The Ligand-induced Structural Changes of Human L-Arginine:Glycine Amidinotransferase

A MUTATIONAL AND CRYSTALLOGRAPHIC STUDY*

Erich Fritsche‡, Andreas Humm, and Robert Huber

From the Department of Structural Research, Max Planck Institute for Biochemistry, Abteilung Strukturforschung, Am Klopferspitz 18a, D-82152 Martinsried, Germany

Human L-arginine:glycine amidinotransferase (AT) shows large structural changes of the 300-flap and of helix H9 upon binding of L-arginine and L-ornithine, described as a closed and an open conformation (Humm, A., Fritsche, E., Steinbacher, S., and Huber, R. (1997) EMBO J. 16, 3373–3385). To elucidate the structural basis of these induced-fit movements, the x-ray structures of AT in complex with the amidino acceptor glycine and its analogs γ-aminobutyric acid and δ-aminovaleric acid, as well as in complex with the amidino donor analogs L-alanine, L-α-aminobutyric acid, and L-norvaline, have been solved at 2.6-, 2.5-, 2.37-, 2.3-, 2.5-, and 2.4-Å resolutions, respectively. The latter three compounds were found to stabilize the open conformer. The glycine analogs bind in a distinct manner and do not induce the transition to the open state. The complex with glycine revealed a third binding mode, reflecting the rather broad substrate specificity of AT. These findings identified a role for the α-amino group of the ligand in stabilizing the open conformer. The kinetic, structural, and thermodynamic properties of the mutants ATΔM302 and ATΔ11 (lacks 11 residues of H9) confirmed the key role of Asn300 and suggest that in mammalian amidinotransferases, the role of helix H9 is in accelerating amidino transfer by an induced-fit mechanism. Helix H9 does not add to the stability of the protein.

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‡ To whom correspondence should be addressed. Tel.: 49-898578-2660; Fax: 49-898578-5165; E-mail: fritsche@biochem.mpg.de.

The aim of the work described here was to establish a role for the

of AT (1), which catalyzes the rate-limiting step of creatine biosynthesis: the transfer of an amidino group from L-arginine to glycine, resulting in L-ornithine and guanidinoacetic acid, the immediate precursor of creatine. AT is regulated in a variety of ways, including product inhibition of its catalytic activity by ornithine (5), end product repression of its synthesis at a pretranslational level by creatine (6–10), induction by growth hormone and thyroxine (9, 11), and repression during embryonic development (12–14). Deficiencies of L-ornithine:2-oxoacid amidotransferase (EC 2.6.1.13) result in hyperornithinemia (15), which is associated with type II muscle fiber atrophy and gyrate atrophy of the choroid and retina, a disease that progressively leads to blindness (16). Because ornithine is a strong competitive inhibitor of AT, its 10–20-fold increased plasma concentration in hyperornithinemia results in inhibition of AT, suggesting impaired de novo creatine biosynthesis as the cause of the aforementioned pathogenesis of the eye and muscle (16, 17).

AT exhibits a rather broad substrate specificity and can utilize a wide variety of amidino donors, such as L-canavanine, 4-guanidinobutyrate, 3-guanidinopropionate, and hydroxyguanidine, and amidino acceptors, such as L-canaline, 4-aminobutyrate, 3-aminopropionate, and hydroxylamine, in addition to the physiological substrates (1). Biochemical data suggested a ping-pong mechanism for the transamidination reaction (18, 19), including a transient covalent attachment of an amidino group to Cys407 (20–22). The crystal structure of human AT at high resolution revealed that the nucleophilic Cys407 is located at the bottom of an narrow active site channel (23). The enzyme adopts open and closed conformations during the reaction cycle. The crystal structures of AT in complex with L-arginine and L-ornithine showed that binding is accompanied by large conformational changes, with shifts of up to 5 Å for the 300-flap and for helix H9 compared with the unliganded enzyme (Fig. 1) (23). However, highly homologous amidinotransferases are also found in certain strains of Streptomyces sp., in which they catalyze two transamidination reactions in the biosynthesis of the streptomyacin family of antibiotics (24–27). These amidinotransferases lack the flexible helix H9 in human AT as the most prominent difference, indicating distinctly different ligand and intermediate binding. The crystal structure of L-arginine:inosamine-phosphate amidotransferase StrB1 from Streptomyces griseus (StrB1) was solved recently at 3.1 Å (28) and revealed a fold closely related to AT. However, major changes were found in loops surrounding the active site, resulting in an open and solvent exposed cavity. These differences can be largely attributed to the deletion of AT helix H9 and to the deletion of one residue in the AT 300-loop. The conformation of the 300-loop implies that StrB1, in contrast to AT, adopts a single conformation during catalysis.

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the induced-fit movements of AT and in particular for AT helix H9. In addition, the properties of a ligand molecule inducing the large conformational changes upon binding should be clarified. The crystal structures of AT in complex with the amido acceptor analogs γ-amino butyric acid and δ-aminovaleric acid on the one hand, and the amido donor analogs l-alanine, L-α-aminobutyric acid, and l-norvaline on the other hand (Fig. 2), revealed two distinct binding modes. A third binding mode was found for glycine, reflecting the rather broad substrate specificity of AT. The ability of these compounds to trigger the induced-fit movements of AT and in particular for AT helix H9 is de-luted mass spectra revealed major peaks for wild-type AT of 45,812 Da (electrospray ionization MS, calculated mass, 45,777 Da), for ATΔM302 of 45,659 Da (electrospray ionization MS, calculated mass, 45,646 Da), for ATΔM302 of 44,540 Da (matrix assisted laser desorption/ionization MS, calculated mass, 44,491 Da), and for StrB1 of 40,170 Da (electrospray ionization MS, calculated mass, 40,163 Da). The yields of electropherotically homogeneous protein per liter of culture were 2–3 mg for AT and ATΔM302 and 0.2–1 mg for ATΔ11 and StrB1.

**TABLE I**

| Crystal | GLY | GABA | DAVA | ALA | AABA | NOR | AT ΔM302 |
|---------|-----|------|------|-----|------|-----|---------|
| a (%)   | 2.6 | 2.5  | 2.37 | 2.3 | 2.5  | 2.4 | 2.9     |
| c (%)   | 83.69 | 83.62 | 83.73 | 83.76 | 83.81 | 83.80 | 83.71 |
| Total reflections | 200.33 | 200.38 | 200.16 | 199.95 | 199.71 | 199.60 | 200.41 |
| Unique reflections | 71864 | 73220 | 113088 | 97489 | 117304 | 109575 | 73369 |
| Completeness (%) | 93.7 | 98.5 | 90.4 | 91.6 | 99.5 | 98.6 | 97.7 |
| Last shell | 93.5 | 90.7 | 89.4 | 97.7 | 99.9 | 96.2 | 98.8 |
| Last shell | 11.5 | 8.2 | 10.5 | 8.4 | 8.4 | 9.5 | 10.4 |
| Bond length (Å) | 33.1 | 35.0 | 27.6 | 33.2 | 34.5 | 35.3 | 36.5 |

| Crystal | GLY | GABA | DAVA | ALA | AABA | NOR | AT ΔM302 |
|---------|-----|------|------|-----|------|-----|---------|
| a (%)   | 16.7 | 16.4 | 17.9 | 17.6 | 17.8 | 17.7 | 17.3 |
| c (%)   | 2938 | 2938 | 2938 | 2938 | 2938 | 2938 | 2938 |
| Total reflections | 20118 | 21443 | 25723 | 28573 | 24436 | 27030 | 15236 |
| Unique reflections | 145 | 144 | 125 | 128 | 114 | 133 | 51 |
| Root mean square deviation from ideal geometry | 0.011 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 |
| Bond angle (°) | 1.57 | 1.56 | 1.58 | 1.61 | 1.62 | 1.61 | 1.57 |

| Crystal | GLY | GABA | DAVA | ALA | AABA | NOR | AT ΔM302 |
|---------|-----|------|------|-----|------|-----|---------|
| a (%)   | 140 | 140 | 140 | 140 | 140 | 140 | 140 |
| c (%)   | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Total reflections | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Unique reflections | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Completeness (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Last shell | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Last shell | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Bond length (Å) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Bond angle (°) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

*AT ΔM302, AT deletion mutant ΔM302. Complexes were obtained by soaking crystals in harvesting buffer (20% polyethylene glycol 6000, 267 mm HEPES-NaOH, pH 7.0) containing the corresponding ligands. GLY, 0.5 mM glycine, 16 h; GABA, 0.1 mM γ-amino butyric acid, 50 mM; DAVA, 1.0 mM γ-aminovaleric acid, 32 h; ALA, 0.5 mM l-alanine, 40 h; AABA, 0.5 mM l-α-aminobutyric acid, 15 h; NOR, saturated l-norvaline, 24 h. 

**Protein Determination and Activity Measurements**—The protein concentration was determined spectrophotometrically using a specific absorption coefficient at 280 nm of 2.0 cm^2 mg^-1 for AT and its variants, and 1.46 cm^2 mg^-1 for StrB1. The activity of AT and StrB1 was measured by the method of Van Fossen et al. (30), by incubating the enzyme with 20 mM l-arginine and 20 mM of glycin or hydroxylysine, as amido donor and acceptor, respectively, and determining the formed l-ornithine by a ninhydrin color reaction. One enzyme unit is defined as the amount of enzyme that releases 1 μmol of l-ornithine/h at 37 °C.

**Crystallization**—The purified proteins were concentrated by ultracentrifugation (Centrifuge-30, Amicon) to approximately 13 mg/ml. Crystals of AT and ATΔM302 were grown by vapor diffusion using the hanging drop method. The droplets were made from 7 μl of a protein solution (13 mg/ml in 30 mM HEPES-NaOH, 0.5 mM EDTA, 0.5 mM GSH) and 14 μl of a reservoir solution (3% (w/v) polyethylene glycol 6000, 40 mM HEPES-NaOH, pH 7.0) and equilibrated against the reservoir solution. Tetragonal-bipyramidal-shaped crystals of AT (approximately 0.7 × 0.4 × 0.4 mm) appeared after 2 days. Crystals from the mutant ATΔM302 grew from precipitated protein within 5 days. ATΔM302 crystallized iso morphously to the wild-type enzyme in the tetragonal space group P4_2_2_1, with lattice constants of a = b = 83.71 Å, c = 200.41 Å, and a = b = c = 90° (Table I). The asymmetric unit contained one molecule with a solvent content of 68% (V_m = 3.83 Å^3/Da) (31).
267 nm HEPES-NaOH, pH 7.0) containing either glycine (0.5 mM), γ-aminobutyric acid (0.1 mM), δ-aminovaric acid (1.0 mM), L-alanine (0.5 mM), L-α-aminobutyric acid (0.5 mM), or L-norvaline (saturated), and soaked for 15–50 h. The chemical structures of the soaked compounds are presented in Fig. 2.

Data Collection and Processing—X-ray measurements were performed on a MAR Research imaging plate detector system at 18 °C mounted on a Rigaku RU200 rotating anode x-ray generator with λ = CuKα = 1.542 Å. Images were processed with MOSFLM (32) and data were scaled and reduced using ROTAVATA/AGROVATA/TRUNCATE (33). The statistics of data collection are given in Table I.

Refinement and Quality of the Final Model—As the crystals of the AT deletion mutant AT1M302 and of all complexes were isomorphous with those of the wild-type protein, the wild-type structure was used as a model for initial Fourier (F2 - F) and difference Fourier (F2 - F) maps. Subsequently, the substrates and inhibitors were fitted to the electron density using FRODO (34) on an ESV-30 Graphic system workstation (Evans & Sutherland, Salt Lake City, UT), and the model was refined with XPLOR (35) using the parameter set of Eng and Huber (36). The final model comprises residues 64–423. The region from residue 230 to residue 242 displays average main chain B values, which are about 0.1 Å2 (AT in closed conformation) and 45 Å2 (AT in open conformation) above the B values for the whole protein and correlate with poor electron density. Water molecules were built into 1 σ peaks of a 2Fo - Fo map. Water molecules with B factors > 80 Å2 were removed from the model prior to the last refinement. The models show good geometry with R-factors below 20% and root mean square deviations for a bond length of 0.010–0.011 Å and for bond angles of 1.56–1.62°. Two of the three Ramachandran outliers (37) (Met174 and His333) are well defined and are observed as outlier solely in the structures adopting an “open” state. The final refinement statistics and stereochemical parameters are presented in Table I.

Circular Dichroism Measurements—Far-UV circular dichroism spectra were recorded on a Mark-IV (Jobin Yvon) spectropolarimeter. The spectra were recorded from 195 to 250 nm in a thermostated 0.01-cm quartz cuvette at 10 °C. A protein concentration of about 0.15 mg/ml in phosphate-buffered saline (39), pH 7.4, 0.5 mM EDTA, and 1 mM GSH was used. All spectra were accumulated four times and corrected for the buffers. Mean molar residue ellipticities (θθ) were calculated based on a mean residue molecular weight of 113. The thermal unfolding curves were determined by monitoring the changes in dichroic intensity at 216 nm as a function of temperature. The measurements were carried out in the temperature range of 10–80 °C with a temperature gradient of 0.5 °C/min using an oil-thermostatted cylindrical 0.01-cm quartz cell connected to a thermoprogrammer. The Tm values were derived by fitting the temperature-unfolding curves using the Gibbs-Helmholtz equation,

\[
\Delta G(T) = \Delta H_T \cdot (1 - T/T_m) - \Delta C_P \cdot (T_m - T) + T \cdot \ln(T/T_m) 
\]

(1)

where \(\Delta G\) is the free energy of unfolding, \(T_m\) is the temperature at which 50% of the protein molecules are unfolded, \(\Delta H_T\) is the enthalpy of unfolding at \(T_m\), and \(\Delta C_P\) is the heat capacity. A \(\Delta C_P\) value of 2.7 kcal mol\(^{-1}\) K\(^{-1}\) was estimated from the amino acid composition (38). Because the thermal transitions of all studied proteins were irreversible, a quantitative evaluation of thermodynamic parameters was not possible.

Size Exclusion Chromatography—Size exclusion chromatography was performed using a Superose 6 PC 3.2/30 column mounted on a SMART\textsuperscript{TM} system (Amersham Pharmacia Biotech). For all measurements a flow rate of 40 μl/min and phosphate-buffereed saline, pH 7.4, 0.1 mM EDTA, 0.5 mM dithiothreitol as buffer was used. The following marker proteins were used for calibration: bovine serum albumin (66 kDa), ovalbumine (45 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa).

Analytical Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium experiments were performed in a Beckman Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Double-sector cells with sapphire windows were used in an An-G rotor. Initial protein concentrations were about 0.2 mg/ml (AT1M302 and AT111) and 0.27 mg/ml (Str1B), corresponding to an initial absorbance at 280 nm of 0.4. Prior to the experiments, the protein samples were equilibrated against phosphate-bufered saline, pH 7.4, 0.5 mM EDTA, 0.5 mM dithiothreitol. Sedimentation coefficients were determined at 20 °C and 44,000 rpm, plotting ln(r) versus time. The scanning wavelength was 280 nm. The partial specific volume was calculated using the partial specific volumes of the component amino acids, yielding a value of 0.735 ml/g. The solvent density was estimated to be 1.000 g/ml. High-speed sedimentation equilibria measurements (40) were performed at 22 °C and 16,000 rpm. The molecular weight was calculated from log r versus r\(^2\) plots using a computer program written by G. Bohm (University of Regensburg). The scanning wavelengths were 230 and 280 nm.

RESULTS AND DISCUSSION

Crystal Structures of AT in Open Conformation—The crystal structures of AT and of its complexes with L-arginine and L-norvaline have been described (33), and they show that AT exists in at least two different conformations: (i) a closed conformation of ligand-free AT, and (ii) an open conformation induced by the binding of L-arginine (to AT mutant C407S) and L-norvaline (Fig. 1). In order to evaluate the molecular basis of these ligand-induced conformational changes, the structures of the binary complexes of AT with the amidino donor analogs L-norvaline, L-α-aminobutyric acid, and L-alanine (Fig. 2) were solved at resolutions of 2.4, 2.5, and 2.3 Å, respectively. These amino acids differ in the number of side chain carbon atoms, and they are not substrates because they lack the terminal amino group. In the presence of substrate concentrations of 10 mM, only L-norvaline was found to inhibit amidine transfer (41, 42). The x-ray structures of L-norvaline, L-α-aminobutyric acid, and L-alanine provide a clue for the inhibition mechanism at atomic resolution. All these ligands induce the transition from the closed to the open state and bind similar to the L-ornithine and L-arginine co-crystal structures (Fig. 1). The carboxylate and α-amino groups of these compounds would clash with the side chain of Asn302 in the closed conformer. This leads to a displacement of the 300-flap (residues Asp398, Pro399, Asn302, Pro401) toward helix H9 (residues Val322–Glu324). A concomitant repositioning of helix H9 prevents sterical collision of Pro299 with His336 and of Asn302 with Arg335. The shifts are as large as 5.1 Å for the C\(_n\) position of Pro299 and about 3.3 Å for the C\(_n\) positions of helix H9 (Fig. 1). These motions open the active site and may facilitate ligand binding and dissociation of reaction products. In agreement with the canonical binding, the carboxylate oxygen OT\(_2\) of L-norvaline accepts a hydrogen bond from Ser355 O\(_1\) (2.8 Å) and is fixed via a bifurcated hydrogen bond to N\(_\alpha\)-Aminobutyric acid and L-alanine.

Crystal Structures of AT in Closed Conformation—The crystal structures of unligated AT, as well as of AT in complex with the amidino acceptor or analogs glycine, γ-aminobutyric acid, and δ-aminovaric acid (Fig. 2), were found in closed conformation. γ-aminobutyric acid and δ-aminovaric acid bind differently to L-norvaline, L-α-aminobutyric acid, and L-alanine. The carboxylate group of the ligand is shifted 1.2 Å away from Asn302, which allows binding without inducing conformational changes (Fig. 3B). The carboxylate oxygen OT\(_1\) of δ-aminovaric acid is a hydrogen bond acceptor to N\(_\gamma\) of Arg223 (3.2 Å). OT\(_2\) shares a hydrogen bond to Ser355 O\(_1\) (2.5 Å), to the amide nitrogen of Ser355 (2.9 Å), and to N\(_\delta\) of Asn302 (2.9 Å). A buried
would sterically interfere with the side chain of Asn 300 in the closed yellow conformation and induces the displacement of the 300-loop in a conformation of helix H9 and of the 300-loop in open conformation (AT in the open (2.8 Å), and to Ala306 N (2.9 Å) and His305 O (2.7 Å), respectively. A simultaneous location of the carbonyl oxygen of Pro301 of AT302 could substitutes for the carbonyl oxygen of the deleted residue Met302, accepting a hydrogen bond from the α-amino group. These findings suggest that AT302 does not switch between an open and closed state but is permanently locked in the closed conformation.

Evidence for Structural Integrity of AT ΔM302 and AT Δ11—The structural integrity of the mutants AT ΔM302 and AT Δ11 was demonstrated by x-ray crystallography (for AT ΔM302), analytical ultracentrifugation experiments, and circular dichroism spectroscopy. The far-UV circular dichroism spectra of these mutants are very similar. Sedimentation coefficients and molecular masses of AT ΔM302 and AT Δ11, determined by analytical ultracentrifugation, were found to be in good agreement with results for human kidney L-arginine:glycine amidinotransferase (Table II) (43). The sedimentation analysis shows that AT and its mutants AT ΔM302 and AT Δ11, as well as StrB1, form homodimers and that the mutations did not affect dimerization. However, gel filtration experiments for wild-type AT, AT ΔM302, AT Δ11, and StrB1 yielded apparent molecular masses between 52 and 56 kDa for all proteins, probably as a result of adsorption effects on the size-exclusion column.

Kinetic Properties of AT ΔM302 and AT Δ11—A kinetic analysis of the deletion mutant AT ΔM302 revealed an activity of about 0.15 units/mg, corresponding to a 100-fold decrease as compared with the wild-type enzyme (specific activity of about 15 units/mg). Both AT and AT ΔM302 displayed a background arginase activity of about 0.1 units/mg as reported previously (44), resulting from the hydrolysis of the covalent enzyme amidine intermediate. As mentioned above, the new side chain conformation of the key residue Asn300 may allow binding of water molecule bridges OT1 (2.8 Å) to the side chain of Thr307 (2.8 Å), and to Ala306 N (2.9 Å) and His305 O (2.7 Å), respectively. The δ-amino group of δ-aminobutyric acid is surrounded by Asp300, Cys407, His305, and Asp170. A comparison of the structures in complex with γ-aminobutyric acid and δ-aminobutyric acid with l-norvaline, l-δ-aminobutyric acid, and l-alanine revealed that the α-amino group and not the number of carbon atoms is the principal determinant for the movement of the 300-loop in concert with helix H9.

For glycine, a completely different binding mode was observed. Its carboxylate group is not involved in ion pair formation with Arg322 but makes a bifurcated hydrogen bond to His303 N61 (3.4 Å) and to a water molecule (3.0 Å), which hydrogen bonds to Tyr164 OH (3.1 Å) (Fig. 3C). The amino group donates a hydrogen bond to Ser354 Oγ (3.3 Å) and is 4.6 Å apart from the nucleophilic cysteine 407. A simultaneous binding of l-arginine and glycine is not possible, in accordance with a double-displacement (ping-pong) mechanism.

Structure of AT ΔM302—A comparison of the amino acid sequences of AT and StrB1 revealed a one residue deletion at position 302. The corresponding deletion ΔM302 was introduced into the AT gene by site-directed mutagenesis, and the variant was recombinantly expressed and crystallized. This variant differs from the wild-type structure only at the site of the Met302 deletion, which results in a new path for residues (Asn300-Pro301-Met302), whereas the flanking residues Pro299 and His303 are at topologically identical positions to the wild-type structure. Most remarkably, the side chain of Asn300 takes an alternative position, which would not interfere with l-ornithine binding, and in fact could contribute a hydrogen bond to the α-amino group (Fig. 3D). The carbonyl oxygen of Pro301 of AT ΔM302 could substitutes for the carbonyl oxygen of the deleted residue Met302, accepting a hydrogen bond from the α-amino group. These findings suggest that AT ΔM302 does not switch between an open and closed state but is permanently locked in the closed conformation.

Induced-fit Mechanism of Human Amidinotransferase

![Fig. 1. The ligand-induced structural changes of AT. A, superimposition of helix H9 and of the 300-loop in open conformation (blue) and “closed” AT (yellow). Binding of an l-ornithine molecule (green) would sterically interfere with the side chain of Asn300 in the closed conformation and induces the displacement of the 300-loop in a concerted fashion with H9. B, comparison of the 300-loop and helix H9 of AT in the open (blue) and closed (yellow) conformations. The orientation is the same as in A. The open conformation is stabilized by hydrogen bonds from Pro300 O and Asn300 Oδ1 to the guanidine group of Arg302 (dashed lines). The coordinates were taken from Ref. 23, and the figure was produced with SETOR (46).](image)

| Protein  | S (Å) | kDa  |
|----------|-------|------|
| AT ΔM302| ND    | 90.0 ± 2.8 |
| AT Δ11   | 5.14  | 90.1 ± 2.0 |
| StrB1    | 4.93  | 68.9 ± 6.1 |
AT\textsuperscript{11} showed a residual activity of 1.0 units/mg. This corresponds to a 15-fold reduced activity and lies in the range of StrB1, which naturally lacks helix H9. Obviously, the conformational flexibility of helix H9 is important for efficient catalysis and may stabilize the open conformation of the 300-flap. Indeed, the residue Arg\textsuperscript{235} of H9 donates two hydrogen bonds to Pro\textsuperscript{299} O and one to Asn\textsuperscript{300} O\textsuperscript{51}, thereby fastening H9 to the 300-flap (Fig. 1B).

Additional evidence that the ligand-induced conformational changes increase the catalytic activity stems from activity measurements of AT, AT\textsuperscript{M302}, and AT\textsuperscript{11} in the presence of L-arginine and \(\delta\)-aminovaleric acid as amidino donor and acceptor, respectively. The activity of the wild-type enzyme was found to be reduced about 5-fold, reflecting weaker binding of \(\delta\)-aminovaleric acid and/or impaired dissociation. In contrast, no reduction of enzymatic activity was observed for AT\textsuperscript{M302} and AT\textsuperscript{11}. A likely explanation is that the dissociation of L-ornithine from these closed variants is hampered and limits the overall velocity.

**Stability Measurements**—In order to evaluate a possible role of helix H9 in protein stability, temperature-induced transition curves were measured for AT\textsuperscript{11} and compared with AT and AT\textsuperscript{M302}. The thermal transitions were reversible and did not allow determination of thermodynamic data. AT and AT\textsuperscript{M302} show similar midpoints of transition at \(T_m = 51.9\) and 51.2 \(^\circ\)C, respectively. For AT\textsuperscript{11}, the \(T_m\) value (53.1 \(^\circ\)C), as well as the cooperativity of the transition, is slightly increased (Fig. 4). These results suggest that neither residue Met\textsuperscript{302} nor helix H9 adds to the stability in vertebrate AT.

**Implications for the Induced-fit Mechanism**—The crystal structures of AT in complex with L-alanine, L-\(\alpha\)-aminobutyric acid, L-norvaline, glycine, \(\gamma\)-aminobutyric acid, and \(\delta\)-aminovaleric acid showed three different binding modes that lead to an understanding of the broad substrate specificity. It is known from L-arginine analogs that only L-arginine (chain number of carbon atoms \(n = 5\)) and L-canavanine (the guanidinoxy analog of arginine) can serve as amidino donor. Longer and shorter arginine analogs, such as homoarginine \((n = 6)\), \(\alpha\)-amino-\(\gamma\)-guanidinobutyric acid \((n = 4)\), and \(\alpha\)-amino-\(\beta\)-guanidinobutyric acid \((n = 3)\) are not substrates for AT\textsuperscript{1} (1). These amino acid derivatives are supposed to bind to AT via their \(\alpha\)-amino and carboxylate groups, but only compounds with the appropriate chain length reach the nucleophilic cysteine and are able to transfer their amidino groups. The same holds true for L-norvaline and related inhibitors, such as L-\(\alpha\)-aminobutyric acid, and L-alanine, which bind in a similar manner but lack the terminal guanidino/mino group. In contrast, the chain length is not important for glycine analogs functioning as amidino acceptors as long as they are shorter than \(n = 6\). Glycine \((n = 2)\), \(\beta\)-aminopropionic acid \((n = 3)\), \(\gamma\)-aminobutyric acid \((n = 4)\), and \(\delta\)-aminovaleric acid are substrates for AT, albeit inferior to glycine (1). We showed that the amidino acceptor analogs \(\gamma\)-aminobutyric acid \((n = 4)\) and \(\delta\)-aminovaleric acid \((n = 5)\) bind differently to amino acid derivatives and do not induce the displacement of the 300-loop and of helix H9. Acceptors with fewer than \(n = 4\) carbon atoms are proposed to use different binding modes. This was observed for glycine, which binds in a completely distinct manner, closer to the nucleophilic cysteine 407. It was noted before (45) that arginine reacts with both AT and StrB1, whereas glycine is not a substrate for StrB1. A comparison of the two active sites revealed that residues interacting with L-arginine are all conserved but that most residues involved in glycine binding are not conserved. The residues Tyr\textsuperscript{164}, Ser\textsuperscript{154}, and Asn\textsuperscript{300} are topologically replaced by residues His\textsuperscript{102}, Ala\textsuperscript{224}, and Cys\textsuperscript{279} in StrB1, disrupting the hydrogen bond network observed for AT (Fig. 3C). Clearly, in AT, the active site is optimized to accommodate glycine. Its function in creatine biosynthesis is assisted by the flexible 300-loop and helix H9, resulting in a much more restricted active site. The kinetic and crystallographic analysis of AT and of its deletion mutants suggested that these key differences have led to (i) a strong increase of the catalytic turnover, which is required for creatine biosynthesis, (ii) a slightly diminished arginase side reactivity, and (iii) an increased substrate specificity. Stability measurements showed that H9 may increase the pH stability but has no function in temperature stability.
FIG. 3. The binding modes of L-norvaline (A), δ-aminovaleric acid (B), and glycine (C) and the active site topology of the mutant ATΔM302 (D). A, the binding mode of L-norvaline. AT is found in the open conformation. The residue Asn₃₀₀ is displaced upon binding of L-norvaline (see Fig. 1B). B, the δ-aminovaleric acid binding site. AT is found in the closed conformation. Asn₃₀₀ hydrogen bonds to the ligated δ-aminovaleric acid molecule. C, the glycine binding site. Only the hydrogen bonds to Ser₃₅₄, Wat¹¹¹, and His₂⁰⁵ are within hydrogen bond distances; the other interactions indicated are in the range of 3.9 Å. For A–C, the $F_o - F_c$ difference electron density maps contoured at the 1σ level are shown in red and superimposed on the refined models. D, active site topology of mutant ATΔM302. The final $2F_o - F_c$ electron density map contoured at 1σ (green) is superimposed on the refined model. For comparison, the corresponding residues of closed AT are represented as black sticks. A modeled L-norvaline inhibitor molecule is shown in cyan. The deletion of residue Met₃⁰₂ results in a new side chain conformation of Asn₃⁰₀, which allows binding without obvious steric restrictions. The figure was produced with SETOR (46).

FIG. 4. Thermal unfolding curves for AT (●), ATΔM302 (○), and ATΔ11 (×). The change in dichroic intensity at 216 nm as a function of temperature was monitored. The heating rate was 0.5 °C/min. Measurements were done using 0.15 mg/ml of protein in phosphate-buffered saline, pH 7.4, 0.5 mM EDTA, 0.5 mM dithiothreitol. See under “Experimental Procedures” for details.
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