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Topical Review

Structure and Function of Mitochondrial Anion Transport Proteins

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Introduction

The mitochondrial anion transporters confer a highly selective permeability upon the mitochondrial inner membrane. To date, at least fourteen anion transporter activities have been clearly identified (*see* Table 1). These carriers catalyze a high magnitude flux across the inner membrane and as such occupy a particularly prominent position within eukaryotic cell intermediary metabolism. For example, the exchange of cytoplasmic ADP for mitochondrial ATP on the adenine nucleotide transporter, as well as the import of phosphate on the phosphate carrier, are essential in order to provide the cell with ATP made via oxidative phosphorylation. Citrate efflux from mitochondria on the citrate transporter provides the cytoplasm with a carbon source that supports fatty acid and sterol biosyntheses. Additionally, this efflux likely provides a supply of NAD^+ (via the concerted action of ATP-citrate lyase and malate dehydrogenase) for use by the glycolytic pathway. Malate efflux from mitochondria on the dicarboxylate carrier represents a required step in the gluconeogenic pathway. Alternatively, in yeast this carrier may catalyze the influx of malate, thereby providing an anaplerotic function. Pyruvate influx into mitochondria via the pyruvate transporter is a required step for: (i) the complete oxidation of glucose and amino acids (thereby providing the energy source for a substantial portion of the cell's ATP); and (ii) the supply of carbon precursor for the gluconeogenic as well as the triacylglycerol and sterol biosynthetic pathways. Finally, the α-ketoglutarate and the aspartate/glutamate carriers play essential roles in the malate/aspartate shuttle, as well as in gluconeogenesis. The well-established anion transporters and their metabolic importance are listed in Table 1. The progress to date in the study of these transporters is summarized in Table 2.

Because of the importance of the mitochondrial anion transporters in physiology and disease, considerable effort has been expended to extend our understanding of these carriers to the molecular level. This review will summarize the current state of knowledge concerning the molecular basis underlying the functioning of the major mitochondrial anion transport proteins. Emphasis will be placed on the structure-function relationships that have been elucidated to date, and on the more recent approaches that have been developed which will likely guide future efforts. Since a goal of this review is to be brief, I will primarily focus on developments during the last 5 years and citations to literature will be selective. For earlier information, as well as information regarding other (i.e., the neutral and cationic) metabolite mitochondrial transporters, the reader is referred to several insightful earlier reviews (LaNoue & Schoolwerth, 1979; LaNoue & Schoolwerth, 1984; Kramer & Palmieri, 1989; Kramer & Palmieri, 1992; Walker, 1992). Also, detailed information on mitochondrial carrier sequences

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[1] The abbreviations used are: CTP, citrate transport protein; DTP, dicarboxylate transport protein; EPR, electron paramagnetic resonance; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; MTSES, sodium (2-sulfonatoethyl)-methanethiosulfonate; MTSET, [2(Trimethylammonium)ethyl]-mathane-thiosulfonate bromide; NiEDDA, Nickel(II) ethylenediaminediacetate; PTP, phosphate transport protein; TMD, transmembrane domain; and UCP, uncoupling protein.

Table 1. Mitochondrial anion transport proteins

Transporter name	Physiological substrates	Metabolic significance
ADP/ATP	ADP,ATP	Oxidative phosphorylation
Phosphate	Phosphate	Oxidative phosphorylation
ATP-Mg/phosphate	ATP-Mg, phosphate	Matrix adenine nucleotide content
Citrate	Citrate, isocitrate, *cis*-aconitate, malate, phosphoenolpyruvate, succinate	Fatty acid and sterol biosynthesis, gluconeogenesis, isocitrate-α-KG shuttle
α-ketoglutarate	α-ketoglutarate, malate, succinate, oxaloacetate	Malate/aspartate shuttle, gluconeogenesis, isocitrate-α-kg shuttle, nitrogen metabolism
Dicarboxylate	Phosphate, malate, succinate, oxaloacetate, sulfate, sulfite	Gluconeogenesis, anaplerosis, urea synthesis, sulfur metabolism
Pyruvate	Pyruvate, other monocarboxylates, ketone bodies	Citric acid cycle, gluconeogenesis
Aspartate/glutamate	Aspartate, glutamate, cysteine-sulfinate	Malate/aspartate shuttle, gluconeogenesis, urea synthesis, cysteine catabolism
Glutamate	Glutamate	Urea synthesis
Branched chain α-keto acid	Branched chain α-keto acids	Branched chain amino acid catabolism
FAD	FAD	Supply reducing equivalents to e transport chain
Succinate/fumarate	Succinate, fumarate	Gluconeogenesis
Oxaloacetate	Oxaloacetate, sulfate, thiosulfate	Anaplerosis, sulfur metabolism
Uncoupling protein	H^+, halide anions	Thermogenesis

Table 2. Summary of progress to date on the mitochondrial anion transporters[a]

Progress	Mitochondrial anion transporter													
	AAT	UCP	Cit	αKG	Phos	Dicarb	Succ/Fum	Oxalo	Asp/Glu	Pyr	FAD	Glu	BCK	ATP-P_i
Purification	+	+	+	+	+	+	+	+	+	±	−	−	−	−
Functional reconstitution	+	+	+	+	+	+	+	+	+	+	−	−	+	−
Kinetic analysis	+	+	+	+	+	+	±	±	+	±	−	±	+	±
Identification of gene or open reading frame	+	+	+	+	+	+	+	+	−	−	+	−	−	−
Topology	±	±	±	±	±	−	−	−	−	−	−	−	−	−
Oligomeric state	+	+	+	+	+	−	−	−	−	−	−	−	−	−
Overexpression	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Site-directed mutagenesis	±	±	±	±	±	−	−	−	−	−	−	−	−	−
Human chromosomal location	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Crystallization	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] The following abbreviations are used for individual anion transporters: AAT, ADP/ATP; UCP, uncoupling protein; Cit, citrate; αKG, α-ketoglutarate; Phos, phosphate; Dicarb, dicarboxylate; Succ/Fum, succinate/fumarate; Oxalo, oxaloacetate; Asp/Glu, aspartate/glutamate; Pyr, pyruvate; FAD, flavin adenine dinucleotide; Glu, glutamate; BCK, branched chain α-keto acid; and ATP-Pi, ATP-Mg/Phosphate. **Note:** A positive sign (+) denotes that this aspect of the problem either has been completed or that much work has been done. A negative sign (−) indicates that little or no significant progress in this area has been published. A (±) sign indicates that significant progress has been made, but that additional work is in order.

from diverse organisms has been compiled and analyzed by D.R. Nelson and can be accessed at the following internet address: *http://drnelson.utmem.edu/homepage.html*. Finally, it should be noted that it is likely that mitochondria contain other anion transporters in addition to those listed in Table 1. In fact, experimental evidence has been obtained indicating the presence of uptake systems for coenzyme A (Tahiliani & Neely, 1987), thiamine pyrophosphate (Barile, Passarella & Quagliariello, 1990) and N-acetylglutamate (Meijer et al., 1982). However, conclusive proof at the molecular level for the existence of discrete transport proteins catalyzing these activities has not yet been obtained, and thus while this remains an important area for future research,

these putative transport functions will not be further considered in this review.

Primary Structure

To date, the primary structure has been determined for the ADP/ATP (Aquila et al., 1982), phosphate (Runswick et al., 1987; Ferriera, Pratt & Pedersen, 1989), citrate (Kaplan, Mayor & Wood, 1993), dicarboxylate (Palmieri et al., 1996*b*; Kakhniashvili et al., 1997), α-ketoglutarate (Runswick et al., 1990), carnitine (Indiveri et al., 1997), ornithine (Palmieri et al., 1997*a*), FAD (Tzagoloff et al., 1996), succinate/fumarate (Palmieri et al. 1997*b*), and oxaloacetate (Palmieri et al., 1999) transporters, as well as the uncoupler protein (Aquila, Link & Klingenberg, 1985). It should be noted that although the carnitine and ornithine carriers are not "anion" carriers, they are included in this list for the sake of completeness. With many of these carriers their sequence has been determined from multiple sources, thereby enabling the development of a consensus sequence of residues conserved across species. These have been, and will continue to be, useful in focusing site-directed mutagenesis studies on the subset of residues essential and/or unique to a given carrier's function. In addition, in the yeast *Saccharomyces cerevisiae* a complete set of putative mitochondrial carrier sequences have been identified (Mayor et al., 1997; Moualij et al., 1997; Palmieri et al., 2000). Although, in many cases, proof that a given open reading frame does in fact encode a functional mitochondrial transporter still remains to be obtained.

Without exception, at the level of their primary structure, all of the mitochondrial metabolite carriers of known function display several common characteristics, despite the fact that the level of sequence identity between carriers of different function is rather limited (i.e., approx. 25%). The set of common characteristics include: (i) a mature transporter size of approximately 300 residues with a basic isoelectric point; (ii) the presence of three homologous 100 amino acid domains (as revealed by dot matrix analysis) that show homology not only with respect to each other, but also with respect to all the domains present within other mitochondrial carriers; (iii) a signature sequence motif consisting of Pro-X-(Asp/Glu)-X-(Val/Ile/Ala/Met)-(Lys/Arg)-X-(Arg/Lys/Gln/Ala)-(Leu/Met/Phe/Ile) which usually repeats two-three times; and (iv) the presence of 6 putative membrane-spanning domains (as revealed by hydropathy analysis). Based on these sequence similarities, it is thought that the mitochondrial transporter superfamily is derived from a common ancestral gene that encoded approximately 100 amino acid residues which consisted of two membrane-spanning domains separated by a hydrophilic loop (Saraste & Walker, 1982; Aquila, Link & Klingenberg, 1987; Runswick et al., 1987; Klingenberg, 1989; Walker, 1992; Walker & Runswick, 1993) . The tripartite structure likely arose via two tandem duplications of a common ancestral gene.

Topology

As mentioned above, based on the recognized tripartite structure and hydropathy analysis, it was proposed early on that each of the 100 residue domains contains two transmembrane α-helices, resulting in six helices per monomer (Saraste & Walker, 1982; Aquila, Link & Klingenberg, 1987; Runswick et al., 1987). The two helices within a given repeat element are connected by a longer, hydrophilic loop in comparison to the connections between the three repeating domains by shorter, hydrophilic loops. Figure 1 depicts a topology model for the mitochondrial citrate transport protein (Kaplan et al., 2000). The general features of this anion carrier model are thought to characterize all of the mitochondrial transporters. However, it should be noted that the exact amino acid residue at a given position will vary from one carrier to another. Obtaining experimental validation of this general model has been the subject of active investigation. With the different carriers, most features of the model have been experimentally verified using a variety of membrane impermeable reagents including peptide-specific antibodies, proteases, and chemical labeling agents. For example, with the citrate carrier, immunological studies (Capobianco et al., 1995) indicate that the amino and carboxy termini are exposed to the cytoplasmic side of the inner membrane, thereby supporting an even number of transmembrane domains as predicted in the above model. With the UCP[1], Miroux et al. (1992, 1993) have employed a library of fusion proteins to select antibodies directed against short segments of the carrier. This has permitted extensive topology studies to be carried out. These studies enabled a determination of the orientation of the N-terminal extremities of TMDs I–IV. Prior studies had localized both the N- and C-terminal ends of TMDVI. In combination, this work provided experimental validation for the orientation and delineation of the six TMDs depicted in Fig. 1. With the α-ketoglutarate carrier, the cytosolic exposure of the N- and C-termini, and loop *B* has been experimentally verified, whereas hydrophilic loops *A* and *C* protrude into the matrix (Bisaccia et al., 1994, 1995). Similarly, with the phosphate carrier, the exposure of the N- and C-termini, as well as loops *B* and *D* to the cytosolic side has been demonstrated, as has the exposure of loop *C* to the matrix (Ferriera, Pratt & Pedersen, 1990; Capobianco, Brandolin & Palmieri, 1991; Palmieri et al., 1992; Palmieri et al., 1993). Finally, with the ADP/ATP transporter considerable experimental evidence has been obtained, utilizing a battery of approaches, in support of the above model (*see* Klingenberg (1993) for a detailed review of

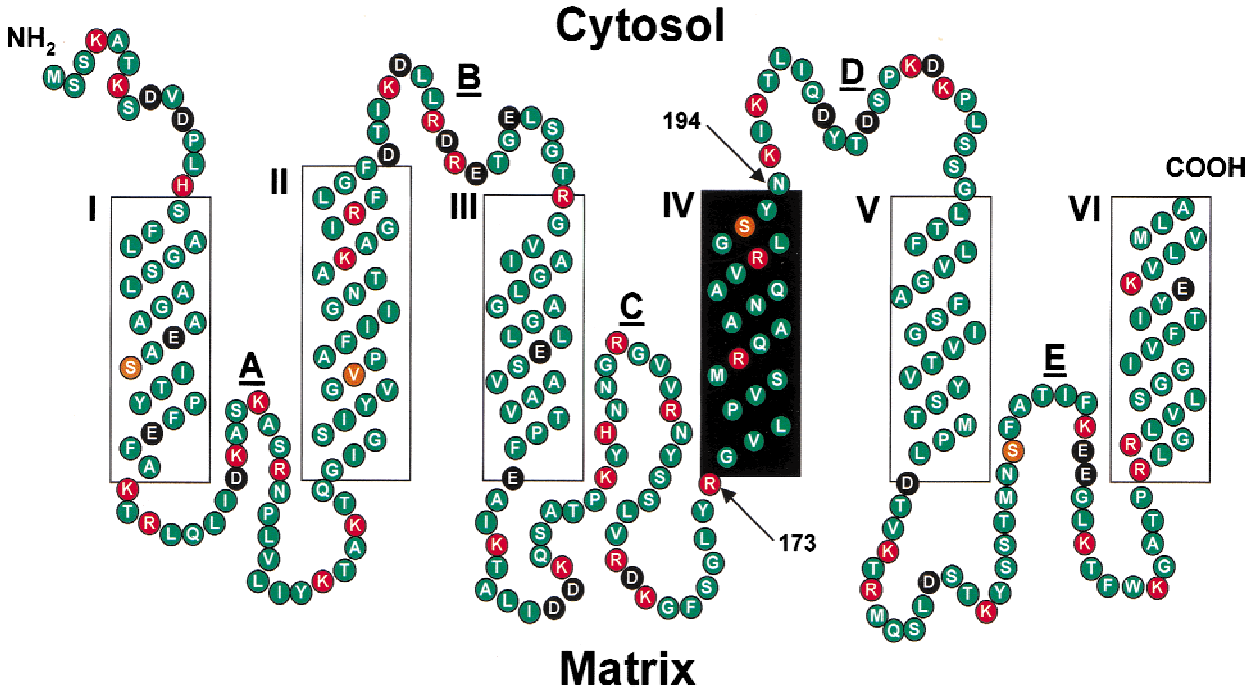

Fig. 1. Proposed transmembrane topography of the cys-less yeast mitochondrial citrate transport protein monomer. For the purpose of this review, the proposed model, which depicts the cys-less citrate transport protein, serves as a general model for the mitochondrial carrier superfamily. However, the exact residue at a given location will vary from one transporter to another. Six transmembrane α-helical domains are depicted (I–VI) which are connected by five hydrophilic loops (*A–E*). Whether these loops are external to, or instead partially penetrate the membrane remains an open question. Positively charged residues are denoted in red, negatively charged residues in black, and the wild-type cysteines, which are replaced with Ser or Val in the Cys-less CTP construct, in orange. TMD IV is denoted in black. Reproduced from Kaplan et al. (2000a) with permission from *J. Biol. Chem.*

these findings). In summary, the validity of the model depicted in Fig. 1 has been clearly demonstrated. However, it should be emphasized that with many of the TMDs the precise location of the membrane/aqueous interface remains in question.

Kinetic Mechanism

From the functional viewpoint, kinetic analyses carried out in either intact mitochondria and/or with purified mitochondrial transporters in reconstituted liposomal systems have indicated that the citrate (Bisaccia et al., 1993), α-ketoglutarate (Sluse, Sluse-Goffart & Duyckaerts, 1989; Palmieri et al., 1990; Indiveri et al., 1991; Palmieri et al., 1992), dicarboxylate (Indiveri et al., 1993), glutamate-aspartate (Dierks, Riemer & Kramer, 1988; Sluse et al., 1991), and possibly the ADP/ATP (Sluse, Sluse-Goffart & Duyckaerts, 1989) carriers all operate by an identical kinetic mechanism, namely, a sequential type mechanism including the formation of a ternary complex of two substrate molecules with the transport protein *prior to the translocation step.* Moreover, substrate binding occurs via a rapid-equilibrium, random mechanism. Thus, there is not an obligatory order in the binding of internal *vs.* external substrate. This kinetic mechanism has important structural consequences, in that two substrate binding sites must exist, on opposite sides of the membrane, and these sites must be exposed and occupied prior to the translocation event (Kramer & Palmieri, 1992). In principle, this could be accomplished by a dimeric structure with each monomer containing a separate binding site and transport pathway. In fact, our molecular modeling studies with the citrate transporter suggest such a structure (Kotaria et al., 1999). Finally, it should be noted that controversy exists with respect to the mechanism of the ADP/ATP transporter. Klingenberg contends that this carrier operates via a "single binding center gated pore" mechanism in which a single binding site is accessible during different steps in the transport cycle to one side of the membrane and then the other (Klingenberg, 1989; Klingenberg, 1992). This mechanism is consistent with a variety of inhibitor and substrate binding studies (for a review *see* Klingenberg, 1989). He has further proposed that a single translocation pathway is formed at the interface of two monomers (Klingenberg, 1981; 1992). Moreover, crosslinking studies conducted by Terada's group (Majima et al., 1995; Hashimoto et al., 1999) have been interpreted along the lines of the single binding center model (*see*

below). However, while this model is consistent with substantial data, it is inconsistent with the observed kinetics. Resolution of this controversy will require detailed structural information at the molecular level.

Dimeric Structure

An important issue related to the molecular mechanism(s) of this carrier family concerns the oligomeric state of functional mitochondrial transporters. As discussed above, both hydropathy analysis and considerable experimental evidence support the idea of 6 membrane-spanning domains per transporter monomer. However, numerous transporters from a variety of other membranous sources are somewhat larger and are thought to consist of 10–12 membrane-spanning domains in a single monomeric polypeptide chain (Maloney, 1990; Griffith et al., 1992). Moreover, it has been suggested that with other transporters, the monomer has a substructure such that it may in fact function as a dimer (Maloney, 1990). Thus, the concept that the mitochondrial carriers act as functional dimers, in effect containing 12 membrane-spanning domains per functional unit, is an appealing one that would enable a uniform structural principle to provide the physical basis for transporter function with a wide variety of transport proteins from diverse sources.

Thus, the question arises as to whether experimental evidence supports a dimeric state for the mitochondrial carriers. With several of the transporters, the evidence is clearly in favor of a functional dimer. For example, crosslinking studies conducted with the uncoupling protein (Klingenberg & Appel, 1989) and the α-ketoglutarate carrier (Bisaccia et al., 1996*b*), using the agent Cu^{2+}-phenanthroline, clearly indicate the formation of a dimer via the formation of a disulfide bond in both instances. Moreover, the extent of crosslinking was observed to be independent of carrier concentration and could be prevented by the presence of SDS, thus indicating that crosslinking occurred between two native monomers that preexisted in a dimeric state. With the uncoupler protein, additional evidence in favor of a functional dimer includes a nucleotide binding stoichiometry of one nucleotide per two monomers (Lin & Klingenberg, 1982), as well as hydrodynamic measurements carried out with the isolated protein (Lin, Hackenberg & Klingenberg, 1980). With the ADP/ATP transporter, hydrodynamic (Hackenberg & Klingenberg, 1980), crosslinking (Klingenberg et al., 1979), and inhibitor binding stoichiometry (Riccio, Aquila & Klingenberg, 1975; Klingenberg, Riccio & Aquila, 1978), all point to the translocase functioning as a dimer. Finally, with the citrate transporter, using both size-exclusion chromatography and charge-shift native polyacrylamide gel electrophoresis, Kotaria et al. (1999) have shown that following overexpression and solubilization, the yeast citrate carrier exists as a dimer. Interestingly, a cys-less variant of the citrate carrier also exists as a dimer, thereby indicating that disulfide bonds are not required for either the formation or stabilization of the dimeric state.

Channel-like Properties of Mitochondrial Anion Transporters

Defining characteristics of mitochondrial anion carrier function include high substrate specificity, high activation energy (which indicates substantial conformational change during the catalytic cycle), and a relatively slow turnover number (Dierks, Stappen & Kramer, 1994). In addition, with the antiporters, movement of one substrate is strictly coupled to the movement of the counter substrate. In contrast to these properties, evidence has now been obtained with several mitochondrial transporters indicating that under certain conditions they display channel-like function. For example, with the glutamate-aspartate carrier, covalent modification with certain cysteine-specific reagents results in the conversion of the strict antiport function to solute uniport (i.e., efflux) (Dierks et al., 1990; Dierks, Salentin & Kramer, 1990; Dierks, Stappen & Kramer, 1994). In this uniport mode, there is a dramatic increase in the K_m for internal substrate (>2,000-fold) and a marked decrease in the substrate specificity such that many different ions of different sizes can now be transported. Interestingly, the transporter displays a similar activation energy and first order rate constant. Thus, the modified carrier functions as a fairly unspecific channel which displays energetic and certain kinetic properties that are carrier-like. It is thought that the induced uniport function does not have a physiological role. However, it does provide insight into the molecular entities underlying transporter function. Thus it has been proposed that chemical modification has severely disrupted the gating function of the transporter, thereby knocking out the feature of substrate recognition (Dierks, Stappen & Kramer, 1994). However, the rate limiting conformation changes that likely occur within the channel during substrate passage are unchanged, and hence a constant activation energy was observed.

This antiport to uniport conversion which is induced by chemical modification and correlates with the appearance of certain channel-like functions has been observed in other mitochondrial carriers as well, including the ADP/ATP translocase (Dierks et al., 1990), the carnitine (Indiveri et al., 1992) and the phosphate (Schroers, Kramer & Wohlrab, 1997) carriers. Most interestingly, using site-directed mutagenesis, the residue modified within the phosphate carrier was identified as Cys-28 in the first transmembrane domain. Thus this residue is involved in an important gating function at the matrix side of the phosphate carrier. Related to this, it has been proposed that two functional domains exist within the mi-

tochondrial carriers (Dierks, Stappen & Kramer, 1994; Gonzalez-Barroso et al., 1997; Gonzalez-Barroso et al., 1999). A channel domain and a gating domain. The channel domain would consist of the transmembrane α-helices, and the gating domain would be comprised of the interconnecting hydrophilic loops. Moreover, the idea advocated by Schroers et al. (1997) and others (Nikaido & Saier, 1992; Kramer, 1994; DeFelice & Blakely, 1996) is particularly relevant, namely, that the mechanisms of coupling among antiporters, symporters, and uniporters, as well as differences between carrier and channel function likely reflect very subtle structural differences. Evidence with mitochondrial, as well as other carriers, increasingly indicates channel-like function. Thus, the distinction between "carrier" and "channel" mechanisms are likely to reflect minimal differences in structural elements.

Recent Structure/Function Studies With Individual Anion Transporters

At this point, we will now proceed with a detailed discussion of the results obtained and the conclusions derived from recent structure/function studies that have been conducted with several of the mitochondrial anion transporters.

ADP/ATP Transporter

The ADP/ATP transporter catalyzes an electrogenic, obligatory 1:1 exchange of ADP for ATP. This highly abundant inner membrane transporter is essential for ATP production and hence has been intensively studied (for detailed reviews *see* Klingenberg, 1989, 1992, 1993). It was the first carrier protein to be isolated in an intact state and the first for which a primary structure was established. The ADP/ATP transporter has been identified in numerous species with a total of 39 sequences having been identified including multiple isoforms. Early experiments identified two distinct conformations known as the c- and the m-states. In the c-state the substrate binding site faces the cytosol and in the m-state it faces the matrix. Recent developments have focused on the use of chemical crosslinking and site-directed mutagenesis in the quest to unravel its structure/function relationships.

A central issue with regard to the functioning of the ADP/ATP transporter involves the location and functional role of the three matrix-oriented hydrophilic loops *A*, *C*, and *E* (*see* Fig. 1). Terada's group has carried out an elegant series of studies (Majima et al., 1993, 1995, 1998; Hashimoto et al., 1999) aimed at addressing this issue by examining both the ability of a series of crosslinking agents to crosslink endogenous Cys within the transporter, and the effect of substrate and inhibitors on the formation of such crosslinks. Their results can be summarized as follows: (i) each monomer in the homodimeric form of the transporter (i.e., the functional form) contains one hydrophilic loop A (within which Cys56 resides) that protrudes into the matrix when the carrier is in the m-state. As part of the homodimeric state of the transporter these two loops can be crosslinked by a variety of agents added at the matrix side, and therefore these loops are accessible from the matrix side in the m-state. However, when the carrier is in the c-state conformation these loops are not crosslinked by any of the reagents tested, thereby suggesting that the two Cys are no longer accessible to reagents added from the matrix side but rather now protrude into the membrane; (ii) since crosslinking of the pair of A loops when the carrier is in the m-state conformation causes an inhibition of transport, it is concluded that fluctuation of the A loops is likely to be decisively important in transporter function, and that crosslinking serves to lock the carrier in the m-state conformation thereby prohibiting its conversion to the c-state; (iii) the inability to form crosslinks involving loops *C* or *E* (even though both contain Cys residues) suggests that these loops are inaccessible to the agents tested, most likely because rather than project into the matrix they in fact intrude into the membrane; and (iv) based on the findings that eosin-5-maleimide, an SH-reactive fluorescein derivative, specifically labels Cys159 in loop *C*, and that eosin Y, a non-SH reactive analogue of the above compound, inhibits ADP transport by binding to the same Cys in submitochondrial particles and can be displaced by transporter substrates, it has been concluded that loop *C* represents the primary binding domain within the translocase. Based on the above findings, Hashimoto et al. (1999) have proposed that a pair of *A* loops (which are quite positively charged) may function as the transporter gate which fluctuates widely from residing a very short distance from each other to a distance of separation of at least 17Å. The movement of this pair of *A* loops from the matrix into the membrane may serve to attract negatively charged substrates to the primary substrate binding domain within loop *C*. They propose that such a cooperative swinging loop model accounts for the mechanism of the translocase. Finally, it should be noted that the model developed by these authors postulates a single transport pathway per dimer and is consistent with the single binding center-gated pore mechanism discussed earlier.

Extensive site-directed mutagenesis, carried out by Klingenberg's and Nelson's groups, has revealed important information regarding the roles of individual residues in ADP/ATP transporter function. Their investigations have focused on 5 types of residues. **The first group** consisted of Cys residues within the translocase. Despite earlier literature suggesting the presence of an

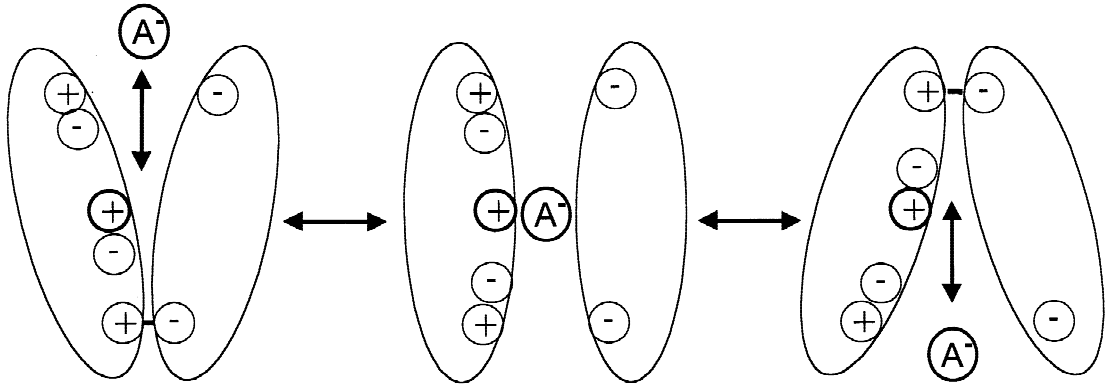

Fig. 2. The charge-oscillating model for gating of the ADP/ATP transporter. This figure was constructed based on the model presented by Klingenberg (1992). A^- denotes the anionic substrate.

essential Cys (Hoffmann et al., 1994), they found that following mutation of each endogenous Cys to Ser, one at a time, substantial reconstituted transporter function was retained (Klingenberg & Nelson, 1994). **A second group** of mutations focused on arginine residues located within the α-helical TMDs (Heidkamper et al., 1996). R96A (TMDII) and R204L (TMDIV) mutations totally suppressed translocase function. In contrast, an R294A mutation (TMDVI) was well tolerated. Interestingly, with regard to R96, other mitochondrial anion transporters contain arginines in homologous positions, suggesting that this site plays an essential role in the functioning of this family of transporters, rather than a role that is specific to the translocase. **A third group** of mutations focused on the R252, 253, 254 triplet which is located within matrix loop *E* (Heidkamper et al., 1996). Translocase function is largely abolished (greater than 95%) by mutation at these sites. Interestingly, with all of these arginine mutations, ATP transport is more drastically inhibited than is ADP transport. This finding suggests that these residues may interact (either directly or indirectly) with the negative charge on the substrate. **The fourth group** consisted of lysine and arginine residues located in either the transmembrane or the matrix loop domains (Muller et al., 1997). Thus, mutation of K38, a TMDI residue which is conserved among AACs, but not among other mitochondrial carriers, to Ala, substantially, but not completely, inactivated the translocase. Residues K48 and R152 are part of a motif (+×+) which occurs at a similar location in each of the three repeat matrix loops (i.e., loops *A, C,* and *E* in Fig. 1). The K48A mutation caused nearly complete suppression of transport, whereas the R152A mutation resulted in normal ADP/ADP exchange, but drastic reduction in exchange modes involving external ATP. These results, together with findings mentioned above for the R252-254 matrix triad (i.e., +++) point to the importance of this recurring motif in translocase function. Finally, mutation of residues K179 and K182 located in matrix loop C had little effect on function. **The fifth group** of residues studied consisted of the helix terminating acidic residue triad, E45, D149, and D249. These residues are thought to terminate TMDs I, III, and V, respectively and reside at the membrane/matrix compartment interface. Upon mutation of E45 to G a partial inhibition of transporter function resulted, whereas D149S and D249S mutations rendered the translocase inactive.

The above findings lend support to an earlier model proposed by Klingenberg (1992) to account for gating and substrate binding in the AAC. This model is depicted in schematic form in Fig. 2. The transporter gates consist of ion pairs that may form and result in closure of a given gate across the channel. Negative residues on both sides of the binding center are postulated to be mobile such that they can either pair with the positive residue of the binding center or the more external positive residues present in either gate. In the absence of substrate the central positive charge of the binding center is free and will pair with either the internal or external mobile negative charge. By neutralizing one mobile negative charge the binding center residue causes release of one positive charge at a gate, which can now form an ionic bond across the channel resulting in gate closure. Concomitantly, the opposite gate is opened since its positive residue is neutralized by the mobile negative charge. Thus in the absence of substrate, one gate is closed and the other is open. Upon substrate binding, the central positive residue is sequestered and the released mobile negative charge moves toward the outside and pairs with the positive charge of the gate. Therefore, substrate binding is propagated to the gates by the two mobile negative residues such that in the transition state both gates are open. A mobile negative charge then displaces the substrate from the binding center which enables release of the substrate and closing of the near-side gate. It is further proposed that positively charged clusters seen in loops *A, C,* and *E* serve to steer negatively charged substrate into the channel. Thus, there likely

exists an annular positive field around the channel entrances. These positive charges likely interact either with substrate or negatively charged amino acid side chains. Also, rings of negative and positive charge may alternate and participate in the propagation of the anion through the protein. While this gating model was initially conceived as a feature of the single binding center-gated pore mechanism, it is conceivable that it could also account for gating in a sequential type mechanism in which a dimeric carrier contains two monomers each with one substrate binding site and one transport channel.

Finally, Nelson's group (Nelson, Felix & Swanson, 1998) has utilized a regain-of-function approach to study charge pair interactions within the translocase. With this strategy mutants of the ADP/ATP transporter which could not grow on glycerol/ethanol were selected for spontaneous revertants. Thus, upon construction of an R294A (inactivating) mutant, a revertant mutation in E45 to either Gly or Gln resulted in a regain of function, thereby suggesting that a charge-charge interaction occurs between R294 and E45. Utilizing this strategy, data for the following additional charge pair interactions were obtained: K38-E45, R152-E45, R152-D149, and D149-R252 (note: in all cases the parent (inactivating) mutation is listed followed by the revertant (reactivating) mutation). These observations indicate that charge-pairs exist both within the 100 amino acid domains as well as between domains. This has led to the proposal that a concerted shift from inter- to intradomain charge-pairs may be a part of the transport cycle. The fact that certain sites are involved in more than one interaction stresses the dynamic nature of these interactions. Finally, it is noteworthy that these charge-pair interactions occur among residues that are part of the mitochondrial carrier defining sequence motif PX(−)XX(+) and thus are likely to play a fundamental role in the transport mechanism of all the mitochondrial anion carriers.

It has been observed that under certain conditions, the translocase can display channel-like properties (Brustovetsky & Klingenberg, 1996; Ruck et al., 1998). Thus patch-clamp experiments performed with isolated, reconstituted translocase yield single-channel measurements indicating the presence of a large slightly cation-selective channel with a mean channel conductance of 300–600 pS. The channel was usually in the open state with little current fluctuation up to the holding voltage of 80–100 mV. Ca^{2+} induced the transporter to form the high conductance channel. The large conductance suggests that the channel is relatively wide. It has been proposed that the Ca^{2+} mediates this channel-forming effect by binding to cardiolipin and effectively competing with and displacing transporter lysine residues (Brustovetsky & Klingenberg, 1996). The displaced positively charged side chains might trigger an intramolecular repulsion which then opens the gates of the preformed transport pathway. It has been further proposed that the ADP/ATP transporter may comprise the key channel-forming unit within the mitochondrial permeability transition pore. In summary, the ADP/ATP transporter appears to be able to adopt two conformations that cause it to function either as the specific ADP/ATP obligatory exchanger, or as an unspecific channel. For issues related to this, see earlier discussion.

The Uncoupling Protein

The uncoupling protein (UCP1) from brown adipose tissue mitochondria transports H^+ from the cytosol into the matrix, thereby dissipating redox energy and generating heat. UCP1 has been included in this review because: (i) its primary structure clearly places it within the mitochondrial anion transporter family (e.g., its signature sequence motif, triplicate repeat structure, 6 predicted membrane-spanning domains), and (ii) it transports both protons as well as a variety of monovalent anions including halides, nitrate, oxohalogenides, pyruvate, alkylsulfonates, and fatty acid anions (for detailed recent reviews, refer to Klingenberg & Huang, 1999; Ricquier & Bouillaud, 2000).

To date, three UCP isoforms have been discovered. UCP1 is restricted to brown fat mitochondria and provides a source of heat via dissipation of the proton gradient. UCP2 displays an amino acid sequence that is 59% identical with UCP1, and is expressed in all tissues. UCP3 is 54% identical to UCP1 and is expressed primarily in skeletal muscle, as well as in brown adipose tissue in certain species. Upon overexpression and functional reconstitution in a liposomal system, UCPs 2 and 3 catalyze an electrophoretic flux of protons and alkylsulfonates, and the proton flux exhibits an obligatory requirement for fatty acids. Thus, all three UCP isoforms display nearly identical functional properties, suggesting that each isoform may have a role in energy dissipation.

Despite considerable study, disagreement remains concerning the molecular mechanism of the UCP. Two rather different mechanisms have been proposed. **Mechanism 1,** advocated by Klingenberg (and discussed in detail in Klingenberg & Huang, 1999), posits that UCP1 transports protons with fatty acids providing an essential carboxyl group which makes proton transport possible and/or more efficient. The proton pathway through the membrane consists of a combination of fatty acid carboxyl groups (primarily near the cytosolic surface of the membrane) and proton donor/acceptor amino acid residues within UCP1. **Mechanism 2,** proposed by Garlid et al. (1996), involves a cycling of fatty acids in the membrane, with UCP1 ensuring transport of the anionic form of fatty acids. In this fatty acid protonophoretic model, depicted schematically in Fig. 3, UCP1 does

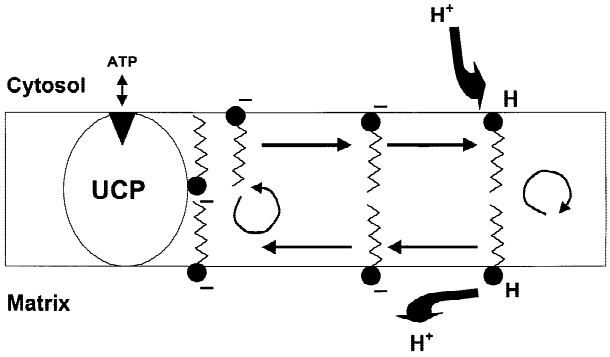

Fig. 3. Proposed fatty acid protonophoretic mechanism of the uncoupling protein. This figure was constructed based on the model presented by Garlid et al. (1996).

not transport H^+, but rather transports fatty acid anions. Thus, the fatty acid anion partitions into the lipid bilayer (from the matrix side), diffuses laterally to reach the UCP, and binds most likely to the outer surface of the UCP. The fatty acid substrate is then transported across the membrane via a flip-flop mechanism. It subsequently diffuses away from the UCP, is protonated, and rapidly flip-flops again across the bilayer (i.e., towards the matrix side) as the undissociated fatty acid. It then releases its proton to the mitochondrial matrix thereby completing the transport cycle. Consequently, H^+ is transported in the opposite direction to the fatty acid anion. While a number of experiments using a variety of approaches have been performed which support each of the two models (*see* previously cited reviews for additional detail), to date neither model has been proven unequivocally.

Site-directed mutagenesis has provided important insight into UCP functioning. One issue that mutagenesis experiments have sought to clarify involves the molecular basis for the well-documented inhibition of UCP function by purine nucleotides. Thus, Bouillaud et al. (1994) showed that deletion of amino acids 267–269, which are located in hydrophilic matrix loop *E* (Fig. 1), abolished nucleotide inhibition of the UCP. Gonzalez-Barroso et al. (1997) subsequently showed that enlarging the deletion to include residues 261–269, converts the UCP into a nonspecific pore that facilitates the permeation of molecules of up to 1 kDa in size. They then demonstrated that construction of the analogous deletion mutants in matrix loops *A* and *C* caused a similar loss in nucleotide regulation (Gonzalez-Barroso et al., 1999). They concluded that the mutational data support the involvement of all three matrix loops in a gating function. It was further proposed that the matrix loops form a hydrophobic pocket that accommodates the purine ring of the nucleotide. Finally, they suggested that the tripartite structure seen at the level of the amino acid sequence is reflected in the functional organization of the UCP.

Garlid and coworkers demonstrated that the three Arg residues, located in TMDs II, IV, and VI, are essential for nucleotide inhibition, thereby implying that these three helices are involved in forming portions of the nucleotide binding site (Modriansky et al., 1997). They propose that these helical domains form the walls of an aqueous pocket for nucleotides, which enter from the cytosolic side and interact with matrix loop *E*. Klingenberg's group has identified Glu190 (TMDIV) and His2l4 (located at the TMDV/cytosolic interface), as the pH sensors for the nucleotide binding site, which are proposed to function by controlling access of the phosphate moiety to its binding cleft (Echtay, Bienengraeber & Klingenberg, 1997; Echtay et al., 1998). Based on the totality of the above findings, a potential mechanism was elaborated (Huang & Klingenberg, 1996) and extended (Modriansky et al., 1997) to explain the nucleotide inhibition. It consists of the following steps. First, the nucleotide enters an aqueous pocket within the UCP such that the sugar-base moiety binds to matrix loop *E* and the nucleotide β-phosphate binds to Arg182. Concomitantly, Glu190 may bind to Arg83 to form a salt bridge. The sum of these interactions represents an initial loose conformation. Upon protonation of Glu190, Arg83 is freed and made available to bind to the second charge on the β-phosphate of diphosphates and the γ-phosphate of triphosphates giving rise to a tight-binding conformation. The nucleotide is now correctly positioned such that the α-phosphate can bind to Arg276, thereby inducing a final conformational change that occludes an internal fatty acid binding site and results in an inhibition of transport.

Finally, mutagenesis experiments have also aimed at identifying a proton transport pathway through the UCP. Thus mutation of His145 and His147, located in hydrophilic matrix loop *C*, to neutral residues, causes a substantial reduction in H^+ transport (Bienengraeber, Echtay & Klingenberg, 1998). This led to the proposal that these residues may be located at the matrix-side entrance to the H^+ translocation channel in UCP1. Garlid et al. (1999) have argued that these findings are equally supportive of the fatty acid protonophore model wherein the histidines in UCP1 form part of the surface binding site in the fatty acid anion transport pathway. Asp27, located in TMDI, and conserved in all UCPs is another potential proton donor/acceptor. Mutation of this residue to Asn reduced proton transport by 80% (Klingenberg & Huang, 1999).

This section will be concluded with a note on the role of the UCPs in physiology. Since this topic has recently been extensively reviewed (Ricquier & Bouillaud, 2000) it will be mentioned only briefly. It has been clearly established, utilizing a variety of experimental approaches including the use of knockout experiments, that the role of UCP1 is to maintain body temperature via induction of respiratory uncoupling and thermogenesis in

brown fat mitochondria. In contrast, the physiological roles of UCP2 and UCP3 are less well established. Suffice to say that they do not seem to be involved in adaptation to cold. Increased expression of UCP2 and UCP3 genes in response to a number of thermogenic hormones and numerous other factors suggest a possible role for these UCPs in thermogenic mechanisms of fever, and perhaps in temperature regulation in specific parts of the body. Moreover, UCP2 and UCP3 may play a role in resting energy expenditure. However, these associations are speculative and this is currently an area of active investigation.

The Citrate Transport Protein

The citrate transport protein (CTP) catalyzes an electroneutral exchange across the inner membrane of a tricarboxylate (i.e., citrate, isocitrate, *cis*-aconitate) plus a proton for either another tricarboxylate-H^+, a dicarboxylate (i.e., malate, succinate), or phosphoenolpyruvate. In addition to playing a central role in intermediary metabolism (*see* Table 1), altered CTP function is characteristic of certain disease states. For example, CTP function is decreased in type 1 diabetes (Kaplan, Oliveira & Wilson, 1990*b*) in a manner that can be corrected via administration of exogenous insulin (Kaplan et al., 1991). Moreover, the human CTP gene maps within a region on chromosome 22q11 associated with allelic losses in several clinical syndromes including DiGeorge syndrome, velo-cardio-facial syndrome, and a subtype of schizophrenia (Stoffel et al., 1996). This finding suggests that the CTP may be involved either in the biological etiology of these syndromes, or perhaps may play a modifying role (Goldmuntz et al., 1996).

At the molecular level, initial studies focused on the purification (Bisaccia, De Palma & Palmieri, 1989; Kaplan et al., 1990*a*) and kinetic characterization (Bisaccia et al., 1993) of the reconstituted, purified carrier. Shortly thereafter, a full-length cDNA encoding the mature rat liver mitochondrial CTP was cloned (Kaplan, Mayor & Wood, 1993). Subsequently, cDNA sequences encoding the CTP have been isolated and sequenced from a variety of sources. The CTP amino acid sequence indicates that the carrier displays the structural properties characteristic of the other carriers in this family. Recently, we identified the yeast homologue of the higher eukaryotic protein via the overexpression, reconstitution approach (Kaplan et al., 1995). An advantage afforded by the yeast CTP (in contrast to the rat liver CTP (Xu et al., 1995) is that following overexpression and purification, its function can be reconstituted in a liposomal system with high specific activity. Thus, the yeast CTP represents ideal material for a comprehensive structure/function analysis.

To this end, my laboratory has embarked upon an extensive program of site-directed mutagenesis in combination with biochemical and biophysical approaches in order to molecularly dissect CTP function. Initial studies focused on the construction of a cys-less CTP (Xu et al., 2000). The wild-type yeast mitochondrial CTP contains 4 endogenous cysteines. Sequential replacement of each Cys with Ser (or in the case of Cys-73 with Val) yielded a Cys-less CTP (i.e., a C28S/C73V/C192S/C256S) that displays a K_m, V_{max}, and substrate specificity that closely resemble the wild-type values. These results therefore validate the use of the Cys-less CTP for structure/function studies, the conclusions of which should be directly applicable to the wild-type protein.

With the Cys-less CTP in hand, we then utilized a Cys-scanning mutagenesis approach both to identify essential residues, as well as to probe the environment of a given replacement Cys. Our initial studies have focused on TMDIV. This TMD was of particular interest because of the presence of two arginine residues (i.e., Arg181 and Arg189) which, if one assumes an α-helical conformation for this domain, would reside on the same face of the helix with a distance of separation that would enable pairing with two of the negative charges on citrate, thereby effectively neutralizing the transported substrate. Accordingly, each of 22 consecutive residues (i.e., residues 173–194) were replaced one at a time, with cysteine. The single-cys variants were overexpressed in *E. coli*, isolated, and functionally reconstituted in a liposomal system. We obtained significant activity with all single cys variants, with the exception of the R181C and R189C mutants which were totally inactive. These results therefore indicated that with the exception of the R181C and R189C mutants: (i) other residues within TMDIV are not essential for CTP function; and (ii) CTP overall structural and functional integrity has been preserved with this panel of single Cys mutants.

The role of Arg181 and Arg189 in CTP function was further probed. We observed that upon replacement of either Arg with Lys, there was partial retention of CTP function. Interestingly, replacement of either Arg with Cys, followed by chemical modification of the Cys with MTSEA, a reagent that imparts positive charge to its site of attachment, also partially rescued function. Other MTS derivatives did not. In combination, the above findings indicate that while positive charge at positions 181 and 189 is essential for CTP function, the chemistry of the guanidinium residue is not. Moreover, the precise three-dimensional orientation of the positively charged side-chain and/or its bulk is important.

We have recently applied a strategy (Kaplan et al., 2000*a*), that had previously proven successful in deciphering structure/function relationships in channel proteins (Akabas et al., 1992; Akabas et al., 1994; Kurz et al., 1995), to our studies of the CTP. This approach involves determining the accessibility of engineered

single-cys residues to methanethiosulfonate (MTS) reagents. In particular we made use of the fact that: (i) two of these reagents (i.e., MTSET and MTSES) are extremely hydrophilic and have clearly been shown by ourselves (Kaplan et al., 2000*a*) and others (Holmgren et al., 1996) to be impermeable to a lipid bilayer in the absence of a transport or channel protein; and (ii) these reagents are similar in size to citrate. Thus, within a TMD, only those residues that are located at a water-accessible surface of the protein will rapidly react with these agents. Within the CTP, we contend that such residues are likely to comprise a portion of the citrate translocation pathway. Accordingly, we determined the pseudo first order rate constant for inhibition of citrate transport by reaction of each of three separate MTS reagents with our panel of single Cys mutants. The data are depicted in Fig. 4 and clearly indicate several points. **First,** the pseudo first order rate constants for transport inhibition differ by over seven orders of magnitude depending upon residue position. **Second,** for the stretch of residues 177–193, a periodicity in the accessibility to the MTS reagents of approximately 4 is observed, thereby providing the first evidence supporting an α-helical secondary structure for this domain. In fact, to our knowledge, this is the first experimental evidence obtained with any of the anion carriers, that a TMD is in fact α-helical. **Third,** based on the assumption that the engineered Cys residues occupy similar positions as do the wild-type side chains, the rate constant data indicate a highly accessible face of the helix which consists of residues Pro-177, Gln-182, Asn-185, Gln-186, Leu-190, and Tyr-193. We contend that this water-accessible face represents a portion of the citrate translocation pathway. In contrast, a highly inaccessible face is defined which consists of: Ser-179, Met-180, Ala-183, Ala-184, Ala-187, Val-188, and Ser-192 and likely faces the lipid bilayer. **Fourth,** superimposed on the α-helical periodicity, there exists a gradient of decreasing accessibility from the near side to the far side of the membrane. Figure 5 depicts a computer model of TMDIV based on the MTS reactivity data. In summary, I believe that this is a very powerful approach that will enable the mapping of the CTP translocation pathway in detail.

Another approach we have recently utilized to complement and extend the MTS reactivity data involves the use of EPR to probe the environment of engineered single Cys residues (Kaplan et al., 2000*b*). This strategy has proven quite successful in deciphering structure/function relationships in other membrane proteins such as the lac permease (Kaback & Wu, 1997) and bacteriorhodopsin (Altenbach et al., 1990). Following attachment of a spin label to each engineered Cys, analysis of the lineshapes in the spectra of a given mutant in combination with a determination of the accessibility of the attached spin label to hydrophobic (i.e., O_2) and hydrophilic (i.e., NiEDDA) molecules has yielded additional information regarding the secondary structure and topography of TMDIV. Briefly, the EPR data confirm the existence of a periodicity in TMDIV consistent with an α-helical structure and allow extension of this structure to residue 173. Moreover, spin label at positions 175, 176, 179, 180, 183, 184, 187, 188, 191, and 192 exhibits both high accessibility to molecular O_2 and intermediate-rapid motion, and thus likely face the lipid bilayer. Spectra at residues 173, 174, 177, 178, 181, 182, 185, 186, 189, 190, 193, and 194 indicate reduced accessibility to O_2 and/or high accessibility to NiEDDA. In addition, the spectra of many of these residues suggest extensive tertiary contact with other portions of the protein. Thus, the data are consistent with a highly structured aqueous translocation pathway. Interestingly, residues 182–189 demonstrate a reduced accessibility to NiEDDA, suggesting a physical barrier to diffusion of the probe through the pathway during the EPR time scale (i.e., EPR is measuring collision frequencies on a microsecond time scale). We believe it likely that via the EPR approach we will be able to probe the CTP gating mechanism.

Finally, a combination of hydropathy analysis, the MTS reactivity data, the EPR data, and in depth computer modeling (Kotaria et al., 1999) has permitted the construction of the first detailed three-dimensional structural models of the TMDs for any member of the mitochondrial carrier protein family. These will serve as a guide for the generation of new site-specific mutants in order to probe the CTP structure/function relationships via the above approaches.

The α-Ketoglutarate Transporter

The α-ketoglutarate transporter catalyzes an electroneutral obligatory exchange of α-ketoglutarate for malate or other dicarboxylates across the inner membrane. It was the first of the mitochondrial transporters to be overexpressed in *E. coli* and subsequently refolded to a reconstitutively active form (Fiermonte, Walker & Palmieri, 1993). Its sequence is now known in seven species. With regard to its structure/function relationships, recent research has focused primarily on its oligomeric structure and the role of Cys residues in this structure, and secondarily on the use of site-directed mutagenesis to probe the roles of individual amino acid residues in the transport mechanism.

In an elegant study, Bisaccia et al. (1996*b*) utilized a crosslinking approach to investigate the oligomeric state of the α-ketoglutarate transporter. They demonstrated that reagents such as Cu^{2+}-phenanthroline and diamide were able to crosslink the transporter to dimers. The extent of crosslinking was increased 4–5-fold by inclusion of certain solvents such as acetone and isopropanol.

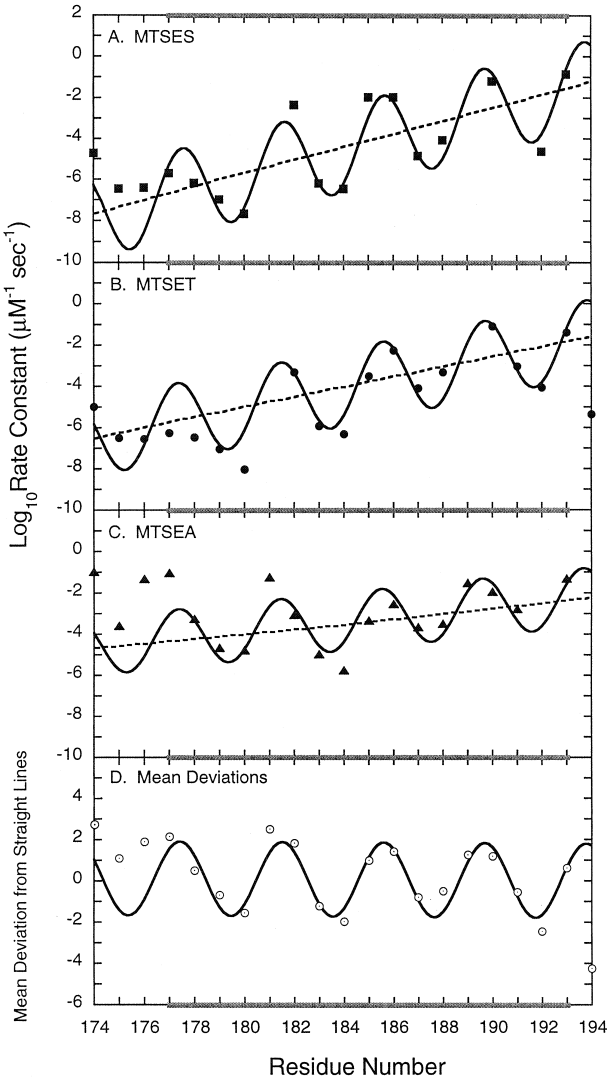

Fig. 4. Rate constants for inactivation of citrate transport by MTS reagents vs. location of engineered cysteine. Time course data for inactivation (activation) of a given single-Cys CTP variant by the MTS reagents were fitted to a simple exponential function by unweighted Marquardt nonlinear least squares: $r_t = (r_0 - r_\infty)\cdot e^{-m.c.t.} + r_\infty$ where r_t is the observed activity remaining at time t (seconds), r_0 is the initial activity, r_∞ is the asymptotic activity at $t = \infty$, c is reagent concentration (μM^{-1}) and m is the rate constant for inactivation ($\mu M^{-1}sec^{-1}$). Estimates of m (which range over 7 orders of magnitude) are reliable as judged from replicate experiments producing standard errors less than 20% of each estimate. The rate constant for inactivation, m, can be interpreted as a measure of a residue's accessibility to the MTS reagent. The board range of estimates of m dictated that further analyses be confined to Log_{10} transformed values. For each data set (MTSES, MTSET and MTSEA) a Fourier analysis, as implemented in Mathematica, revealed a regular periodicity of 4 between residues 177 and 193 with a noticeable loss of signal when flanking sites were included. Therefore, further analyses were restricted to residues 177 through 193. For each MTS reagent, estimates of Log_{10} m were fitted to the periodic function $Log_{10}\ m = sn - i + a\cdot Sin((n - x)\cdot 2\pi/p)$ using unweighted Marquardt nonlinear least squares. The magnitude of the rate constant for inactivation (Log_{10} m) is a function of position (residue number, n) with the straight line ($(sn - i)$) describing the slope (s) and intercept (i) of the overall trend in a data set and the trigonometric function ($a\cdot Sin((n - x)\cdot 2\pi/p)$) describing the periodicity either side of the line (p is the number of residues per 2π radians, a is amplitude and x a constant setting the register with respect to position, n). Reproduced from Kaplan et al. (2000*a*) with permission from *J. Biol. Chem.*

Interestingly, reconstituted transport activity was increased 20–30% by pretreatment with these solvents, and this elevation was maintained after crosslinking. As much as 90% of the total protein could be crosslinked to the dimeric form. The extent of crosslinking was independent of the transporter concentration, thereby indicating that crosslinking occurred with preexisting dimers. Fragmentation of the dimer into peptides, followed by reduction with β-mercaptoethanol and amino terminal sequencing of the newly generated band, indicated that crosslinking arose via disulfide formation between Cys184 (TMDIV) in each monomer. Importantly, this result suggests that Cys184 within the two monomers are close to each other in the native dimer structure. Furthermore, they have shown that in the absence of reducing agents Cys184 is the only Cys labeled with sulfhydryl reagents, and that Cys221 and Cys224 are linked by a disulfide bridge (Bisaccia et al., 1996*a*). The latter finding is consistent with the prediction that the two Cys residues reside on the same face of the TMDV α-helix. Finally, the presence of substrate increased the binding of sulfhydryl reagent to the carrier, a result which is consistent with the observation that substrate binding causes a change in the tertiary structure of the transporter (Capobianco et al., 1996).

Site-directed mutagenesis has been used to probe the roles of individual residues in the functioning of the α-ketoglutarate transporter. A cys-less variant of this carrier has been constructed, which displays near native kinetics and affinity for substrate, again demonstrating that Cys residues are not essential for mitochondrial transporter function (Palmieri et al., 1996*a*). The roles of 4 intramembranous Arg residues have been probed. Mutation of any of the Arg to Leu resulted in the inactivation of transport. Interestingly, substitution of Lys for Arg at positions 90 and 288 in TMDs II and VI, respectively resulted in the retention of significant transport. In contrast, Lys did not support transport when substituted for Arg at positions 98 and 190 in TMDs II and IV, respectively. In combination, the data point to the requirement for *intramembranous* positive charge at several sites, a characteristic shared by several of the mitochondrial transporters. Moreover, the specific requirement for Arg at two positions suggests a rigid steric and/or charge distribution requirement at these positions. Finally, since the α-ketoglutarate transporter does not contain intramembranous negative charge, it is thought that the positive charges are not involved in helix stabilization, but rather in binding the substrate and/or the charged residues present in the interconnecting loops (Palmieri & van Ommen, 2000).

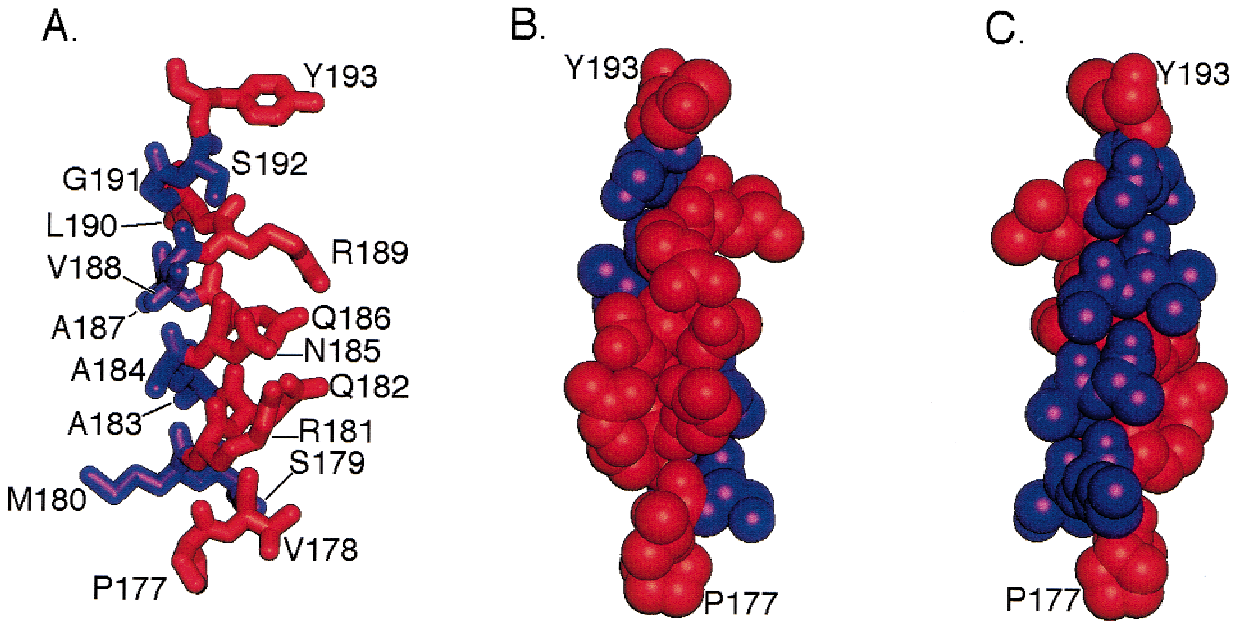

Fig. 5. Graphical representation of residues 177-193 within TMDIV of the CTP colored on the basis of reactivity toward the MTS reagents. Residues were colored based on the mean of the deviations from the straight lines as depicted in Fig. 4, Panel *D*. Residues with values less than zero are colored blue, while those with values greater than zero (i.e., more reactive and therefore more accessible) are colored red. The sequence was modeled initially as a standard α-helix, with N- and C-terminal residues capped as amides. The structure was lightly minimized using the CHARMm force field (Brooks et al., 1983) to allow adjustment of the backbone around Pro177. (*A*) Stick figure representation of the modeled helix, side view, with less reactive residues on the left and more reactive residues on the right. (*B*) Space-filling representation, showing the face of the helix containing the more reactive residues (i.e., water-accessible). (*C*) Space-filling representation, showing the face of the helix containing the less reactive residues (i.e., water-inaccessible). Reproduced from Kaplan et al. (2000*a*) with permission from *J. Biol. Chem.*

The Dicarboxylate Transport Protein

The dicarboxylate transport protein (DTP) catalyzes an electroneutral exchange across the inner membrane of a dicarboxylate for either inorganic phosphate or certain sulfur-containing compounds. Molecular studies on the DTP have focused primarily on identification and characterization of its gene in different species followed by overexpression and functional characterization of the corresponding protein product. Thus, several years ago the gene encoding the yeast mitochondrial DTP was identified for the first time via overexpression of its gene product in *E. coli* followed by functional reconstitution in liposomes (Palmieri et al., 1996*b*; Kakhniashvili et al., 1997). The overexpressed protein product displayed kinetic properties, substrate specificity, and inhibitor sensitivity that were quite similar to those found with the native transporter in isolated mitochondria. Moreover, the deduced amino acid sequence displayed the mitochondrial transporter signature motif, three homologous 100 amino acid domains, and six-predicted membrane-spanning regions. Thus, it was concluded that based on both structural and functional considerations, the yeast DTP is assignable to the mitochondrial carrier family. The ability to obtain high quantities of purified, functional DTP renders this carrier ripe for a detailed structure/function analysis, which so far has yet to be carried out.

In an important recent development, Fiermonte et al. (1998) used a rather novel extension of this approach in order to identify the mammalian homologues of the yeast DTP. The impediment that needed to be overcome was the fact that the sequence divergence between yeast and mammalian mitochondrial transporters is typically too great to permit accurate identification of the latter sequences. The solution consisted of identification of a DTP sequence from an organism of intermediate evolutionary distance. In this case, a *C. elegans* sequence was identified which was 37% identical to the yeast DTP. Following confirmation that this sequence was in fact a DTP (utilizing the overexpression and functional reconstitution approach), comparison of the confirmed *C. elegans* sequence with protein sequences encoded by mammalian expressed sequence tags permitted identification of the murine, rat and most recently the human DTP sequences (Fiermonte et al., 1998, 1999). The identity of the protein encoded by the rat open reading frame was confirmed via the overexpression approach. This strategy will likely have utility in identification of the transport functions encoded by other putative mammalian mitochondrial transporter genes.

A second noteworthy development involves the creation of a yeast mutant strain in which the DTP gene has been deleted (Palmieri et al., 1999*b*). Interestingly, the deletion strain failed to grow on ethanol or acetate, but was viable on other nonfermentable (pyruvate, lactate,

glycerol, and oxaloacetate) and fermentable (glucose, galactose) carbon sources. Moreover, growth on ethanol or acetate was restored by addition of low concentrations of aspartate, glutamate, fumarate, citrate, oxoglutarate, oxaloacetate, and glucose. These results have been interpreted to indicate that the main function of the DTP is an anaplerotic one, in which dicarboxylates (e.g., succinate) are shuttled from the cytoplasm into the mitochondrial matrix, rather than to direct carbon flux toward gluconeogenesis by exporting mitochondrial malate into the cytoplasm. Once in the matrix the dicarboxylates fuel the Krebs cycle thereby providing for the oxidation of acetyl-CoA produced from ethanol or acetate. The question as to whether the DTP is important for gluconeogenesis remains an open one, although Palmieri et al. (1999*b*) suggest that this may mainly be the role of the succinate-fumarate carrier. Finally, it should be noted that this knockout approach is likely to provide important information concerning the physiological roles assumed by the other mitochondrial anion transporters.

On another note, recent results suggest that the DTP may play an important role in detoxification. For example, it has been shown that in both rat kidney mitochondria, as well as following reconstitution of partially purified Triton X-114 extracts in liposomes, the dicarboxylate transporter (and to a lesser extent the α-ketoglutarate transporter) are able to catalyze the uptake of glutathione (Chen & Lash, 1998; Chen, Putt & Lash, 2000). Since glutathione is the primary intracellular nonprotein thiol, and plays an important role in detoxification, this finding imparts important physiological significance to the DTP. In addition, Masereeuw and coworkers (1996) have demonstrated an important role for the DTP in kidney cortex mitochondria. Organic anions that are actively secreted by the kidney accumulate in the proximal tubule. Within these cells, mitochondria represent one of the principal intracellular stores. These authors have shown that fluorescein, a fluorescent organic anion, as well as other organic anions that are substrates for the plasma membrane renal organic anion transport systems, are substrates for the mitochondrial dicarboxylate carrier. These findings likely have important clinical implications since nephrotoxicity as a consequence of the accumulation of a variety of organic anion drugs in the proximal tubule has been reported (Goldstein, 1993).

The Phosphate Transport Protein

The mitochondrial phosphate transport protein (PTP) is responsible for catalyzing a phosphate/proton cotransport from the cytosol into the mitochondrial matrix, thereby supplying sufficient substrate for oxidative phosphorylation. Gene sequences encoding the PTP have been identified from several species and at least two isoforms exist. The transporter has been overexpressed (Wohlrab & Briggs, 1994) and recent efforts have focused on the extensive use of site-directed mutagenesis to decipher its structure/function relationships. Thus Wohlrab and coworkers have focused on the roles played by acidic and basic residues residing either within the TMDs or near the membrane/aqueous compartment interface (Phelps et al., 1996; Briggs, Mincone & Wohlrab, 1999). Five residues have been found to be essential for transport. These include: His-32 (TMDI), Glu-126 and -137 (TMD III), Asp-39 and -236 (at the matrix ends of helices I and V, respectively). Their investigations have led to the proposal that Glu126, His32, and Glu137 may form a proton cotransport pathway that displays key elements that are similar to the proton transport pathway found in bacteriorhodopsin (Phelps et al., 1996). Furthermore, this proton transport pathway may be coupled, possibly via His-32 to the phosphate transport pathway. They have proposed a model in which two copies each of TMDs I and III form such a pathway in the homodimeric phosphate carrier structure. This model is further supported by their earlier finding that under oxidizing conditions, an intersubunit, transport-inhibitory disulfide bond is formed between Cys-28 in associated monomers (Phelps & Wohlrab, 1993). Finally, it is of interest to note that of the 5 residues that were identified as being critical for transport, three are either strictly conserved (Asp-236) or conservatively substituted (Asp-39 and Glu-137) in several other mitochondrial anion carriers, suggesting they assume a common structural and/or mechanistic role in multiple carriers.

Concomitant with their mutagenesis studies, Wohlrab and coworkers (Briggs et al., 1999) noticed that the efficiency of incorporation of the transporter into liposomes varied markedly depending upon the location of the mutation. Incorporation efficiencies ranged from a low of 1.5% to a high of 39.2%, with the wild-type value being approximately 16%. They propose that in their system, incorporation efficiency is a measure of dramatic global conformational change which may reflect alterations in the interactions at the interface between the subunits in the dimer. Moreover, they postulate that monomeric PTP is likely to display a lower incorporation efficiency, compared with the dimer. Assessment of the effect of numerous mutations on both reconstituted transport activity and liposomal incorporation efficiency has led to their proposal that TMDs I, IV, interhelical loop *C*, and the C-terminal domain beyond TMDVI are involved in formation of the subunit interface of the homodimer.

In an elegant series of studies, Schroers et al. (1998) have developed a protocol by which they can dissociate dimeric PTP into two monomers and then combine two differentially labeled PTP monomers in order to form defined homo- and heterodimeric PTPs. Utilizing this approach they have been able to demonstrate that mono-

meric PTP is nonfunctional, and that transport catalyzed by dimeric PTP involves functional crosstalk between the two monomers, such that inactivation of one monomer yields a nonfunctional dimer. The strategy of differentially mutating a residue in one monomer of the dimer has tremendous utility for studying the number, composition and dynamics of the translocation pathway(s) within the PTP. This approach should also prove extremely effective with other members of the mitochondrial anion transporter family.

Other Mitochondrial Anion Transporters

With respect to other identified anion transporters (e.g., the glutamate/aspartate, glutamate, pyruvate, ATP-Mg/phosphate, α-keto acid transporters), there has been little recent progress on their molecular characterization. Clearly this represents an area that is ripe for future investigation.

Identification of New Mitochondrial Carrier Activities

As mentioned above, several years ago a procedure was devised to overexpress the mitochondrial α-ketoglutarate carrier in *E. coli* (Fiermonte, Walker & Palmieri, 1993). The transporter localized into the inclusion body fraction and could then be solubilized with the zwitterionic detergent sarkosyl. The overexpressed transporter refolded to a native state as demonstrated by reconstitution of function in a liposomal system. This procedure was then used to overexpress the yeast mitochondrial phosphate carrier protein (Wohlrab & Briggs, 1994). Shortly thereafter, Kaplan et al. (1995) utilized this approach to identify, for the first time, the transport function encoded by a yeast open reading frame which based on the deduced amino acid sequence was thought to potentially encode a member of the mitochondrial carrier family. Thus, the yeast mitochondrial citrate transporter gene was identified via overexpression and functional reconstitution of its protein product. Shortly thereafter both the Kaplan (Kakhniashvili et al., 1997) and the Palmieri/Walker (Palmieri et al., 1996*b*) groups succeeded in identifying the yeast gene encoding the mitochondrial dicarboxylate carrier. This represented the first identification of the DTP gene and its primary sequence from any organism. Several points are noteworthy. First, the overexpression/functional reconstitution approach has now proven applicable to most, if not all members of the yeast mitochondrial transporter family (Mayor et al., 1997; Palmieri et al., 2000). Second, the power of the approach is that not only is the gene encoding a given transport function identified, but abundant quantities of transport protein are also produced, thereby overcoming the chief impediment to studying the carriers utilizing a variety of structure based approaches.

Recently, this strategy has been utilized to identify new mitochondrial carrier functions in yeast. For example, heretofore unknown mitochondrial succinate-fumarate and oxaloacetate (or alternatively sulfate) transporters have been identified (Palmieri et al., 1997*b*, 1999*a*). The former transporter catalyzes the exchange of cytosolic succinate for intramitochondrial fumarate and likely serves to connect the production of succinate by the glyoxylate cycle in the cytoplasm with the mitochondrial tricarboxylic acid cycle and electron transfer by complex II. The latter transporter appears to use the proton-motive force to drive the uptake of oxaloacetate (produced via cytosolic pyruvate carboxylase) into mitochondria. Similarly, via this approach, the protein encoded by the yeast ARG11 gene has been identified as the mitochondrial ornithine carrier (Palmieri et al., 1997*a*).

Finally, it should be noted that the Palmieri/Walker groups have been constructing knockout strains of yeast in which a given carrier gene has been deleted (for review *see* Palmieri et al., 2000). Examination of the growth characteristics of the resulting knockout strains is likely to shed important insight into the physiological importance of a given transporter's function.

Perspectives and Future Directions

During the last several years major progress has been made in mitochondrial carrier research. Thus, development of procedures to overexpress and reconstitute many of the anion transporters has enabled the: (i) initiation of comprehensive mutagenesis studies in order to understand the transport mechanisms involved; (ii) identification of the carrier function encoded by a given gene sequence thereby resulting in knowledge of the primary structure that corresponds to a known carrier function; (iii) identification of previously unknown carrier activities; and (iv) procurement of sufficient quantities of purified transporter to initiate a variety of structural studies.

During the next five years, I think tremendous progress in mitochondrial transporter research will continue and accelerate in two main areas. The first concerns the identification of the transport function encoded by a given gene. This will likely lead to the determination of a complete set of mitochondrial anion transporters. Based on the facts that: (i) the yeast genome contains 27–35 genes that putatively encode mitochondrial transporters and the complexity of the human genome suggests an even greater number; and (ii) mitochondria are known to catalyze an impressively diverse set of metabolic reactions that require the transport of many different types of anions across the inner membrane, we suggest the existence of many, as yet undiscovered, trans-

port functions within the inner membrane. Moreover, there are several identified transport activities of substantial importance (*see* Table I) for which the corresponding genes have not yet been identified and thus the primary structure of the protein remains unknown. In the view of this author, based on their metabolic importance, the pyruvate and glutamate/aspartate transporters are at the top of this list, and substantial effort should be put forth to fill this gap in knowledge.

The second area in which major breakthroughs are likely during the next several years concerns the continued elucidation of the structural basis underlying mitochondrial anion transporter function. It is the opinion of this author that the following approaches are likely to prove most successful. **First,** the use of the cys-scanning mutagenesis of TMDs in combination with a quantification of the ability of membrane impermeable MTS derivatives to inhibit transport should provide a definition of the residues that each TMD contributes to the transport pathway, as well as evidence regarding the secondary structure of a given TMD. **Second,** the use of cys-scanning mutagenesis in combination with electron paramagnetic resonance and/or disulfide crosslinking will permit a determination of distances and tilt angles between transmembrane helices, as well as the distances between the helices and the hydrophilic loops. These approaches have proven quite successful with the lac permease (Kaback & Wu, 1997) and bacteriorhodopsin (Altenbach et al., 1990). **Finally,** in view of the facts that several major impediments have been overcome, and structures are beginning to appear for channel proteins (Chang et al., 1998; Doyle et al., 1998), I believe that the use of X-ray crystallography to solve the structures of members of this important class of protein is likely to prove successful in the near future. What is needed is an intense, creative, and sustained effort, plus a little luck.

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