# IODINATION OF PERIPHERAL MITOCHONDRIAL MEMBRANE PROTEINS IN CORRELATION TO THE FUNCTIONAL STATE OF THE ADP/ATP CARRIER

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**Summary**

Iodination of intact mitochondria with $^{125}I$ results in the labeling of essentially one polypeptide with an approximate MW of 30 000. This polypeptide seems to be a component of the inner boundary membrane as it can not be removed from the mitochondria by procedures which destroy the outer membrane (e.g. incubation with digitonin). The amount of the radioactive label which can be bound to this polypeptide is determined by ADP, atractylate, and bongkrekate, components which act on the functional state and the position of the ADP/ATP carrier in the membrane. [1,2]

Recently it was shown that the inner boundary membrane of mitochondria differs from the crista membrane in protein and phospholipid composition which suggests a different function of this part of the inner membrane. [3,4] Some of these specific protein and (or) phospholipid components may perform the contacts between the outer and inner boundary membranes as described by Hackenbrock [5].

In this paper we describe a protein which is suggested to be located within the contact sites. It can be labeled at the periphery of intact mitochondria and it seems to be connected in some way to the proteins of the inner membrane because of two properties: 1.) it can not be removed from the mitochondria by digitonin; 2.) the amount of label which can be bound to the protein depends on the orientation of the ATP/ADP car-

rier within the inner membrane as described in the reorientation mechanism by Klingenberg [1,2].

## METHODS

Mitochondria from rat liver were isolated in a buffered sucrose medium (0.3 M sucrose, 10 mM triethanolamine, 2 mM EDTA, pH 7.4) as described recently [6]. Mitochondria of Tetrahymena pyriformis (strain GL amicronnelate) were isolated from logarithmically growing cells by a modified method of Kobayashi [7].

### Iodination of mitochondrial membranes

Iodination of intact mitochondria and fragments of mitochondrial membranes was performed according to [8]. The incubation mixture contained mitochondrial material suspended in sucrose medium pH 7.4, KI 0.01 mM, $^{125}I$ 500 µCi, glucose 0.5 mM, lactoperoxidase 0.1 mg, glucose oxidase 0.5 µg. The reaction was initiated by addition of glucose. When indicated iodination of intact mitochondria was also performed at pH 6.5 in the presence of atractylate 5 µmol, bongkrekate 10 µmol and ADP 20 µmol per g mitochondrial protein. After incubation for 10 min at room temperature the reaction mixture was diluted ten fold with cold 0.3 M sucrose and the mitochondria or fragments of mitochondrial membranes were collected by centrifugation.

### Treatment with digitonin

In order to separate inner and outer membranes the iodinated mitochondria were suspended in sucrose medium to a concentration of 10 mg protein per ml. This suspension was incubated for 20 min at 4°C with 0.12 mg digitonin per mg mitochondrial protein. The reaction was stopped by a 10 fold dilution with sucrose medium. Mitoblasts were collected by centrifugation for 10 min at 5 000 xg. The supernatant was centrifuged for 20 min at 27 000 xg. The resulting pellet was discarded and the supernatant subjected to a high speed centrifugation to sediment the outer membrane fragments (90 min at 300 000 xg). The mitoblast fraction was again incubated with 0.5 mg digitonin in order to separate remnants of the outer membrane and was collected as described above. Soluble proteins and non-convalently bound radioactive label were removed from mitochondria, mitoblasts and outer membrane by the following procedure: the sediments were washed twice in 10 ml 0.01 mM KI buffered with triethanolamine 10 mM, pH 7.6. Subsequently they were sonicated with a Branson sonifier B12 for 40 sec at 50 watt output in 0.15 M KCl and dialysed against the same medium overnight. After dialysis the suspensions were subjected to a high speed centrifugation as mentioned above. The pellets were subjected to electrophoresis and autoradiography.

### Acrylamide gel electrophoresis

Sodium dodecylsulphate gels contained 12.5 % and 3 % acrylamide in the separating and stacking gel, respectively, and were prepared and run as described by Laemmli [9]. The gels were stained with coomassie brilliant blue, scanned in a spectrophotometer at 620 nm and subsequently cut into slices which

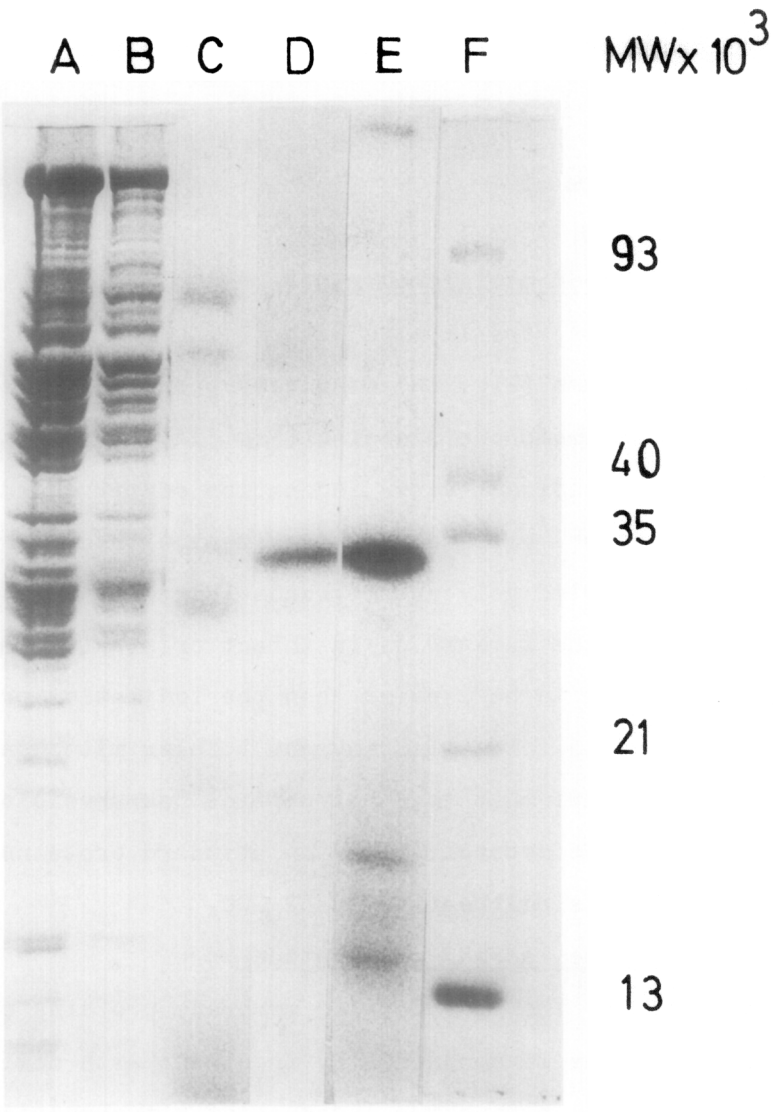


Fig. 1
Electrophoretic pattern of coomassie blue stained membrane proteins from rat liver mitochondria (A) and mitoblasts (B) after iodination with $^{125}I$ and corresponding autoradiograms (C: autoradiogram of B, D: autoradiogram of A). The gels were loaded with equal amounts of radioactivity (Gel A loaded with five times more protein than gel B). E: autoradiography of gel electrophoresis from intact tetrahymena mitochondria. F: enzymes of known MW, phosphofructokinase (93 000), aldolase (40 000), lactate dehydrogenase (35 000), myokinase (21 000), cytochrome C (13 000).

correspond to the different polypeptide bands. Radioactivity in the gel sections was determined in a Packard counter. After staining and drying, the gels were autoradiographed on Kodak X-ray films.

Enzyme determination

Succinate dehydrogenase (EC 1.3.99.1) and monoamine oxidase (EC 1.4.3.4) were determined as in reference [4].

## RESULTS AND DISCUSSION

### Iodination of mitochondrial membranes

Intact mitochondria were labeled with $^{125}I$ using lactoperoxidase. Autoradiograms (Fig. 1D) of electrophoretically separated mitochondrial membrane proteins (Fig. 1A) reveal only one band with a high radioactivity. Iodination of mitochondria with ruptured outer membranes (by treatment with digitonin) leads to five labeled polypeptide bands (Fig. 1B and C). A polypeptide with the same MW as in intact crista type mitochondria was found to be labeled when the iodination procedure was applied to intact Tetrahymena tubular mitochondria (Fig. 1E). We assume that this polypeptide has physiologically access to the cytosol. Based on standard proteins the apparent MW of this polypeptide is 32 000.

### Localization of the labeled polypeptide

We checked whether the $^{125}I$ labeled membrane proteins could be removed from the mitochondria by treatment with digitonin. As shown in Table 1 even after exposure of mitochondria to digitonin concentrations as high as 0.5 mg per mg protein (which remove 90 % of the monoamine oxidase activity) the specific radioactivity in the intensively washed mitoblast membranes increases by a factor of 1.5 as compared to the total mitochondria. A similar increase of specific activity is observed for succinate dehydrogenase. On the other hand in the outer membrane fraction where the specific activity of monoamine oxidase increases by a factor of 6, the specific radio-

Table 1
Specific activity of marker enzymes and specific radioactivity of total mitochondrial membranes as well as separated outer and inner membrane fractions after labeling of intact mitochondria from rat liver with $^{125}I$. The outer membrane fraction was prepared by incubation of the mitochondria with 0.12 mg digitonin per mg mitochondrial protein whereas the inner membrane fraction was obtained after incubation with 0.5 mg digitonin per mg. Values are means of three experiments.

| | monoamine oxidase | | succinate dehydrogenase | | radio-activity | |
|---|---|---|---|---|---|---|
| | mU/mg | % | U/mg | % | CPM/mg $\times 10^{-3}$ | % |
| total mitochondrial membranes | 19.8± 1.3 | 100 | 1.35±0.4 | 100 | 4.0±1.7 | 100 |
| mitochondrial membranes labeled in the presence of atractylate + ADP | -- | -- | -- | -- | 6.7 | 100 |
| outer membrane | 127.0±46 | 643 | 0.42±0.2 | 31 | 13.1±6.8 | 328 |
| outer membrane labeled in the presence of atractylate + ADP | -- | -- | -- | -- | 16.0 | 238 |
| inner membrane | 1.7± 0.4 | 9 | 2.2±0.4 | 163 | 6.1±1.9 | 153 |
| inner membrane labeled in the presence of atractylate + ADP | -- | -- | -- | -- | 19.5 | 291 |

activity increases only three fold. The increase in specific radioactivity of both membrane fractions may be explained by a loss of unlabeled protein through the digitonin treatment especially from the outer membrane fraction.

Quantitative analysis of radioactivity in the electrophoresis of intact mitochondria reveals that the radioactive label is

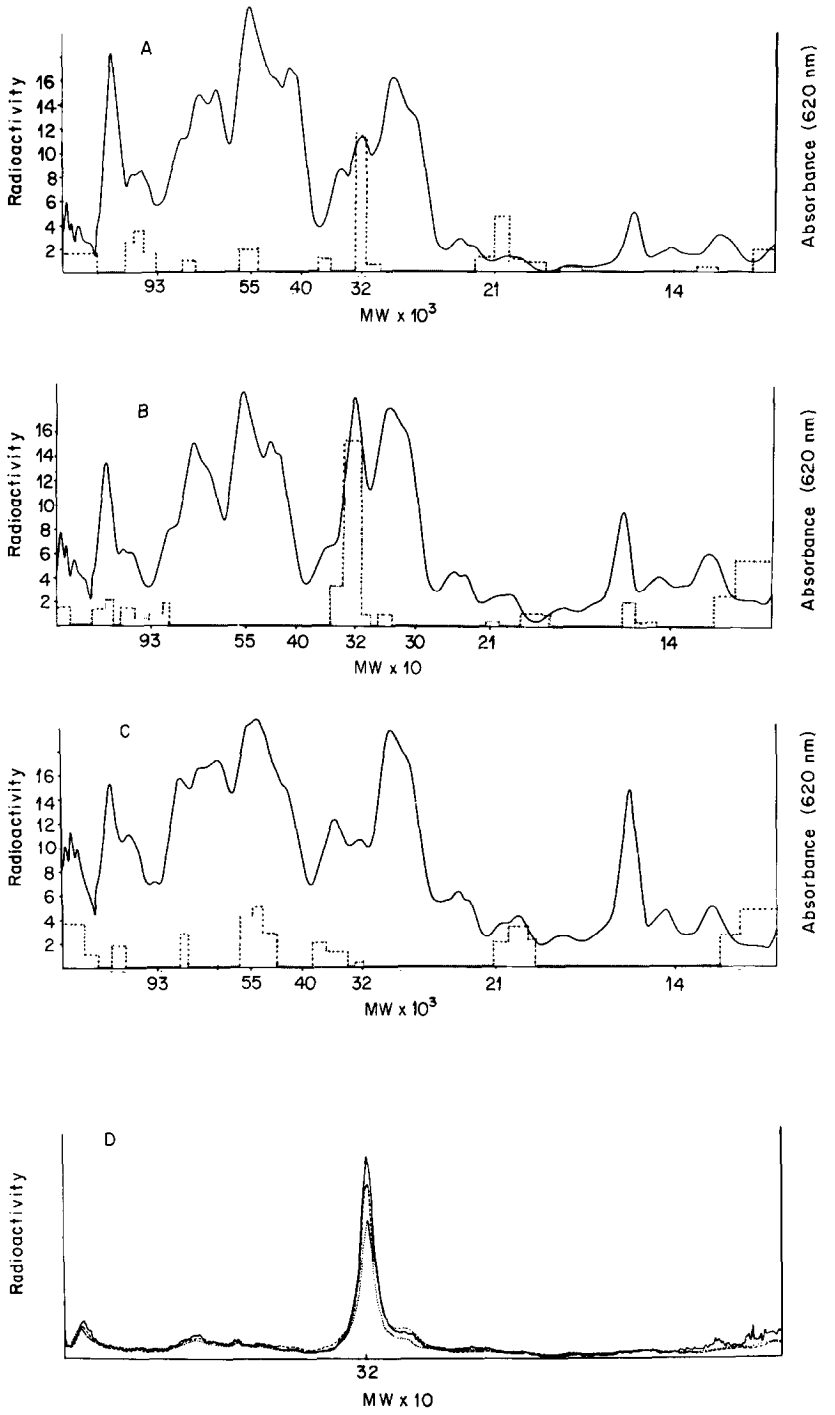


Fig. 2
Densitometric scans of SDS polyacrylamidegelelectrophoresis: membranes of intact mitochondria A, the inner membrane fraction B (after incubation with 0.5 mg digitonin) and the outer

mainly concentrated within one band of MW 32 000 (Fig. 2A) as already shown by autoradiography (Fig. 1A and D). The radioactivity and the intensity of stain increases in the 32 000 MW region of the gel when proteins of the inner membrane from the mitoblast fraction are loaded (Fig. 2B). On the other hand the radioactivity and the protein concentration in the 32 000 MW region of the gel decreases in the polypeptide pattern of the outer membrane fraction (Fig. 2C).

The results presented above suggest the existence of a polypeptide fraction of MW 32 000 which can be labeled in intact mitochondria and remain fixed to the mitoblast membrane after removal of the outer membrane. Huber and Morrison [10] described a 33 000 MW polypeptide in rat liver mitochondria with the same properties. They argued that this polypeptide fraction may contain a component located at the contact sites between the two boundary membranes [5]. Hackenbrock reported that after treatment of the mitochondria with digitonin remnants of the outer membrane remain attached to the inner boundary membrane at their contact sites [11,12]. In addition Boxer [13] described the iodination of a MW 29 500 polypeptide at the periphery of intact ox heart mitochondria and suggested

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membrane fraction C (after incubation with 0.12 mg digitonin). Before separation of the different membrane fractions the intact mitochondria were labeled with $^{125}I$. The gels were cut into pieces corresponding to the different polypeptide bands. Radioactivity within the gel sections was determined and is expressed relatively to the mean radioactivity (50 CPM). The height of the columns represents relative radioactivity, the basis of the columns shows length of the different gel sections. D autoradiography of SDS gel electrophoresis separating membrane proteins of intact mitochondria labeled with $^{125}I$ in the presence of ADP ..... , in the presence of ADP and atractylate ———— , and in the absence of any ligand ------- . The autoradiograms are scanned at 720 nm.

Table 2
Specific radioactivity which remains fixed to the mitochondrial membranes after iodination with $^{125}I$ of intact mitochondria at pH 6.5 in the presence of different ligands of the ADP/ATP carrier. Unspecifically bound radioactivity and soluble proteins are removed as described in Methods. Radioactivity is given in absolute values and in percent of the label bound in the presence of ADP.

| | radioactivity CPM/mg x $10^{-3}$ | % of CPM bound in the presence of ADP |
|---|---|---|
| without ligands | 8.0 | 143 |
| + ADP | 5.6 | 100 |
| + ADP<br>+ atractylate | 11.6 | 207 |
| + ADP<br>+ bongkrekate | 2.8 | 50 |

this polypeptide to be a component of the inner boundary membrane. It seems difficult to decide to which of the two boundary membranes the labeled compound belongs. However the amount of radioactivity which can be bound to intact mitochondria depends on the presence of substances which affect the functional state of the ADP/ATP carrier.

Function of the labeled polypeptide

As shown in Table 2 the amount of radioactivity bound to the mitochondrial membranes increases by a factor of two when the ADP/ATP carrier is trapped at the outer side of the inner membrane by atractylate in the presence of ADP. At pH 6.5 bongkrekate rapidly enters the mitochondria and in the presence of ADP the carrier is trapped at the inner side of the

inner membrane [1,2]. The radioactivity found in mitochondrial membranes under these conditions amounts to only 50 % of that found in the presence of ADP. When mitochondria were iodinated in the absence of any ligand more label is bound to the membranes than in the presence of ADP. This finding aggrees with the results of Klingenberg showing that the carrier accumulates at the outer surface of the membrane when no ligands are added [14].

The mitochondrial membranes labeled under different states of the ADP/ATP carrier are analyzed by electrophoresis and autoradiography. As shown in Fig. 2D the protein band of MW 32 000 is labeled to a higher extent when the carrier is trapped at the outer side of the inner membrane (e.g. in the presence of atractylate and in the absence of any ligand). In agreement with this the specific radioactivity of the separated inner membrane increases when intact mitochondria are labeled in the presence of ADP and atractylate (Table 1).

It remains to be proven whether the radioactive polypeptide is a component of the ADP/ATP transport system or a polypeptide which might be exposed to the iodination system depending on the different positions of the ADP/ATP carrier. Scherer et al. [14] have been able to correlate conformational changes of the mitochondria to the location of the carrier at different sides of the membrane. On the other hand the carboxyatractylate binding protein isolated by Riccio et al. has a MW of 30 000 [15] which is very similar to that of the iodinated protein.

The amount of $^{125}I$ applicable to a peripheral membrane protein is changed in correlation to the functional state of a typical inner membrane component (e.g. the ADP/ATP carrier)

and it may thus be suggested that the proteins at the periphery of intact mitochondria are in some way connected to the inner membrane proteins. Most probably these interactions between proteins of the two boundary membranes take place within the contact sites described by Hackenbrock [5].

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