Altered chromatographic behaviour of mitochondrial ADP/ATP translocase induced by stabilization of the protein by binding of 6'-O-fluorescein-atractyloside

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Atractyloside (ATR) is a high-affinity specific inhibitor of the mitochondrial ADP/ATP translocase (AAT). The binding of a fluorescent derivative, 6'-O-fluorescein-ATR (FATR), to mitochondria has been characterized. The binding constants obtained are in agreement with previously published values for ATR, demonstrating that FATR is a suitable probe of the AAT. AAT inhibited by FATR (FATR-AAT) was solubilized in dodecyl maltoside and purified by two separate ion-exchange chromatography steps at different pHs, which allowed FATR-AAT to be purified to homogeneity. The presence of the bound fluorescent probe enabled the inhibited AAT to be distinguished from the unliganded protein during chromatography, as they were markedly different in their chromatographic behaviour. The puri-

fied FATR-AAT was dimeric and in a single major conformation containing 1 mole FATR per mole of AAT dimer. In contrast, uninhibited AAT was monomeric and conformationally unstable. Use of the fluorescent ATR derivative in the development of the protocol enabled the stable dimeric AAT to be monitored directly and purified more effectively. The purification protocol was repeated using non-derivatized ATR, and highly pure AAT was obtained that was devoid of other members of the mitochondrial carrier family.

Key words: ADP/ATP translocase, atractyloside (ATR), mitochondrial transport protein, protein conformation.

INTRODUCTION

The mitochondrial ADP/ATP translocase (AAT) catalyses the exchange, across the inner membrane of the organelle, of cytosolic ADP for ATP, produced in the matrix by ATP-synthase. The AAT is the most highly expressed member of the mitochondrial carrier family (MCF) [1,2] and it has been used as a model to establish certain principles of transport mechanism [3]. The AAT undergoes substantial conformational changes during transport [4–8]. This feature renders the protein unstable when it is extracted from the membrane, and makes it particularly susceptible to proteolysis [9]. Based on its sequence and topological studies, the AAT is predicted to contain six transmembrane helices, with three repeat domains each containing two helices [10–15]. However, very little is known about the detailed three-dimensional structure of the AAT or, indeed, any member of the MCF.

Two naturally occurring heteroglucosides, atractyloside (ATR) and its carboxylated derivative carboxyatractyloside (CATR), inhibit the AAT with high affinity and high specificity. ATR is a competitive inhibitor [dissociation constant (K_D) $\approx$ 50 nM], whereas CATR binds with a much higher affinity (K_D = 2–10 nM) and is considered a quasi-irreversible inhibitor of the AAT [16–19]. Titration of radiolabelled CATR against the AAT, revealed a binding ratio of 1 mole CATR per 2 moles AAT [20,21]. The ATR-inhibited AAT is dimeric in the detergent Triton X-100 [22,23]. Significantly, inhibition of the AAT with ATR 'locks' the protein into a single stable conformation [24]. Although no natural ligands of the AAT give rise to a distinct optical signal that could be used to study their interaction with the AAT, a number

of fluorescent ATR derivatives have been used to investigate the binding sites and structure of the AAT [21,25,26].

Structural analysis of the protein by either biophysical or crystallographic techniques requires sufficient amounts of a highly pure preparation. Not only must the AAT be isolated from other mitochondrial proteins, but all sources of microheterogeneity must also be eliminated. Possible sources of heterogeneity include: proteolytic modification of the polypeptide, variation in the aggregation or oligomeric state of the protein, conformational instability arising from the dynamic nature of the molecule, and partial denaturation of the sample [27]. The AAT has been purified previously as the CATR complex [28,29] and as the unliganded protein [30]. Both methods involve the application of a Triton X-100 extract of mitochondria on to a hydroxyapatite (HA) column. CATR-inhibited AAT does not bind to hydroxyapatite, whereas most other proteins in the extract do. Unliganded AAT is unstable and appears to unfold within hours of solubilization, then binding strongly to the HA support. This method has provided a convenient source of AAT, used in many kinetic and biochemical analyses, but the preparation contains significant levels of homologous members of the MCF and, in addition, the AAT is probably partially unfolded. Therefore, it does not meet the stringent requirements of biophysical and, more especially, crystallization methods.

In this study we describe the characterization of a novel fluorescent ATR derivative, 6'-O-fluorescein-ATR (FATR), and its use as a probe to monitor the purification of FATR-AAT (AAT inhibited by FATR). FATR contains one fluorescein residue linked to the ATR moiety at the 6'-O position. FATR is likely to have the

Abbreviations used: AAT, ADP/ATP translocase; ATR, atractyloside; BCIP/NBT, 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium; CATR, carboxyatractyloside; DDM, *N*-dodecyl β -D-maltoside; DTT, dithiothreitol; F_{527} , fluorescence emission at 527 nm; FATR, 6'-O-fluorescein-ATR; FATR-AAT, AAT inhibited by 6'-O-fluorescein-ATR; HA, hydroxyapatite; K_{av} , partition coefficient; K_D , dissociation constant; K_i , inhibitor concentration at which transport activity is reduced by half; MCF, mitochondrial carrier family; m/z , mass-to-charge ratio; SMP, sub-mitochondrial particle.

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same inhibitory properties as ATR, since it is known that the only group of ATR that can be modified without alteration of its biological properties is the primary alcohol of the glucose disulphate moiety [31]. This ATR derivative was used to monitor the purification of the AAT from bovine heart mitochondria by two-step ion-exchange chromatography. The chromatographic behaviour of FATR-ATR differs significantly from that of the uninhibited AAT. Analysis of the two conformational states of the protein provides a structural basis for these chromatographic differences.

EXPERIMENTAL

Materials

Mitochondria were isolated from fresh bovine hearts as described previously [32]. FATR was a gift from Mitokor (San Diego, CA 92121, U.S.A.). CATR was purchased from Calbiochem (San Diego, CA 92039, U.S.A.) and ATR from Sigma. [^{14}C]ADP, and chromatography columns and media were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). *N*-Dodecyl β -D-maltoside (DDM) was supplied by Anatrace (Maumee, OH 43537, U.S.A.). Dialysis membrane (Medicell International, London, U.K.) and Centriprep concentrators (Amicon, Glos., U.K.) had a 10 000 Da molecular mass cut-off. Protein concentrations were determined by the bicinchoninic acid assay [33] with a kit supplied by Pierce (Rockford, IL 61105, U.S.A.). All other chemicals were purchased from Sigma.

Measurement of ADP/ATP transport in isolated mitochondria

ADP/ATP transport in isolated mitochondria was measured by the direct-exchange procedure [34]. The reaction buffer contained 20 mM Tris/HCl, pH 7.4, 250 mM sucrose and 1 mM EDTA. Mitochondria (1 mg of protein) were pre-incubated with FATR at 0 °C for 45 min. The reaction was started by the addition of [^{14}C]ADP and terminated by the addition of 20 μM CATR. Then mitochondria were harvested immediately from the reaction buffer by filtration through a Millipore filter and washed with buffer. The radioactivity of the filter was measured by liquid scintillation counting. Results were analysed using an unweighted least-squares fit of the data directly to a hyperbola using the Prism 3.0 program supplied by GraphPad Software (San Diego, CA 92121, U.S.A.).

FATR binding by fluorescence spectroscopy

Mitochondria (0.5 mg of protein) were resuspended in 1 ml of standard fluorimetry buffer containing 20 mM Mops, pH 7.4, 80 mM glycerol and 1 mM EDTA, and kept on ice for 45 min with increasing concentrations of FATR. Unbound FATR was removed by centrifugation at 10 000 *g* for 10 min followed by resuspending the mitochondria three times in buffer, then assaying bound FATR spectrofluorimetrically. After chromatographic separation, unbound FATR was removed rapidly from fractions containing AAT by passage through a PD-10 desalting column, equilibrated with fluorimetry buffer at 4 °C. The protein concentration was adjusted to 50 $\mu\text{g}/\text{ml}$. After exchange of the sample into the standard buffer to ensure constant pH (as fluorescein exhibits pH sensitive fluorescence), fluorescence measurements were recorded. Samples (0.1 mg of protein) were analysed at 10 °C using an excitation wavelength of 488 nm and by measuring fluorescence emission at 527 nm (F_{527}). In these experiments, the total concentration of FATR was approx. that of free FATR, enabling values for K_D to be determined. The K_D values and the

number of ligand-binding sites were obtained by an unweighted least-squares fit of the data to a hyperbola using the GraphPad program Prism 3.0.

Extraction of AAT from bovine heart mitochondria

Isolated mitochondria were resuspended to 20 mg/ml in ice-cold buffer containing 20 mM Tricine, pH 7.4, 250 mM sucrose, 150 mM Na_2SO_4 , 1 mM EDTA, 0.02 % sodium azide and 0.5 ml of mammalian protease inhibitor cocktail (Sigma). FATR was added to a concentration of 5 μM , and the suspension was kept on ice for 45 min. Sub-mitochondrial particles (SMPs) were produced by sonicating the mitochondria on ice. Intact mitochondria were removed by centrifuging at 10 000 *g* for 10 min, and then the SMPs were collected by centrifuging the decanted supernatant at 140 000 *g* for 1 h. They were resuspended in buffer to a concentration of 50 mg/ml and 2 vol. of the same buffer containing 3 M guanidinium hydrochloride were added to strip extrinsic proteins from the membranes. The sample was stirred at 4 °C for 2 min, and then 3 vol. of buffer, containing no guanidinium hydrochloride, were added. The stripped SMPs were centrifuged at 140 000 *g* for 1 h. The pellet was resuspended in ice-cold buffer at a protein concentration of 10 mg/ml. Proteins were solubilized by adding an equal volume of the same buffer containing 4 % (w/v) DDM and gently stirring on ice for 20 min. Insoluble material was removed by centrifuging at 140 000 *g* for 1 h.

Ion-exchange chromatography

The solubilized proteins were dialysed overnight at 4 °C against 20 vol. of QA buffer containing 10 mM Tricine, pH 7.4, 0.1 M sucrose, 1 mM EDTA, 1 mM DTT (dithiothreitol) and PMSF (0.02 %, w/v). The sample was applied to a column of Q-Sepharose HP 26/10 equilibrated at 4 °C in QA buffer containing 0.1 % (w/v) DDM, and the flow-through containing the AAT was collected. The column was eluted with a gradient of 0–100 % of buffer QB [QA containing 0.1 % (w/v) DDM and 1 M NaCl]. The flow-through was dialysed overnight at 4 °C against 20 vol. of CMA buffer containing 10 mM sodium acetate, pH 4.8, 0.1 M sucrose, 1 mM EDTA, 1 mM DTT and PMSF (0.02 %, w/v). After dialysis, FATR (1 μM) was added and the sample was applied to a column of CM-Sepharose HP 26/10 equilibrated with CMA buffer plus 0.1 % (w/v) DDM and 1 μM FATR. The column was eluted with a gradient of 0–100 % of buffer CMB (CMA containing 0.1 % (w/v) DDM, 1 μM FATR and 1 M NaCl).

Size-exclusion chromatography

Fractions from the CM-Sepharose column were concentrated to 10 mg/ml and applied to a column of Superdex 200 26/60 equilibrated at 4 °C in buffer containing 20 mM Tricine, pH 7.4, 0.1 % (w/v) DDM, 0.1 M sucrose, 150 mM Na_2SO_4 , 1 mM EDTA and 0.02 % sodium azide. The column was calibrated with the following protein standards: ferritin, aldolase, albumin, ovalbumin and ribonuclease A (molecular masses 440, 158, 67, 43, and 13.7 kDa, respectively). The void volume was determined to be 108 ml with Blue Dextran 2000, and the total bed volume was 318 ml. A plot of K_{av} (partition coefficient) versus log(molecular mass) gave a straight line after linear regression (results not shown). It was used to calculate the apparent molecular mass of the AAT.

Protein analysis

Protein samples were analysed by SDS/PAGE [35] on gradient gels containing 12–22 % (w/v) acrylamide [acrylamide:

bis-(acrylamide), 30:0.8 (w/w)] with a 4 % (w/v) stacking gel. The separating and stacking gels were prepared in buffers containing 0.375 M Tris/HCl (pH 8.8) and 0.1 % (w/v) SDS, and 0.125 M Tris/HCl (pH 6.8) and 0.1 % (w/v) SDS, respectively. The running buffer consisted of 0.25 M glycine, 25 mM Tris/HCl (pH 8.3) and 0.1 % (w/v) SDS. Sample buffer containing 0.3 M Tris/HCl (pH 6.8), 10 % (w/v) SDS, 25 % (v/v) glycerol, 5 mM 2-mercaptoethanol and 0.015 % (w/v) Bromophenol Blue was added to the protein samples (typically 10 μ g). Gels were stained with Coomassie Blue.

Limited trypsinolysis and immunodetection of the AAT

Trypsin was added to the protein sample (100 μ g) at a trypsin:protein ratio of 1:100 (w/w). After incubation at 10 °C for 4 h, digestion was stopped by adding bovine pancreatic trypsin inhibitor [2-fold excess (w/w)]. Fragments were separated by SDS/PAGE and transferred on to a PVDF membrane. They were detected with polyclonal primary rabbit antibodies raised against SDS-denatured bovine AAT. Membranes were blocked with a solution containing 50 mM Tris/HCl, 150 mM NaCl, 2 % Marvel milk powder and 0.5 % Tween 20 at 22 °C and then incubated with the primary antibody (1:5000 dilution in blocking buffer) at 22 °C for 2 h. The membrane was washed and then incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate for 2 h. Then the washed membranes were developed with a solution of BCIP/NBT (5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium) made with tablets purchased from Sigma and following the manufacturer's instructions.

N-terminal sequence analysis

Protein samples immobilized on PVDF membranes were deacetylated [36] and analysed by automated Edman degradation using an Applied Biosystems protein sequencer (Model 494) as described previously [37].

Electrospray-ionization MS

Proteins were purified for MS by reverse-phase HPLC on a PRP-3 RP column (Hamilton, Bonaduz, Switzerland). Samples (~300 pmol of protein) were mixed with an equal volume of 6 M guanidinium hydrochloride in 0.1 % (v/v) formic acid then centrifuged at 10 000 *g* for 15 min. The column was equilibrated with 60 % aqueous formic acid and samples were eluted with a linear gradient of propan-2-ol. The molecular mass of protein purified by reverse-phase HPLC was measured with a Sciex API III⁺ triple quadrupole mass spectrometer equipped with electrospray ionization calibrated with poly(propylene)glycols. Samples were introduced into a stream (3 μ l/min) of 50 % (v/v) aqueous acetonitrile and spectra were recorded by scanning the first quadrupole from 600 to 1600 *m/z* (mass-to-charge ratio) over 10 s.

RESULTS

Binding of FATR-ATR to isolated mitochondria

At an excitation wavelength of 488 nm, FATR exhibited an emission maximum at 533 nm (Figure 1A). The emission maximum and fluorescence yield were unaffected by the addition of mitochondria (0.5 mg of protein). Because of the high fluorescence yield at 533 nm, the purification of the FATR-AAT was monitored by emission at 527 nm. Since the fluorescence emission

Figure 1 Fluorescence emission of FATR

(A) Emission spectra of FATR in the presence (solid line) and absence (broken line) of mitochondria (0.5 mg of protein). (B) F_{527} in response to increasing concentrations of FATR. Samples in standard fluorimetry buffer (see Experimental section) were excited at 488 nm.

is insensitive to the presence of protein, and is consistent in the buffer used, the F_{527} could be calibrated against the concentration of FATR. A linear relationship between F_{527} and FATR concentration is shown in Figure 1(B), whereby $F_{527} = 67.66 \times \mu\text{M FATR}$ [R^2 (goodness of fit) = 0.9975]. The binding of FATR to bovine heart mitochondria is shown in Figure 2(A). Non-specific binding to mitochondria was determined in the presence of 20 μM CATR, a potent and highly specific inhibitor of the AAT, and at 0.25 μM FATR, it accounts for approx. 75 % of the binding. The curve for specific binding (Figure 2A, inset) is hyperbolic, as was reported for ATR binding [18]. In these experiments, the total concentration of FATR was approximately that of free FATR, and so a value for K_D could be determined. Fitting the data to the hyperbola by least-squares analysis, revealed a K_D of 53 ± 8 nM ($n = 4$). The number of high affinity sites was 0.21 ± 0.04 nmol/mg protein. Scatchard analysis (Figure 2B) demonstrated a single type of binding site corresponding to the high affinity binding observed with ATR and other fluorescent derivatives [38]. Since CATR prevented the binding of FATR, it is likely that FATR interacted specifically with the AAT.

Inhibition of nucleotide transport by FATR

ADP transport in bovine heart mitochondria was inhibited by increasing concentrations of FATR. Inhibition increased sharply

Figure 2 Binding of FATR to bovine heart mitochondria

(A) Mitochondria (0.5 mg of protein) were incubated in 1 ml of standard fluorimetry buffer with increasing concentrations of FATR. Unbound FATR was removed by washing three times in buffer. Non-specific binding (●) was assessed in the presence of 20 μ M CATR. The inset shows specific binding obtained by the subtraction of non-specific binding (●) from the total FATR bound (○). The data were analysed by a least-squares fit to a hyperbola, from which a K_D value of 53 ± 8 nM ($n = 4$) was obtained. (B) Scatchard analysis of specific binding of FATR to mitochondria demonstrating a single type of high-affinity binding.

at FATR concentrations up to approx. 70 nM, becoming less pronounced at higher concentrations. This pattern of inhibition reflects the specific binding of FATR to mitochondria (Figure 2A, inset). The inhibition plots converged to the same intercept at the ordinate, suggesting that FATR acts as a competitive inhibitor (Figure 3). At lower ADP concentrations, the slopes relative to inhibited transport diverged slightly from linearity. Non-linearity has been described for tightly binding inhibitors, where the concentration of free inhibitor varies significantly depending on the concentration of the added substrate [39]. After least-squares analysis, fitting the data to a hyperbolic function, the K_i (inhibitor concentration at which transport activity is reduced by half) was determined to be 50 ± 13 nM ($n = 3$), which corresponds to the K_D for FATR binding. The stability of binding was determined by transferring mitochondria into buffer containing no exogenous FATR and measuring ADP uptake at various time intervals. The inhibition of ADP uptake remained at constant levels for over a week, indicating a very low 'off-rate' for FATR.

Figure 3 Kinetics of inhibition of ADP transport in bovine heart mitochondria by FATR

Mitochondria (1 mg of protein) were pre-incubated at 0 °C for 45 min with the following concentrations of FATR: 0.0 μ M (■), 0.1 μ M (▲) and 0.2 μ M (▼). ADP transport was started by addition of [14 C]ADP at concentrations ranging from 2.5 to 40 μ M. Velocity (v) is expressed as nmol/min/mg protein.

Table 1 Purification from bovine heart mitochondria of FATR-AAT

Values shown are the average of six independent purifications. Gdm, guanidinium hydrochloride.

Purification step	Total protein (mg)	Protein yield (%)	FATR-binding activity (μ mol)	Specific FATR-binding activity (nmol of FATR/mg of protein)
Isolated mitochondria	4000	100.0	8.0	2.0
SMPs	2200	54.9	5.5	2.7
Gdm-treated SMPs	1080	26.8	3.6	3.3
DDM extract	640	16.1	2.4	3.8
Q-Sepharose	160	4.0	2.3	14.4
CM-Sepharose	125	3.1	1.9	15.2
Superdex 200	102	2.6	1.6	15.7

Extraction of proteins from stripped SMPs

In order to achieve a high level of purification of inhibited AAT, it was found that several enrichment procedures were required before solubilizing the AAT in detergent. Matrix and many outer-membrane proteins were removed during the preparation of SMPs. Treatment of SMPs with 2.0 M guanidinium chloride removed extrinsic membrane proteins, such as the F_1 domain of the ATP synthase, which otherwise were found to co-chromatograph persistently with the inhibited AAT. After solubilization with DDM, over 70% of the total FATR bound to mitochondria had been separated from the AAT (Table 1), which correlates approximately with the proportion of non-specific binding to mitochondria shown in Figure 2. Over 30% of the bound FATR was removed by the preparation of SMPs, suggesting that it was bound to lipid or protein components of the outer-membrane or inter-membrane space. Similarly, it can be deduced that 25% of FATR bound to mitochondria had associated with extrinsic proteins of the inner membrane, and a further 15% was bound either to proteins that were not solubilized or with the bilayer itself.

Figure 4 Cation-exchange chromatography of mitochondrial extracts after partial purification by Q-Sepharose chromatography

(A) Elution profile of flow-through fractions from the Q-Sepharose column (pH 7.4) chromatographed on a CM-Sepharose column after adjusting the pH to 4.8. FATR (1 μ M) was added to the column buffers and the sample. Left-hand axis, absorbance at 280 nm ($A_{280\text{ nm}}$); right-hand axis, NaCl concentration (M). (B) Fluorescence emission of fractions eluted from the CM-Sepharose column. Left-hand axis, F_{527} ; right-hand axis, the approximate FATR content determined using the standard curve shown in Figure 2B. (C) SDS/PAGE analysis of fractions eluted from the CM-Sepharose column. Fractions are labelled across the top of the figure: Q, Q-Sepharose flow-through fractions applied to the CM-Sepharose column; 1–6, fractions from peaks 1–6 shown in Figure 4(A). Molecular mass markers (kDa) are indicated on the left-hand side.

Ion-exchange chromatography of FATR-inhibited AAT

The desalted soluble extract was applied to a Q-Sepharose anion exchange column. The AAT did not bind to this column over the pH range 5.0–9.0, and the addition of FATR did not affect the chromatographic behaviour of the AAT. However, purification was most efficient at pH 7.4, where approx. 75% of the protein in the sample bound to the column with virtually no loss of FATR-binding activity in the flow-through fractions which contained the AAT (Table 1). The residual contaminants included other members of the MCF (Figure 4C, lane Q). Q-Sepharose chromatography was the most efficient purification step in the protocol, resulting in a nearly 4-fold increase in specific activity.

Unbound material from the Q-Sepharose column was dialysed to adjust the pH, and exogenous FATR (1 μ M) was added to both the sample and the CM-Sepharose column buffer. At pH values from 4.5–8.5, over 90% of the AAT did not bind to the CM-Sepharose column. At pH values above 5.5, very little purification was achieved as the flow-through was contaminated

Figure 5 Size-exclusion chromatography of AAT obtained by CM-Sepharose chromatography

(A) Elution profile of AAT from CM-Sepharose peak 1 (solid line) and peak 2 (broken line) chromatographed on a Superdex 200 column. The inset shows SDS/PAGE analysis of protein concentrated from Peak 1, revealing a single band containing AAT. (B) F_{527} of fractions eluted from the column (left-hand axis) and bound FATR concentration (right-hand axis). CM-Sepharose peak 1 (●) and peak 2 (○). The column was calibrated as described in the Experimental section and a plot of K_{av} versus $\log(\text{molecular mass})$ was used to calculate the apparent molecular mass of the samples (results not shown).

with other proteins. However, at pH values below 5.0 virtually all contaminants were removed from the flow-through, including, significantly, other carrier proteins (Figure 4 and Table 1). The small proportion of AAT that bound to the column eluted across the salt gradient as five discrete peaks. The protein in these peaks exhibited no FATR-binding activity (Figure 4B). Re-application of desalted protein from any of the peaks 2–6 yielded the same chromatographic profile of five peaks, with no protein eluting in the flow-through fractions, suggesting that the population of CM-Sepharose-binding AAT molecules were not in a single, stable conformation. The chromatographic profile differed significantly when exogenous FATR was omitted. All of the AAT was bound to the column and it exhibited the same elution profile as the material that had bound in the presence of FATR. Therefore, stabilization of the AAT with FATR is required to prevent it from binding to the column.

Determination of oligomeric state

Fractions from the CM-Sepharose column that contained AAT were analysed by size-exclusion chromatography on a Superdex 200 column (Figure 5). FATR-inhibited AAT from peak 1 eluted at a volume corresponding to an apparent molecular mass of 150 kDa. Estimation of the oligomeric state of the inhibited AAT, taking into consideration the bound detergent, suggests the presence of a dimer in solution supported by approx. 140 molecules of DDM per AAT dimer. This amount of bound detergent is similar to that found for other membrane proteins such as bacteriorhodopsin, cytochrome oxidase and bacterial photosynthetic reaction centres [40]. Analysis of ATR-inhibited AAT demonstrated that it was dimeric in Triton X-100 [22,23]. The molar ratio of bound FATR:AAT was approx. 1 mole of FATR per AAT

Figure 6 Tryptic digestion of the AAT

(A) Time-course for digestion of membrane-bound uninhibited AAT. Numbers on the left-hand side indicate the molecular mass (kDa) and the numbers across the top show the length of the reaction in min. (B) Effect of FATR on trypsinolysis of membrane-bound AAT in the uninhibited state (–) and inhibited with FATR (+). (C) Trypsinolysis of the AAT from peak 1 and peak 2 after CM–Sepharose chromatography. (D) Model of the cleavage pattern shown in (A) showing cleavage at Lys⁴² (K42) and Lys²⁴⁴ (K244) and the N-terminal sequences obtained from the derived fragments. Numbers on the left-hand side indicate the molecular mass (kDa) of the fragments.

dimer, which is in agreement with previous ratios determined for the binding of CATR [20], and other fluorescent ATR derivatives [21]. In contrast, AAT from CM–Sepharose peaks 2–6 eluted at a volume corresponding to an apparent molecular mass of 87 kDa, which corresponds to the AAT monomer with approx. 90 associated DDM molecules.

Analysis of the conformation of the AAT by limited trypsinolysis

Partial trypsinolysis of the AAT revealed a specific pattern of trypsinolysis of the membrane-bound unliganded AAT (Figure 6A). After 5 min, two anti-AAT immunoreactive bands appeared with molecular masses of approx. 25 kDa and 20 kDa. After a further 10 min, first the 25 kDa band and then the 20 kDa band had been digested. N-terminal sequence analysis of the bands (Figure 6D) revealed that the 25 kDa band was a mixture of two fragments with N-terminal sequences starting with Ser¹ and Gln⁴³ (Figure 6D). The 20 kDa band contained a single fragment with Gln⁴³ at its N-terminus. After 2 h digestion, no immunoreactive bands remained and the AAT appeared to have been digested completely. Cleavage at Lys²⁴⁴, which is 54 residues from the C-terminus, removed a 5 kDa fragment from the C-terminal end of the AAT and produced the 25 kDa Ser¹ fragment. Cleavage at both Lys⁴² and Lys²⁴⁴ yielded a 20 kDa fragment (Figure 6D). Although the AAT has 23 trypsin cleavage sites distributed throughout the protein, initial digestion always occurred at Lys⁴² and Lys²⁴⁴ located in loops 1 and 3, respectively.

Treatment of the membrane-bound AAT in the presence and absence of FATR with trypsin for 4 h, revealed that pre-incubation with FATR protects the AAT from digestion (Figure 6B). Protein from CM–Sepharose peaks 1 and 2 was similarly treated. The FATR-inhibited AAT in peak 1 was resistant to trypsinolysis whereas the uninhibited AAT in peak 2 was completely digested (Figure 6C). The major conformational change induced by the binding of FATR to the AAT was the occlusion of these two loops, which in the uninhibited AAT are exposed to bulk solvent and susceptible to proteolysis [12].

Analysis of purified AAT

N-terminal sequence analysis after de-acetylation of CM–Sepharose fractions containing FATR-inhibited AAT yielded the sequence Ser-Asp-Gln-Asp-Ser-Phe- identifying the protein as the AAT [41]. Analysis of samples not subjected to deacetylation revealed no sequence information, demonstrating the absence of other proteins. Mass spectrometric analysis of the same samples resulted in a single series of sharp peaks arising from ions containing 25–45 protons. A molecular mass of 32925.2 Da with a standard deviation of 3.2 was obtained from these ions, which is in agreement with the molecular mass calculated from the amino acid sequence. Other than the series of ions arising from the AAT, no significant peaks were present in the spectrum, demonstrating the purity of the AAT and the absence of microheterogeneity arising from proteolysis or partial modification of the protein.

DISCUSSION

AAT is the most highly expressed member of the mitochondrial transporter family in the inner membranes of mitochondria [1,2]. ATR-inhibited AAT has been purified previously by chromatography on HA [28–30], and this preparation has been the subject of extensive kinetic and biochemical analysis. However, this purification method has proved to be inadequate for producing pure protein for structural analysis using biophysical or crystallographic techniques, because trace amounts of other members of the MCF, particularly the phosphate carrier, oxoglutarate/malate carrier and aralar, persist. In addition, microheterogeneity in the protein itself, arising most significantly from the inability of HA chromatography to reliably separate the highly stable enzyme complexed with ATR from free AAT, which has been shown to be conformationally unstable [5–9,15]. HA is a crystal form of calcium phosphate that has been applied in the purification of numerous proteins. Depending on the ionic state of the column, HA can function as a cationic or anionic matrix, but the precise mechanism through which proteins interact with HA remains ambiguous [42,43].

The use of a two-step ion-exchange chromatography protocol has enabled the pH to be adjusted to optimize interactions between the cationic and anionic supports. Negative Q–Sepharose and CM–Sepharose chromatography at pH 7.4, purified the protein to a level similar to the traditional HA method, in which both FATR–AAT and uninhibited AAT did not bind to either support. However, by adjusting the pH to 4.8 for CM–Sepharose chromatography, it was found that the unliganded AAT and other persistent mitochondrial carriers were removed. These interactions most likely occurred through charged loops 1 and 3 which were exposed in the uninhibited AAT. Inhibition by FATR induced conformational changes in which the loops are occluded within the protein, thus preventing it binding to the columns. The use of FATR as a fluorescent probe for monitoring the AAT during chromatography enabled the purification to be more specific by isolating different conformational states of the protein. FATR–AAT isolated by this method was highly pure and lacked heterogeneity.

Conclusions

The two-stage ion-exchange chromatography method described in this study yields a highly pure preparation of FATR–AAT suitable for biophysical or structural analysis (Figure 4A, inset and Table 1). Binding of FATR locks the dimeric AAT in a single stable conformation characterized by a tightly packed helical bundle burying the loops within. With very few charged residues exposed,

the binding of the protein to either cationic or anionic matrices is precluded. A small proportion of the FATR-AAT complex dissociates during the purification, resulting in monomeric AAT. Loss of FATR from the AAT renders the protein conformationally unstable and able to bind to the anionic matrix through exposed positive residues. The separation of these molecules from the inhibited AAT by CM-Sephadex chromatography provides pure, stable FATR-AAT. The purification has been routinely reproduced using non-derivatized ATR with the same results.

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