Journal of Bioenergetics and Biomembranes, Vol. 25, No. 5, 1993

Chemical, Immunological, Enzymatic, and Genetic Approaches to Studying the Arrangement of the Peptide Chain of the ADP/ATP Carrier in the Mitochondrial Membrane

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Received March 30, 1993

In the process of oxidative phosphorylation, the exchange of cytosolic ADP^{3-} against mitochondrial ATP^{4-} across the inner mitochondrial membrane is mediated by a specific carrier protein. Two different conformations for this carrier have been demonstrated on the basis of interactions with specific inhibitors, namely carboxyatractyloside (CATR) and bongkrekic acid (BA). The two conformations, referred to as CATR and BA conformations, are interconvertible, provided that ADP or ATP are present. The functional ADP/ATP carrier is probably organized as a tetramer. In the presence of CATR or BA the tetramer is split into two dimers combined with either of the two inhibitors. The amino acid sequence of the beef heart carrier monomer (297 residues) contains three repeats of about 100 residues each. Experimental results obtained through different approaches, including photolabeling, immunochemistry, and limited proteolysis, can be interpreted on the basis of a model with five or six transmembrane α helices per carrier monomer. Two mobile regions involved in the binding of nucleotides and accessible to proteolytic enzymes have been identified. Each of them may be visualized as consisting of two pairs of short amphipathic α helices, which can be juxtaposed to form hydrophilic channels facilitating the nucleotide transport. Mutagenesis in yeast is currently being used to detect strategic amino acids in ADP/ATP transport.

KEY WORDS: ADP/ATP carrier; carboxyatractyloside; bongkrekic acid; mitochondrial carrier.

INTRODUCTION

The mitochondrial ADP/ATP carrier is a key energetic link between the mitochondrial and the cytosolic compartments of aerobic eukaryotic cells. It is located in the inner mitochondrial membrane, and its concentration amounts to 10% of the protein of this membrane in heart mitochondria. Under physiological conditions, it catalyzes the transmembrane exchange between cytosolic ADP and mitochondrial ATP generated by oxidative phosphorylation. Only the free forms of these nucleotides are transported. The one-to-one stoichiometry of the exchange allows the mitochondrial pool of adenine nucleotides to be maintained at a constant level. Because of the difference of charges of ADP^{3-} and ATP^{4-} at physiological pH, and the absence of compensation of the charge difference by a movement of protons, the ADP^{3-}/ATP^{4-} exchange is electrogenic. As a consequence, it must be driven by the membrane potential of the mitochondrial membrane. It has been calculated that the energy which is required for export of ATP in the course of oxidative phosphorylation corresponds to 30% of the energy delivered by mitochondrial respiration in terms of protonmotive force.

The low turnover of the ADP/ATP carrier (1000 to 2000 per minute at 25°C) is compensated by its high concentration, so that the transport capacity of ADP and ATP across the mitochondrial membranes can

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            1                                                                                                 10
bovANC1     MSDQA----------------------------------------------------------------------------------LSFLKDFLAGGVAA
bovANC2     MTEQA----------------------------------------------------------------------------------ISFAKDFLAGGIAA
hANC1       MGDHA----------------------------------------------------------------------------------WSFLKDFLAGGVAA
hANC2       MTEQA----------------------------------------------------------------------------------ISFAKDFLAGGIAA
hANC3       MTDAA----------------------------------------------------------------------------------LSFAKDFLAGGVAA
moANC3      MTDAA----------------------------------------------------------------------------------VSFAKDFLAGGVAA
ratANC1     MGDQA----------------------------------------------------------------------------------LSFLKDFLAGGIAA
ratANC3     MTDAA----------------------------------------------------------------------------------VSFAKDFLAGGVAA
anoANC1     MTKKADP--------------------------------------------------------------------------------YGFAKDFLAGGISA
yANC1       MS-----------------------------------------------------------------------------HTETQTQQSHFGVDFLMGGVSA
yANC2       MSSNAQVKTPL-------------------------------------------------------------------PPAPAPKKESNFLIDFLMGGVSA
yANC3       MSSDAK------------------------------------------------------------------------------QQETNFAINFLMGGVSA
ncANC       MAEQQK---VL--------------------------------------------------------------------------GMPPFVADFLMGGVSA
zmANC1      MADQANQPTVLHKLGGQFHLRSIISEGVRAR--NICPSVSSYERRFATRNYMTQSLWGPSMSVSGGINVPVMQT-PLCANAPAEKGGKNFMIDFMMGGVSA
zmANC2      MADQANQPTVLHKLGGQFHLSSSFSEGVRAR--NICPSFSPYERRFATRNYMTQSLWGPSMSVSGGINVPVMPT-PLFANAPAEKGGKNFMIDFMMGGVSA
chlrANC     MAKEEKN----------------------------------------------------------------------------------FMVDFLAGGLSA
ricANC1     MAEQANQPTVLQKFGGQFHLGSSFSEGVRAR--NICPSVSSYDRRFTTRSYMTQGL------VNGGINVPMMSSSPIFANAPAEKGGKNFMIDFLMGGVSA
potANC1     MADMNQHPTVFQKAANQLDLRSSLSQDVHARYGGV--QPAIYQRHFACGNYSNAGL--QRGQATQDLSLITSNASPVFVQAPQEKGLAAFATDFLMGGVSA
artANC      MVEQTQHPTILQKVSGQL-LSSSVSQDIRG-YASASKRPATYQKHAAYGNYSNAAF--QYPLVAA--SQIATTTSPVFVQAPGEKGFTNFAIDFMMGGVSA
            *                                                                                     *   .*. **..*

            |        30         |         50         |         70         |         90         |        110
bovANC1     AISKTAVAPIERVKLLLQVQHA-SKQISAEKQYKGIIDCVVRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDRHKQ-FWRYFAGNLA
bovANC2     AISKTAVAPIERVKLLLQVQHA-SKQIAADKQYKGIVDCIVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKRTQ-FWRYFAGNLA
hANC1       AVSKTAVAPIERVKLLLQVQHA-SKQISAEKQYKGIIDCVVRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQLFLGGVDRHKQ-FWRYFAGNLA
hANC2       AISKTAVAPIERVKLLLQVQHA-SKQIAADKQYKGIVDCIVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKHTQ-FWRYFAGNLA
hANC3       AISKTAVAPIERVKLLLQVQHA-SKQITADKQYKGIVDCIVRIPKEQEVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKRTQ-FWRYFAGNLA
moANC3      AISKTAVAPIERVKLLLQVQHA-SKQITADKQYKGIIDCVVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKRTQ-FWRYFAGNLA
ratANC1     AVSKTAVAPIERVKLLLQVQHA-SKQISAEKQYKGIIDCVVRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDRHKQ-FWRYFAGNLA
ratANC3     AISKTAVAPIERVKLLLQVQHA-SKQITADKQYKGIIDCVVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKRTQ-FWRYFAGNLA
anoANC1     AVSKTAVAPIERVKLLLQVQAA-SKQIAVDKQYKGIVDCFVRIPKEQGIGAFCGGNLANVIRYFPTQALNFAFKDVYKQVFLGGVDKNTQ-FWRYFLGNLG
yANC1       AIAKTGAAPIERVKLLMQNQEEMLKQGSLDTRYKGILDCFKRTATHEGIVSFWRGNTANVLRYFPTQALNFAFKDKIKSLL--SYDRERDGYAKWFAGNLF
yANC2       AVAKTAASPIERVKLLIQNQDEMLKQGTLDRKYAGILDCFKRTATQEGVISFWRGNTANVIRYFPTQALNFAFKDKIKAMF--GFKKE-EGYAKWFAGNLA
yANC3       AIAKTAASPIERVKILIQNQDEMIKQGTLDKKYSGIVDCFKRTAKQEGLISFWRGNTANVIRYFPTQALNFAFKDKIKLMF--GFKKE-EGYGKWFAGNLA
ncANC       AVSKTAAAPIERIKLLVQNQDEMIRAGRLDRRYNGIIDCFKRTTADEGVMALWRGNTANVIRYFPTQALNFAFRDKFKKMF--GYKKDVDGYWKWMAGNLA
zmANC1      AVSKTAAAPIERVKLLIQNQDEMIKSGRLSEPYKGIVDCFKRTIKDEGFSSLWRGNTANVIRYFPTQALNFAFKDYFKRLF--NFKKDRDGYWKWFAGNLA
zmANC2      AVSKTAAAPIERVKLLIQNQDEMIKSGRLSEPYKGIADCFKRTIKDEGFSSLWRGNTANVIRYFPTQALNFAFKDYFKRLF--NFKKDRDGYWKWFAGNLA
chlrANC     AVSKTAAAPIERVKLLIQNQDEMIKQGRLASPYKGIGECFVRTVREEGFGSLWRGNTANVIRYFPTQALNFAFKDKFKRMF--GFNKDKE-YWKWFAGNMA
ricANC1     AVSKTAAAPIERVKLLIQNQDEMIKAGRLSEPYKGIGDCFGRTIKDEGFASLWRGNTANVIRYFPTQALNFAFKDYFKRLF--NFKKDKDGYWKWFGGNLA
potANC1     AVSKTAAAPIERVKLLIQNQDEMLKAGRLSEPYKGIGECFGRTIKEEGFGSLWRGNTANVIRYFPTQALNFAFKDYFKRLF--NFKKDRDGYWKWFAGNLA
artANC      AVSKTAAAPIERVKLLIQNQDEMLKAGRLTEPYKGIRDCFGRTIRDEGIGSLWRGNTANVIRYFPTQALNFAFKDYFKRLF--NFKKDKDGYWKWFAGNLA
            *..**. .****.*.*.* *      .         * ** .* *     ..   ..  ** ***.************.*  * ..     . . . .   **.
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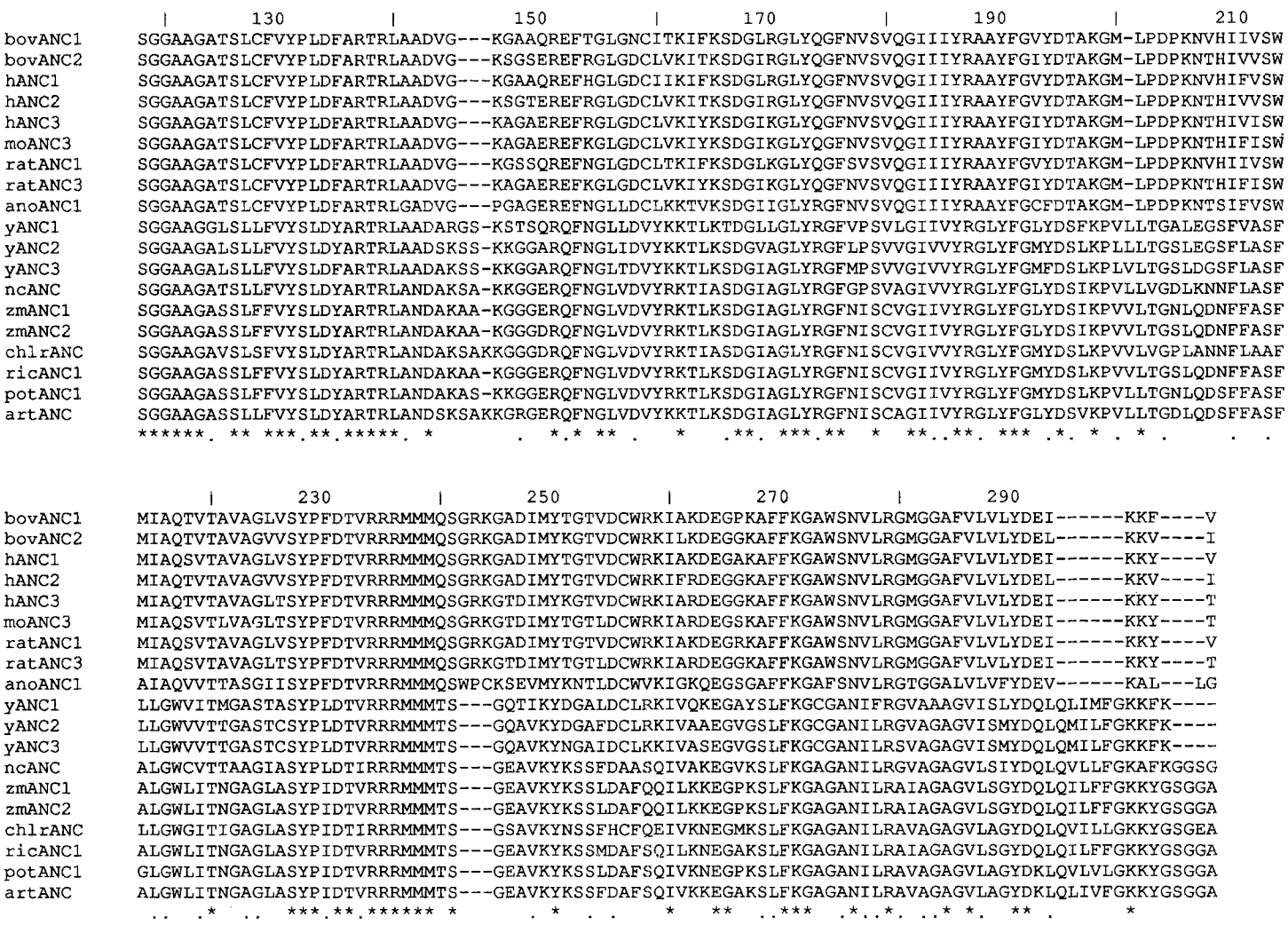

Fig. 1. CLUSTAL V multiple sequence alignment of ANC proteins. Sequences were aligned with the computer program CLUSTAL V (Higgins *et al.*, 1992) using the Dayhoff protein weight matrix PAM250. Asterisks indicate identities while dots highlight similar residues (PAM250 score of 8 or more). Sources of ANC proteins: h, human (Cozens *et al.*, 1989; Battini *et al.*, 1987; Houldsworth and Attardi, 1988), mo, mouse (X70847); rat, rat (D12770, 12771); bov, bovine (Aquila *et al.*, 1982; Powell *et al.*, 1989); ano, *Anopheles gambiae* (L11617); y, yeast (Adrian *et al.*, 1986; Lawson and Douglas, 1988; Kolarov *et al.*, 1990); nc, *Neurospora crassa* (Arends and Sebald, 1984); zm, *Zea mays* (Winnings *et al.*, 1991); chlr, *Chlamydomonas reinhardtii* (Sharpe and Day, 1993); ric, rice (D12637); pot, potato (Emmermann *et al.*, 1991); art, *Arabidopsis thaliana* (Schuster *et al.*, 1993). Accession numbers (EMBL Data Bank) are indicated for unpublished sequences.

easily cope with the energy requirement of the cell in terms of exported mitochondrial ATP. Among the different reactions participating in the ATP cycle in eukaryotic cells and controlling the activity of this cycle, ADP/ATP transport has a strategic function. The control strength exerted by the ADP/ATP carrier depends on the tissue and on metabolic conditions. For example, the ADP/ATP carrier exerts a partial control over the rate of oxidative phosphorylation in liver, but not in heart where the major limiting factor is the capacity of the respiratory chain. The understanding of a number of physiological and structural features of the ADP/ATP carrier has benefited from two exceptional peculiarities, namely the abundance of this carrier, which allows its purification in good yield, and the specific and powerful inhibition of ADP/ATP transport by two types of toxic compounds, atractyloside (ATR) and carboxyatractyloside (CATR), on one hand, and bongkrekic acid (BA) and isobongkrekic acid on the other, that bind specifically to the cytosolic and to the matrix face of the carrier, respectively. These considerations on the physiological features of mitochondrial ADP/ATP transport were reviewed in detail a few years ago (Vignais *et al.*, 1985; Klingenberg, 1985). A recent detailed investigation of the kinetics of ADP/ATP transport in rat heart mitochondria explored with a rapid filtration system led us to conclude on the existence of a microcompartmentation of the intramitochondrial adenine nucleotides (Brandolin *et al.*, 1990). This compartmentation is of a kinetic nature in that the entire pool of mitochondrial adenine nucleotides is exchangeable over a sufficient period of time, but only a part of it is rapidly mobilizable.

In the following, we shall address the problem of the topography of the ADP/ATP carrier through different approaches, namely chemical, immunological, enzymatic, and genetic approaches. A large number of results have been obtained through studies carried out with the beef heart mitochondrial carrier. In more recent studies, dealing with mutagenesis, the yeast ADP/ATP carrier has been used.

COMPARISON OF SEQUENCES OF ADP/ATP CARRIERS

Multiple isoforms of the ADP/ATP carrier protein, encoded by specific genes, have been characterized in mitochondria originating from a variety of cells. For example, three isoforms of the carrier have been identified by analysis of human cDNA libraries and shown to be differently expressed, depending on the tissues. Expression of the isoforms of the ADP/ATP carrier might be regulated by the overall energetic requirements of the cell, possibly in connection with different stages of development (Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Houldsworth and Attardi, 1988; Lunardi *et al.*, 1992). It cannot be excluded that heterodimers or heterotetramers of the carrier might be involved in the modulation of kinetic properties of ADP/ATP transport. In the yeast *S. cerevisiae*, three genes encoding the ADP/ATP carrier have been identified. Only one gene, *ANC2*, is essential for the cells to grow on a nonfermentable carbon source (Lawson and Douglas, 1988). A mutated form of this gene was found to encode a carrier protein with an R96H change, defective in nucleotide transport across the mitochondrial membrane (mutation op_1) (Kolarov *et al.* 1990, Lawson *et al.*, 1990; Lauquin, unpublished). More recently, a third gene has been characterized, whose transcription is paradoxically required for growth of yeast cells under anaerobic conditions (Kolarov *et al.*, 1990; Drgon *et al.*, 1991). The relative contribution of each carrier to the ADP/ATP transport activity is not yet established.

The amino acid sequences of 19 adenine nucleotide carriers are presently available. Alignment of these sequences with the CLUSTAL V program is shown in Fig. 1. By simple visual inspection, it is possible to distinguish two groups of sequences, one for mammalian carriers and the other for plants, fungi, and yeast. Analysis of the second group reveals that the adenine nucleotide carriers from plants (*Solanum tuberosum, Zea mays, Oriza sativa,* and *Arabidopsis thaliana*) specifically display an N-terminal-extended region which is absent in the *Chlamydomonas reinhardtii* carrier (Sharpe and Day, 1993). As higher plants have probably evolved from the group of green algae, it can be hypothesized that this amino-terminal extension results from a specific acquisition which has been fixed after divergence of the higher plant lineage. At present there is no indication of the function of this extra sequence. A striking peculiarity of all ADP/ATP carriers is the presence of the RRRMMM cluster in the C-ter region of the carriers.

Looking at the isoforms of the ADP/ATP carrier in yeast, referred to as Anc1, Anc2, and Anc3 and comparing their sequences to those of carriers from other organisms, it is interesting to note that only eight amino acid residues of the yeast Anc1 sequence are different from the otherwise absolutely conserved

residues of the 18 other ADP/ATP carriers. These residues are G/A 40, L/I 95, L/F 115, F/A 132, G/A 139, T/S 183, F/L 293, and G/A 298 (the numbers correspond to the Anc2 sequence). These substitutions are rather conservative except the F/A 132 substitution. Gawaz *et al.* (1990) have reported that the molecular activity of the yeast Anc1 protein is about 42% of that of the Anc2 isoform. It is therefore tempting to speculate that one or more of these substitutions are responsible for the less efficient activity of Anc1.

In addition to the 19 ADP/ATP carrier sequences, nine sequences for other mitochondrial carriers have been reported, namely phosphate carriers (Runswick *et al.*, 1987; Ferreira *et al.*, 1989; Phelps *et al.*, 1991; Dolce *et al.*, 1992), bovine oxoglutarate/malate carrier (Runswick *et al.*, 1990), and proton carriers (Aquila *et al.*, 1985; Bouillaud *et al.*, 1988, 1989; Balogh *et al.*, 1989). These sequences have been aligned with the CLUSTAL V program (data not shown). In all sequences, 10 residues are totally conserved and 32 are substituted in a highly conservative manner. This suggests that these residues may play a decisive role in the peptide chain arrangement in the membrane and in the transport mechanism. Among the conserved residues are four glycine and two proline, corresponding to G72, G175, G265, G272 and P27 and P229 in the case of the beef heart carrier T1. Two basic residues (K32 and R139), one acidic residue (D231), and a glutamine residue (Q36) constitute the rest of the conserved residues. These proline and glycine residues may have been critical for the scaffolding of a common ancestral mitochondrial carrier, and may have provided the necessary flexibility for the corresponding domains of the polypeptide chain, an essential requirement for the functioning of carriers. The P247G mutation introduced in the yeast Anc2 resulted in a 40% decrease in the carrier molecular activity (Klingenberg *et al.*, 1992), indicating that this conserved proline, although involved in the control of the turnover of the carrier, is not absolutely essential for the transport activity. The conserved acidic and basic amino acids and the glutamine residue could be involved in structurally important charge interactions and hydrogen bonding.

THE CATR AND BA CONFORMATIONAL STATES OF THE BEEF HEART MITOCHONDRIAL ADP/ATP CARRIER PROTEIN

There exist two distinct conformations of the beef heart ADP/ATP carrier protein that can bind atractyloside and carboxyatractyloside or bongkrekic acid, and these conformations, referred to as CATR and BA conformations, are characterized by different reactivities of well-defined regions of the protein to chemical, enzymatic, and immunochemical reagents (for review, see Vignais *et al.*, 1985). It has been demonstrated that these two different conformations of the carrier protein preexist in the mitochondrial membrane and are only stabilized by the inhibitory ligands (Block *et al.*, 1983).

The importance of the conformation of the ADP/ATP carrier for the accessibility and reactivity of specific groups to chemical reagents is abundantly documented. A first clue was that some tyrosyl residues in the membrane-bound carrier in mitochondria are iodinated to a larger extent in the presence of CATR than in the presence of BA, probably as a result of a conformation-dependent modification of the accessibility of these tyrosyl residues to the reagent (Brdiczka and Schumacher, 1976). Later, it was found that the conformational state of the ADP/ATP carrier can be probed by specific antibodies directed to the CATR-carrier complex or to the BA-carrier complex. Antibodies directed to the CATR-carrier complex do not exhibit reactivity toward the BA-carrier complex or the denatured carrier protein (Buchanan *et al.*, 1976; Boulay *et al.*, 1986). Conformational changes occurring in the N-terminal region of the carrier, and depending on the binding of CATR or BA, have been demonstrated with the use of antibodies generated against a peptide corresponding to the N-ter sequence (Brandolin *et al.*, 1989). Further, the selective alkylation of cysteine 56 by *N*-ethylmaleimide (Boulay and Vignais, 1984) requires the presence of ADP or ATP and is enhanced by BA, but prevented by CATR. In other words, cysteine 56 is not readily accessible in the CATR conformation but is unmasked in the BA conformation. Located close to cysteine 56, a K42-Q43 bond is susceptible to proteolysis in the BA conformation but not in the CATR conformation (Marty *et al.*, 1991). In summary, depending on the presence of CATR or that of BA, some specific regions of the ADP/ATP carrier are unmasked and become accessible to chemical reagents, antibodies, or proteolytic enzymes. Taken together, these results afford evidence for the occurrence of two different conformations of the ADP/ATP carrier that can be trapped by CATR or BA. They were indicative of a role of ADP or ATP in the transition between the CATR and BA

conformations, which was fully demonstrated through the use of fluorescent nucleotides and by determination of changes in intrinsic fluorescence.

KINETICS OF THE TRANSITION BETWEEN THE CATR AND BA CONFORMATIONS OF THE ADP/ATP CARRIER STUDIED BY SPECTROFLUORIMETRY

Through the use of two fluorescent, nonpermeant, derivatives of ADP and ATP, namely naphthoyl-ADP (N-ADP) and naphthoyl-ATP (N-ATP), respectively, it has been possible to follow the kinetics of the transition between the CATR and the BA conformations of the ADP/ATP carrier. It was first shown that the membrane-bound ADP/ATP carrier in mitochondria or in inside-out submitochondrial particles (SMP) is able to bind, but not to transport, N-ADP and N-ATP, and that each carrier unit contains several binding sites (Block *et al.*, 1982a). The fluorescence was greatly decreased upon binding of N-ADP to the carrier; conversely, release of bound N-ADP upon addition of CATR or BA resulted in an increase of fluorescence. These properties were used to monitor the transition of the carrier between the CATR and the BA conformers (Block *et al.*, 1983). N-ADP bound at random to either of the two conformers in mitochondria. In the absence of any added transportable nucleotide, only N-ADP bound to the BA conformer was rapidly displaced by BA, and conversely only N-ADP bound to the CATR conformer was rapidly displaced by CATR. However, upon addition of a micromolar amount of ADP or ATP, a concentration too low to displace the bound N-ADP by direct competition, the CATR/BA conformational transition was considerably accelerated and complete release of bound N-ADP was achieved in a few seconds upon addition of either BA or CATR. Thus, the rapid CATR/BA conformational transition depended on the presence of ADP or ATP. Nucleotides capable of binding to the ADP/ATP carrier, but unable to be transported, were inefficient, indicating that the transition between the CATR and the BA conformations probably reflects molecular events inherent in the nucleotide transport process itself. In addition, the ability for externally added ADP or ATP to trigger the rapid reversible CATR/BA conformational transition led to the interesting conclusion that external ADP and ATP are able to bind to both the CATR conformer and the BA conformer, which contradicts the postulate of the single reorientating site (Klingenberg, 1985) alternatively opened to the inside (*m* state) or the outside (*c* state). In this single-site model, the same site is supposed to bind ADP or ATP and, in addition, CATR when the site is opened to the outside, and BA when it is opened to the inside. In the *m* state, the nucleotide binding site would be irreversibly opened to the inside and thereby unable to bind any external nucleotide. As a consequence, the postulated *c* and *m* states are clearly not equivalent to the CATR and BA conformations. Moreover, the single-site mechanism cannot be reconciled with the existence of several binding sites per carrier unit, as demonstrated with the membrane-bound carrier and with the isolated carrier in detergent (Dupont *et al.*, 1982; Brandolin *et al.*, 1982; Block and Vignais, 1984).

Another way to follow the time course of the CATR/BA conformational transition was to explore the intrinsic fluorescence of the ADP/ATP carrier solubilized in detergent. The intrinsic fluorescence is in fact very sensitive to the addition of ADP or ATP. The nucleotide-induced signal was due to changes in the environment of tryptophanyl residues. Among the adenine nucleotides tested, only those which are transported, i.e., ADP and ATP, were effective. Addition of ADP or ATP to the beef heart carrier protein isolated in the presence of laurylamidodimethylpropyl aminoxide (LAPAO) resulted in a fluorescence increase (Brandolin *et al.*, 1981, 1985). The fluorescence increase was prevented by CATR added before ADP (ATP) and reversed by CATR added after ADP (ATP); surprisingly, it was enhanced by BA. A detailed kinetic analysis of the ADP (ATP)-induced fluorescence changes was interpreted in terms of the coexistence of carrier units in the CATR conformation and carrier units in the BA conformation in the absence of any added ligand. In the presence of transportable nucleotides which made the CATR/BA conformational transition possible, the binding of CATR or BA to the respective conformers resulted in the formation of either a low-fluorescence CATR-carrier complex or a high-fluorescence BA-carrier complex. Recent experiments carried out with yeast mitochondria show that the ADP/ATP carrier in yeast also responds by fluorescence changes to the addition of ADP or ATP (Brandolin *et al.*, 1993).

The nature of the detergent used to solubilize the ADP/ATP carrier protein is an important parameter which determines the basal conformational state of the purified carrier and which may be due to the micellar properties of the detergent. For instance,

the beef heart carrier was stabilized in the CATR conformation when prepared in the presence of LAPAO, and in the BA conformation when LAPAO was replaced by cholamidopropyldimethylammoniopropane sulfonic acid (CHAPS), a zwitterionic detergent (Block and Vignais, 1986). It remains the case that, whatever the detergent employed, LAPAO or CHAPS, the transition of the ADP/ATP carrier from one conformer to the other requires the presence of transportable nucleotides.

OLIGOMERIC STATE AND NUCLEOTIDE BINDING SITES OF THE BEEF HEART ADP/ATP CARRIER

The oligomeric state of the beef heart ADP/ATP carrier using the CATR- and BA-carrier complexes has been explored by analytical centrifugation (Hackenberg and Klingenberg, 1980) and neutron scattering (Block *et al.*, 1982b). The CATR-carrier complex was more stable than the BA-carrier complex, but in both cases the inactive carrier was found to be dimeric. From nucleotide binding experiments, it appears that the native carrier in the mitochondrial membrane has a higher degree of oligomerization than the inhibited carrier. Each carrier, either in the membrane-bound state or solubilized in detergent, contains more than one binding site (Dupont *et al.*, 1982; Brandolin *et al.*, 1982; Block and Vignais, 1984). An equal number of low-affinity sites and high-affinity sites were demonstrated for the binding of ADP or ATP. In addition, the total number of CATR binding sites is twice that of the high-affinity ADP/ATP binding sites (Block and Vignais, 1984). Since only one CATR binds per carrier dimer in the CATR-carrier complex (Klingenberg *et al.*, 1978; Block *et al.*, 1986), an unescapable conclusion is that the functional ADP/ATP carrier is organized as a tetramer. Each tetrameric carrier would thus contain two CATR binding sites accessible to CATR from the cytosolic face of the membrane and two BA binding sites accessible to BA from the mitochondrial matrix, and in addition two nucleotide binding sites on each face of the carrier tetramer. The putative tetrameric organization of the functional carrier is consistent with the positive cooperativity demonstrated for CATR or BA binding to the membrane-bound and the solubilized carrier (Vignais *et al.*, 1971; Brandolin *et al.*, 1985). The carrier tetramer is probably less stable than the carrier dimer, and inactivation or inhibition by CATR and BA may be viewed as resulting from the splitting of the functional tetramer into two stable inactive CATR-carrier dimers or BA-carrier dimers. Of particular interest is the fact that all subunits in the oligomeric ADP/ATP carrier are asymmetrically oriented, in a parallel—and not antiparallel—manner. This holds not only for the carrier in mitochondria or in SMP, but also for the isolated carrier reinserted in liposomes (Brandolin *et al.*, 1980).

EXPERIMENTAL APPROACHES TO THE TOPOGRAPHY OF THE ADP/ATP CARRIER PROTEIN IN THE MEMBRANE OF BEEF HEART MITOCHONDRIA

Severe difficulties encountered in the crystallization of membrane transport proteins have led to the development and the refinement of theoretical and experimental methods in an attempt to decipher some features of the topography of these proteins. These methods include prediction of the secondary structure from the amino acid sequence, specific or unspecific chemical modifications with permeant and nonpermeant modifiers, controlled proteolysis, and specific immunological detection of extramembranous regions of the peptide chain (for review, see Ovchinnikov, 1987). These approaches have been applied to investigate the topography of the beef heart ADP/ATP carrier trapped either in the CATR or the BA conformation. Interestingly, some segments of the peptide chain of the beef heart carrier were shown to undergo significant movement during the transition between the CATR and the BA conformations and thus probably in the course of the transport process.

1. Immunochemical reactivity. The sidedness of the N-ter part of the ADP/ATP carrier protein in beef heart mitochondria was probed with rabbit antibodies raised against an 11-residue synthetic peptide corresponding to the N-ter sequence of the carrier (anti-N-ter antibodies). The specificity of N-ter antibodies was first checked by Western blot analysis of mitochondrial lysates and by ELISA with the purified carrier in detergent, coated onto microtiter plates. The cytosolic exposure of the N-ter region of the membrane bound ADP/ATP carrier was evidenced in direct ELISA using mitoplasts coated on the plates and in indirect ELISA (Brandolin *et al.*, 1989). The reactivity of the N-ter region of the carrier to the anti-N-ter antibodies was exacerbated when the mitochondria were incubated in the presence of CATR but, on the contrary, decreased considerably when CATR was replaced by

BA. At present, interpretation of these data in terms of structural changes at the molecular level is essentially speculative. Since the CATR and nucleotide binding sites of the beef heart carrier are localized by photolabeling in the C-ter half of the peptide chain, a propagated change of conformation from the CATR binding site within the same monomer is a plausible explanation. One may also imagine that there exist interactions between mobile segments of monomers in an oligomeric carrier, and more particularly between the N-ter region of a given monomer and segments of adjacent monomers containing the CATR and nucleotide binding sites.

In contrast to the N-ter region, no definite conclusion about the cytosolic or matrix exposure of the C-ter segment of the ADP/ATP carrier could be obtained by immunological or limited proteolysis approaches. Possibly, the C-ter end of the carrier is not freely accessible to the hydrophilic medium surrounding the membrane because it is folded in such a manner that it interacts with polar segments of the peptide chain located within the membrane and lining a translocation channel (see last section); it would then become accessible on one side of the membrane or the other, depending on experimental conditions.

2. Limited proteolysis. Site-specific proteases allow the determination of the sidedness of restricted segments of membrane proteins when used under controlled conditions since they are nonpermeant reagents. In the case of the beef heart ADP/ATP carrier, the limited proteolysis technique was combined with immunodetection of the proteolysed fragments, using a set of antipeptide antibodies and microsequencing of these fragments for definite identification (Marty *et al.*, 1991). To determine the regions of the carrier exposed to the cytosol or to the matrix, two types of mitochondrial particles were used, namely right-side-out mitoplasts and SMP. Both mitoplasts and SMP were pretreated with CATR or with BA to trap the carrier in the CATR or the BA conformation, respectively. The ADP/ATP carrier protein in mitoplasts was unexpectedly resistant to relatively high concentrations of proteases like trypsin and endo-Arg and endo-Lys specific proteases which, on the other hand, could easily cleave the carrier in SMP pretreated with BA. A control test carried out with SMP pretreated with CATR was negative, stressing the difference between the conformations stabilized by CATR and BA. A limited number of peptide bonds exposed to the matrix when the carrier was stabilized in the BA conformation were determined, namely R30-V31 and/or R59-I60, K42-E43, K146-G147, and K244-G245. Two of the bonds that are cleaved in a conformation-sensitive manner are located close to regions involved in the binding of atractyloside and ADP or ATP. These bonds are therefore located in movable segments of the peptide chain of the carrier that probably participate in conformational changes during the transport process.

3. Photolabeling. Mapping of the specific nucleotide and CATR binding sites of the ADP/ATP carrier has been achieved with radiolabeled azido derivatives of ADP and atractyloside, respectively (Boulay *et al.*, 1983; Dalbon *et al.*, 1988). Preliminary tests led to the conclusion that the two photoprobes fulfilled the requirements for topological studies, i.e., they were nonpermeant and highly specific. Moreover, they were able to bind to high-affinity sites unambiguously accessible from the cytosolic side of the carrier. 2-Azido (α^{32}P)ADP was chosen as photoprobe because it is predominantly in the *anti*-conformation, like ADP, in contrast to 8-azido ADP which is essentially in the nonnatural *syn*-conformation. Two segments of the peptide chain of the carrier, spanning residues F153 to M200 and Y250 to M281, were covalently labeled, predominantly at positions K162, K165, I183, V254, and K259 (Dalbon *et al.*, 1988). The first segment was also photolabeled by azido atractyloside (Boulay *et al.*, 1983). Whether the two segments labeled by 2-azido ADP belong to the same carrier monomer, or result from the binding of 2-azido ADP to a site formed by the juxtaposition of two interacting monomers, is an open question. However, because of the probable organization of the functional carrier in two interacting dimers, each one being able to bind one molecule of 2-azido ADP, we favor the second hypothesis. Mapping the ADP/ATP carrier of yeast mitochondria with 2-azido (α^{32}P)ADP led to the labeling of the region G172-M210 (Mayinger *et al.*, 1989), a region equivalent to the photolabeled region F153-M200 of the beef heart carrier (Dalbon *et al.*, 1988). The uncoupling carrier protein, a proton carrier protein belonging to the mitochondrial carrier family, was photolabeled by 2-azido ADP in a region homologous to the Y250-M281 region of the ADP/ATP carrier (Winkler and Klingenberg, 1992).

4. Chemical labeling. The first convincing evidence for the occurrence of conformational changes depending on the binding of inhibitors to the ADP/ATP carrier stemmed from experiments dealing with

Table I. Use of Antipeptide Antibodies, Proteases, Photoactivable Analogues of ADP and Atractyloside, and Chemical Modifiers to Probe Accessible Regions of the ADP/ATP Carrier in Beef Heart mitochondria and Inside-out Submitochondrial Particles[a]

Probes	Accessible regions of the membrane-bound ADP/ATP carrier	References
Antipeptide antibodies directed to the N-ter	N-ter region exposed to cytosol in the CATR conformation	Brandolin *et al.* (1989)
Trypsin Endo Arg-protease Endo Lys-protease	Exposure of the peptide bonds R30-V31 or R59-I60, K42-E43, K146-G147, and K244-G245 to the matrix in the BA conformation	Marty *et al.* (1991)
Azido-ADP	Cysotolic accessibility of segments F153-M200 and T250-M281	Dalbon *et al.* (1988)
Azido atractyloside	Cytosolic accessibility of the segment F153-M200	Boulay *et al.* (1983)
N-ethylmaleimide	Reactivity of C56 to the permeant SH reagent *N*-ethylmaleimide, but not the nonpermeant SH reagents	Vignais *et al.* (1975) Boulay and Vignais (1984)
Pyridoxal phosphate	Reactivity of K22, K42, K48, K106, and K162 to the nonpermeant PLP	Bogner *et al.* (1986)

[a] For details, see text.

iodination of tyrosyl groups (Brdiczka and Schumacher, 1976) and alkylation of cysteine 56 with *N*-ethylmaleimide (Boulay and Vignais, 1984). Covalent modifications of the carrier with arginyl- and tryptophanyl-specific reagents demonstrated that the amino acid sequences of the CATR and BA binding sites of the carrier were not identical (Block *et al.*, 1981a, b), a result which was difficult to reconcile with the single binding site concept (Klingenberg, 1985). Pyridoxal phosphate (PLP) has been used to probe the distribution of lysine groups in the ADP/ATP carrier on each side of the mitochondrial membrane (Bogner *et al.*, 1986; Klingenberg, 1989). Although PLP is thought to be nonpermeant, a surprisingly high number of lysine groups were found to be accessible from both sides of the membrane, and it was suggested that lysine groups at positions 22, 42, 48, 106, and 162 belong to the translocation path. Paradoxically, lysine 9, which is obviously exposed to the cytosol when the carrier is stabilized in the CATR conformation as shown by immunochemical labeling (see above), was not reactive to PLP. All these results are summarized in Table I.

TOPOGRAPHY AND FUNCTIONING OF THE YEAST ADP/ATP CARRIER: THE MUTAGENESIS APPROACH

Only one *in vivo* mutation has been characterized in the yeast ADP/ATP carrier, the op1 mutation which is a R96H substitution (Kolarov *et al.*, 1990; Lawson *et al.*, 1990). This mutation results in the inability of the yeast cell to grow on nonfermentable substrates like glycerol, lactate, or ethanol. Nevertheless the translocation activity was not completely abolished by this mutation; reconstitution experiments have shown that affinity constants for ATP and ADP were not significantly modified while the transport activity was reduced to about 15% of the wild type. On the other hand, site-directed mutagenesis of the Anc2 carrier (see nomenclature in the section on comparison of sequences) have been actively investigated. Several substitutions of the R96 residue have been made, namely R96-D, -L, and -P (Nelson *et al.*, 1992; Klingenberg *et al.*, 1992), and R96-K, -N, and -Q (Lauquin's group, unpublished results). None of them were able to rescue the growth on glycerol medium. They led to the biosynthesis of ADP/ATP carrier proteins with very poor activity if any. Mutants in which arginine residues 204 or 294 have been replaced by leucine or in which arginine residues 252, 253, and 254 have been substituted by isoleucine were unable to grow on glycerol or lactate (Nelson *et al.*, 1992; Klingenberg *et al.*, 1992). In addition, it was shown that the transport activity of the R252I mutated carrier, determined after *in vitro* reconstitution, amounted to only 8% of that of the wild type carrier. These results indicate that the function of the

arginine triplet is crucial for transport activity. Nelson *et al.*, (1992) have isolated several intragenic revertants from mutants of the arginine triplet R252, 253, and 254. Unexpectedly, the mutations R253I and R254I yielded 15 distinct second-site revertants whose mutations were scattered along the polypeptide chain at different distances from the original arginine mutation. Another interesting observation was that the site-directed mutagenesis of cysteine 73, corresponding to cysteine 56 in the beef heart carrier, did not drastically affect transport (Klingenberg *et al.*, 1992), which led to the conclusion that inactivation of the beef heart ADP/ATP carrier by *N*-ethylmaleimide alkylation of cysteine 56 (Boulay and Vignais, 1984) is due to some steric hindrance of the substrate binding site. A similar situation has also been described for the *N*-ethylmaleimide-sensitive cysteine in mitochondrial phosphate carriers from different species (Phelps and Wohlrab, 1991). In the case of a site-directed mutagenesis of tryptophan 235, it was found that the mutant with the W235F substitution was unable to grow on glycerol (Nelson *et al.*, 1992) in contrast with the W235Y substitution, which yielded an active carrier (unpublished data). The goal of current mutagenesis experiments carried out in the author's groups is to detect the effect of conformational changes on the environment of specific tryptophanyl residues.

PREDICTED ARRANGEMENT OF THE PEPTIDE CHAIN OF THE ADP/ATP CARRIER IN THE MITOCHONDRIAL MEMBRANE

In addition to the experimental data, a theoretical approach based on analysis of the distribution analysis of hydrophobic and hydrophilic residues along the peptide chain has been used in an attempt to portray the arrangement of the ADP/ATP carrier in the mitochondrial membrane. Applied to the mitochondrial carriers with a known sequence, this method led to the conclusion that the sequences of all these carriers consist of three repeats of about 100 amino acid residues each with alternation of regions rich in hydrophobic residues and in hydrophilic residues (Saraste and Walker, 1982; Runswick *et al.*, 1987, 1990). Assuming that sequence homologies result in folding homologies, it has been postulated that all mitochondrial carriers are folded in a similar way, the specificity of each carrier for the transported species being imposed by variable amphipathic segments of the peptide chain (Walker, 1992).

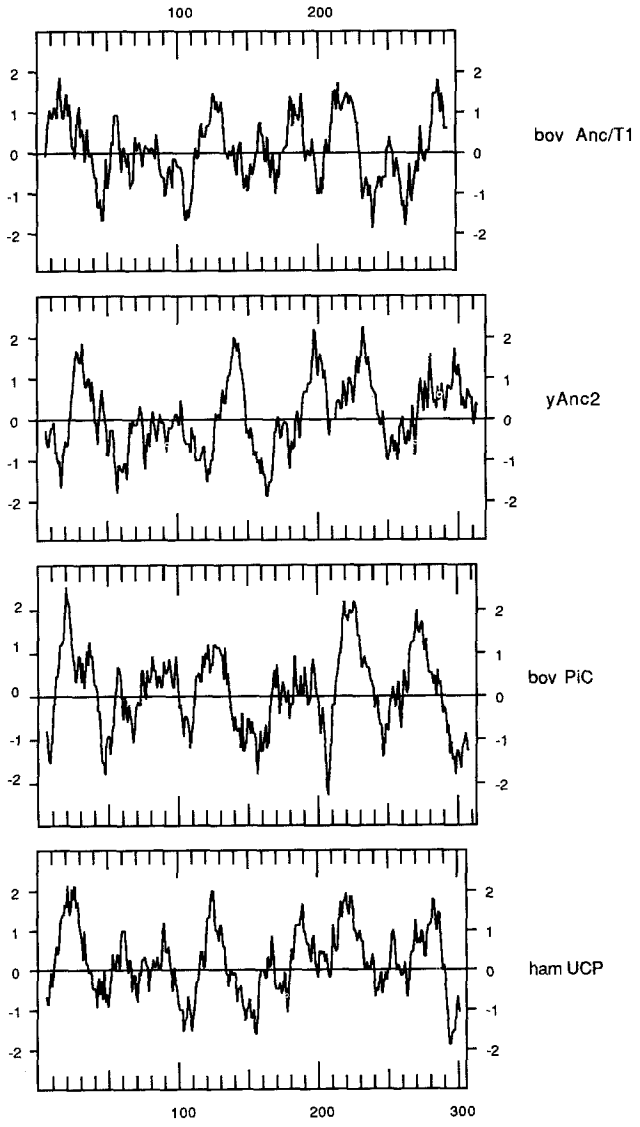

Fig. 2. Comparison of hydropathy plots of four mitochondrial carriers. Hydroplots of the ADP/ATP carriers from beef heart mitochondria (bov Anc/T1) and *S. cerevisiae* mitochondria (yAnc2), the bovine phosphate carrier (bov PiC), and the uncoupling protein from hamster brown fat mitochondria (ham UCP), according to Kyte and Doolittle (1982), with a window of 11 amino acids.

The homology principle between the mitochondrial carriers was taken as a guide to compensate for the lack of experimental data. On the basis of this principle, it has been suggested that the C-ter region of the ADP/ATP carrier, for which the experimental data were not conclusive, might be exposed to cytosol because of the structural analogy of the ADP/ATP carrier with the proton carrier uncoupling protein

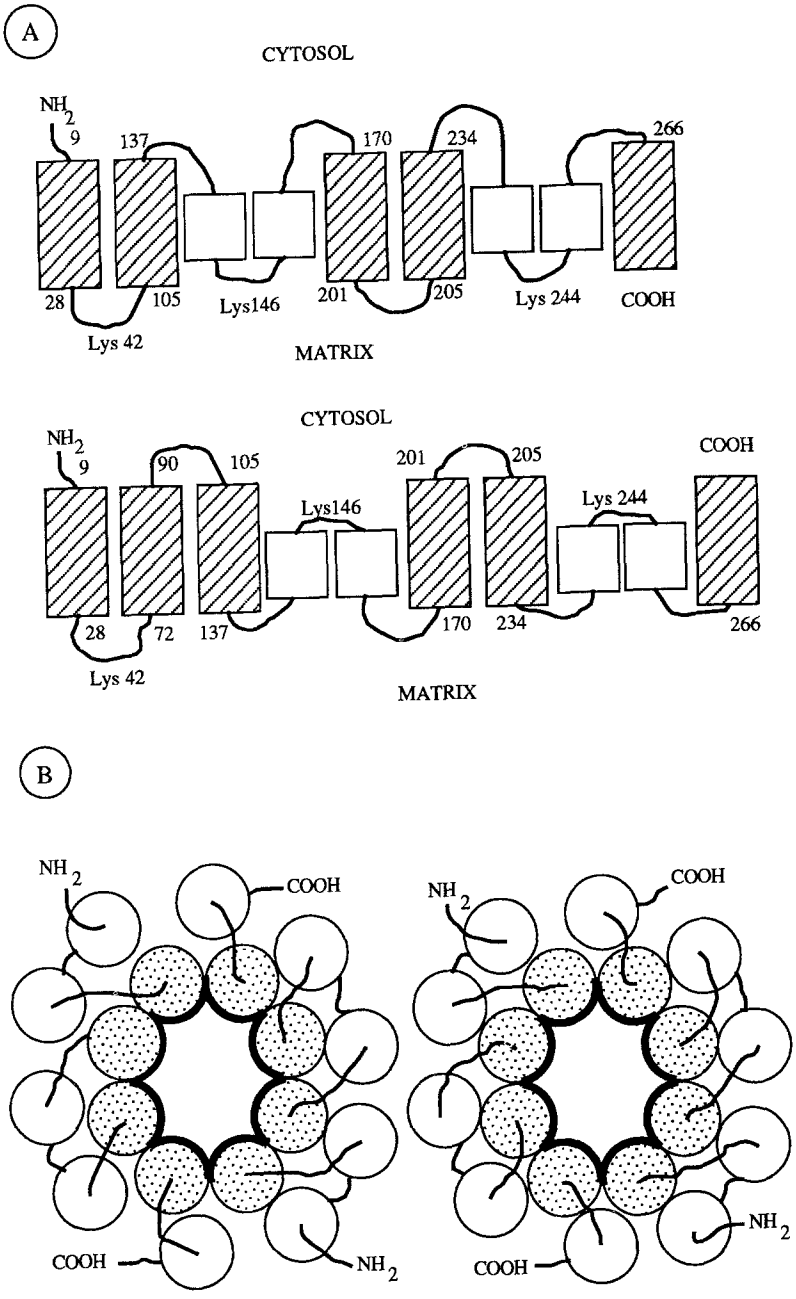

Fig. 3. Postulated arrangement of the ADP/ATP carrier in the mitochondrial membrane. (A) front view of the monomeric carrier with five or six transmembrane helices and with four short amphipathic helices. (B) Top view of a possible tetrameric organization of the carrier, showing hydrophilic paths (highlighted) delineated by the polar faces of the short amphipathic helices. Either five- or six-helix monomers might participate in such an arrangement. Adapted from Marty *et al.* (1991), with permission.

(Klingenberg, 1989). In keeping with the threefold symmetry postulate, each block of about 100 amino acid residues in any mitochondrial carrier can be visualized as containing two membrane-spanning α helices connected by a hydrophilic loop. However, on the basis of hydropathy plots of the N-terminal block of about 100 residues in the ADP/ATP carrier, it is clear that there is only one segment, span-

ning residues 9–28 in the case of the beef heart carrier and residues 20–50 in that of the yeast Anc2 carrier, that is hydrophobic enough to cross the membrane as α helix, all the remaining portion of the N-terminal block being too largely hydrophilic to do so (Fig. 2). The possibility that another segment of the N-terminal block crosses the lipid bilayer relies on a pairing association with a partner segment, possibly belonging to another carrier monomer. In such a case the ADP/ATP carrier would be dimeric and its symmetry would be twice threefold. The only experimental evidence in favor of a six-helix arrangement in an ADP/ATP carrier monomer stems from the demonstration of three equidistant cleavable bonds, K42–E43, K146–G147, and K244–G245, accessible from the matrix compartment in the BA-carrier complex (Marty *et al.*, 1991). On the other hand, although the cytosolic exposure of regions 153–200 and 250–281, which were photolabeled by azido derivatives of atractyloside and ADP, appears to be consistent with a five-helix model, it cannot be excluded that the photolabeled regions belong to hairpin-shaped peptide segments inserted into the bilayer and connected to α helices on the matrix side of the membrane. Determination of the sidedness of the C-terminal extremity of the peptide chain of the ADP/ATP carrier would ascertain the validity of carrier monomer models with an even or an odd number of transmembrane segments since with an even number of spanning segments, both extremities of the peptide chain would be exposed to the cytosol. One must recognize that despite the numerous results obtained on the topography of the ADP/ATP carrier by different approaches, the experimental data were not informative enough to allow a definite choice between the five- and six-helix models of carrier monomers. At present, the phosphate carrier is the only mitochondrial carrier in which a threefold symmetry is convincing, based on the demonstration that both the N- and the C-terminal regions of the Pi carrier face the cytosolic face of the mitochondrial membrane (Capobianco *et al.*, 1991), which is consistent with the hydropathy plot (Fig. 2).

A key element for understanding the molecular mechanism of ADP/ATP transport is the identification of peptide segments lining the nucleotide translocation pathway. Obvious candidates correspond to the amphipathic segments 137–170 and 234–266, which contain the binding sites for azido-atractyloside and 2-azido ADP as well as two proteolytic cleavage sites K146–G147 and K244–G245,

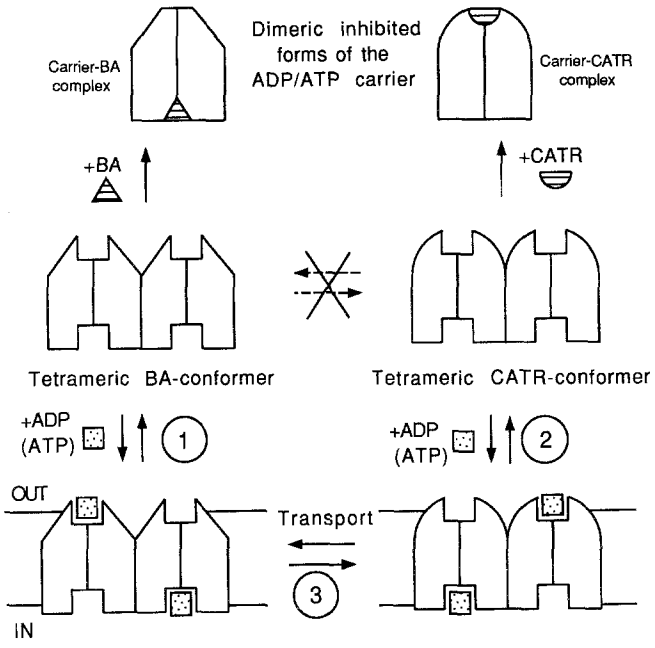

Fig. 4. Scheme illustrating the conformational states of the ADP/ATP carrier and their involvement in the transport process. The postulated tetrameric CATR and BA conformers of the ADP/ATP carrier are schematized. In the absence of ADP or ATP, the CATR/BA conformational transition is not allowed. Only after ADP (ATP) binding to inward- and outward-facing sites either on CATR or BA conformers (steps 1 and 2), and formation of a ternary complex, is the transport of nucleotides possible (step 3). The inhibitory effect of CATR and BA on the nucleotide transport is viewed as the splitting of the functional tetrameric carrier into inhibited dimers. Note the different locations of the CATR and BA binding sites and the overall asymmetry of the carrier with respect to the plane of the membrane.

unmasked in the BA conformation. These regions are characterized by an overall positive charge, consistent with the binding of the negatively charged ADP or ATP. In the scheme of Fig. 3A, they are shown to connect α helices on one side of the membrane, the cytosolic side in the five-helix model, or the matrix side in the six-helix model. They might be inserted into the lipid bilayer possibly as hairpin structures, flexible enough to undergo conformational changes. A closer inspection of the sequence of these amphipathic segments reveals that each of them may be visualized as consisting of two short α helices, spanning residues 140–151 and 153–167, on the one hand, and 237–249 and 253–267, on the other, with the polar residues clustered on one face of each short helix. These features are illustrated in the scheme of Fig. 3B, which shows a top view of a functional ADP/ATP carrier, made up by the association of two

dimers, each containing a hydrophilic translocation channel delineated by the short amphipathic helices. Possibly, the conformational changes undergone by these helices participate in the control of the channeling of ADP and ATP. The existence of short amphipathic segments within the folding of multispanning membrane-bound proteins has been postulated by Lodish (1988) on the basis of sterical considerations; it would result in a local reduced thickness of the membrane with a reduced length of the transport pathway, which in the present case would be favorable to the transfer of bulky molecules such as ADP or ATP.

As discussed by Duyckaerts *et al.* (1980), ADP/ATP transport proceeds by a sequential mechanism in which both the external substrate and the internal substrate bind to the carrier before the exchange occurs. This implies the formation of a ternary complex which can be easily accommodated by a tetramer model. As illustrated in Fig. 4, one of the dimers of the tetrameric carrier binds and partially engulfs an externally added adenine nucleotide, whereas the other dimer proceeds in the same manner with an internal nucleotide. The two conformers involved in ADP/ATP transport are referred to as CATR and BA conformers. In the absence of transportable nucleotide, the transition between these two conformers is virtually forbidden. The experimentally demonstrated dimeric CATR-carrier and BA-carrier complexes would be the result of a decoupling process resulting in the quasi-irreversible partition of the tetramer into two dimers stabilized by the binding of either of the two inhibitors.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Eamon Rooney for critical reading of the paper.

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