Dialectics in Carrier Research: The ADP/ATP Carrier and the Uncoupling Protein

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A concise review is given of the research in our laboratory on the ADP/ATP carrier (AAC) and the uncoupling protein (UCP). Although homologous proteins, their widely different functions and contrasts are stressed. The pioneer role of research on the AAC, not only for the mitochondrial but also for other carriers, and the present state of their structure–function relationship is reviewed. The function of UCP as a highly regulated H^+ carrier is described in contrast to the largely unregulated ADP/ATP exchange in AAC. General principles of carrier catalysis as derived from studies on the AAC and UCP are elucidated.

KEY WORDS: ADP/ATP carrier; uncoupling protein; transport; mitochondria.

INTRODUCTION

The inner mitochondrial membrane is the locale of an intensive traffic of metabolites between the cytosolic and matrix space catalyzed by a family of mitochondrial carriers. These carriers must have developed with the symbiosis of the ancestor prokaryote in the eukaryotic host. Other components of the mitochondrial energy machinery, such as cytochromes and the ATP synthase, are carried over from the prokaryote. As a result the most characteristic components of mitochondria are the solute carriers. Several reviews on the mitochondrial carriers have appeared from 1970, to mention only a few (Klingenberg, 1970; LaNoue and Schoolwerth, 1979; Krämer and Palmieri, 1992).

Work in our laboratory has been concerned primarily with the subgroup involving energy transfer, the ADP/ATP carrier (AAC), the phosphate carrier, and the uncoupling protein (UCP). We shall here review some of our research on the AAC and UCP. Although similar in structure, many of their features are contrasting (Klingenberg, 1985a). Whereas AAC provides free energy to the cytosol, UCP degrades free

energy into heat. Whereas AAC is a fundamental carrier of mitochondria in all cells, UCP is a highly specialized carrier, occurring exclusively in mammalian brown adipocytes. Whereas AAC must have developed together with the mitochondria in the earliest eukaryotes, UCP developed very late with mammalians only, and thus it is probably the latest addition to the mitochondrial family.

THE PARADIGM ROLE OF THE ADP/ATP TRANSPORT SYSTEM

The ADP/ATP exchange is the most active transport system in most aerobic cells. Therefore it was no accident that the ADP/ATP transport system and the underlying AAC pioneered important steps in the research of biological substrate transport in general.

The ADP/ATP exchange was the first transport system to be defined in mitochondria (Pfaff *et al.*, 1965; Klingenberg and Pfaff, 1966). By studying this transport, which had the advantage of dealing with highly specific and well-defined substrates, the matrix space containing the mitochondrial pool of adenine nucleotides and solutes was first defined (Heldt *et al.*, 1965). Thus, the inner mitochondrial membrane was recognized as the locale for solute transport and an osmotic barrier. A clear case was made that the

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ADP/ATP exchange is modulated and driven by the membrane potential (Pfaff and Klingenberg, 1968; Klingenberg and Rottenberg, 1977; LaNoue et al., 1978). Research then penetrated to the molecular level of a carrier for ADP and ATP by defining the carrier sites with ADP or ATP binding (Weidemann et al., 1970a). Also the dynamics of a carrier binding site and its conversion to the inside and outside could be demonstrated (Erdelt et al., 1972; Klingenberg and Buchholz, 1973). This was achieved first alone with ADP and ATP and then fully exploited by the fortunate occurrence of highly specific inhibitors, such as atractylate, which was found to bind to the carrier from the outside, and bongkrekate only from the inside. With these tools, for the first time the translocational events of a carrier could be demonstrated on a molecular level using competitive binding studies between these various ligands (Erdelt et al., 1972; Klingenberg and Buchholz, 1973). Thus, the single-binding center-gated pore mechanism (SBCGP) was defined, which is now accepted to be the basic mechanism for most biological carriers (see also Klingenberg, 1974, 1976).

Because of its abundance and under protection with carboxyatractylate, the AAC could be isolated as an intact protein, as the first carrier from any biological membranes (Riccio et al., 1975). With this protein, well-defined reconstitution studies were performed in which for the first time the number and the sidedness of incorporated carrier molecules were accounted for by using site specific inhibitors (Krämer and Klingenberg, 1977, 1979, 1980).

The AAC marked another important step at the frontier of biomembrane research when it produced the first primary structure of a biomembrane carrier protein (Aquila et al., 1982). Because of its abundant availability as an intact membrane protein, it was one of the earliest candidates for crystallizing a membrane protein. However, the failure illustrated the general experience that typical membrane proteins cannot be crystallized.

With this background, our interest was aroused in another protein in the mitochondrial membrane, the UCP from brown adipose tissue mitochondria. Our suspicion that we may deal with a similar protein was based essentially on two observations: a similar molecular weight as seen by azido-ATP labeling in the mitochondrial membrane (Heaton et al., 1978) and the interaction also with nucleotides (Rafael and Heldt, 1976). With this working hypothesis, we adjusted the AAC isolation procedures for purification of intact UCP (Lin and Klingenberg, 1982).

Similar to AAC (Hackenberg and Klingenberg, 1980), the UCP also turned out to be a homodimer in hydrodynamic structures (Lin et al., 1980). Surprisingly, also UCP exhibited the phenomenon of only one binding center for the nucleotides per two subunits. The sequencing of UCP fully confirmed the suspected similarities to the AAC (Aquila et al., 1985). It was also the first indication that a mitochondrial carrier family of similar proteins may exist. Again, by amino acid sequencing of the phosphate carrier (Aquila et al., 1987), the primary structures of the trio of energy subgroup carriers established that these carriers form a family.

In the last 10 years the research in our laboratory has concentrated on structure–function relationships in the two homologous carriers UCP and AAC. Their comparison proved to be very illuminating and document the old wisdom that the comparative approach is the main motor of biological research.

FUNCTION OF AAC AND UCP

The AAC and UCP differ extremely with respect to the transported solutes (Table I). With ADP and ATP, AAC transports one of the largest solutes among any biological carriers, whereas with H^+, UCP transports the smallest and most elementary solute (Table I). Not only the extreme size difference between both solutes but also the contrast between the solute–protein interaction in both cases poses challenging problems to the structure–function relationship. In AAC the translocational process can be expected to involve large conformational changes of the single-binding center-gated pore mechanism (SBCGP) which have actually been observed (Aquila et al., 1978; Klingenberg, 1985b). In the AAC these conformational changes between the well-defined c- and m-states may even rock the protein from one side of the membrane to the other and cause drastic changes in the configuration of the mitochondrial membrane (Scherer and Klingenberg, 1974). During the translocation of H^+ by UCP the conformational changes should be only small, and none could be detected so far. The idea has been proposed (opening lecture of first EBEC Meeting, Urbino, 1980) that the size of the conformational changes is inversely related to the diameter of the solutes. These again are related to the log of the basic translocation rate. Within the homologous mitochondrial carrier family this may apply to the three carriers involved in energy transduction. In fact, the molecule

Table I. Similarities and Contrasts in AAC/UCP Function

Function	AAC	UCP	Score[a]
	Transport		
Solute	ADP/ATP	H^+/OH^-	−
Transport mode	Exchange	Unidirectional	−
Regulation	Autonomous	External	−
		(Fatty acid, ND, TP)	
	Nucleotide interaction		
Specificity	ADP/ATP	A/G–DP, A/G–TP	−
Transport activity	Activation	Inhibition	−
Active transport	$\Delta\Psi$-driven	$\Delta\Psi$-driven	+
Protein stability	Decreased	Increased	−
pH Dependence	Low	Strong	−
Sidedness of binding	c- and m-side	Only c-side	−
Affinity	Low ($< 10^5\,M^{-1}$)	High ($< 10^6\,M^{-1}$)	−
Binding to purified protein	17 (CAT^b)	15 (GTP, ATP)	+
(μmol/g protein)			
Equivalent (functional)	59	62	+
Mr (kD)			
Functional dimer	Half-site reactivity		+

[a] Score: −, constrast; +, similarity.
[b] CAT, carboxyatractylate.

transport activity, $8 \times 10^2\,\text{min}^{-1}$ for AAC, $5 \times 10^3\,\text{min}^{-1}$ for phosphate carrier, $2.5 \times 10^4\,\text{min}^{-1}$ for UCP, is related to the exponential of the solute diameter D, $v \approx a\exp(-bD)$.

Until the early 1960's it was not known whether a solute traffice across the mitochondrial membrane was necessary and whether it needed carriers, i.e., membrane-bound proteins. Two features provided strong evidence for carrier function: the transport of ADP and ATP by tightly coupled exchange reactions (Pfaff et al., 1965), which was highly specific for free ADP and ATP, and subsequently the identification of inhibitors for the ADP/ATP exchange first of atractylate (Heldt et al., 1965) and then of bongkrekate (Weidemann et al., 1970b). In contrast, H^+ transport in the brown adipose tissue mitochondria is unidirectional and, together with the small solute size, at first sight it does not typify a carrier function. However, the specific inhibition by purine nucleotides gave it carrier status, which was then confirmed by the identification and isolation of UCP (Lin and Klingenberg, 1982).

A common feature of both transport systems is their dependence on $\Delta\Psi$. For the ADP/ATP exchange, initially it was controversial that the membrane potential affects the charge difference between ATP^{4-} and ATP^{3-} and was also difficult to prove (LaNoue et al., 1978). In the UCP the unidirectional H^+ transport is dependent on $\Delta\Psi$ for its uncoupling function (Klingenberg and Winkler, 1985). In both cases $\Delta\Psi$ affects the maximum rate of transport rather than the K_M. However, there is a marked difference between the relation of $\Delta\Psi$ and the rate. In AAC $\Delta\Psi$ affects the logarithm of the exchange rate ($v^{>< } = k\exp\Delta\Psi$) (Krämer and Klingenberg, 1982), whereas in UCP it is linearly correlated ($v = k\Delta\Psi$) (Klingenberg and Winkler, 1985). For the AAC, this has been interpreted that the potential difference $\Delta\mu$ controls the carrier distribution between the discrete translocation steps according to the SBCGP. In UCP the linear relation reflects ohmic resistance of a more channel-type transport without such well-defined discrete steps. This is in line with the extreme differences in the size of the solutes as discussed above.

STRUCTURE

The isolated AAC is packed in a large Triton micelle, which according to a number of hydrodynamic studies would be best characterized as an oblate ellipsoid. The AAC dimer binds a large excess of detergent amounting to about 170 molecules/dimer (Hackenberg and Klingenberg, 1980). A similar structure also was determined for the isolated UCP micelle (Lin et al., 1980). The large detergent envelope explains the exclusion of these proteins from hydroxyapatite during the isolation. It suggests that the proteins are very hydrophobic. However, when the primary structure of AAC emerged, it was a surprise
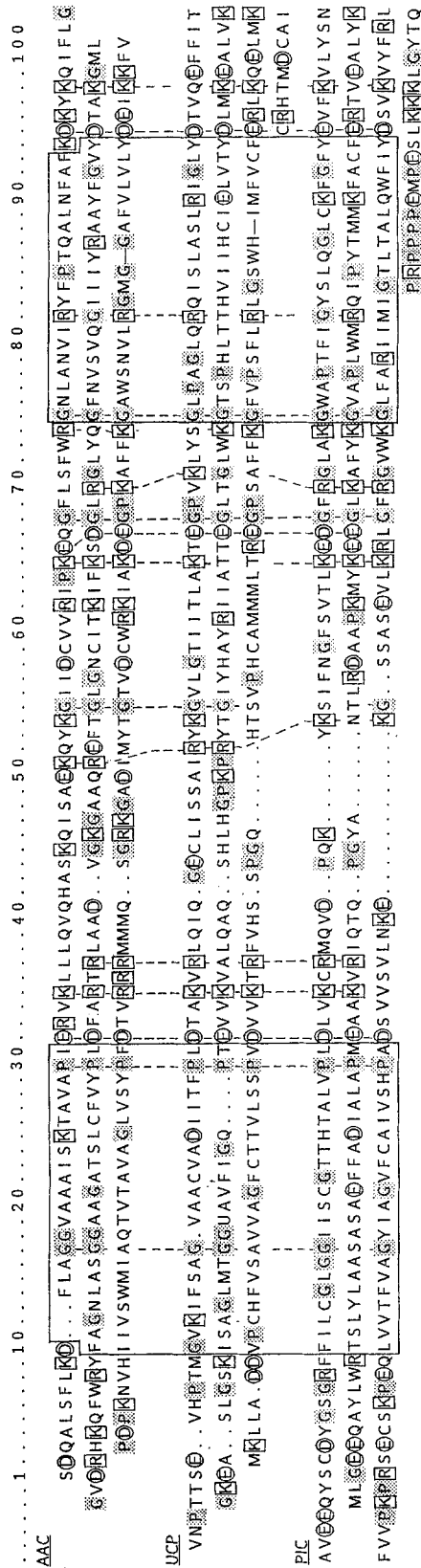
AAC

UCP

PIC

Fig. 1. The sequence of three bovine mitochondrial carriers arranged to emphasize the threefold similarity between the repeat domain structures. The C-terminal segments for the Pi-carrier are separately printed. The boxed areas are putative transmembrane α-helices.

that it had a relatively low degree of hydrophobicity. Hydrophobicity analysis for transmembrane helices identified only weakly hydrophobic sections (Aquila *et al.*, 1985; Saraste and Walker, 1982). The segmentation of the sequence into three similar repeats about 100 residues long strongly facilitated the assignment of hydrophobic sections. This was amplified when further sequences first of UCP and then of the phosphate carrier became known (Aquila *et al.*, 1987) (Fig. 1). With the introduction of algorithms for amphiphatic helices or even amphiphatic β-structures, it was possible to find that in each repeat domain the second helix is more amphiphatic (Aquila *et al.*, 1985). By vertical alignment of the repeat segments, not only within each sequence but also among the sequences of other carriers, the possible α-helix spans were more precisely delineated (Klingenberg, 1989).

Within the three second helices of each repeat conspicuous arginine residue and more selectively for UCP and PiC, D and E are also found. In the first helix lysine (AAC) and aspartate (UCP) may be involved in transport (see below). At the C-terminal of the α-spans, strikingly conserved acidic residues are positioned. These are the most conserved residues seen among the whole mitochondrial carrier family. The first helix in each segment is terminated by an acidic residue in the strikingly conserved motive

"allbnb" (a = acidic, 1 = lipophilic, b = basic, n = neutral), and in the AAC in the third segment even by all bbb with an R triplet. This strong ionic cluster hinges the α-span in the membrane. They are contained in the central hydrophilic segment between the two helices in each repeat of about 30 to 40 residues length. The two hydrophilic linkages between the repeats and the N and C-terminals are mostly shorter. The central segments contain high concentrations of charged residues several of which are well conserved in the vertical alignment. Less conservation is observed in the hydrophilic linkers. Also strikingly conserved in the central segments are glycine and proline in the helices. They obviously mark some structural features which are important for their common function of the mitochondrial carriers.

TOPOLOGY IN THE MEMBRANE

In the determination of the folding or topology of the carrier sequence, difficulties were encountered because most of the carrier sequences appeared to be located within the membrane, and no clear proteolytic cleavage sites were found for some time. After considerable uncertainties, a consensus was reached that both the N- and C-terminals protrude to the cytosolic site of the membrane (Klingenberg, 1989;
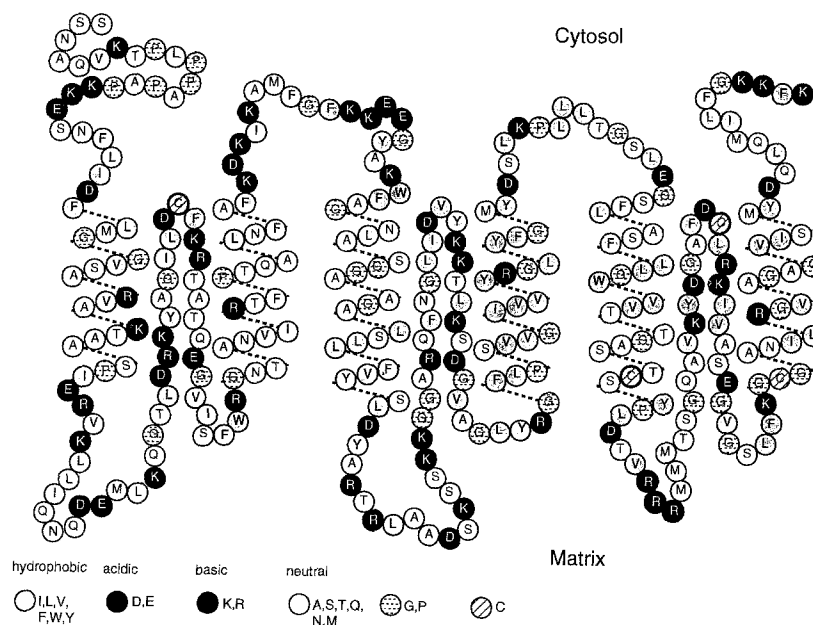

Fig. 2. The folding of a mitochondrial carrier, exemplified with the AAC-2 from *Saccharomyces cerevisiae*. The three-repeat structure is emphasized. Each repeat contains two transmembrane α-helices and a nonhelical loop protruding into the membrane space from the matrix side.

Eckerskorn and Klingenberg, 1987; Boulay et al., 1986; Brandolin et al., 1989) (Fig. 2). This agrees with the three-repeat model of six membrane-spanning α-helices. Antibody studies, crosslinking, and also some proteolytic studies sustain this folding model. Probing the numerous lysines in the AAC with membrane impermeant reagents, such as pyridoxal-phosphate, was also instrumental for assigning hydrophilic segments to the outer and inner face of the membrane (Bogner et al., 1986). However, several lysine groups could be probed both from the inside and outside, their accessibility being dependent on the translocational state of the carrier. It was concluded that these lysines are located in the translocational pathway penetrating the membrane. As a consequence, a part of the hydrophilic central section of each domain was proposed to loop from the matrix site into the membrane and to line the translocational channel. Subsequent support for this folding model came from affinity labeling with nucleotides in the AAC and in the UCP (Dalbon et al., 1988; Mayinger et al., 1989; Mayinger and Klingenberg, 1992). In both cases the central sections of either the second or the third repeat was labeled, although the nucleotides were accessing the proteins only from the cytosolic site.

According to these data, the loop would involve about 15 residues at the upstream portion of the central segment in each repeat. Whether this loop penetrates the whole membrane or only part of the membrane remains to be seen. In the meantime, the pore of cation channels has also been proposed to be lined by hydrophilic hairpins. First for the Na^+-channel (Guy and Seetharamulu, 1986) and now also for the K^+- and Ca^+-channel, intramembrane β-hairpin elements were proposed (for review, see Miller, 1992). Again what was first found for the AAC and UCP, i.e., the involvement of intramembrane nonhelical structures in the translocation channel, may turn out to be of significance for other translocating systems.

The high amount of basic residues particularly in the central sections of the AAC and UCP results in an overall basicity of these proteins. There are at least four ways in which the basic residues can be involved, the binding with the anion substrate, the binding of cardiolipin, the gating process, and the positive charge fields in the vestibule. It is highly probable that part of the excess of cationic charges is compensated by cardiolipin which is localized primarily on the matrix side of the inner membrane. For the AAC, an unusually high amount of tightly bound cardiolipin (6 mol/mol AAC) has been determined (Beyer and Klingenberg, 1985). Additional non-tightly bound cardiolipin is also interacting specifically with AAC (Drees and Beyer, 1988). Since most of the other mitochondrial carriers also probably interact with cardiolipin, a major portion of the inner membrane cardiolipin is probably associated with the inner mitochondrial membrane carriers. A complete dependence of the transport on cardiolipin has been shown with mutant AAC (Hoffmann, 1991).

Another possible role of the positive and negative charge clusters in the central segments is the gating of the pore. The gates are considered to consist of ion pairs which may form on closing of the gate across the channel. With the "charge relay" model of the gating process, a mobile charge translates signals from the binding center which contains a positive charge for anionic substrates (Klingenberg, 1992). On occupying the binding center from one side through an open gate, the substrate releases a negative charge from the binding center which then can swing to the positive charge of the closed gate. Paired with this positive charge the gate is opened. In the intermediate states both gates are open but the pathway is blocked by the substrate. This again would be in line with the carrier being potentially continuous pore-forming proteins.

BINDING SITES

The nucleotide binding sites in AAC and UCP were probed by affinity labeling with nucleotide derivatives. Photoaffinity labeling with 2-azido-ATP marked in bovine heart AAC the central region of the second repeat and to a lesser extent also of the third repeat (Dalbon et al., 1988). In yeast AAC, both with 8-azido and 2-azido ATP, the central section only of the second repeat was labeled (Bogner et al., 1986). Obviously, labeling at the third repeat is unspecific. It seems that in the AAC the purine binding site is in the nonhelical central loop.

In UCP three different groups could be labeled by using the 2- and 8-azido ATP, and the fluoronitrophenyl derivative of ATP (Winkler and Klingenberg, 1992; Mayinger and Klingenberg, 1992). All these residues are localized in the central section of the third repeat. Complementing these data, a regulatory site which controls the nucleotide binding in UCP by protonization was identified as E262, close to the azido-labeled T259 and T264 (Winkler, unpublished). The binding of the nucleotides to different

repeat domains in AAC and UCP agrees with their different, even opposite, function in both carriers. In AAC the nucleotide protein interaction triggers the translocational event by inducing the gating process and the conformational changes. In UCP the nucleotide binding inhibits H^+ transport and acts from a binding site different from the translocation channel. The inhibitory binding site in UCP is accessible only from the cytosolic surface. Yet, the identification of three or four binding sites in the central hydrophilic segment of the last repeat domain provides the strongest evidence yet that this portion is forming some sort of an intramembrane loop.

The nucleotide interaction illustrates best the contrasts between both homologous carriers (Table I). It induces opposing conformational changes as probed, for example, by proteolytic enzymes (see Table I). In the AAC, ADP or ATP renders the structure labile so that it becomes more accessible to trypsin digestion (Klingenberg et al., 1978). In UCP, ATP binding tightens up the structure, as seen by protection against trypsin digestion (Eckerskorn and Klingenberg, 1987). This homologous antinomy is an impressive example for illustrating that activation of the translocational process opens up the structure whereas inhibition induces rigidity. Further, in AAC, binding of the inhibitors atractylate or carboxyatractylate stabilizes the AAC against denaturation by detergents or against proteolytic attack (Aquila et al., 1978).

STRUCTURE–FUNCTION RELATIONSHIPS

The identification of AAC genes in yeast provided an excellent opportunity for the manipulation of the AAC structure (Adrian et al., 1986; Lawson and Douglas, 1988). In yeast three genes for the AAC have been found. Of these, AAC2 is normally expressed under aerobic conditions and AAC3 seems to be expressed under anaerobic conditions (Kolarov et al., 1990). On elimination of the AAC2 gene, AAC1 is expressed, although only poorly. In yeast strains from which AAC1 and AAC2 genes have been deleted, both AAC2 and AAC1 genes were expressed on plasmid vectors (Lawson et al., 1990; Gawaz et al., 1990). Thus, it became possible to isolate both isoforms separately and compare for the first time the functional differences of isoforms of the AAC. The major difference was an about 2/3 lower molecular activity of AAC1 as compared to AAC2. The K_M's for ADP and ATP were unchanged.

Site-directed mutagenesis is concentrated on the normally expressed AAC2 (Klingenberg et al., 1992). Each of the domains contains a cysteine. C73 in the first domain was considered to be essential since alkylation inhibited transport. However, the mutation C73S did not cause inactivation of the AAC. C73 does not appear to be involved in transport, but the alkylated SH group either blocked the translocation path or blocked a hydrogen bond. The nonessentiality of an inhibitory SH group is found also in other carrier proteins. Further conspicuous residues to be mutated were the three arginines located in the second helix of each repeat domain. Among these, R96H mutation was found to be identical with the point mutation in the natural op1 mutant. The mutants of all these three arginines with neutral residues were unable to grow on glycerol and indicative of damage in the AAC. However, the isolated AAC turned out, in the reconstituted system, to be only partially incapacitated. Probably there are other reasons why the phenotype is more strongly affected than the AAC function itself. One suggestion would be that the three arginines are involved in the protein transport pathway.

A striking feature in the AAC is the triplet R252, 253, 254 followed by a methionine triplet (Nelson and Douglas, 1993). Also these arginines are essential, and mutants in the reconstituted system quite inactive. According to our folding model they are localized on the matrix site, but not within the translocation channel. It seems improbable that this positive charge cluster is involved in binding of the transport. Our suggestion has been that its function is to steer negatively charged solutes into the channel. Together with the positive residue doublets in the other repeats, they may form an annular positive field in the vestibule around the channel entrance.

The mutants K179A and K182A are both phenotypically and functionally active. In our model both groups K179 and K182 are localized in the hairpin of the second repeat domain and therefore should be involved in the translocation. The only lysine localized within a transmembrane α-helix is K39. The mutant K39A is completely inactive also in the reconstituted system. So far, this is the strongest case of an essential group among our screening program. It supports the early results with bovine AAC that K23 was the only lysine group which was 100% protected by carboxyatractylate against labeling with pyridoxalphosphate.

Another project was to find second-site revertants starting from some well-defined mutants
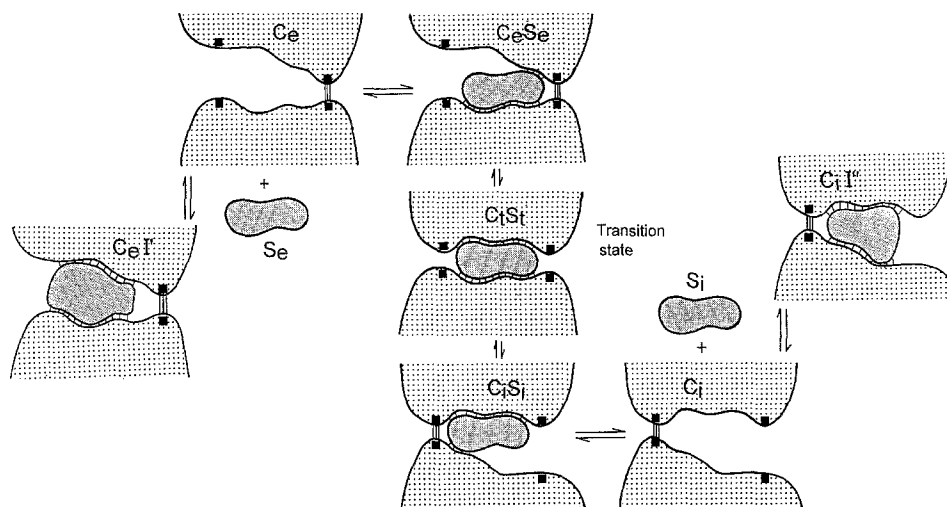
Fig. 3. The single-binding center-gating pore (SBCGP) mechanism, emphasizing the substrate–protein interaction by the induced transition fit (ITF) as a driving force for the transport catalysis (from Klingenberg, 1991b). C = carrier, I = inhibitor, S = solutes, i = internal, e = external, t = transition state.

(Nelson et al., 1993). Most productive has been the R254I mutant from which 11 different second-site revertants have been obtained. Strikingly, these mutations seemed to be all on the opposite cytosolic side of the membrane. In helical wheel projections it was possible to show that the mutations fall within half of each helix and probably are involved in helix contacts near the cytosolic phase. Second-site mutations seem to be a promising tool in detecting structure–function relationships between residues.

PRINCIPLES OF CARRIER CATALYSIS AS ILLUSTRATED BY AAC AND UCP

The analysis of transport mechanisms is a multi-layered approach. There is a structural understanding of the molecular events, and there is the kinetic and thermodynamic description of the transport catalysis. The two approaches are interlinked so that the structure and analysis should allow an ultimate understanding of the molecular forces which control the translocational events. After the basic mechanisms of carrier action are elucidated with the AAC, we have turned our attention, also for the principles of carrier catalysis, to AAC. Because nucleotide protein interaction is such a ubiquitous and well-described phenomenon, it is not surprising that the principles of carrier catalysis were also first described with AAC (Klingenberg, 1985c; Klingenberg, 1991b). The same phenomena will also apply to other carriers, but the discrete catalytic steps may be not as well defined.

This treatment of the transport catalysis will also predict why in carriers with small and weakly interacting substrates a unidirectional transport is possible. Again the antinomy between AAC and UCP will illustrate the principle.

A scheme employing the important elements in our induced transition fit model (ITF) of carrier catalysis is given in Fig. 3 (Klingenberg, 1987, 1991b). The scheme employs three conformation states of the carrier, the c- and m-state as well as the transition state, although the flexibility of the binding center should permit several substrates. In either the c- or the m-state, the carrier-binding center is not substrate-like and therefore the interaction force between substrate and protein is comparatively weak. Yet, this interaction triggers the flexibility of the binding center and drives it into the transition state where the binding-center conformation is more substrate-like and therefore produces a larger substrate–protein interaction force. The driving force is an induced fit between the substrate and the protein. Each conformation change also affects the gates on both sites of the binding center. In the transition state both gates are considered to be open, in line with the charge relay model of the gating system as described above.

The ITF of carrier catalysis is quasi-opposite to the substrate–protein interactions in enzyme catalysis. Whereas in the transition state in enzymes the substrate is distorted, in carriers the substrate remains intact, but the protein conformation is distorted. Whereas in enzymes the binding center in the

ground state has a transition-state like conformation, in carriers it is different from the transition state. Whereas in enzymes induced fit distracts from catalysis (Jencks, 1980) and only increases specificity, in carriers induced fit is the essence of catalysis.

The energy profile for the translocational step should produce a smooth profile without major energy differences between the states (Fig. 3). The conformational change into the transition state requires considerable energy, which is provided by the intrinsic binding energy of the solute. In the external or internal binding state only small amounts of binding energy are provided and only small conformational changes are induced. Thus, a smoothing of the energy levels is obtained to assure good catalytic activity. The theory also states that the binding-center configuration in the c- and m-state is more inhibitor-like than substrate-like. In these "ground" states the inhibitor–carrier interaction releases the full intrinsic binding energy without the subtraction of conformational energy. As a result, inhibitor binding is tight. In other terms, the conformation of the binding center in the two ground states can be sensed by inhibitors.

The AAC provides impressive examples with CAT fitting into the binding center in the c-state, and BKA to the different conformation of the binding center in the m-state. Accordingly, the structure of the inhibitors is quite different although both have in common a minimum of three negative charges as the substrate ligands. Thus, these types of inhibitors utilize the substrate unlike conformation of the binding center, analogous to transition-state inhibitors in enzymes. They fix the binding center in the most opposite conformations. Therefore at least two structurally different types of inhibitors may exist, whereas in enzymes they fix the binding center in the transition state.

One interesting consequence of the ITF is the understanding of the differences of the transport types between AAC and UCP. In AAC the activation barrier for the conformation changes to their transition state is much larger than in UCP because it deals with larger substrates. Only the high energy released by the nucleotide–protein interaction can compensate this barrier. Therefore, a unidirectional transport is nearly impossible because it requires transition through the uncompensated energy barrier. In UCP the conformational changes, the solute–protein interactions, and the catalytic barrier to be compensated are small. Thus, the unidirectional transport does not encounter a major obstacle. In fact, the rate difference for unidirectional

transport between AAC and UCP is about 2×10^4, which would correspond to a difference in activation energy barriers of 20 kcal (see also Fig. 1).

Another conclusion to draw from the ITF is the nonexistence of vectorial group translocation (Klingenberg, 1991a). Vectorial group translocation has been the basis for a concept of chemiosmosis and was early postulated by Peter Mitchell to be involved in transport through the membranes (e.g., Mitchell, 1979). In this case, both chemical and translocational catalysis are combined and interdependent. However, the ITF mechanism implies that substrate–protein interactions are opposite in the chemical and vectorial catalysis of proteins. They cannot be fitted into one protein–ligand interaction. Originally, the vectorial group translocation was an attractive hypothesis for active transport because it seemed to provide energy for the vectorial process. However, it ignored substrate–protein interaction, which is necessary for any type of catalysis, whether chemical or vectorial.

H^+ TRANSLOCATION BY UCP

Among the large family of H^+ translocating systems, whether involved in primary or secondary active transport, UCP can be considered to be functionally the simplest yet known. UCP only catalyzes H^+ transport driven by the membrane potential without any additional machinery involved. We have classified UCP as an amputated H^+-substrate cotransporter (Klingenberg, 1985c).

Although H^+ is a simple and ubiquitous solute, its transport is not a trivial matter. The main problem is the low concentration of H^+ with physiological pH as compared to other cations. Probably for this reason a seemingly simple H^+ transport by UCP becomes more complicated. On withdrawal of all endogenous fatty acids, H^+ transport by UCP becomes inhibited and can only be activated by addition of fatty acids (Rial et al., 1983). There is no competition between the activation by fatty acids and the inhibition by GTP, ATP, etc. The specificity calls for fatty acids from C_{10} up but is quite tolerant with respect to substitutions at the acyl chains. A steep maximum is observed at C_{14} which is probably due to the limited solubility of fatty acid C_{16} and higher (Winkler and Klingenberg, 1993).

H^+ transport can be measured in the reconstituted system in both directions in the uptake or

release mode, depending on how the membrane potential is imposed by the K^+ gradient (Klingenberg and Winkler, 1985). Most important for understanding the function of fatty acids are the pH dependence in H^+ transport. The pH influence inside and outside of the liposomes can be interpreted to show that fatty acids are necessary on the H^+ receiving site of the UCP in the membrane, i.e., in the efflux system on the outside and in the influx in the inside. All these results taken together indicate that fatty acids are directly involved in the H^+ transfer of UCP. Fatty acids may provide the central H^+ translocating carboxyl group, which would be the simplest but less likely mechanism (Klingenberg, 1990). More in line with these data is the concept that fatty acids provide carboxyl groups in the access path to a central constituent H^+ translocating group. The extremely low concentrations of H^+ necessitates that a central H^+ transferring group is supplied with H^+ delivering carboxyl groups in the entrance and exit paths. The existence of these carboxyl buffering groups has been demonstrated for bacteriorhodopsin on both sides of the central H^+ translocating switch (Butt et al., 1989). Whereas in bacteriorhodopsin these groups are constituents, in UCP they would be provided by the free fatty acids. This permits a regulation of the H^+ transport activity of UCP required in the physiological function of the brown adipose tissue.

With these mechanisms, UCP is a highly regulated H^+ carrier under the control of pH and nucleotide and fatty acid concentrations. Again, here UCP and AAC differ from each other. AAC as a more elementary carrier is unregulated as far as we know. Of course, both are under autonomous control by $\Delta\Psi$.

PERSPECTIVES

The mechanism of carrier action is a major challenge in biomembrane research. The unusual simplicity of the mitochondrial carrier structures provides a good chance to remain on the frontier in our understanding of carrier mechanism. However, one of the main barriers to progress is the structure resolution of these carriers. As typical membrane proteins go, they cannot be crystallized. Therefore, structure analysis is confined to computer-aided analysis of structurally characteristic elements in the sequence and to methods applicable to the solubilized or membrane-localized molecules. Perhaps NMR will have to replace X-ray or electron diffraction analysis. The

genetic approach, despite its limitations, has also been very helpful. Here the mitochondrial carriers are at a disadvantage to bacterial carriers because of the self-defeating limitations in the expression of negative mutations. Heterologous expression in bacteria requires renaturation of the protein and so far has not been successful for membrane proteins. The insect cell system again has other severe disadvantages.

In the meantime, the functional approach will continue to give important information concerning the catalytic mechanism of solute transfer. UCP is a system in which elementary steps of the H^+ transfer in the biological system are represented. AAC is a peculiar example for the ubiquitous and all important nucleotide–protein interaction which is utilized so widely for energy transfer and protein conformation change.

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