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# The mitochondrial ATP/ADP carrier: Interaction with detergents and purification by a novel procedure

Manuel Rojo *, Theo Wallimann

*Institute for Cell Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland*

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**Abstract**

The interaction of several classes of detergents with mitochondrial ATP/ADP carrier (AAC) was studied. The detergents that were best suited for solubilization of active AAC differed in several physico-chemical properties, but contained relatively rigid or planar hydrophobic (sub)moieties. Based on specific binding of AAC to Blue Sepharose, a novel method for the purification of the AAC was developed. The new method gave AAC samples which were devoid of non-essential lipids and allowed to purify AAC isoenzymes from several species and tissues to a significantly higher degree of purity than that achieved up to now. Western blot analysis of purified AACs with an antiserum against chicken heart AAC confirmed that immunological variability is more important between tissues than between species. In contrast to liver and kidney AACs, brain AAC displayed similar antigenic properties to heart AAC.

*Keywords:* ATP/ADP carrier; Detergent; Purification

## 1. Introduction

The ADP/ATP carrier (AAC) catalyzes the exchange of ATP and ADP across the inner mitochondrial membrane [1] and belongs to the large family of mitochondrial anion carriers [2,3]. This intrinsic membrane protein was one of the first membrane transport proteins to be purified [4] and has been thoroughly characterized since then [1]. However, the precise transport mechanisms as well as the nature of the functional oligomeric unit are still unknown. In addition, since AAC crystallization has not yet been achieved, information on its structural organization is derived only from the primary sequence [5,6], from the differential reactivity of various amino acid residues to specific reagents [7] and from the proteolytic susceptibility of certain (extramembranous) domains [8]. The final goal of this work was to obtain AAC preparations that would be suited for conducting extensive crystallization studies in the future.

Over the last years several membrane proteins have been crystallized (in three or two dimensions). Success in crystallization was partly due to the availability and use of several newly developed detergents and amphiphiles (see, e.g., [9–11]). Although various detergents have been the subject of systematic studies concerning multiple aspects of their interaction with membranes and membrane proteins, still no rationale exists that allows to identify a priori the detergent or detergent class that is suited best for a particular protein and/or application. Thus, detergents have to be selected on the basis of empirical studies. In this work we have measured the ability of various classes of deter-

Abbreviations: AAC, ATP/ADP carrier; BS, Blue Sepharose; CAT, carboxyatractyloside; $C_{10}E_5$, decyl pentaoxyethylene ether; $C_{12}E_9$, dodecyl nonaoxyethylene ether (Thesit); CHAPS, 3-[(3-choloamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediamine tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; HTP, hydroxyapatite; LDAO, lauryldimethylamine oxide; NP-40, Nonidet P-40; octyl-Glc, octyl β-D-glucopyranoside; octyl-POE, octyl polyoxyethylene ether; Pipes, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; PVDF, polyvinylidene difluoride; RA, Red agarose (Reactive Red 120); SB 3–12, *N*-dodecylsulfobetaine (Zwittergent 3–12); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TX-100, Triton X-100.

* Corresponding author. Present address: Université de Genève, Département de Biochimie, Sciences II, 30, quai Ernest-Ansermet, CH-1211 Genève 4, Switzerland. Fax: +41 22 7026464. E-mail: rojo@sc2a.unige.ch


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gents to solubilize AAC transport activity in order to compare their effect on AAC.

The availability of purified protein remains the basic requirement for extensive and successful crystallization and reconstitution studies. Up to now, AAC has been purified by hydroxyapatite (HTP) chromatography from bovine heart [4] and, after selective preextraction and solubilization procedures, also from other tissues and organisms [12,13]. Using this simple and rapid method AAC is co-eluted with mitochondrial lipids and, due to the similar chromatographic properties of several mitochondrial carriers and porins towards HTP [2,14], also with some of these mitochondrial proteins. In order to improve the purity of AAC samples, we developed a new purification procedure based on specific binding of solubilized AAC to Blue Sepharose. Binding to Blue Sepharose resulted in the separation of AAC from other mitochondrial carriers and from excess mitochondrial lipids and allowed exchange of the detergent used for solubilization. Using this procedure, we purified AAC isoenzymes from different species and tissues to a significantly higher degree of purity than that achieved up to now.

## 2. Materials and methods

### 2.1. Materials

HTP (Bio Gel HTP) was from Bio-Rad, Blue Sepharose from Pharmacia LKB, Dowex 1 × 8 from Serva, Red Agarose (Reactive Red 120), Carboxyatractyloside (CAT) and turkey egg yolk phospholipids (phosphatidylcholine type XII-E, No. P8077) from Sigma, [$^{14}$C]ATP (No. CFB.91) from Amersham. Detergents were purchased of the highest available quality (peroxide- and carbonyl-free resp. membrane research quality): TX-100 from Sigma, $C_{12}E_9$ (= Thesit) and NP-40 from Boehringer, octyl-Glc, octyl-POE and $C_{10}E_5$ from Bachem, LDAO from Calbiochem, SB 3–12 and CHAPS from Fluka. Lipids were dissolved in methanol/chloroform (1:4) and kept at −20°C under $N_2$. Detergent stock solutions (10–20% w/v in water) were stored at 4°C: CHAPS and octyl-Glc for 1 week, other detergents for months. Polyoxyethylene detergents were stored under $N_2$.

### 2.2. Preparation of mitochondria

All steps were performed at 4°C. Mitochondria were prepared by differential centrifugation after mechanical disruption of the different tissues with a glass-teflon homogenizer (all rat tissues) or a polytron tissue blender (bovine and chicken cardiac muscle) in isotonic MSH-buffer (220 mM mannitol, 70 mM sucrose, 10 mM Hepes (pH 7.4), 0.05 mM EDTA). The mitochondrial fraction of brain and heart was subjected to an additional density gradient centrifugation step in 20% Percoll [15]. Mitochondria were centrifuged in a tabletop centrifuge, the pellets (5–30 mg protein) frozen in liquid nitrogen and kept at −70°C. Only if mitochondria were used for AAC purification with HTP and BS chromatography, mitochondria (10 mg/ml) were incubated in 40 μM CAT in MSH prior to storage (CAT-treated mitochondria).

### 2.3. Reconstitution and measurement of ATP transport

Bovine heart mitochondria (10 mg/ml) were solubilized in NTE-buffer (150 mM $Na_2SO_4$, 10 mM Tricine (pH 8.0), 0.2 mM EDTA) with 3% detergent by repeated sucking of the detergent/membrane solution (0.2–0.5 ml) through a pipette tip. The solubilisate was subjected to ultracentrifugation (Airfuge™ Beckman, 4 min, 30 psi) and the supernatant (30 μl) was immediately added to 0.6 ml of preformed liposomes (60 mg/ml phospholipid in 30 mM $Na_2SO_4$, 20 mM ATP, 20 mM Pipes (pH 7.0)). Preformed liposomes were obtained by resuspension of evaporated turkey egg yolk phospholipids in 30 mM $Na_2SO_4$, 20 mM ATP, 20 mM Pipes (pH 7.0) and extensive (30 min) sonication under $N_2$ gas in ice/$H_2O$ [16]. The AAC was reconstituted by the freeze-thaw sonication procedure [16] and the amount of [$^{14}$C]ATP in the vesicles was measured as described [16]. The activity parameters were calculated as follows: after addition of [$^{14}$C]ATP to the proteoliposome suspension, AAC transport activity mediates the equilibration of the specific activities of ATP inside and outside the proteoliposomes. Due to the strict counterexchange catalyzed by AAC [1], the increase of internal [$^{14}$C]ATP is described by the equation:

$$cpm_{in}(t) = cpm_{in}^{max} \times (1 - e^{-kt})$$

Where $cpm_{in}(t)$ is the amount of [$^{14}$C]ATP in the vesicles at the time $t$, $cpm_{in}^{max}$ (= the maximal transport capacity) is the amount of internalized label after complete equilibration of internal and external specific activities (i.e., $cpm_{in}(t = \infty)$), and $k$ is the transport rate of the AAC in $min^{-1}$. $cpm_{in}^{max}$ and $k$ were fitted to the data using the fitting procedure provided by the KaleidaGraph™ program. The maximal transport capacity $cpm_{in}^{max}$ was also determined by measuring the amount of internalized [$^{14}$C]ATP after the time required for complete equilibration of internal and external specific acitivites ($t \geq 30$ min). It is important to remark that $cpm_{in}^{max}$ is proportional to the number of vesicles that have reconstituted active AAC molecules.

### 2.4. AAC purification

All steps were performed at 4°C. Mitochondria were preextracted and solubilized at a protein concentration

of 10 mg/ml with 0.5% and 3% detergent, respectively, in NHE-buffer (150 mM $Na_2SO_4$, 20 mM Hepes (pH 7.0), 0.2 mM EDTA) for 5 min with a glass-teflon homogenizer rotating at 180 rpm. Solubilized membranes were subjected to ultracentrifugation (70 Ti rotor, 20 min, 70000 rpm) and the supernatant applied to HTP-columns (20 mg protein/g dry HTP) which had been preequilibrated with NHE-buffer containing 0.5% detergent. The columns were eluted with NHE-buffer containing 0.5% detergent. The flow-through fractions were analyzed by SDS-PAGE and those containing highly enriched AAC were pooled (= HTP-eluate) and concentrated by ultrafiltration. When required, the $Na_2SO_4$ concentration was adjusted by dilution with HE-buffer (20 mM Hepes (pH 7.0), 0.2 mM EDTA) containing 0.5% detergent. Experiments with BS and other affinity matrices were performed with commercially available chromatography columns or with Pasteur pipettes clogged with cotton-wool. Columns were eluted by gravity and/or with peristaltic pumps at a flow rate $\leq 0.75$ ml $min^{-1} cm^{-2}$.

### 2.5. SDS-PAGE and immunoblot

SDS-PAGE was performed as described by [18]. To achieve optimal resolution of the proteins of the HTP-eluate, the separation gel contained 17.5% acrylamide with an acrylamide/bisacrylamide ratio of 150:1 [14] (instead of the normal ratio of 37.5:1). Staining was with Coomassie brilliant blue R-250. Proteins were transferred onto nitrocellulose or PVDF membranes using a semi-dry transfer chamber at a constant current of 1 mA/$cm^2$ for 1 h. Primary antibodies were decorated with peroxidase-labeled goat anti-rabbit IgG antibodies coupled to horseradish peroxidase. Peroxidase activity was detected by incubation of the nitrocellulose sheet in PBS containing 0.03% chloronaphthol plus 0.03% $H_2O_2$. Stain intensity of gels and immunoblots was quantified by densitometry with a Shimadzu CS-930 scanner. For the generation of anti-AAC antiserum, purified chicken heart AAC (150 $\mu$g) was emulsified with complete Freund's adjuvant and injected intracutaneously at multiple sites on the back of rabbits. A first boost was given after 28 days and a second one after an additional 14 days with 75 $\mu$g of protein each. 14 days after the last boost the animals were killed, bled and the sera stored frozen.

### 2.6. Other methods

Ultrafiltration was performed with membranes from Amicon (PM/YM, with pore size 10 or 30). Proteins were precipitated from samples containing high amounts of detergent and/or lipid with methanol/chloroform [17]. Protein was determined with the bicinchoninic acid (BCA) assay of Pierce Chemical Co. including 0.25% SDS and using bovine serum albumin (BSA) as a standard. Proteins were analyzed by Edman degradation using an Applied Biosystems 470A sequencer. Lipid phosphorus was measured by the method of Fiske and SubbaRow [19] after destruction of phospholipids with 70% perchloric acid.

## 3. Results

### 3.1. Interaction of AAC with detergents

Detergents determine the oligomeric state and conformation of solubilized membrane proteins, can lead to their irreversible denaturation and may influence their chromatographic properties (e.g., the mitochondrial porin VDAC does not bind to HTP when solubilized in TX-100, but binds when solubilized in LDAO [20]). Since up to now AAC has been preferentially solubilized in TX-100 [1], we decided to also test other detergents in our purification attempts. In order to select appropriate, 'benign' detergents, several classes of detergents were investigated for their ability to solubilize the active AAC. Earlier work had revealed that the ability to reconstitute AAC transport is rapidly lost after solubilization [21], and we therefore reasoned that the ability of a detergent to solubilize the AAC in an active state would reflect its 'mildness' towards the native structure of this protein molecule.

Bovine heart mitochondria were solubilized with several classes of detergents; the solubilization conditions (detergent concentration, detergent-to-protein ratio and ionic strength) were chosen for maximal solubilization of membrane proteins [22]. Indeed, except for $C_{12}E_9$, all detergents displayed a similarly high solubilization capacity of mitochondrial proteins (Fig. 1A). The amount of solubilized AAC in these mitochondrial solubilisates was determined in a semi-quantitative manner by densitometric analysis of immunoblots decorated with an antiserum raised against chicken heart AAC (immunoblot not shown; for characterization of the anti-AAC antiserum see Fig. 4). It appeared that detergents differed in their ability to solubilize AAC (Fig. 1B): TX-100 had the highest AAC solubilization capacity, followed by NP-40, CHAPS and LDAO (60–80% of that of TX-100). $C_{12}E_9$, $C_{10}E_5$, octyl-POE and SB 3–12 had a lower capacity to solubilize AAC (30–40% compared to that of TX-100), and octyl-Glc solubilized a far lower amount of AAC (approx. 10% of that solubilized by TX-100).

In order to determine to what extent the solublized AAC remained active, the mitochondrial solubilisates were subjected to a reconstitution procedure and the reconstituted maximal transport capacities were measured. With the protein-to-lipid ratio used in these reconstitution experiments the number of AAC

molecules is limiting, and therefore the maximal transport capacity is proportional the number of reconstituted active AAC molecules. It appeared that the maximal transport capacity reconstituted from the TX-100-, NP-40- and CHAPS-solubilisates was significantly higher than that of the other solubilisates (Fig. 1C). The values of the reconstituted activity (maximal transport capacity, Fig. 1C) and of the amount of solubilized AAC (Fig. 1B) were used to calculate relative specific activities (specific maximal transport capacity, Fig. 1D). This latter parameter is a measure of the ratio of active versus inactive AAC molecules in the mitochondrial solubilisates. The relative specific activities of AAC solubilized with octyl-Glc, TX-100, NP-40 or CHAPS were the highest, followed by those of AAC solubilized with $C_{12}E_9$, $C_{10}E_5$ or octyl-POE. The relative specific activities of AAC solubilized with LDAO or SB 3–12 were significantly lower (Fig. 1D).

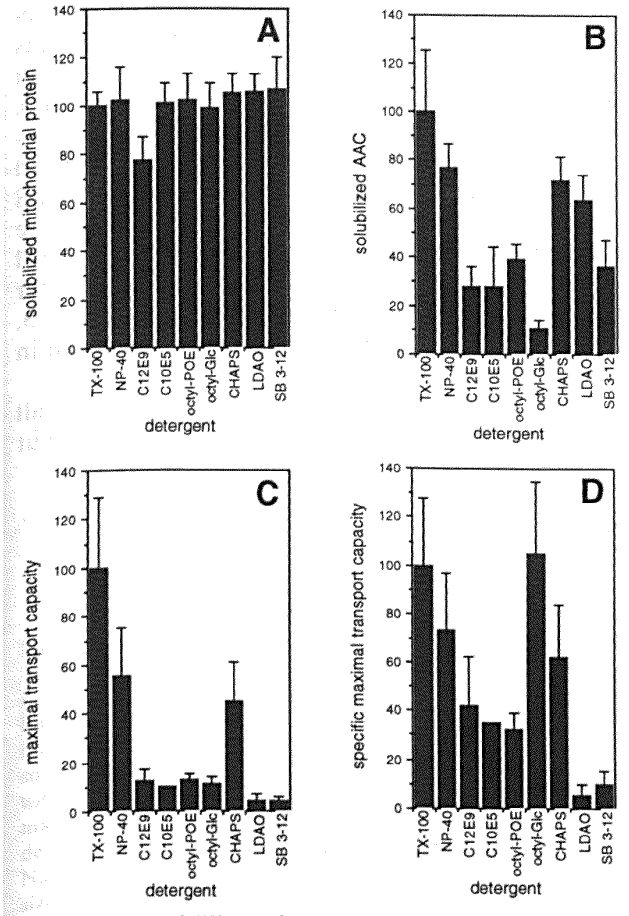


Fig. 1. Capacity of different detergents to solubilize and reconstitute AAC. Bovine heart mitochondria were solubilized with different detergents and ultracentrifuged. The protein (A) and AAC (B) content of each supernatant was determined. Aliquots of the supernatants were immediately subjected to a reconstitution procedure and their maximal transport capacity determined (C). The values of the maximal transport capacities were divided by the values of the AAC content to express the specific maximal transport capacity (D). All values are the mean of three experiments and are expressed as percentage of the values obtained with TX-100: 84.5±5% solubilized mitochondrial protein (A); 100±25 arbitrary units (B); 16.4±4.7 nmol ATP/ml (C); 100±28 arbitrary units (D). Bars indicate the standard deviation.

The AAC transport rates could only be measured where relatively high maximal transport capacities had been reconstituted (i.e., with TX-100, NP-40 or CHAPS). These transport rates varied largely between different series of reconstitution experiments ($k = 0.1$–$1\ min^{-1}$). However, within an experimental series, no significant differences were observed between the transport rates of different AAC preparations (TX-100, NP-40 and CHAPS; not shown).

In view of these results, three detergents were selected for further studies on the purification of AAC: TX-100, CHAPS and octyl-POE. TX-100 and CHAPS were selected because they combined a high AAC solubilization capacity with a high AAC specific activity. Octyl-POE was selected instead of NP-40 (which appeared to have more favorable properties) because our interest was to conduct the purification attempts with detergents of different classes. In contrast to NP-40, which closely resembles TX-100 in several aspects, octyl-POE differs from TX-100 and other 'classical' polyoxyethylene detergents in the size of its hydrophylic moiety (octyl-POE, $n = 5$; $C_{12}E_9$, $n = 9$; TX-100, $n = 10$; NP-40, $n = 11$ polyoxyethylene units). These features may be relevant for our purposes because the large polyoxyethylene moieties of 'classical' detergents might hide the small hydrophilic domains of AAC molecule [1] and render them inaccessible for protein–matrix or protein–protein interactions [20].

### 3.2. Purification of AAC

HTP-chromatography has been the method of choice for the purification of AAC [4] and of several other mitochondrial carriers and porins [2]. However, since at least five mitochondrial membrane proteins, including AAC, do not adsorb to HTP [14], it is often difficult to achieve complete purification of these carriers by a single HTP chromatography step. In this work, samples were analyzed with an SDS-PAGE system that, contrary to standard SDS-PAGE, allows to resolve several protein components of the HTP-eluate [14]. We found that AAC samples obtained by HTP chromatography (HTP-eluates) were always contaminated to a variable extent with other proteins (Fig. 2, lanes a, d and g), and that the extent of contamination was higher when mitochondria from other tissues than bovine heart were used as a starting material (not shown).

In order to obtain AAC samples of higher purity, different affinity matrices were tested for their ability to bind AAC. HTP-eluates were applied to affinity

columns (0.4 mg protein/ml column material) preequilibrated with 0.5% detergent in NHE-buffer and the columns were washed with the same buffer. It appeared that AAC was not retained by ATP-hexane-agarose or 5′-AMP-Sepharose (not shown) but bound to Blue Sepharose (BS) and Red agarose (RA). This interaction appeared to be specific because, in contrast to AAC, the other proteins of the HTP-eluate were collected in the flow-through (Fig. 2, lanes b, e and h). Due to the ability of BS and RA to bind several dehydrogenases and kinases, it was inferred that AAC binding also depended on a structural similarity between the ligands of BS and RA and AAC substrates. However, in contrast to the elution properties of some kinases (e.g., mitochondrial creatine kinase [23]), nucleotide-containing buffers were not suited for the elution of AAC, and therefore high ionic strengths, chaotropic salts or SDS had to be used (see below).

The strong binding of AAC to BS and RA prompted us to investigate whether other matrices would also bind AAC in a specific, yet weaker manner, so that AAC could be displaced in the presence of its substrates. It was shown earlier that several anthraquinones, including the ligand of BS (Cibachron blue) are inhibitors of AAC [24]. Therefore, anthraquinone dyes whose inhibition type, in contrast to that of Cibachron blue, had been shown to be competitive for ADP (Nuclear Fast Red and Uniblue A [24]) were coupled to Sepharose. However, these matrices failed to retain AAC even at very low ionic strengths (not shown). Considering that the ligand of Red agarose, Procion red, is not an anthraquinone- but a naphthol-derivative, it appears as if the combination of numerous sulfonate and aromatic groups in the affinity ligands, and not a structural similarity of these ligands to nucleotides or anthraquinone dyes, is required for AAC binding (for discussion see, e.g., [25]).

It was known that the solubilized AAC is more stable when bound to its specific inhibitors carboxyatractyloside (CAT) or bongkrekate [21]. However, since CAT-binding could have disturbed the interaction of AAC with affinity matrices, all previously described experiments were done with the free AAC. We decided to proceed with the purification of the AAC using the chromatographic matrix BS and found that the binding of CAT by AAC did not affect its interaction with BS. Therefore, all subsequent experiments were performed with mitochondria that had been preincubated with CAT (CAT-treated mitochondria). Briefly, CAT-treated mitochondria were washed, pre-extracted and solubilized. The solubilisate was applied to HTP-columns and the HTP-eluates loaded onto BS columns ($\leq$ 1.5 mg protein/ml BS). BS columns were then eluted sequentially with loading buffer, a NaCl gradient (0.15–3 M) and an SDS-containing buffer (representative elution profiles are shown in Fig. 3, further details are given in the Methods section and in the figure legends).

Binding of the AAC occurred in the presence of all three detergents (TX-100, CHAPS and octyl-POE), but

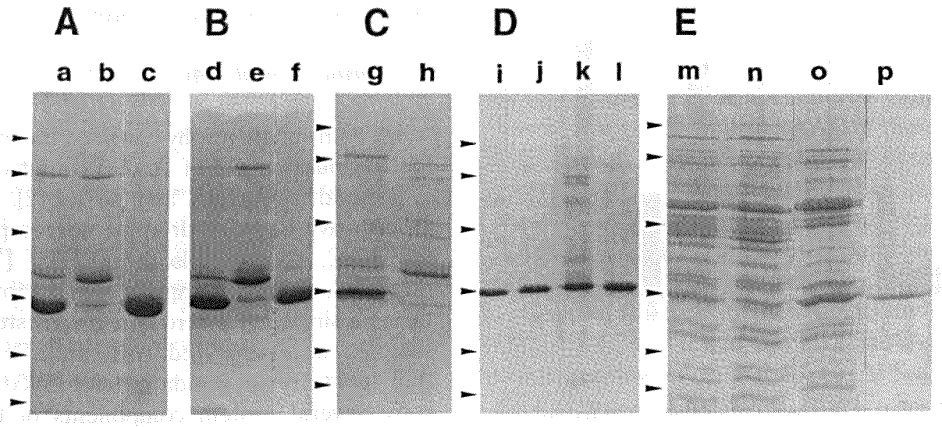


Fig. 2. SDS-PAGE analysis of AACs at different stages of purification. Mitochondria from bovine heart (A, B, C, E) or different rat tissues (D) were solubilized in 3% TX-100 (A), CHAPS (B) or octyl-POE (C, D, E) and either passed through HTP-columns and the HTP-eluates applied to BS-columns (A–D) or directly applied to BS-columns (E). All BS-columns were sequentially eluted with loading buffer, a NaCl-gradient and SDS-containing buffer as described in Fig. 3. A, B, C and E show different fractions of individual purification experiments. D shows equivalent fractions of four independent experiments. Lanes a, d and g: HTP-eluates; lanes b, e and h: flow-through of the BS-columns (equivalent to fractions 1–5 of the experiments described in Fig. 3); lanes c and f: homogeneously pure AACs eluted from BS (equivalent to fractions 27–36 of the experiments described in Fig. 3B and C). Lanes i–l: pure AACs from rat heart (i), brain (j), liver (k) and kidney (l) eluted from BS (equivalent to fractions 46 and 47 in experiments equal to that described in Fig. 3A). Lane m: bovine heart mitochondria; lanes n, o and p: flow-through of the BS-column, fraction eluted with 1 M NaCl and fraction eluted with SDS respectively (i.e., equal to fractions 1–5 (n), 21–23 (o) and 46 + 47 (p) of an experiment equal to that described in Fig. 3A, except that solubilized bovine heart mitochondria instead of a HTP-eluate was applied to BS). Lanes a–f, 10 $\mu$g; lanes g–l and p, 6 $\mu$g; lanes m–o, 30 $\mu$g protein. Arrowheads indicate the positions of molecular mass markers (97, 66, 43, 31, 22 and 14 kDa).

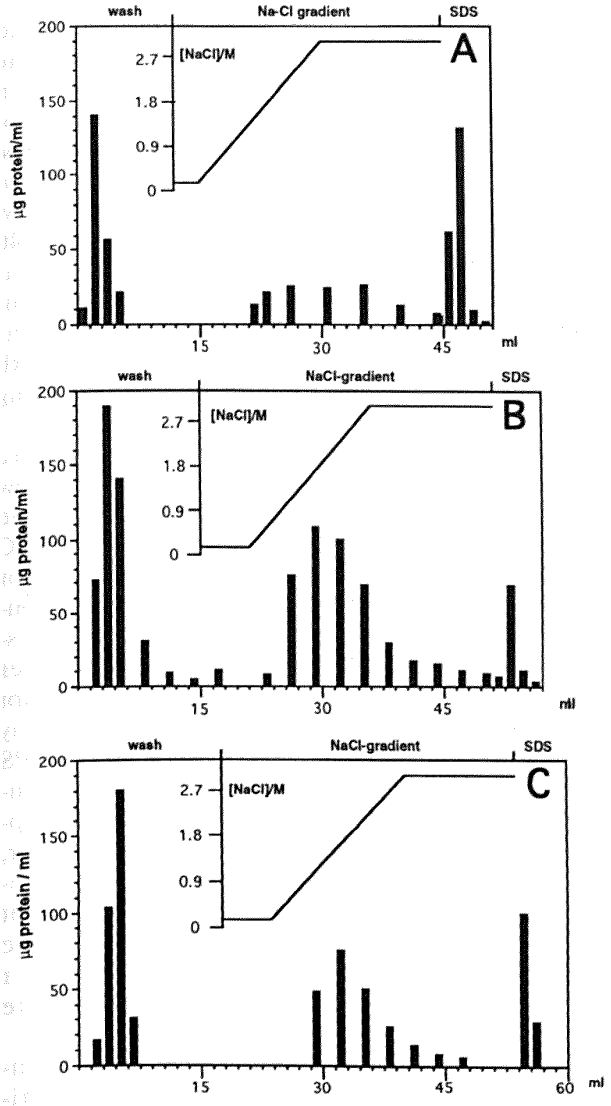


Fig. 3. Elution profiles of BS-chromatography with octyl-POE (A), TX-100 (B) and CHAPS (C). HTP-eluates of bovine heart (A, C) or rat kidney (B) were applied to BS-columns equilibrated with NHE buffer with 0.5% detergent (A, B) or with 25 mM $Na_2SO_4$ in HE buffer with 0.5% detergent (C). After loading, the columns were successively eluted with the respective equilibration buffer (wash), with a NaCl gradient (NaCl-gradient) and then with 0.5% SDS in 150 mM NaCl (SDS). For the NaCl gradient, HE-buffer containing 0.5% detergent was supplemented with the indicated concentration of NaCl. The 1.5 ml BS-columns were eluted with 0.3 ml/min, fraction size was 1 ml. Columns were equilibrated to room temperature prior to elution with 0.5% SDS in 150 mM NaCl.

a lower ionic strength ($\leq$ 25 mM instead of 150 mM $Na_2SO_4$) was required for quantitative binding when using CHAPS. The detergent also appeared to influence the conditions of elution: while with TX-100 and CHAPS a major fraction of BS-bound AAC was already eluted with the NaCl gradient (Fig. 3B and C), AAC in octyl-POE could only be eluted in the SDS fraction (Fig. 3A). Routinely, 6–9% of mitochondrial protein was recovered after HTP-chromatography and 3–5% after BS-chromatography (i.e., the recovery of the BS step was about 50%). As judged by SDS-PAGE, the AAC eluted from BS was homogeneously pure (Fig. 2, lanes c and f). The amount of phospholipid that co-eluted with AAC was in the range of 0.1–0.3 $\mu$mol lipid phosphorus/mg protein; this approximately corresponds to the amount of phospholipid (mostly cardiolipin: 0.18–0.25 $\mu$mol/mg) that was found to co-sediment in sucrose-density gradients with AAC [26].

Due to the stronger binding of AAC when solubilized in octyl-POE, it was also possible to establish a one-step purification procedure for AAC. Bovine heart mitochondria were solubilized with octyl-POE, directly applied to BS columns ($\leq$ 15 mg protein/ml BS) and the columns sequentially eluted as described (loading buffer, a NaCl gradient and SDS-containing buffer). Although the SDS-PAGE patterns of mitochondria (Fig. 2, lane m) and the flow-through fraction (Fig. 2, lane n) appeared to be quite similar, numerous mitochondrial proteins bound to BS (20–30% of the applied material). While several bound proteins were eluted with increasing salt concentrations (Fig. 2, lane o), only AAC remained bound to BS (3–4% of applied protein) and was eluted in a highly pure form (Fig. 2, lane p) with SDS-containing buffer.

### 3.3. Analysis of purified AAC isoenzymes

Having established a protocol that allowed purification of bovine heart AAC to homogeneity, we then proceeded to purify AAC from different sources for comparative studies. We purified AACs from bovine and chicken heart and from rat heart, brain, liver and kidney (for the SDS-PAGE patterns of the rat AAC isoenzymes see Fig. 2, lanes i–l). We generated a rabbit antiserum against the purified chicken heart AAC. The anti-AAC antiserum was used to reveal AAC in blots of total mitochondrial proteins from chicken heart and to compare the immunogenic properties of AACs purified from all the different sources (see Fig. 4B for protein staining with Poinceau red). The antiserum reacted in a highly specific manner because it decorated almost exclusively the 30 kDa band of AAC on blots of total chicken heart mitochondrial protein (Fig. 4A, lanes a and a'). The antiserum decorated all AACs with marked tissue- and species-specific differences. The strongest signal was obtained with AAC from chicken heart (Fig. 4A, lanes a and a': mitochondria, lanes b and b': purified AAC). The signal intensities obtained with the bovine heart, rat heart and rat brain AACs (Fig. 4A, lanes c, d and e, respectively) were lower and almost identical amongst each other. Finally, while rat liver AAC gave almost no signal (Fig. 4A, lane f), rat kidney AAC gave a weak signal (Fig. 4A, lane g).

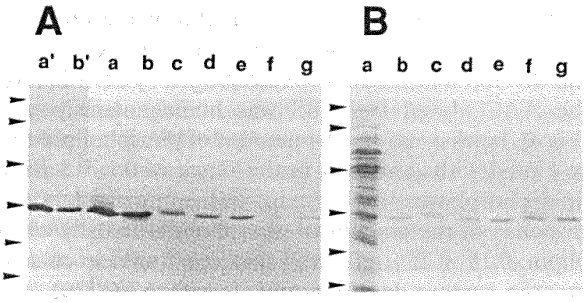


Fig. 4. Analysis of the purified AACs by immunoblot. Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose sheets. The nitrocellulose sheets were stained with Poinceau red (B), destained, and decorated with an antiserum raised against purified chicken heart AAC (A). Lanes: a and a′, chicken heart mitochondria; b and b′, chicken heart AAC; c, bovine heart AAC; d, rat heart AAC: e, rat brain AAC; f, rat liver AAC: g, rat kidney AAC. The anti-AAC antiserum was diluted 1:500 except for lanes a′ and b′, where it was diluted 1:2000. Antibodies were visualized with goat anti-rabbit antibodies coupled to horseradish peroxidase. Lanes a and a′, 15 μg; other lanes, 1.5 μg protein. Arrowheads indicate the positions of molecular mass markers (97, 66, 43, 31, 22 and 14 kDa).

It is not yet clear whether the antigenic diversity of AAC isoenzymes arises from differential gene expression, alternate RNA splicing or post-translational modifications. Since at least two different AAC genes are expressed in a tissue-specific manner in mammals [27,28] and since the differences between the highly homologous AAC genes are clustered at the N-terminus [27], we tried to obtain N-terminal protein sequence information for the different AACs. The AACs from different rat tissues (heart, brain, kidney, liver) were subjected to SDS-PAGE, blotted onto PVDF membranes and subjected to Edmann degradation followed by amino acid analysis. No amino acids could be detected, suggesting that the N-termini of all AACs might be blocked, perhaps by the N-terminal acetylation found for the bovine heart isoenzyme [5].

## 4. Discussion

The studies on the interaction of the AAC with several classes of detergents revealed that TX-100, NP-40 and CHAPS were best suited for solubilization of AAC from mitochondria: they solubilized high amounts of AAC that retained a high specific activity. It is remarkable that these three detergents display similar properties towards the AAC because TX-100 and NP-40 (both are nonionic polyoxyethylene detergents with an aromatic residue in their hydrophobic moiety) differ from CHAPS (zwitterionic detergent with a hydrophobic moiety similar to that of bile acids) in several physico-chemical properties (molecular structure, charge of the polar moiety, type and aggregation number of the micelle) and even in the solubilized AAC conformation they induce [29]. In many cases the effects of detergents on membrane proteins have been shown to depend mainly on the nature (charge, size) of the 'polar head' and/or the size of the 'hydrophobic tail' (e.g., [30]). Here it appears as if the presence of cyclic structures in the hydrophobic detergent moiety, which is the only common feature of TX-100, NP-40 and CHAPS, is most relevant for efficient solubilization and reconstitution of active AAC. This apparent 'mildness' of detergents with relatively rigid or planar hydrophobic (sub)moieties has been also found with other membrane proteins, but the reasons underlying this effect are still debated (for review see, e.g., [30]).

The zwitterionic detergents LDAO and SB 3–12 solubilized high amounts of AAC with a very low specific activity. Since the loss of activity might be due to an (irreversible) effect of these detergents on AAC structure, these detergents might not be suited for conducting structural studies on the AAC. The nonionic detergents $C_{12}E_9$, $C_{10}E_5$ and octyl-POE displayed intermediate properties: they solubilized lower amounts of AAC than the other detergents (except octyl-Glc) with a specific activity that was halfway between that obtained with TX-100/NP-40/CHAPS and with LDAO/SB 3–12. In contrast, octyl-Glc solubilized significantly lower amounts of AAC that appeared to have a very high specific activity. However, we cannot rule out that this high specific activity results from the amplification of errors that are inherent to the measurement of very low values. Therefore, the interaction of AAC with octyl-Glc requires further characterization before definitive conclusions are drawn.

With the novel purification procedure AAC isoenzymes from several species and tissues could be purified to a much higher degree than that obtained up to now. The increase in purity was accompanied by a reduced yield of AAC. Since the yield after HTP-chromatography is aproximately 60% (as determined by other authors that followed protein-bound [$^{35}$S]CAT through different steps of purification [4]) and the recovery of the BS-step was 50%, we estimate that the fraction of mitochondrial AAC recovered after BS-chromatography is approximately 30%. This yield is in the same range as that achieved when purifying mitochondrial creatine kinase by BS-chromatography (47%) [23]. Since the purified AAC samples were devoid of non-essential lipids and since binding of AAC to BS allows exchange of the detergent used for solubilization, this new method will enable one to perform new reconstitution and crystallization experiments with controlled detergent and lipid composition. It has to be remarked that crystallization of membrane proteins could frequently be achieved only with a particular isoenzyme, and that the detergent and lipid environ-

ment were instrumental in rendering crystallization possible (for review see [9–11]).

Western blot analysis of the purified AACs confirmed that immunological variability is more important between tissues than between species and showed that, in contrast to liver and kidney AACs, brain AAC displays similar antigenic properties to heart AAC. Previous work of other authors had revealed that antisera raised against bovine heart AAC cross-react (1) similarly well with heart AACs of different mammals (rat, cow and pig), (2) with a lower affinity with AAC from rat and bovine kidney and (3) with the lowest affinity with AAC from rat and bovine liver [31–33]. The fact that in this work an antiserum raised against chicken heart AAC discriminated between AACs from different mammalian tissues with the same specificity as sera raised against mammalian heart AACs (1) shows that chicken and mammalian heart AACs have similar antigenic properties, (2) suggests that the antigenic tissue-diversity of mammalian AACs also applies to chicken (avian) AACs and (3) confirms that immunological variability is more important between tissues than between species. It is remarkable that, in contrast to the rat liver and kidney AACs, rat brain AAC displayed similar antigenic properties to rat heart AAC.

From the high specificity of AAC–BS interaction we infer that BS-chromatography could also be used for purification of AAC from other sources than those tested here, e.g., from *Escherichia coli* extracts (the expression of recombinant AAC in *E. coli* has been reported recently [34]) and for specific removal of AAC from protein mixtures (e.g., mitochondria or carrier preparations). The latter use is relevant because, due to the relative abundancy of AAC in mitochondria and to the similar chromatographic properties of several mitochondrial carriers towards HTP [2,14], AAC is often present in preparations of other mitochondrial carriers as a minor but persistent contamination.

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