FRAGMENTATION OF THE ADP/ATP CARRIER PROTEIN FROM BEEF HEART MITOCHONDRIA

Localisation of the atractyloside binding site in a peptide obtained by cyanogen bromide cleavage

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1. Introduction

Covalent photolabeling of the mitochondrial ADP/ATP carrier protein in situ by a photoactivable derivative of atractyloside, referred to as arylazido atractyloside, has been described in [1,2]. Photolabeling is a convenient approach to analyse the localisation of the atractyloside binding site. Atractyloside is known as a non-penetrant inhibitor [3]; therefore its photoactivable derivative is expected to bind covalently to a domain of the carrier protein which is at the outer surface of the mitochondria.

The experiment described here was designed to map the atractyloside binding site. In principle, such an experiment consists in photolabeling intact mitochondria by [^{3}H]arylazido atractyloside, followed by extraction and purification of the photolabeled carrier. Peptide bonds in the isolated carrier protein are then subjected to specific cleavage by chemical or enzymatic means. The labeled peptide isolated after cleavage presumably contains the atractyloside binding site that was originally exposed to cytosol in the inner mitochondrial membrane. Here, we show that cyanogen bromide cleavage of the photolabeled carrier protein from beef heart mitochondria (mol. wt 30 000 yields only one labeled cleavage product with mol. wt 23 000.

Abbreviations: Mops 3-(*N*-morpholino propane sulphonic acid); SDS, sodium dodecyl sulfate; Arylazido atractyloside, *N*-4-azido-2-nitrophenyl aminobutyryl atractyloside; CNBr, cyanogen bromide

2. Materials and methods

2.1. *Materials*

[4-^{3}H]Aminobutyric acid (45 Ci/mmol) was obtained from the Commissariat à l'Energie Atomique, Saclay.

Sephadex G-50 (fine) was purchased from Pharmacia, guanidine–HCl and iodoacetamide from Pierce, acrylamide and bis-acrylamide from Eastman-Kodak, linear polyacrylamide (mol. wt 5×10^6) from British Drug Houses and cyanogen bromide from Merck.

2.2. *Synthesis of photoactivable derivatives of ADP and atractyloside*

N-4-Azido-2-nitrophenylaminobutyryl atractyloside (arylazido atractyloside) was prepared in its radioactive form as in [1] with spec. radioact. 2.0×10^{11} dpm/mmol.

2.3. *Preparation of the beef heart carrier protein*

The ADP/ATP carrier protein was routinely purified from beef heart mitochondria as in [4], with some modifications including washing with organic solvents. Mitochondria (~ 1 g protein) were suspended in 160 ml 0.12 M KCl, 1mM EDTA, 60 μM carboxyatractyloside, 5 mM Mops (pH 6.8) and incubated at 4°C for 30 min. They were lysed by Triton X-100

and subjected to hydroxyapatite chromatography. The pass-through fraction (~ 30 mg protein) was concentrated on a PM 10 Amicon membrane. This concentrated fraction (20 ml) was treated by 5 vol. acetone at −20°C for 1 night. After centrifugation, the precipitate was washed twice by 30 ml diethyl-oxide containing 1% formic acid at −20°C to remove bound lipids. At this stage, the material subjected to SDS–polyacrylamide gel electrophoresis gave a single band with a mol. wt 30 000, after staining by Coomassie blue.

Protein was measured with the Folin-Ciocalteu Reagent [5] in the presence of SDS; bovine serum albumin was used as a standard.

2.4. *Cleavage by cyanogen bromide*

CNBr cleavage was performed according to [6]. The detailed procedure is given in section 3.1. The cleavage products were separated either by chromatography on Sephadex G-50 fine equilibrated with 30% acetic acid or by high pressure liquid chromatography using a peptide separation column equilibrated with 70% acetic acid.

2.5. *Electrophoresis of cleavage products of the carrier protein. Detection of the [^{3}H]-labeled product by fluorography*

Electrophoresis was made on 20% polyacrylamide slab gel in the presence of 0.1% Na-dodecyl sulfate as in [7]. After fixation in a mixture of 10% trichloro-acetic acid –20% methanol, the proteins were colored with Coomassie blue R250. ^{3}H-labeled peptides in polyacrylamide gels were detected by fluorography as in [8], after impregnation with a solution of 2–5 diphenyloxazole in dimethylsulphoxide. After drying, the gel was exposed for 4 weeks to an hypersensitive Kodak RPX-Omat film at −70°C [9].

2.6. *Amino acid analysis*

The carrier protein and the peptides resulting from CNBr cleavage were hydrolysed with 6 N HCl for 18 h and 48 h at 110°C in evacuated sealed tubes. Hydrolysates were analyzed using a Technicon amino acid analyzer. The content in tryptophan was established after hydrolysis with methane sulfonic acid [10]. Cysteine residues were determined as carboxylmethyl-cysteine after treatment with iodoacetamide or as cysteic acid after performic acid oxidation.

3. Results

3.1. *CNBr cleavage of the purified ADP/ATP carrier protein*

The purified carrier protein (10 mg in 2 ml 6 M guanidine–HCl, 2 mM EDTA and 200 mM Tris–HCl (pH 8.5) was treated by 3 mM dithiothreitol under N_2 at 40°C for 1 h then carboxymethylated in the dark in the presence of 15 mM iodoacetamide for 15 min. The reaction was terminated by addition of 30 mM dithiothreitol. After dialysis in the dark against 2 l distilled water at 4°C overnight, the protein solution was incubated for 3 h at 37°C in 0.2 M thioglycolic acid to reduce possible methionine sulfoxide groups. The carrier protein was then precipitated with acetone at −20°C, followed by 3 washings with a mixture of acetone and water (80/20, v/v). After evaporation of acetone under vacuum in the cold, the protein was subjected to CNBr cleavage. At this point a major difficulty inherent to the CNBr cleavage technique itself had to be solved, namely the non-complete cleavage of methionyl peptide bonds because of methionine oxidation that generates methionyl sulfoxide. Thioglycolic acid is able to both prevent the formation of methionyl sulfoxide and reduce methionyl sulfoxide into methionine [11]; thioglycolic acid was therefore systematically introduced in the medium. In fact, its omission resulted in the formation of a supplementary peptide with mol. wt 25 000. Routinely, 10 mg alkylated protein was treated with a 500-fold excess of CNBr (with respect to the methonine residues) in 70% (v/v) formic acid for 36 h at 37°C [6]. The reaction was terminated by addition of 5 vol. distilled water. The cleavage products, recovered after freeze drying, were dissolved in a 1 ml mixture of 6 M guanidine–HCl, 5% butanol and 30% acetic acid, then separated by chromatography on a column of Sephadex G-50 fine (90 × 1.5 cm) equilibrated with 30% acetic acid. Elution by 30% acetic acid was followed by spectrophotometry at 254 nm. Four clearly separated peaks were obtained (fig.1). They included a peak corresponding to a large molecular weight product (23 000) (peak I) and three others (II–IV) corresponding to small peptides (mol. wt < 5000). SDS–polyacrylamide gel electrophoresis indicated that peak I contained only one peptide ($CNBr_1$) migrating as a single band (mol. wt 23 000) whereas peaks II, III and IV contained a hetero-

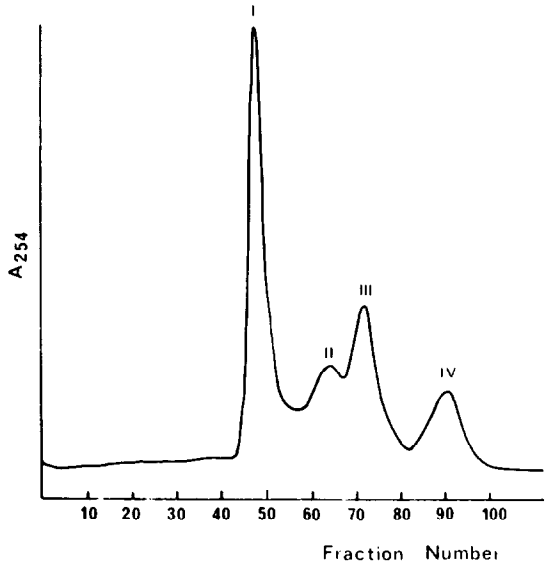

Fig.1. Gel filtration of the CNBr cleavage products of the ADP/ATP carrier protein. Detailed conditions are given in section 3.1. Peaks I–IV were analyzed by SDS–polyacrylamide gel slab electrophoresis (see fig.2).

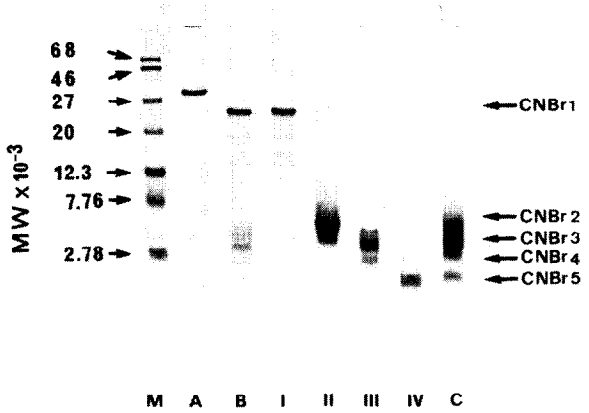

Fig.2. SDS–polyacrylamide slab gel electrophoresis of CNBr cleavage products of the ADP/ATP carrier. Gel M corresponds to migration of standards. The molecular standards used were bovine serum albumin (68 000), ovalbumin (46 000), triosephosphate isomerase (27 000), trypsin inhibitor (20 000), cytochrome *c* (12 300), cytochrome *c* CNBr fragments (7800 and 2780). Gel A corresponds to the isolated ADP/ATP carrier protein, gel B to CNBr cleavage products, gel I–IV to CNBr cleavage products present in peaks I–IV recovered by Sephadex G-50 chromatography (cf. fig.1), gel C to the sum of the CNBr cleavage products ($CNBr_2$–$CNBr_5$).

Table 1
Amino acid composition of the ADP/ATP carrier protein and the CNBr peptide carrying the ADP and atractyloside site

	CNBr product I mol. wt 23 000 (residues/mol)	ADP/ATP carrier mol. wt 30 000 (residues/mol)
Asp	16	22–24
Thr	9	14
Ser	11	16
Glu	17	19
Pro	6	7–8
Gly	23	28–29
Ala	25	28 ± 1
Val	14	19
(Cys-)	3	4
Met	1	6
Ile	10	14–15
Leu	19	22
Tyr	9	11–12
Phe	17	19
Trp	1–2	4
Lys	15	17
His	3	4
Arg	12	14–15

Residues/mol of assumed mol. wt 23 000 and 30 000: nearest integer established after different hydrolysis times

geneous material corresponding to at least four peptides ($CNBr_2$–$CNBr_5$) (fig.2). The mol. wt 23 000 product was also obtained with good yield by high pressure liquid chromatography, starting from the CNBr cleavage products solubilized in 26% formic acid, 70% ethanol and 4% water (data not shown).

Amino acid determinations of the mol. wt 23 000 product and of the non-degraded protein are presented in table 1. The polarity index of the mol. wt 23 000 product calculated as in [12] was found to be the same as that of the whole carrier molecule (40%). No N-terminal amino acid residue could be identified both in the carrier protein and in the mol. wt 23 000 cleavage, suggesting that the mol. wt 23 000 peptide and the intact carrier protein have in common the same blocked N-terminal amino acid. Consequently the small peptides ($CNBr_2$–$CNBr_5$) arising from CNBr cleavage and thereby most of the Met residues are located in the C-terminal moiety of the protein. Some free homoserine was detected in the CNBr cleavage products (~ 4.2 nmol starting from 30 nmol carrier pro-

tein). Free homoserine suggests a Met–Met sequence [6]; the small yield of accumulation of free homoserine (14% of the expected value) can be explained by a residue of Ser or Thr adjacent to the Met–Met sequence [13].

3.2. *Isolation of the photolabeled ADP/ATP carrier protein from beef heart mitochondria photoirradiated in the presence of [^{3}H]arylazido atractyloside. Analysis of the CNBr cleavage products*

[^{3}H]Arylazido atractyloside was added at 5 μM final conc. to beef heart mitochondria (20 mg protein) in 10 ml 120 mM KCl, 5 mM Mops, 1 mM EDTA (pH 6.8). After 30 min incubation in the dark, the mixture was photoirradiated for 20 min at 0°C with an Osram lamp of 250 W. The mitochondria were then sedimented by centrifugation for 10 min at 20 000 × *g* and washed once with 10 ml of the same medium without the arylazido derivative. They were then lysed with Triton X-100 without further treatment, and the lysate was passed on a hydroxyapatite column as for the non-labeled carrier protein. Photolabeled protein (500–800 μg) was recovered in the pass-through fraction. The CNBr cleavage products of the isolated photolabeled carrier were strictly similar to those found for the non-labeled carrier, namely a large mol. wt 23 000 peptide and several small peptides. The ^{3}H radioactivity was located by fluorography after SDS–polyacrylamide gel electrophoresis. Gel 1 (fig.3) corresponds to the separation of the CNBr cleavage products of the carrier protein. The ^{3}H radio activity was found to be essentially located in the mol. wt 23 000 peptide. No radioactivity was detected in the region of the gel corresponding to the small molecular weight products. Gels 2 and 3 (fig.3) correspond to the photolabeled carrier protein and to a crude extract of photolabeled mitochondria, respectively. Gel 2 shows a main radioactive band (b) with mol. wt 30 000 and a faint one with mol. wt ~ 60 000 (band d) which might be a dimer. In gel 3, beside the carrier protein (band b) and its putative dimer (band d), there were supplementary radioactive bands (a,c) with mol. wt ~ 26 000 and ~ 45 000. The significance of these latter bands is not clear.

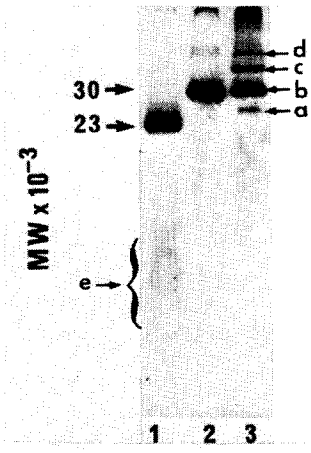

Fig.3. Radioautograph of SDS–polyacrylamide slab gel electrophoresis of photolabeled samples. Identification of the CNBr peptide labeled by [^{3}H]arylazido atractyloside. Beef heart mitochondria were photolabeled as in section 3.2. The ADP carrier protein was then isolated from the photolabeled mitochondria, and the isolated photolabeled protein was treated by CNBr. Gel 1 corresponds to the CNBr cleavage products. Gel 2 corresponds to the ADP/ATP carrier prior to CNBr cleavage and gel 3 to a SDS extract of photolabeled mitochondria. Arrow (b) corresponds to the carrier protein, arrow (a) possibly to a degradation product of mol. wt 27 000. Radiolabeled bands of higher molecular weight, (c,d) correspond to unknown components. The low molecular weight CNBr cleavage products, not detected by autoradiography, are located in a region of the gel designated by arrow (e).

4. Discussion

This work represents the first approach to the study of the arrangement of the polypeptide chain of the ADP/ATP carrier within the inner mitochondrial membrane. We describe here the localisation of the atractyloside binding site using a specific photolabel, [^{3}H]arylazido atractyloside. Covalent photolabeling by [^{3}H]arylazido atractyloside was performed on intact mitochondria. Our approach was based on the fact that atractyloside is a non-penetrant inhibitor of the ADP/ATP carrier and that its binding site is contained in a domain of the carrier protein which is exposed to the outside. The same approach holds for the binding site of arylazido atractyloside, since atractyloside and arylazido atractyloside have the same affinity and specificity [1,2]. Among the CNBr cleavage products of the photolabeled carrier, a large peptide with mol. wt 23 000 was found to contain most of the ^{3}H radioactivity. It had a blocked N-terminal amino acid, as was the case of the intact carrier protein. Several small peptides, at least 4, located

close to the C-terminal end of the carrier protein were apparently devoid of bound radioactivity. These data indicate that at least part of the mol. wt 23 000 peptide fragment corresponds to the outer domain of the carrier protein and that one or several regions of this domain constitute the atractyloside binding site. Hopefully a more precise localisation of the atractyloside binding site may be attained by using other cleavage procedures. Similar photolabeling assays were performed with [^{3}H]arylazido ADP (data not shown) with the same results as for [^{3}H]arylazido atractyloside. This could be expected as arylazido ADP is a non-penetrant inhibitor like arylazido atractyloside and that both ADP and atractyloside may bind to closely related sites.

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