Recombinant expression and isolation of human L-arginine:glycine amidinotransferase and identification of its active-site cysteine residue

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Creatine and its phosphorylated form play a central role in the energy metabolism of muscle and nerve tissues. L-Arginine:glycine amidinotransferase (AT) catalyses the committed step in the formation of creatine. The mitochondrial and cytosolic forms of the enzyme are believed to derive from the same gene by alternative splicing. We have expressed recombinant human AT in *Escherichia coli* with two different N-termini, resembling the longest two forms of the enzyme that we had isolated recently

from porcine kidney mitochondria as a mixture. The enzymes were expressed with N-terminal histidine tags followed by factor Xa-cleavage sites. We established a new method for the removal of N-terminal fusion peptides by means of an immobilized snake venom prothrombin activator. We identified cysteine-407 as the active-site residue of AT by radioactive labelling and isolation of labelled peptides, and by site-directed mutagenesis of the protein.

INTRODUCTION

L-Arginine:glycine amidinotransferase (AT; EC 2.1.4.1) catalyses the transfer of an amidino group from L-arginine to glycine. The resultant guanidinoacetic acid is the immediate precursor of creatine (Figure 1) [1]. Creatine and its phosphorylated form play an important role in the energy metabolism of muscle and nerve tissues, acting as a dynamic reservoir of high-energy phosphate, which buffers the rapid fluctuations of the ATP/ADP ratio during muscle and nerve action [2]. The highest tissue concentrations of creatine and phosphocreatine are found in skeletal muscle, heart, spermatozoa and photoreceptor cells of the retina [3]. Most creatine is not synthesized in those tissues that have the largest pools of creatine and phosphocreatine, but is taken up from the blood. The major sites of creatine biosynthesis are pancreas, kidneys and liver, where the amidinotransferase is located in the cytoplasm and in the intermembrane space of the mitochondria [4–6]. It has been suggested that AT activity in these tissues is regulated in a number of ways including induction by growth hormone and thyroxine [7], inhibition of the enzyme by ornithine [8] and repression of its synthesis by creatine [9,10]. Transfer of amidino groups is also known in *Streptomyces* bacteria where L-arginine:inosamine phosphate amidinotransferases catalyse two reactions in streptomycin biosynthesis [1,11].

In the original preparations of AT from porcine kidney mitochondria, the enzyme was partially degraded at the N-terminus and showed two major fractions. The N-terminus of the longer form started at position 38, and the N-terminus of the shorter form at position 56 of the protein sequence derived from the cDNA of human mitochondrial AT [12]. Austruy et al. (E. Austruy, L. Bellay, P. Millasot, C. Junien and C. Jeanpierre) submitted a second cDNA sequence of AT derived from human kidney to the EMBL database (accession number X86401). The protein sequence derived from this cDNA was 32 amino acids shorter at the N-terminus and differed in its amino acids 1–5 from the amino acids 33–37 of the corresponding part of the mitochondrial enzyme. After this position, both cDNA sequence and derived amino acid sequence were completely identical with those of the mitochondrial enzyme. Possibly this form, lacking

the mitochondrial leader sequence, is the cytosolic form of the enzyme which may be derived from the same gene by alternative splicing of the same mRNA precursor.

AT is inhibited in gyrate atrophy of the choroid and retina with hyperornithinaemia due to the absence of L-ornithine-2-oxoacid aminotransferase (EC 2.6.1.13), resulting in 10–20-fold increased plasma and urinary ornithine concentrations [8]. AT expression is down-regulated in Wilms tumor [13] and may also play an important role in limb-girdle muscular dystrophy type 2A (LGMD2A). The AT gene was mapped to the locus D15S109E [13] on the distal part of the chromosome band 15q15.3 [14] and lies in a region showing significant linkage disequilibrium for LGMD2A [14–16]. Mutations in the calpain 3-gene are thought to cause LGMD2A but they cannot explain the occurrence of multiple independent mutations in the small inbred population of La Reunion Island LGMD2A patients [17]. Thus the involvement of other genes of the LGMD2A region such as the gene for AT should also be considered as a possible cause of this disease.

In the present report we describe the recombinant production of human AT as a fusion protein with an N-terminal histidine tag, the cleavage of the histidine tag by an immobilized snake venom protease and the identification of the active-site cysteine by chemical methods and site-directed mutagenesis.

MATERIALS AND METHODS

Plasmid construction

In order to express recombinant AT in the periplasmic space of *Escherichia coli*, the plasmid pET12a (Novagen, Madison, WI, USA) was digested with *Sal*I and *Bam*HI and a linker coding for a six-histidine tag was included. The derived peptide sequence after insertion was ASTMAHHHHHHGS including the flanking *Sal*I and *Bam*HI restriction sites. The vector was now called pET12aL and contained, at the inserted methionine residue, a new *Nco*I restriction site. By PCR cloning using cloned AT cDNA [12], the selected AT gene was inserted at the *Bam*HI

Abbreviations used: AT, AT38, AT56, L-arginine:glycine amidinotransferase in general or starting at amino acid 38 or 56 of the sequence of the mitochondrial preprotein respectively; LB, Luria-Bertani; LGMD2A, limb girdle muscular dystrophy type 2A; β -ME, 2-mercaptoethanol; NTA, nitrilotriacetate.

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Figure 1 Enzyme reactions leading to the formation of creatine

site with an N-terminal factor Xa-cleavage site (IEGR) directly after the *Bam*HI site and immediately in front of the AT sequence starting at either codon 38 or 56 of the mitochondrial sequence. The last AT codon in the PCR fragment was followed by two stop codons, a *Nhe*I site and a *Bam*HI site. The correctness of the clones was proven by sequencing of the whole insert in both directions. The plasmids were named pET-12aL38 and pET-12aL56. Since expression from clones containing these plasmids in *E. coli* BL21(DE3)pLysS (Novagen) was so low that it was only detectable in Western blots, the inserts were cut out with *Nco*I and *Bam*HI in two successive partial digests since the AT gene contains an internal *Nco*I site and the histidine tag was to remain on the fragment. These fragments were ligated into pRSET6d [18] digested with *Nco*I and *Bam*HI. Plasmids were named pRSETAT38H and pRSETAT56H. The correctness of the clones was proven as before. In this cloning procedure, the newly introduced *Nco*I site in front of the histidine tag was turned from an internal restriction site into the site coding for the translation start. Cloning procedures were performed as described by Sambrook et al. [19], and sequencing was carried out using the PRISM™ ReadyReactionDyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and a model 373A Sequencing System (Applied Biosystems) according to the manufacturer's instructions. The mutants C407A and C410A were made using the ExSite™ mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions.

Protein expression

Transformation-competent *E. coli* BL21(DE3)pLysS cells were transformed with pRSETAT38H or pRSETAT56H and plated on Luria-Bertani (LB) agar plates, containing 0.1 mg/ml ampicillin, 34 µg/ml chloramphenicol and 1% (w/v) glucose. The plates were incubated at 37 °C for 12–14 h. Then 100 ml of LB medium containing 0.1 mg/ml ampicillin, 34 µg/ml chloramphenicol and 1% (w/v) glucose was inoculated from a single

colony and incubated at 37 °C with shaking until the A_{600} reached a value of 2. For overproduction of AT, 30 ml of this culture was used to inoculate ten 2-litre Erlenmeyer flasks each filled with 900 ml of medium as described above. The *E. coli* cells were grown at 37 °C until the A_{600} was 1.5. The expression of the AT gene was then induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.7 mM, and the cells were grown further at 25 °C for 4 h. The cells were harvested by centrifugation (2800 g; 4 °C; 25 min), resuspended in cold sonication buffer (300 mM NaCl, 2 mM 2-mercaptoethanol (β-ME), 50 mM sodium phosphate, pH 7.8) and frozen at –20 °C.

Protein isolation

The frozen cells were thawed and lysed by T7 lysozyme, coexpressed in this strain. The lysate was sonicated for 10–15 min with the large tip of a Branson sonifier 250 (G. Heinemann, Schwäbisch Gmünd, Germany) and the lysate was centrifuged (2 °C; 1 h; 13000 g) in a L7-55 ultracentrifuge (Beckman, München, Germany). The supernatant was applied at a flow rate of 0.5 ml/min to a column (1.4 cm × 19 cm) containing Ni²⁺/nitrilotriacetate/agarose (Qiagen, Hilden, Germany) equilibrated with sonication buffer. The column was washed with sonication buffer and washing buffer (300 mM NaCl, 2 mM β-ME, 50 mM sodium phosphate, pH 6.2) until the A_{280} fell below 0.7. It was then further washed with a steep gradient of 0–180 mM imidazole in washing buffer, and contaminating proteins were removed with a prolonged washing step of 180 mM imidazole in washing buffer (see Figure 2a). Histidine-tagged AT was eluted from the column with 200 ml of a linear gradient of 180–700 mM imidazole in washing buffer and fractions of volume 7.5 ml were collected. Fractions containing AT (corresponding to 220–420 mM imidazole) were combined and dialysed twice against 5 litres of 30 mM Hepes/2 mM β-ME, pH 8.0, using Spectra/Por 2 (Carl Roth, Karlsruhe, Germany) dialysis tubing.

To produce an immobilized snake venom column, 2.4 ml of *N*-hydroxysuccinimide-activated agarose (Affigel 10, Bio-Rad Laboratories, Munich, Germany) was placed into an empty NAP10 column (Pharmacia Biotech, Freiburg, Germany) and washed with deionized water and Mops buffer (20 mM Mops, 300 mM NaCl, pH 6.5). Taipan (*Oxyuranus scutellatus*) venom (Latoxan, Rosans, France) (22.7 mg dissolved in 1.5 ml of Mops buffer) was applied to the agarose column, which was rotated at 60 rev./min for 4 h at 4 °C to keep the agarose suspended in the venom solution. After removal of the supernatant, the agarose was washed with Mops buffer and the column was filled with 1 M ethanolamine/HCl, pH 7.5. The column was rotated for 1 h at room temperature in order to saturate all activated sites on the agarose that had not been coupled to the taipan venom. After the agarose column had been washed with Mops buffer, it was ready to use and could be stored in Mops buffer with 0.2% NaN₃ at 4 °C for at least 4 months.

The taipan venom column was equilibrated with cleavage buffer (300 mM NaCl, 30 mM Hepes, pH 7.2) for cleavage of the fusion proteins. The fusion protein was applied to the top of the column and CaCl₂ was added to a final concentration of 8 mM. The column was filled with cleavage buffer and rotated at room temperature for 4 h. The resin was allowed to settle and the protein was eluted with 3 column vol. of cleavage buffer; the column was washed thoroughly with cleavage buffer including 0.2% NaN₃ and stored at 4 °C. The reaction products (the cleaved protein and the histidine tag) were separated on the same Ni²⁺/NTA/agarose column as the fusion protein. The mixture

was applied to the column equilibrated in cleavage buffer and washed with cleavage buffer at pH 6.2. The AT was eluted with 20 mM imidazole in cleavage buffer and the histidine tag was removed from the Ni^{2+} -chelate resin with high concentrations of imidazole. Fractions containing the cleaved AT were combined and dialysed against 30 mM Hepes/2 mM β -ME, pH 8.0.

Gel electrophoresis, enzyme activity and protein determination

Enzyme activity was determined as described by Van Pilsum et al. [20]; 1 unit is defined as the amount of enzyme that catalyses formation of 1 μmol of L-ornithine/h at 37 °C. Protein was determined as described by Lowry et al. [21] or from A_{280} using $\epsilon = 91\,900\text{ M}^{-1}\cdot\text{cm}^{-1}$. SDS/PAGE was performed as described by Schagger and von Jagow [22].

Radioactive labelling of the active-site cysteine and peptide isolation

The active-site cysteine was labelled by the method of Grazi and Rossi [23] with L-[guanido- ^{14}C]arginine (2.0 Gbq/mmol; Du Pont de Nemours, Dreieich, Germany). The labelled protein was cleaved in 0.1 M HCl with pepsin at an enzyme/substrate ratio of 1:100 at 23 °C for 16 h. The resulting peptides were separated by reverse-phase HPLC on a C_{18} column (3 mm $\times$ 250 mm, filled with Vydac 300A, C_{18} ; 5 μm material) or a diphenyl column (Vydac 219TP54) using a gradient of 0–42% acetonitrile in 0.1% trifluoroacetic acid in 160 min at a flow rate of 0.25 ml/min. Fractions of volume 200 μl were collected, and 10% of each fraction was mixed with scintillation fluid (Rotiszint 22; Carl Roth) and the radioactivity counted. Radioactive peptides were sequenced with the ABI sequencer 470A following the manufacturer's instructions.

Secondary-structure predictions

Multiple sequence alignments were performed with the Wisconsin Package [24] and formatted with ALSCRIPT [25]. A CD spectrum was recorded for AT38 without the histidine tag in 50 mM potassium phosphate buffer, pH 7.75, using a Jasco J710 CD spectrophotometer (Jasco Deutschland, Grossumstadt, Germany), and secondary-structure elements were predicted with software from Jasco. A further secondary-structure prediction based on the protein sequence was calculated as described by Rost and Sander [26].

RESULTS AND DISCUSSION

Expression and purification of recombinant AT

Two forms of human recombinant AT were produced by transformed *E. coli* cells in a soluble and enzymically active form. One of them, AT38, started at position 38 of the cDNA-derived sequence, and AT56 started at position 56. AT38 corresponds to the longest form isolated recently from porcine kidney mitochondria [12]. Both forms were soluble and active when expression was performed at 25 °C, and they were inactive due to accumulation of the protein in 'inclusion bodies' when expression was performed at 37 °C. The two proteins were expressed with a poly-histidine tag containing six consecutive histidines. This tag was used to isolate the recombinant proteins by metal-chelate affinity chromatography on a Ni^{2+} /NTA/agarose column. The proteins were eluted between 220 and 420 mM imidazole in a linear gradient from 180 to 700 mM (Figure 2a). Fractions containing the enzyme were identified by enzyme assays and

Figure 2 Elution profile of AT38H (top) and AT38 after cleavage of the fusion protein (bottom) on Ni^{2+} /NTA/agarose

Top, the arrows indicate application of sample (1), washing with sonication buffer (2) and washing with washing buffer (3). For further washing steps and for AT elution, the gradient of imidazole in washing buffer is shown. Bottom, the arrows refer to application of the sample (1), washing with 300 mM NaCl/30 mM Hepes, pH 6.2 (2) and washing with 300 mM NaCl/30 mM Hepes/20 mM imidazole, pH 6.2 (3). The following imidazole gradient is shown.

Figure 3 Silver-stained SDS/PAGE showing AT56 with histidine tag (44 039 Da; lane 2), AT38 with histidine tag (45 808 Da; lane 3), AT38 without histidine tag (44 284 Da; lane 4), the mutant protein C407A with histidine tag (45 773 Da; lane 5) and molecular mass markers (lanes 1 and 6)

SDS/PAGE. The latter method also showed that the protein was of high purity (Figure 3). The yield of purified AT was about 2–5 mg/l *E. coli* culture.

The tag was then cleaved off by using a factor Xa-cleavage site, IEGR, which had been introduced into the recombinant proteins immediately in front of residues 38 and 56. We did not use factor Xa for cleavage, however, but a prothrombin activator contained in the venom of the taipan snake, *Oxyuranus scutellatus* [27–29],

Figure 4 Multiple sequence alignment of AT and StrB proteins

Abbreviations are: Hummito, human mitochondrial AT, EMBL accession no. S68805; Humcyto, human cytosolic AT, EMBL accession no. X86401; Pig, pig AT; Rat, rat mitochondrial AT, EMBL accession no. U07971; Sgastrb1, *Streptomyces galbus* StrB1, EMBL accession no. X78973; Sglstrb1, *Streptomyces glaucescens* StrB1, EMBL accession no. X78974; Sblstrb, *Streptomyces bluishii* StrB, EMBL accession no. X78972; Sgrstrb1, *Streptomyces griseus* StrB1, EMBL accession no. S55493/Y00459; Sgrstrb2, *Streptomyces griseus* StrB2, EMBL accession no. X62567;

which has the same specificity as factor Xa but seems to be more active. Because of the apparent absence of other proteases, the unfractionated snake venom, which is much cheaper than purified factor Xa, can be used to cleave fusion proteins at the usual IEGR site.

We used the venom components coupled to agarose. For cleavage, the immobilized venom protease and its substrate were incubated in a rotating column. After incubation the agarose beads were allowed to settle and the cleavage products were eluted with buffer, leaving behind the immobilized venom protease for further use. Our column was used several times over a period of 4 months without any decrease in effectiveness. The cleaved histidine tag was removed from the AT by passing the mixture once again over the Ni^{2+} /NTA/agarose column. The free enzyme bound only weakly to the metal-chelate resin and was eluted at 20 mM imidazole (Figure 2b). The loss of the histidine tag could also be monitored by a small but distinct increase in relative mobility of AT in SDS/PAGE (Figure 3). The identity of the purified products was confirmed by N-terminal amino acid sequence analysis.

Characterization of recombinant AT

The specific activity of both recombinant proteins was rather variable from preparation to preparation (5–21.4 units/mg). However, both forms were only slightly less active than AT isolated from human kidney, which showed a specific activity of 23.3 units/mg [30]. Furthermore both forms were equally active, indicating that residues 38–55 are not involved in the catalytic action of the enzyme. The recombinant enzyme was strongly inhibited by 1 mM Hg^{2+} , 1 mM Zn^{2+} and 10 mM Ni^{2+} . Thus it may be possible that Ni^{2+} ions released from the affinity column bound to the proteins and caused the variable activity levels. In addition, AT may have been irreversibly inhibited by HOC_3^- [31] formed during fermentation in *E. coli*.

CD spectra of AT38 showed 38.5% α -helix, 12.1% β -sheet, 20.3% β -turn and 29.1% random coil when calculated with the Jasco software. Analysis of secondary structure from the sequence, using the method of Rost and Sander [26], predicted 29.3% α -helix, 17.3% β -sheet and 53.4% β -turn and random coil (Figure 4).

Identification of the active-site cysteine

In their work on the reaction mechanism of AT from porcine kidney, Grazi and Rossi [23] have shown that the transamidation reaction occurs via an S-amidinocysteine intermediate that is stable under acidic conditions. After radioactive labelling with L-[guanido- ^{14}C]arginine, cleavage with pepsin and peptide separation by reverse-phase HPLC on a C_{18} column, we found only one radioactive peak with a recovery of about 50% of the applied radioactivity. Analysis of this peak yielded two sequences in about equal concentrations, $_{309}\text{NIIGPGIVLSNP-DRPCHQIDLF}$ and $_{391}\text{IKVNIRNANSLGGGFHCWTCDV}$. Separation of the peptides on a diphenyl column yielded the same peptide mixture. These peptides still contained three of the eight cysteines of the enzyme, Cys-324, Cys-407 and Cys-410. Because the [^{14}C]amidino label was cleaved off at a pH above 7, it was not possible to determine the position of the labelling group during sequence analysis.

In order to examine which of the cysteine residues, Cys-324, Cys-407 or Cys-410, was the active-site cysteine, we produced two mutant proteins in which Cys-407 or Cys-410 were changed into alanine by site-directed mutagenesis (mutants C407A and C410A). With the mutant protein C407A, no activity was detectable whereas protein C410A showed normal activity. Crystals of AT38 and the inactive mutant C407A, both proteins containing the N-terminal histidine tag, showed the same unit cell parameters (A. Humm, F. Fritsche, R. Huber and S. Steinbacher, unpublished work). This suggests that inactivity of the mutant C407A is not due to conformational rearrangements and that Cys-407 is the active-site cysteine residue.

The role of Cys-407 as active-site cysteine is further supported by a comparison of the sequences of AT from various species with the sequences of L-arginine:inosamine phosphate amidinotransferases [11,32] from *Streptomyces* bacteria (Figure 4). In some strains of *Streptomyces* there are two genes, *strb1* and *strb2*, as shown for *Streptomyces griseus* and *Streptomyces glaucescens*. The gene products StrB1 and StrB2 show great sequence similarity but up to now only StrB1 proved to be active in transamidation reactions [6]. Cys-407 is the only cysteine residue that is conserved in the amino acid sequences of AT and StrB1. In the inactive StrB2 proteins this residue is not conserved (Figure 4).

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