Formation of an intramolecular disulfide bond in the mitochondrial adenine nucleotide translocase

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Adenine nucleotide translocase in electron transport particles or in H^+-ATPase preparation from bovine heart mitochondria is capable of forming both inter- and intramolecular disulfide bridges upon reaction with copper-*o*-phenanthroline. We have examined the localisation of the intramolecular disulfide bridge in the protein chain by peptide fragmentation methods. The most likely position of the disulfide bridge is between cysteine 159 and 256, but the possibility of the presence of a second disulfide bridge formed between 129 and 256 cannot be ruled out. Our experimental results support the theoretical model proposed [(1982) FEBS Lett. 144, 250–254] for the topography of the translocase and provide a more accurate description of the arrangement of some of the hydrophilic segments in the molecule.

Adenine nucleotide translocase *Electron transport particle* *H^+-ATPase* *Peptide mapping* *Intramolecular disulfide* *Crosslinking*

1. INTRODUCTION

Adenine nucleotide translocase (Adn) is a major component of the mitochondrial inner membrane. It consists of a single protein chain of 297 amino acid residues, the sequence of which has been determined [1]. The functional unit of the translocase is proposed to be a dimer [2].

We have recently shown that the 29-kDa protein present in electron transport particles as well as in purified H^+-ATPase preparation from bovine heart mitochondria is Adn [3]. We have also shown that treatment of these preparations with Cu-$(OP)_2$ results in the formation of both inter- and intramolecular disulfide bonds in the 29-kDa protein [3].

Since the amino acid sequence of the Adn is known, it seemed possible to identify the cysteines forming the disulfide bridges.

Here, we report the results on localisation of the intramolecular disulfide bridge formed in the translocase molecule upon treatment of the H^+-ATPase preparation with Cu-$(OP)_2$.

2. MATERIALS AND METHODS

H^+-ATPase was prepared from bovine heart mitochondria by the lysolecithin extraction procedure [4].

Aliquots of H^+-ATPase (0.6 mg/ml in 50 mM triethanolamine-HCl buffer, pH 8.0) were incubated with 50 μM Cu-$(OP)_2$ for 10 min at 23°C. The reaction was terminated by adding a 40-fold excess of EDTA. SDS-PAGE was carried out in a

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Abbreviations: H^+-ATPase, H^+-translocating ATPase complex; 2-ME, 2-mercaptoethanol; CNBr, cyanogen bromide; NTCB, 2-nitro-5-thiocyanobenzoate; Cu-$(OP)_2$, copper-*o*-phenanthroline; DTT, dithiothreitol; Adn, adenine nucleotide translocase

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Hoefer SE-500 apparatus according to Laemmli [5] except that 2-ME was omitted throughout the system. The separating gel (0.75 or 1.5 mm × 10.0 cm) consisted of a 12–20% linear polyacrylamide gradient overlaid with a 1-cm, 5% acrylamide stacking gel; 10% glycerol was included in the 20% acrylamide solution. Samples were prepared by incubating suitable aliquots of protein for 30 min at 23°C with 5 mM *N*-ethylmaleimide in sample buffer (50 mM Tris-HCl buffer, pH 6.8, 1% SDS, 10% glycerol, 0.001% Bromophenol blue) and 5% 2-ME was used to reduce samples, where indicated. Gels were stained with Coomassie blue [6] or silver [7].

CNBr cleavage was performed by incubating gel slices for 1 h in 1% CNBr [8]. After the cleavage procedure the gel slices were equilibrated in sample buffer. Protein was extracted by soaking the gel slices in sample buffer for 2 h at 37°C. Supernatants were subjected to SDS-PAGE.

Cyanylation and cleavage at cysteines were carried out essentially as described by Jacobson et al. [9], the modifications were due to the fact that the reaction steps were performed in gel slices. Briefly gel slices were equilibrated in 0.2 M Tris-acetate buffer (pH 8.0) containing 6 M guanidine-HCl, and incubated with 20 mM DTT at 37°C for 1 h. NTCB was added to 100 mM and the reaction was allowed to proceed for 2 h at 37°C after adjusting the pH to 8.0. Next, the gel slices were dialysed in 50% acetic acid overnight and equilibrated with, and incubated in a small volume of 6 M guanidine-HCl–0.1 M sodium borate buffer (pH 9.0) at 37°C for 16 h. Finally 5× concentrated sample buffer was added to the gel slices and after 2 h of soaking, the supernatant of the gel pieces was applied to SDS gel.

All the chemicals were of reagent grade obtained from Sigma.

3. RESULTS

When the H^+-ATPase preparation containing the Adn [3] was treated with Cu-$(OP)_2$, the intramolecularly crosslinked Adn molecule appeared with a slightly increased mobility compared to the non-crosslinked one in the SDS gel (fig.1). So the two species could be separated by SDS-PAGE.

There are four cysteine residues in the translocase molecule in positions 56, 129, 159 and 256; therefore there are six possible ways of forming a disulfide bridge between them as outlined in fig.2.

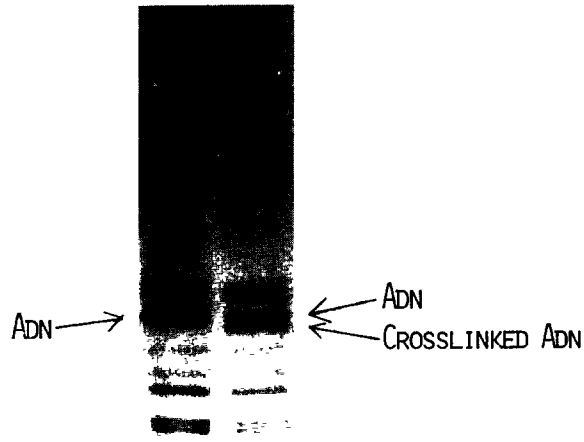

Fig.1. SDS-PAGE profile of: (1) H^+-ATPase, ADN marks adenine nucleotide translocase; (2) H^+-ATPase treated with 50 μM Cu-$(OP)_2$, crosslinked ADN marks the intramolecularly crosslinked adenine nucleotide translocase.

To localise the intramolecular disulfide bridge in the molecule, peptide mapping techniques were used. Fragmentation patterns of each possible location of the disulfide bridge in the intramole-

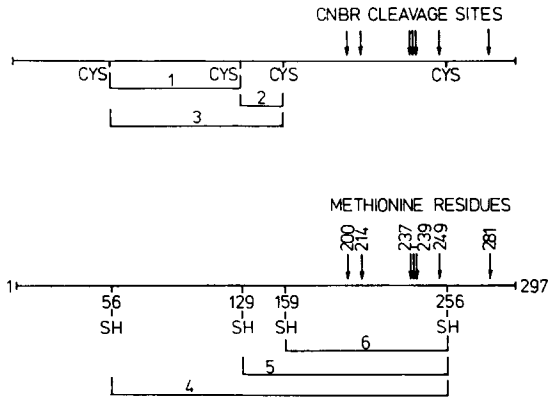

Fig.2. Diagram of the Adn peptide chain. The diagram shows the positions of cysteine and methionine residues and indicates the theoretical combinations (1–6) for the location of the intramolecular disulfide bridge.

cularly crosslinked adenine nucleotide translocase were predicted by calculating the molecular masses of the expected fragments. The experimentally obtained fragmentation profile was then compared with the theoretical versions. Cleavage with CNBr and, in another set of experiments, cyanylation and consequent cleavage at the cysteines were performed. The peptide fragments were analysed by SDS-PAGE.

3.1. *CNBr cleavage*

CNBr cleavage was carried out on the Adn molecule containing the intramolecular disulfide bridge and having the rest of the cysteines blocked by NEM, in gel slices.

In the sequence of the adenine nucleotide translocase there are seven methionine residues in positions 200, 214, 237, 238, 239, 249 and 281. The major fragments deriving from CNBr cleavage are peptides with molecular masses of 20, 21 and 24 kDa (see fig.3, lanes 2 and 3). The low molecular mass fragments do not appear in the SDS gel.

The arrangement of the cysteines is such that 3 out of 4 fall into the 1-200 CNBr fragment (Cys 56, 129 and 159), while 1 (Cys 256) will be found in smaller fragments between 200 and 297. If cysteine 256 is one of the residues involved in the disulfide bridge, these small fragments (3–9 kDa) will remain attached to the 1-200 fragment via the disulfide bridge (see versions 4–6 in fig.2) after cleavage at the methionines. The fragmentation pattern in this case would be expected to lack the 20- and 21-kDa bands; at the same time fragments with molecular masses of 24–29 kDa should appear.

On the other hand, if Cys 256 is not involved in the disulfide bridge (see versions 1–3 in fig.2), the CNBr fragmentation pattern of the crosslinked and non-crosslinked molecules would be expected to be essentially identical.

Fig.3 shows the CNBr cleavage patterns of the crosslinked (lane 1), as well as that of the non-crosslinked Adn resulting from either cleavage of the purified translocase (lane 2), or from the translocase present in the H^+-ATPase preparation (lane 3). The CNBr fragmentation pattern of the crosslinked translocase is altered as compared to the control patterns. The alterations are in accordance with those expected if the disulfide bridge is formed between cysteine 256 and any of the other three cysteines (see fig.2, versions 4–6). If the crosslinked sample is reduced with 2-ME before or after CNBr cleavage (fig.3, lane 4), the fragmentation patterns of the 1 × crosslinked and the control samples are identical.

From the results of the CNBr fragmentation we concluded that out of the six possible types of crosslink, the three types not involving cysteine 256 could be ruled out.

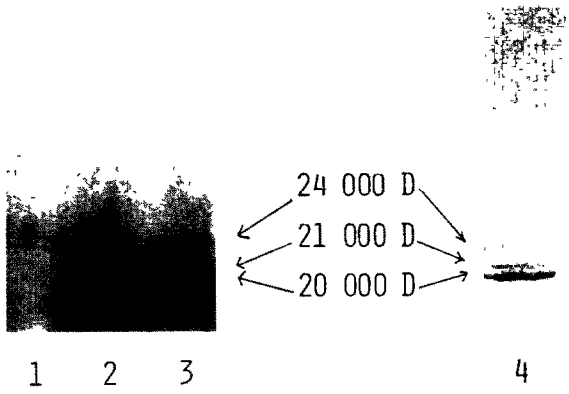

Fig.3. CNBr fragmentation patterns of: (1) intramolecularly crosslinked Adn (non-reduced); (2) purified translocase; (3) the translocase present in the H^+-ATPase preparation; and (4) intramolecularly crosslinked translocase, reduced with 2-ME after CNBr cleavage. Gel slices containing the oxidized or control translocase were excised from slab gels, treated with CNBr (as described in section 2) and the fragments reapplied to SDS-PAGE in reducing or non-reducing conditions.

3.2. *Cyanylation and cleavage at the cysteines*

To distinguish between the remaining three possible types of crosslink, cyanylation and consequent cleavage at the cysteines was carried out as follows: the crosslinked or the control sample was reacted with NEM in SDS-containing buffer to block the SH-groups not involved in the disulfide bridge formation. After SDS-PAGE, the appropriate bands (intramolecularly crosslinked or control translocase) were excised and then the disulfide bridges reduced by DTT. The freed cysteines were then cyanylated and the cleavage at the free cysteines was carried out. In this way cleavage was expected to take place only at the cysteines previously involved in the intramolecular disulfide.

The fragmentation patterns for the three variations (56–256, 129–256, 159–256) were calculated by taking an average molecular mass of 100 kDa for each amino acid and the expected gel-profiles were plotted in fig.4B. Fig.4A shows the experimentally obtained fragmentation patterns from the intramolecularly crosslinked (lane 1), and from the control translocase (lane 2). The CNBr fragments of the translocase and cytochrome *c* were used as molecular mass standards. Bands of 29 and 28 kDa (in lane 1) represent, respectively, the uncleaved and the non-reduced portions of the crosslinked translocase. The presence of the 25-, 16-, 13–14- and 10-kDa bands suggests that the experimentally obtained pattern correlates best with version 159–256. Version 56–256 can be excluded on the basis of the lack of the 20- and 24-kDa bands. The presence of two bands in the 13–14-kDa region is not explained by version 159–256. A mixed pattern could derive from the presence of an additional disulfide bridge, in position 129–256.

4. DISCUSSION

We have previously shown that electron transport particles as well as the H^+-ATPase preparation contain the Adn and have demonstrated that intramolecular disulfide formation in the translocase takes place when electron transport particles are treated with Cu-$(OP)_2$ [3].

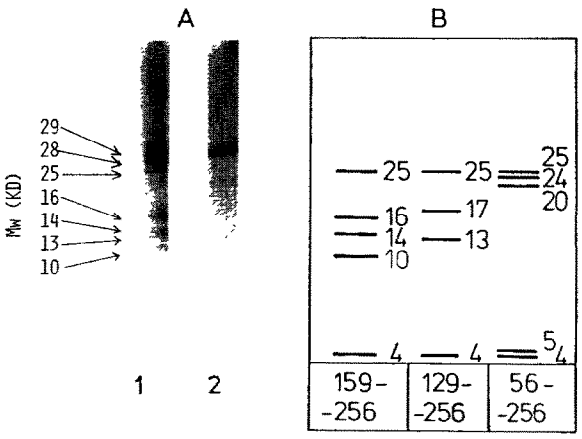

Fig.4. Theoretical and experimental fragmentation patterns after cyanylation and cleavage at the cysteines. (A) Crosslinked (1) and control (2) translocase in gel slices were subjected to NEM treatment, reduction by DTT, cyanylation and cleavage as detailed in section 2. After cleavage the samples were applied to SDS-PAGE and the slab was stained with silver. (B) The calculated fragmentation patterns for the cases in which Cys 256 is linked to Cys 159, Cys 129 or Cys 56.

The results described here show that intramolecular crosslinking of the Adn also occurs in the H^+-ATPase preparation. It seems likely that the purified and reconstituted translocase could be crosslinked in the same way: however, this has not been tested.

We have demonstrated that the intramolecular disulfide bond formed in the reaction with Cu-$(OP)_2$ in the Adn involves Cys 256 and is most likely to be formed between Cys 159 and 256. However, the possibility of an additional disulfide bridge between Cys 129 and 256 could not be ruled out.

Recently, a model for the topography of the adenine nucleotide translocase has been proposed by Saraste and Walker [10]. On the basis of the hydrophobicity plot of the molecule, they have predicted which segments are likely to traverse the membrane or form hydrophilic loops. According to the model, Cys 159 and Cys 256 are located on hydrophilic loops on the same side of the membrane (fig.5A). Our observation of the crosslink formed between these residues supports such an arrangement. The model, however, does not precisely define the relative positions of the different segments of the molecule. Our experimental results show that Cys 159 and Cys 256 are located within a few angstroms of each other (fig.5B).

From our results it is impossible to exclude an additional crosslink between Cys 129 and 256. If such a crosslink were identified between Cys 129 – predicted as being buried in the membrane – and Cys 256 – predicted as aqueous – some modification of the topographical model would be required. In addition, the concurrent crosslinking between 159–256 and 129–256 would require that the segment containing Cys 256 should be capable of swinging between the segments containing Cys 129 and Cys 159.

Formation of a dimer of the adenine nucleotide translocase via a disulfide bridge occurs simultaneously with the formation of the intramolecular disulfide bond in the reaction of electron transport particles with Cu-$(OP)_2$ [3]. We do not know whether the dimer contains in-

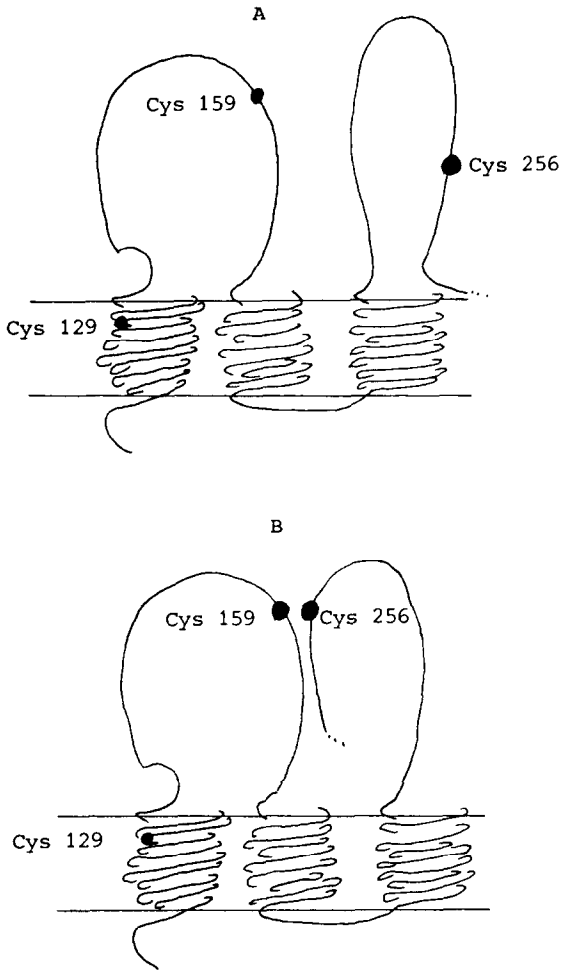

Fig.5. A model for the structure of the adenine nucleotide translocase: (A) as proposed by Saraste and Walker [10]; (B) modified to allow for the crosslink between residues 159 and 256. The positions of the cysteine residues are indicated.

tramolecular disulfide bonds as well. However, the intramolecularly crosslinked species do not undergo dimerization. This may indicate that at least one of the cysteines involved in the intramolecular disulfide bond is also involved in intermolecular disulfide bridges.

Recently, the cysteine, the blockage of which causes inhibition of the transport activity, has been identified as residue 56 in the sequence [11]. Since Cys 56 is not involved in the intramolecular crosslink described here it seems reasonable to conclude that none of the other cysteine residues in the molecule is located sufficiently closely to form a disulfide link with it.

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