IMMUNOLOGICAL CHARACTERIZATION OF THE ADP, ATP TRANSLOCATOR PROTEIN ISOLATED FROM MITOCHONDRIA OF LIVER, HEART AND OTHER ORGANS

Evidence for an organ specificity

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Received 22 December 1976

1. Introduction

The isolation and characterization of a CAT- and BKA-binding protein from bovine heart mitochondria representing different conformational states of the putative ADP,ATP translocator were reported [1–5]. Antibodies against the CAT– and BKA–protein were produced and shown to discriminate strongly between the two conformations [4]. After isolation of the protein from different sources and species, evidence has now been obtained for an organ specificity of the immunological properties of the ADP,ATP translocator. In this paper both, the isolation of the CAT–protein from some other organs and the immunological cross-reactions with the anti-CAT–protein and anti-BKA–protein will be reported.

2. Materials and methods

[^{3}H]CAT was prepared by Na^3HB_4 reduction of CAT-aldehyde (Babel et al. unpublished) [^{35}S]CAT as described earlier [6] and [^{3}H]BKA as recently published [7]. CAT loading of intact mitochondria from different organs and species was done at 0°C in a 10 mM piperazine-*N*,*N*'-bis(2-ethane sulfonic acid) buffer, 0.25 M sucrose, pH 6.8 (buffer A) for 10 min, with 2 μmol labelled CAT/g mitochondrial protein.

BKA loading was carried out at 20°C in buffer A, with 4 μmol [^{3}H]BKA/g protein in the presence of 50 μM ADP for 15 min. Unbound inhibitor was removed by two washes with buffer A. Before solubilization of the translocator protein, mitochondria were pre-extracted with 0.25% Brij 58 at 0°C, in a buffer containing 100 mM Na_2SO_4, 10 mM Tricine, 0.05 mM EDTA, pH 8.0 (buffer B), liver mitochondria at a protein concentration of 50 mg/ml, heart and kidney mitochondria at 10 mg/ml.

After centrifugation at 9500 × *g* for 10 min, the sediment was suspended in buffer B containing in addition 5% Triton X-100 and incubated for 10 min at 0°C, then centrifuged at 143 000 × *g* for 40 min. The supernatant contained the solubilized inhibitor–translocator complex.

Hydroxylapatite chromatography was essentially carried out as described earlier [2] except 100 mM Na_2SO_4, 10 mM Tricine, 0.05 mM EDTA, 0.5% Triton X-100, 0.05% NaN_3, pH 8.0 (buffer C) was used for elution.

Sepharose 6B column chromatography was performed in buffer C. Molecular weight of the isolated translocator proteins was estimated by the SDS–polyacrylamide gel electrophoresis (SPAGE) method with standard calibration proteins (bovine serum albumin, ovalbumin, LDH, triose phosphate isomerase, lactoglobulin, cytochrome *c*).

For antibody production, rabbits received the isolated inhibitor–translocator complex in a solution containing 5 mM Tris, 100 mM NaCl, 5% Triton X-100, pH 7.2.

Prior to injection Freund's incomplete adjuvant was added in a ratio 3:1. Initial injection was 2 mg protein, followed by several booster injections with 1 mg each.

Abbreviations: ATR atractylate, BKA bongkrekate, CAT carboxyatractylate, SPAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

Table 1
Isolation scheme for the CAT–protein complex from calf liver mitochondria

	[^{35}S]CAT content (μmol/g protein)
Calf liver mitochondria + CAT (buffer A) (2 μmol/g protein)	0.4
↓ Extraction with 0.25% Brij 58 (buffer B) (50 mg protein/ml)	
↓ Centrifugation	
Sediment – 5% Triton X-100 (buffer B)	0.8
↓ Centrifugation	
Supernatant → Hydroxylapatite column (buffer C)	
↓	
Pass-through → Sepharose 6B Column (buffer C)	4.9
↓	
Peak-fraction → Sepharose 6B (buffer C)	14.6
↓	
Purified CAT–protein	15.2

Details in Materials and methods.

The presence of antibody was detected first after 4 weeks by the double-diffusion agar plate technique of Ouchterlony, in 1.5% agar plates gelled from a solution containing 56 mM Na-diethylbarbiturate, 56 mM Na-acetate, pH 8.4.

Purification of serum followed the procedure of Masters et al. [8]. Immunodiffusion was carried out overnight at room temperature. After drying the plates, they were stained with Coomassie Brilliant Blue and destained with a solution containing 10% acidic acid and 50% methanol.

3. Results

3.1. *Isolation*

In previous publications we have shown that the ADP,ATP translocator protein can be isolated in two conformational states as CAT–protein complex (*c*-state) and BKA–protein complex (*m*-state) [3,4]. As demonstrated with antibodies, both states have different antigenic properties [4].

In order to investigate organ- and species-specificity of the antibody reactions, the CAT– and BKA–protein complexes were isolated from the mitochondria of various organs. The procedure, originally developed for the isolation of the protein from bovine heart mitochondria, could be successfully adopted in slightly modified versions to mitochondria from other organs. One obtains the inhibitor–protein complex up to 95% pure in Triton X-100 solution. Table 1 shows a flow-sheet for the isolation of the CAT–protein from calf liver mitochondria. The isolated CAT–proteins from calf liver and bovine heart mitochondria migrate in SDS–gel electrophoresis (co-electrophoresis) as one single band at about 30 000 mol. wt. (fig.1). In agreement with the identical molecular weight of the polypeptide from both organs, the amount of CAT bound reaches similar values in both cases.

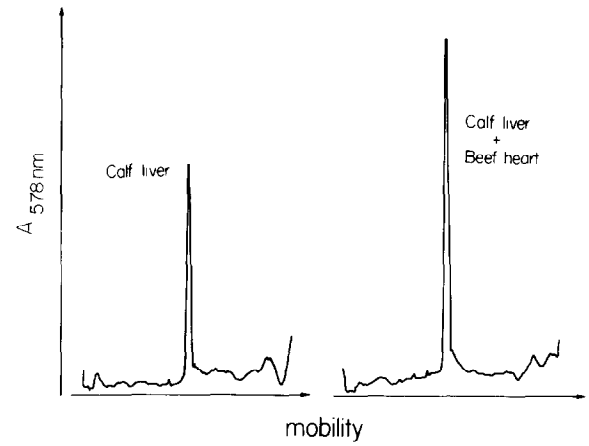

Fig.1. Densitometric traces of SDS–polyacrylamide gel electrophoresis (SDS-PAGE). (Left) purified CAT–protein from calf liver mitochondria (5 μg protein). (Right coelectrophoresis of 5 μg CAT–protein from bovine heart mitochondria and 5 μg CAT–protein from calf liver mitochondria.

In table 2, the binding data are summarized for the various mitochondria and isolated CAT-binding proteins. The amount of CAT bound in the purified proteins ranges from 13–18 μmol/g protein. This variation reflects either a different degree of purity or an incomplete recovery of binding as a result of different stabilities. The enrichment is higher for the isolation of the protein from liver because of the

Table 2
Characteristics of the CAT–protein complex isolated from different organs

Mitochondria source	[^{3}H]- or [^{35}S]CAT bound μmol/g protein		Enrichment factor	Mol. wt (× 10^3) in SPAGE
	Mitochondria	Isolated protein		
Bovine heart	1.8	18.3	10	30
Rat liver	0.35	15.6	45	30
Calf liver	0.4	15.2	38	30
Rat kidney	1.1	13.2	15	30
Neurospora crassa	0.90	15	17	34

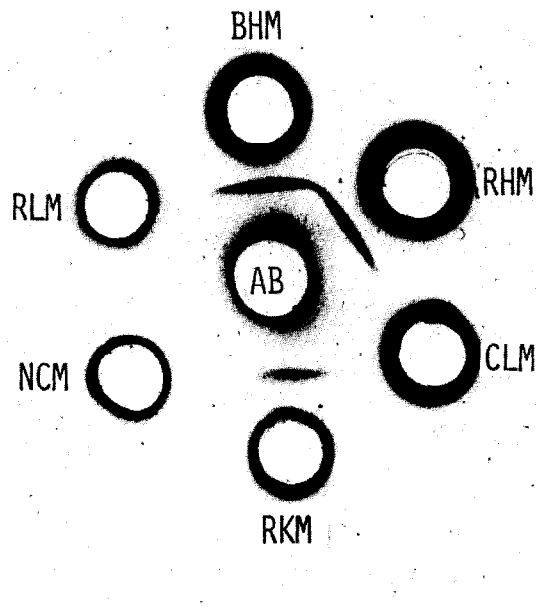

Fig.2. Organ specificity of the antibody against bovine heart mitochondrial CAT–protein in Ouchterlony double diffusion. Precipitation bands are shown after staining agar plates with Coomassie Blue. Center well contained 5 μl of antibody against CAT–protein from bovine heart mitochondria (AB) (69 mg protein/ml). Outer wells, 5 μl of one of the following: CAT–protein (2 mg protein/ml) from bovine heart mitochondria (BHM), purified CAT–protein (2 mg protein/ml) from rat heart mitochondria (RHM), calf liver mitochondria (CLM), rat kidney mitochondria (RKM), *Neurospora crassa* mitochondria (NCM) and rat liver mitochondria (RLM). Each of the solutions in the outer wells contained 0.5% Triton X-100.

lower content of CAT-binding sites in the original mitochondria. The apparent molecular weight in SDS–gel electrophoresis is 30 000 for all proteins with the exception of that for *Neurospora crassa* (H. Hackenberg, diploma thesis, 1975).

The isolation of an ATR-binding protein as claimed by Vignais from rat liver mitochondria using affinity chromatography resulted in a preparation which was only characterized by gel-filtration in detergent (emulphogen). With an estimation of the molecular weight based on gel-filtration in detergent, these authors concluded that the protein exists in a 60 000 and 15 000 mol. wt form which are interconvertible. This preparation differs from the CAT–protein of liver reported here which moves as a single peak in gel-filtration and has a subunit of 30 000 mol. wt in SPAGE.

3.2. *Immunological studies*

The antibodies against CAT–protein from bovine heart mitochondria did not precipitate with the CAT–protein isolated from bovine liver in immunodiffusion on agar plates (fig.2). On the other hand, this anti-CAT–protein reacted with the protein from the same rat organs with corresponding specificity. A clear but weak precipitation was also observed with rat kidney translocator preparation. In accordance to our previous report [4] with the *Neurospora crassa* translocator protein no cross-reaction was seen.

In table 3, the immunological cross-reactions of the anti-CAT–protein and anti-BKA–protein from bovine heart with the ADP,ATP translocator proteins, isolated in various forms and from various organs are shown. In addition to the CAT–protein also the BKA-loaded and -unloaded forms were used as antigens. All forms of the bovine or rat liver are unreactive against bovine heart CAT–protein antibody.

Although the antibody against BKA–protein from bovine heart mitochondria is specific for the carrier conformation in the same organ, it showed a clear, but weak precipitation with all forms of the protein from bovine and rat liver mitochondria.

Because of the weak antigenic activity of the SDS-treated CAT–protein, we did not get sufficiently active antibodies and were therefore unable to study an antigenic specificity of the primary structure of the proteins from different sources.

The results indicate an organ specific reaction between the anti-CAT–protein and the CAT–protein such that there is no cross-reaction between heart and liver and some overlapping reaction between heart and kidney. In contrast the anti-BKA–protein does not react with this selectivity.

4. Discussion

4.1. *Isolation of CAT–protein complex from liver and kidney*

The isolation of the CAT–protein complex from liver requires a higher degree of enrichment than that from bovine heart since the content of CAT-binding

Table 3
Immunological cross reaction of various forms of ADP,ATP carrier protein (Immunodiffusion)

Source	Antigen \ Antibody	Anti-CAT–protein (From beef heart mitochondria)	Anti-BKA–protein (From beef heart mitochondria)
Bovine heart	CAT–protein	++	––
	BKA–protein	––	++
	Unloaded	––	––
	SDS-treated CAT–protein	––	––
Calf liver	CAT–protein	––	+
	BKA–protein	––	+
	Unloaded	––	+
Rat liver	Same results as calf liver		
Rat heart	CAT–protein	++	––
Rat kidney	CAT–protein	+	––
Rabbit organs	Same results as rat organs		

sites was five-times higher in the mitochondria from heart than from liver. Nevertheless essentially the same purification procedure can be applied. Initial pre-extraction with Brij 58 results in a considerable enrichment because of the higher content of soluble proteins in liver and the final purification requires two instead of one gel-filtration step. The purified preparation has a CAT content which is only about 10% lower than that from heart. The purification of the CAT–protein from kidney mitochondria requires a smaller enrichment factor, similar to that of heart, because the kidney mitochondria are relatively rich in CAT-binding sites.

4.2. *Immunological organ specificity*

In a previous publication an astonishing specificity of the antibodies raised against the isolated CAT–protein and BKA–protein was demonstrated. There was nearly no cross-reaction of the anti-CAT–protein with other forms of the protein such as the BKA–protein. A similar somewhat less stringent specificity was observed with the anti-BKA–protein. Apparently consistent with this specificity no reaction with the CAT–protein from *Neurospora* and rat liver was noted.

The present studies reveal that there is no cross-reaction of the anti-CAT–protein from bovine heart with the same antigen from another organ such as liver, even from the same species. On the other hand there is a strong reaction with CAT–protein from the same organ, heart, although from different species such as rat and rabbit. It can be concluded that the antigenic reactivity has a strong organ specificity but at the same time low antigenic differences of this protein exist between these species.

The fact that the anti-BKA–protein from bovine heart reacts with all three forms of the carrier protein from liver, is not necessarily in contradiction with the interpretation for the organ specificity of the anti-CAT–protein, in view of the fact that the anti-BKA–protein preparation is less homogeneous than the anti-CAT–protein because of the greater instability of the BKA–protein used as the antigen. This organ nonspecificity may therefore be related to the less stringent conformation specificity of the anti-BKA–protein preparation.

Antigenic organ specificity of membrane proteins is to our knowledge so far not yet described, except for the mammalian Na^+,K^+-ATPase [9]. The organ specificity of the CAT protein might best be explained in terms of isoenzyme relations such that differences in the primary structure of the heart and liver CAT–

protein exclude common antigenic sites. This exclusion is probably linked to amino acid differences at those sites which are antigenic only in the conformation of the anti-CAT–protein. Therefore, the antigenic organ specificity can be expected to be abolished in the denatured protein. Such a relation between disappearance of organ specificity and denaturation appears not yet sufficiently documented in the literature.

The classical example for organ specific antibodies are those against lactate dehydrogenase where the anti-H–LDH does not react with the isoenzyme M–LDH and vice versa [10]. It has therefore been concluded that the antigenic sites are located at those sites of the surface of native LDH molecules where amino acids are replaced between the two isoenzymes (M. Rossmann, personal communication).

Extending the analogy to LDH further to intermediate hybrid conformations, the situation in the CAT–protein can be visualized such that the CAT–protein, which probably is a dimer, contains identical 'iso-subunits' from heart and liver. The CAT–protein of kidney, with an intermediate antigenicity to anti-CAT–protein from heart, could be a hybrid of heart and liver subunits.

4.3. *Autoimmunological aspects*

In this context an interesting autoimmunological aspect is raised. On the basis of the species unspecificity an autoimmune tolerance in rabbit against the bovine CAT–protein might be expected. In rabbit all CAT–protein related antigenic reactions were the same as in the other animals. This indicates that in the membrane the antigenic determinants of the CAT-binding protein are not accessible. This is in agreement with former studies on the exchangeability of CAT in the CAT–protein – antibody complex from bovine heart [4]. The antigenic sites of the anti-CAT–protein have therefore been considered to be located on the lipophilic interface of the protein which is normally hidden in the membrane. Separation of the carrier protein from the membrane might provoke autoimmunological phenomena in an organism. This was confirmed by an experiment where we found precipitating antibodies against isolated CAT–protein from bovine heart in a serum of a patient with antibodies against mitochondria.

Acknowledgements

This work was supported by a grant from the Sonderforschungsbereich 51 of the Deutsche Forschungsgemeinschaft. During this work W.E. was supported by a fellowship of the Deutsche Forschungsgemeinschaft. The authors thank Professor Berends (Delft) for a gift of bongkrekic acid. The excellent technical assistance of Mrs A. Kriegsmann is gratefully acknowledged.

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