas indicate shared covariant residues between two groups that differ in the third. For example, Asp15 and Ile17 are present only in the intersection of the Csb and Pm sets ($Csb \cap Pm$). The heavily shaded area demarks the 74 residues that are conserved among all three groups ($Csb \cap Pm \cap Ac$). Four Pm, six Ac, and 22 Csb sequences are available. The criteria for including a given amino acid in Figure 6 are that there be no more than three conservative changes or one dissimilar change in the 22 Csb isoforms and no substitutions in the other two groups. There are a large number of additional residues that are conserved in one or two of the classes but show significant variability in the other two or one, respectively. These were omitted from the diagram for space considerations.

EcoRI restriction site within the eAATase promoter. The amplification products were digested with *EcoRI* and *BamHI* and cloned into the corresponding sites of pBluescriptII KS+ (Stratagene) to generate plasmids pKS + AAT, pKS + K68E, pKS + K68M, pKS + E265K, and pKS + E265Q, and the double-mutant plasmids pKS + K68E/E265K and pKS + K68M/E265Q.

Expression and purification

WT and mutant eAATase expression plasmids were introduced into *E. coli* strain MG204, which lacks endogenous AATase activity. The growth conditions for the overexpression of eAATase were essentially as described previously (Onuffer and Kirsch 1994). Cells were harvested by centrifugation at $6000 \times g$ for 15 min. The cell pellet was suspended in 20 mM potassium phosphate, pH 7.2, 5% glycerol, 300 mM KCl, 20 μ M PLP, 10 mM imidazole, 1 mg/mL lysozyme, 2 mM β -mercaptoethanol, and Roche Complete protease inhibitors (Roche Molecular Biochemicals), followed by incubation at 0°C for 20 min. Cell disruption

was performed with a Bead-Beater (Biospec Products Inc.) with 0.1-mm glass beads. Cell debris were removed by centrifugation at $35,000 \times g$ for 30 min.

The recombinant enzymes were purified by a single chromatographic step using Ni-NTA Superflow resin (Qiagen). The column was loaded with the bacterial cell lysate and the nonadherent proteins were removed by rinsing with 20 volumes of wash buffer (20 mM potassium phosphate, pH 7.2, 5% glycerol, 300 mM KCl, 20 μ M PLP, and 10 mM imidazole). The proteins were eluted with a gradient of 10–500 mM imidazole in wash buffer. The purified enzymes were stored at 4°C after dialysis against wash buffer lacking imidazole.

Steady-state enzyme assay

The activity of eAATase with the L-Asp/ α -KG substrate pair was measured by coupling OAA production to the malate dehydrogenase (MDH) reaction (Cronin and Kirsch 1988; Velick and Vavra 1962). The decrease in absorbance at 340 nm was monitored with

a Perkin Elmer Lambda 4B spectrophotometer. Assay conditions were 200 mM TAPS-KOH, pH 8.4, 100 mM KCl, 20 μ M PLP, 150 μ M NADH, 8 units/mL MDH, $0.25\text{--}10 \times K_{M, L-Asp}$ L-Asp, and $0.25\text{--}10 \times K_{M, \alpha-KG}$ α -KG and 25°C. eAATase concentrations were from 5- to 100-nM dependent on the activity of the individual variants. The Michaelis-Menten parameters were determined from a matrix of initial rates obtained from six concentrations of each substrate. These data were fit by nonlinear regression with the NLIN program of the SAS statistical package (SAS Institute) to equations 12–14 for a ping-pong bi bi reaction mechanism (Velick and Vavra 1962). k_{cat} , $K_{M, L-Asp}$, and $K_{M, \alpha-KG}$ were obtained from equation 12, and $k_{cat}/K_{M, L-Asp}$ and $k_{cat}/K_{M, \alpha-KG}$ from equations 13 and 14, respectively.

$$\frac{v}{[E]_T} = \frac{k_{cat}[L-Asp][\alpha-KG]}{K_{M, L-Asp}[\alpha-KG] + K_{M, \alpha-KG}[L-Asp] + [L-Asp][\alpha-KG]} \quad (12)$$

$$\frac{v}{[E]_T} = \frac{(k_{cat}/K_{M, L-Asp})[L-Asp][\alpha-KG]}{[\alpha-KG] + \left(\frac{K_{M, \alpha-KG}}{K_{M, L-Asp}}\right)[L-Asp] + \left(\frac{1}{K_{M, L-Asp}}\right)[L-Asp][\alpha-KG]} \quad (13)$$

$$\frac{v}{[E]_T} = \frac{(k_{cat}/K_{M, \alpha-KG})[L-Asp][\alpha-KG]}{\left(\frac{K_{M, L-Asp}}{K_{M, \alpha-KG}}\right)[\alpha-KG] + [L-Asp] + \left(\frac{1}{K_{M, \alpha-KG}}\right)[L-Asp][\alpha-KG]} \quad (14)$$

The catalytic activity of each eAATase variant was found to be proportional to the concentration of enzyme from 5 to 200 nM. The optimal pH for each mutant was unchanged from that of WT (8.4) (Kuramitsu et al. 1990). Variations of the PLP concentration from 0 to 30 μ M in the assay buffer did not influence the catalytic activity of any variant. The carboxy-terminal His₆-tag on WT eAATase does not significantly affect its catalytic properties.

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