Structure, Function and Regulation of the Tricarboxylate Transport Protein From Rat Liver Mitochondria

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Recent progress is summarized on the structure, function, and regulation of the tricarboxylate (i.e., citrate) transport protein (CTP) from the rat liver mitochondrial inner membrane. The transporter has been purified and its reconstituted function characterized. A cDNA clone encoding the CTP has been isolated and sequenced, thus enabling a deduction of the complete amino acid sequence of this 32.6 kDa transport protein. Dot matrix analysis and sequence alignment indicate that based on structural considerations the CTP can be assigned to the mitochondrial carrier family. Hydropathy analysis of the transporter sequence indicates six putative membrane-spanning α-helices and has permitted the development of an initial model for the topography of the CTP within the inner membrane. The questions as to whether more than one gene encodes the CTP and whether more than one isoform is expressed remain unanswered at this time. Studies documenting a diabetes-induced alteration in the function of several mitochondrial anion transporters, which can be reversed by treatment with insulin, provide a physiologically/pathologically relevant experimental system for studying the molecular mechanism(s) by which mitochondrial transporters are regulated. Potential future research directions are discussed.

INTRODUCTION

The mitochondrial tricarboxylate (i.e., citrate) transport protein plays a major role in intermediary metabolism within the liver parenchymal cell. It catalyzes a 1:1 electroneutral exchange across the mitochondrial inner membrane of a tricarboxylate (e.g., citrate, threo-D_s-isocitrate, cis-aconitate) plus a proton for either another tricarboxylate/H^+, a dicarboxylate (e.g., malate, succinate), or phospho-

enolpyruvate (Robinson *et al.*, 1971; Palmieri *et al.*, 1972; for reviews see LaNoue and Schoolwerth, 1979; Bryla, 1980). By supplying citrate plus a proton to the cytoplasm, the citrate transporter provides a carbon source for the triacylglycerol and sterol biosynthetic pathways, as well as a supply of NAD^+ and NADPH (resulting from the concerted action of malate dehydrogenase and malic enzyme) which support glycolysis and lipid biosynthesis, respectively (Greville, 1969; Watson and Lowenstein, 1970; Brunengraber and Lowenstein, 1973; Denton and Halestrap, 1979; Endemann *et al.*, 1982; Sugden and Williamson, 1982; Conover, 1987; Freed *et al.*, 1988). Because of its importance in cellular metabolism, the structure, function, and regulation of the citrate transport protein (i.e., CTP)[3] have been extensively studied at the molecular level by our laboratory in recent years (Kaplan *et al.*, 1989, 1990a, b, 1991a, b, 1993). This

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[3] The abbreviations used are: bp, base pairs; BTC, 1,2,3-benezene-tricarboxylate; CTP, citrate transport protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SE, standard error of the mean; SZ, streptozotocin.
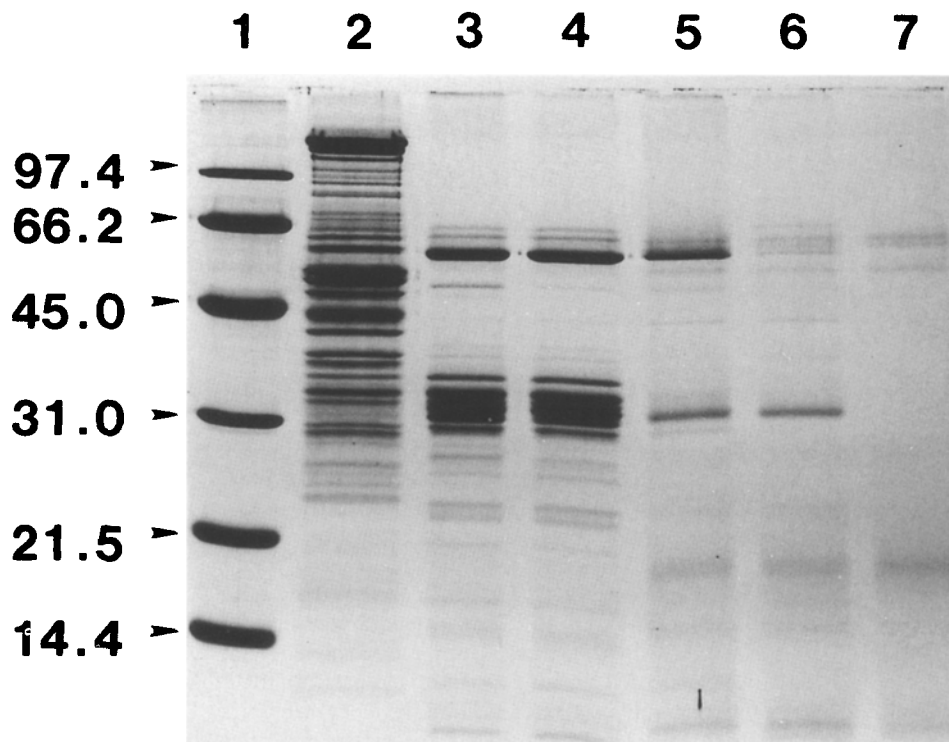
Fig. 1. Coomassie-stained SDS-polyacrylamide gradient gel electrophoretic pattern of the different stages of purification of the functional citrate transport protein from rat liver mitoplasts. Proteins were run in a 4.5% polyacrylamide stacking gel followed by a highly resolving 14–20% linear gradient gel. Lane 1, 1.8 μg of each Bio-Rad SDS-PAGE low-molecular-weight standard protein: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane 2, 15.8 μg (1.3 μl sample) of the Triton X-114 extracted mitoplast protein. Lane 3, 9.6 μg (0.26 ml sample prior to precipitation) of the hydroxylapatite eluate protein. Lane 4, 7.8 μg (0.26 ml sample prior to precipitation) of the Matrex Gel Orange A flowthrough protein. Lane 5, 4.4 μg (1.05 ml sample prior to precipitation) of the Matrex Gel Blue B eluate protein. Lane 6, 3.1 μg (1.05 ml sample prior to precipitation) of the mercaptoethanol-activated Affi-Gel 501 flowthrough protein. Lane 7, 1.1 μg (1.05 ml sample prior to precipitation) of the lipid-containing mercaptoethanol-activated Affi-Gel 501 elution buffer protein. Reproduced with permission from J. Biol. Chem. (Kaplan et al., 1990a).

review is intended to briefly summarize the progress that we have made in these areas and to point out issues of current controversy, as well as questions that are amenable to study in the near future.

PURIFICATION AND FUNCTIONAL RECONSTITUTION OF THE CITRATE TRANSPORT PROTEIN

Several years ago our laboratory developed a procedure for purifying the CTP from rat liver mitoplasts (Kaplan et al., 1990a). The method consists of the extraction of the transporter from mitoplasts with Triton X-114 in the presence of cardiolipin followed by the sequential chromatography of the extract on hydroxylapatite, Matrex Gel Orange A, Matrex Gel Blue B, and Affi-Gel 501. This procedure results in a highly purified preparation of CTP. As depicted in Fig. 1 (lanes 6 and 7), analysis of the purified material by SDS-PAGE indicated the presence of one main mitochondrial protein band with an apparent molecular mass of 32.5 kDa. Upon functional reconstitution in a liposomal system, the purified transporter catalyzes a BTC-sensitive citrate/citrate exchange with a specific activity of 3240 nmol/4 min/mg protein, a value which is enhanced 831-fold relative to the intial extract (Table I). This procedure, which can be performed in approximately 9 hours, results in 10–20 μg of highly purified, functional CTP. It represented the first method to yield purified CTP that both displayed a high specific transport activity and could be

Table I. Purification Of The Citrate Transport Protein From Rat Liver Mitoplasts[a]

	BTC-sensistive citrate/citrate exchange			
	Total activity		Specific activity	
	nmol/4 min	Percent yield	nmol/4 min/mg	Enhancement (- fold)
Triton X-114 extract	386.7	100	3.9	—
Hydroxylapatite eluate	608.7	157	224.6	58
Matrex Gel Orange A flowthrough	523.8	135	222.9	57
Matrex Gel Blue B eluate	103.7	27	2591.7	665
Affi-Gel 501 flowthrough (mercaptoethanol-activated)	64.8	17	3240.0	831

[a] Frozen rat liver mitoplasts (126.9–146.5 mg of protein) were employed as the starting material. Total activity values were normalized to 126.9 mg of starting mitoplast protein. Protein fractions were incorporated into asolectin vesicles, and BTC-sensitive citrate transport was then determined as previously described (Kaplan et al., 1990a). Data represent means of five separate protein isolations. Modified with permission from J. Biol. Chem. (Kaplan et al., 1990a).

obtained in quantities that readily enable further structural as well as functional studies.

We have subsequently investigated several aspects of the functional properties of the purified CTP. Kinetic analysis of the reconstituted CTP yielded a K_m of 0.13 mM and a V_{max} of 1.9 μmol/min/mg protein (see Table II). With regard to its substrate specificity, threo-D_s-isocitrate, malate, and phosphoenolpyruvate effectively inhibited the [^{14}C] citrate/citrate exchange, whereas succinate was considerably less effective, and malonate, α-ketoglutarate, pyruvate, and inorganic phosphate were totally ineffective. Thus, the purified transporter displays a substrate specificity quite similar to that observed for the tricarboxylate transport system in isolated mitochondria. Investigations into the sensi-

tivity of the reconstituted CTP to inhibitors of several mitochondrial transporters indicated that, as expected, BTC, a specific inhibitor of the CTP in isolated mitochondria, effectively inhibited the purified CTP. In contrast, phenylsuccinate, α-cyano-4-hydroxycinnamate, and carboxyatractyloside, which selectively inhibit the α-ketoglutarate, pyruvate, and ADP/ATP transporters in isolated mitochondria (for review, see LaNoue and Schoolwerth, 1979; Bryla, 1980), had little effect on the purified CTP. Somewhat surprisingly, n-butylmalonate, a selective inhibitor of the dicarboxylate transporter, caused a moderate inhibition of the CTP. Taken together, our findings that the purified 32.5-kDa protein displays: (i) a high functional competence, and (ii) near native substrate specificity and inhibitor sensitivity,

Table II. Properties Of The Citrate Transport Protein Purified By Different Laboratories

Source	Purification factor[a] (-fold)	Yield (μg)	Molecular mass[b] (kDa)	K_m (mM)	V_{max}[c] (μmol/min/mg)	Reference
Rat liver mitoplasts	831	13[d]	32.5	0.13[d]	1.9[d]	Kaplan et al. (1990a)
Rat liver mitochondria	1071	2	30.0	0.13	2.0	Bisaccia et al. (1989, 1990)
Rat liver mitochondria	ND[e]	8	38-39	0.04	1.6	Glerum et al. (1990)
Bovine liver mitochondria	ND	35	37-38	0.13	0.4	Claeys and Azzi (1989)

[a] The purification factor is based on reconstituted transport activities and therefore should be considered a crude estimate of the fold purification.

[b] Determined by SDS-PAGE.

[c] V_{max} values have been normalized to a per minute basis. All kinetic constants refer to citrate/citrate exchange except for the data obtained from Glerum et al. (1990), in which citrate/isocitrate exchange was measured.

[d] R. S. Kaplan, previously unpublished data.

[e] ND denotes not determined.

support the conclusion that it represents the complete rat liver mitochondrial tricarboxylate transport system.

COMPARISON OF CITRATE TRANSPORT PROTEINS ISOLATED BY DIFFERENT LABORATORIES

The CTP has been purified in functional form by three laboratories (Bisaccia et al., 1989; Claeys and Azzi, 1989; Kaplan et al., 1990a). A comparison of the properties of these preparations is depicted in Table II. It is noteworthy that similar molecular mass values are obtained by our group (Kaplan et al., 1990a) and the Palmieri laboratory (Bisaccia et al., 1989) (i.e., 32.5 kDa and 30.0 kDa, respectively) and that these values are in the range of values observed for other mitochondrial anion transport proteins (for review, see Kramer and Palmieri, 1989). In contrast, Azzi's laboratory (Claeys and Azzi, 1989; Glerum et al., 1990) has reported a molecular mass value that is significantly higher (i.e., 37-39 kDa) and is atypical with respect to the masses of other mitochondrial inner membrane transporters. One can envision that the higher value obtained by Azzi's group may have arisen from: (1) the existence of a precursor form of the citrate transporter which is proteolytically cleaved to the 30.0–32.5 kDa forms; (2) the purification of a citrate transport protein from a membrane other than the mitochondrial inner membrane; or (3) the expression of a different isoform of the mitochondrial inner membrane citrate carrier. With respect to the first possibility, analysis of the corresponding nucleotide and amino acid sequences of our 32.5-kDa CTP (see below) indicates it unlikely that this protein arose from proteolysis of a significantly larger (i.e., 37–39 kDa) precursor. Regarding the second possible scenario, one should bear in mind that lysosomes, endoplasmic reticulum, and peroxisomes typically contaminate mitochondrial preparations (Pedersen et al., 1978), and that the Azzi group did not employ either mitoplasts [i.e., mitochondria which have been pre-extracted with digitonin, resulting in the removal of the outer mitochondrial membrane and possibly contaminating subcellular organelles as well (Loewenstein et al., 1970)] or purified inner membrane as the starting material. Thus, at present one cannot exclude the possibility that the transporter which they have purified may have arisen from a membrane other than the mito-

chondrial inner membrane, thereby explaining the atypical molecular mass value. Of course, such a finding would be quite interesting in its own right. Clearly data obtained using either mitoplasts or purified inner membrane as the starting material are needed in order to conclusively resolve this issue. Finally, if the third possibility is correct, namely that they have in fact isolated a second isoform of the mitochondrial inner membrane citrate transport protein, this would be an exciting finding indeed.

Table II also indicates that the protein yields for the different preparations vary from 2–35 μg. Thus, while each of the reported procedures provides sufficient protein for functional reconstitution studies, a significant difference exists in their ability to provide the amount of protein needed for various types of structural studies. The different CTP preparations display similar K_m and V_{max} values. It should be noted that the reconstituted K_m values depicted in Table II are similar to the values obtained with isolated mitochondria [i.e., 0.12–0.25 mM (Robinson et al., 1971; Palmieri et al., 1972)]. Additionally, even though the reconstituted V_{max} values cannot be directly compared to the values obtained with isolated mitochondria [i.e., 2–23 nmol/min/mg protein at 9–10°C (Robinson et al., 1971; Palmieri et al., 1972)] since the latter are measured at substantially reduced temperatures using different techniques, they nonetheless are enhanced 831–1071-fold (Bisaccia et al., 1989; Kaplan et al., 1990a) over the values obtained upon reconstitution of the initial detergent extract of the mitochondrial inner membrane.

CHEMICAL MODIFICATION OF THE RECONSTITUTED, PURIFIED CITRATE TRANSPORT PROTEIN

Studies have been conducted to determine the effect of different protein labeling agents (for a review of the specificity of these agents, see Lunblad, 1991) on the purified, reconstituted CTP. We observed (Kaplan et al., 1990a) that N-acetylimidazole, a tyrosine-selective agent, caused only a slight inhibition (i.e., 28%) of the reconstituted BTC-sensitive citrate/citrate exchange. In contrast, reagents selective for cysteine (i.e., N-ethylmaleimide, mersalyl, p-chloromercuribenzoate), histidine (i.e., diethyl pyrocarbonate), arginine (i.e., 2,3-butanedione, phenylglyoxal), and lysine (pyridoxal 5-phosphate) caused substantial inhibition of the reconstituted

transport (i.e., 79–85%, 95%, 71%–115%, and 112% inhibition were observed, respectively). These findings suggest that cysteine residues, as well as residues which carry at least a partial positive charge, are essential for CTP function and may be of mechanistic importance in the transport of this negatively charged substrate plus a proton. Recently, we have initiated studies to characterize more completely the interaction of pyridoxal 5-phosphate with the purified CTP. We find that the pyridoxal 5-phosphate-mediated inhibition displays an IC_{50} value of approximately $110 \mu M$. Additionally, preliminary evidence indicates that citrate substantially protects against the PLP inhibition, whereas negatively charged metabolites which are not significant substrates for the reconstituted CTP, such as succinate and phosphate, provide little protection. These data suggest that PLP may be an active site-directed inhibitor, a possibility which we are currently exploring.

THE SEQUENCE OF THE MITOCHONDRIAL CITRATE TRANSPORT PROTEIN, ITS CORRESPONDING cDNA, AND COMPARISON TO OTHER MITOCHONDRIAL TRANSPORTER SEQUENCES

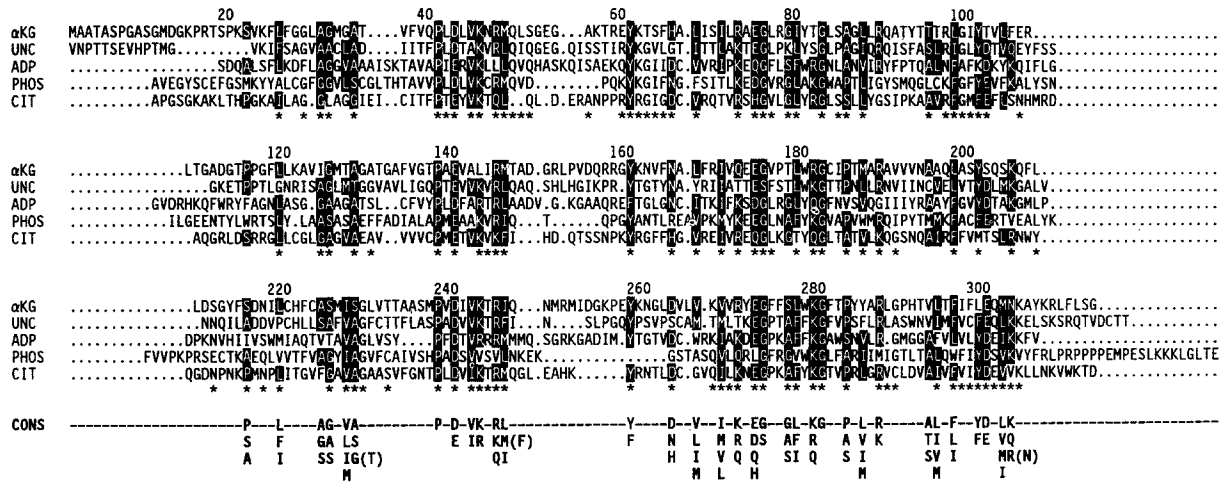We recently reported (Kaplan et al., 1993) the isolation and sequencing of a cDNA clone encoding the entire open reading frame of the CTP which permitted the first determination of the complete amino acid sequence of the rat liver mitochondrial CTP. The CTP cDNA clone consists of a 1927-bp fragment, with 5'- and 3'-untranslated regions of 419 and 572 bp, respectively. The open reading frame encodes a mature protein of 298 amino acids (the calculated molecular mass is 32,557 Da) which is preceded by a presequence of 13 amino acids. The presequence displays characteristics typical of mitochondrial import sequences (Roise and Schatz, 1988) in that it is positively charged, amphipathic, and may possibly exist as an α-helix. The deduced sequence of the mature CTP is in complete agreement with the sequence of 157 residues that was determined by direct protein sequencing. We were able to unambiguously assign the initiating methionine codon (i.e., nucleotides 420–422) based on the facts that: (1) it is the only methionine in the region immediately preceding the amino terminal sequence (the latter was determined by direct sequencing of the transport protein); (2) it is preceded by an in-phase termination

codon; (3) there are six in-frame stop codons between this initiation codon and the next in-frame upstream ATG codon; and (4) it fulfills Kozak's criteria for eukaryotic initiation sites (Kozak, 1986).

Several interesting features of the CTP sequence merit comment. First, the polarity of the CTP (40.3%) is the highest among the mitochondrial anion carriers sequenced to date. Second, FastA and TFastA comparisons of the CTP sequence against the Swiss-Prot and GenBank databases, respectively, reveal that the CTP displays the greatest similarity to other mitochondrial inner membrane transport proteins (i.e., approximately 24–28% identity is observed with respect to the mitochondrial uncoupling protein, ADP/ATP translocase, α-ketoglutarate carrier, and phosphate carrier). Allowing for conservative substitutions, the CTP is 47–53% similar to the above transporters. Dot matrix analysis indicates that the CTP consists of three homologous segments, each of which is approximately 100 amino acids in length. Thus, the CTP displays a tripartite structure similar to that observed with other mitochondrial transporters (Saraste and Walker, 1982; Aquila et al., 1985, 1987; Runswick et al., 1987, 1990; Ferreira et al., 1989; Klingenberg, 1989). Additionally, extensive homology is observed between the repetitive segments within the CTP and those present in each of the other mitochondrial transporters that have been sequenced to date.

Figure 2 presents an alignment of the 15 repetitive sequence domains that comprise the five mitochondrial transport proteins which have been sequenced and reveals several important pieces of information. First, a consensus sequence has been derived which displays 33 positions at which residues are either identical or conservatively substituted in at least 10 of the 15 sequence domains. Second, the CTP sequence contains 102 residues which are identical or conservatively substituted in at least four out of the five mitochondrial transporters that have been sequenced. Third, the CTP sequence displays the mitochondrial carrier-associated motif Pro-X-(Asp/Glu)-X-(Val/Ile/Ala)-(Lys/Arg)-X-(Arg/Lys/Gln)-(Leu/Met/Phe/Ile). Based on the above findings regarding its primary structure, we have concluded that the CTP is a member of the mitochondrial carrier family.

Analysis of the hydropathy of the CTP by the method of Kyte and Doolittle (1982) has indicated the presence of six hydrophobic domains that are of sufficient length to potentially span the inner membrane as α-helices. This has led to the construction

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              20            40                60              80            100
αKG   MAATASPGASGMDGKPRTSPKSVKFLFGGLAGMGAT....VFVQPLDLVKVRMQLSGEG...AKTREYKTSFIA.LISLLRAEGLRGIYTGLSAGLLRQATYTTIRLGINTVLFER.................
UNC   VNPTTSEVHPTMG.........VKILSAGVAACLAD....IITFPLDTAKVRLQIQGEG.QISSTIRYKGVLGT.ITTLAKTEGLPKLYSGLPAGHDRQISFASLRIGLYDTVQEYFSS.................
ADP   ................SDOALSFLKDFLAGGVAAISKTAVAPIERVKLLIQVQHASKQISAEKQYKGIIDC.VVRLPKFRQFLSFWRGNLANVLRYFPTQALNFAFKDKYKQIFLG.................
PHOS  .........AVEGYSCEFGSMKYYALCGFGGVLSCGLTHTAVVPLDLVKCRMQVD........PQKVKGIFNG.FSITLKEDGVRGLAKGWAPTLIGYSMQGLCKFGFTEVFKALYSN.................
CIT   .........APGSGKAKLTHPGKAHLAG.GLACGIEI..CITFPTEYVKTQL..QL.D.ERANPPRYRGIGDC.VRQTVRSHGVLGLYRGLSSLLYGSIPKAAVRGGMHEFLSNHMRD.

              120           140           160              180             200
αKG   ...............LTGADGTPPGFLLKAVIGMTAGATGAFVGTPAEVALIRYTAD.GRLPVDQRRGYKNVFNA.LFRIVQEEGVPTLWRGCIPTWARAVVVNAAQLASISQSKQFL.................
UNC   ...............GKETEPTLGNRISAGLMTGGVAVLIGQPTEYVKVRLQAQ.SHLHGIKPR.VTGTYNA.YRIHATTESFSTLWKGTTPNLLRNVIINCMEIVTYDLMKGALV.................
ADP   ...........GVDRHKQFWRYFAGNLASG.GAAGATSL..CFVYPLDFARTRLAADV.G.KGAAQREITGLGNC.ITKLFKSDGLYKGYYGCLTQKSDGLYKGFYKG.
PHOS  ...........ILGEENTYLWRTSLY.LAASASAEFFADIALAPLEAAKVRIQ...T......QPGWANTLREAVPKYYKEECLNALYKGVAPVWMRQIPYTMKKFACEERTVEALYK.................
CIT   ...............AQGRLDSRRGLLCGLGAGVAEAV..VVVCPMETVKVKFI...HD.QTSSNPKYRGFFEG.VREIVREQGLKETYDGLTATVWKQGSNQALRGFVMTSLRNWY.................

              220           240           260              280             300
αKG   ...............LDSGYFSDNILCHFCASMISGLVTTAASMPVDIVKTRIQ...NMRMIDGKPEYKNGLEVLY.KVVRYEGFFSLWKGFTPYYARLGPHTVLTFIFLEMMKAYKRLFLSG.................
UNC   ...............NNQILADDVPCHLLSAFVAGFCTTFLASPADVVKTRFI...N....SLPGQVPSVPSCAM.TMLTKEGPTAFFKGVPSFLRLASWNVLMFVCFELLKELSKSRQTVDCTT...........
ADP   ...........DPKNVHIIVSWMIAQTVTAVAGLVSY....PFDTVRRRMMMQ.SGRKGADIM.VTGTVDC.WRKJARDEGPKABFKGAWSNVLR.GMGGAFVLVLYDEIKKFV.................
PHOS  .........FVVPKPRSECTKAEQLVVTFVAEYIAGVFCAIVSHPADSVNSVLNKEK...........GSTASQVLQRLGFRGVWKGLFARIIMIGTLTALQWFIYDSVKVYFRLPRPPPPEMPESLKKKLGLTE
CIT   ...............QGDNPNKPMNPLIITGVFEAVAGAASVFGNTPLLVLKTRLQGL.EAHK......MRNTLDC.GVQILKNEGPKAFYKGTVPRLGRVCLDVAINVIYDEVVKLLNKVWKTD.................

CONS  ---------------------P---L----AG-VA---------P-D-VK-RL---------------Y----D--V--I-K-EG--GL-KG--P-L-R-----AL-F---YD-LK----------------
                       S   F     GA LS         E IR KM(F)            F   N L   M R DS  AF R   A V K      TI L  FE VQ
                       A   I     SS IG(T)       QI                   H   I V Q Q   SI Q   S I      SV I   MR(N)
                               M                                        M L   H             M          M    I
```

Fig. 2. Alignment of the citrate transporter repetitive sequence domains with those present in other mitochondrial transporters. The α-ketoglutarate carrier (Runswick et al., 1990), uncoupling protein (Aquila et al., 1985), ADP/ATP translocase (Aquila et al., 1982), and phosphate carrier (Ferreira et al., 1989) were each divided into three sequence domains as described by Runswick et al. (1990). Upon analysis of the CTP sequence, it was similarly divided into three sequence elements. The resulting 15 sequence domains were then compared using the GCG PileUp program, and the alignment is depicted. A consensus sequence was derived based on the criterion that a given residue(s) was identical or conservatively substituted in at least 10 out of the 15 sequence domains. Conserved residues were assigned based on the MDM₇₈ table (Gribskov and Devereux, 1991) and are depicted as white letters on a black background, as well as on the consensus line. The vertical order of conserved residues in the consensus sequence reflects the frequency of occurrence. Residues in parentheses represent alternative substitutions with respect to the adjacent residue. Positions indicated by an asterisk indicate residues that are conserved in at least four out of five sequences within a given domain. Numbers refer to the α-ketoglutarate carrier sequence. Reproduced with permission from J. Biol. Chem. (Kaplan et al., 1993).

of an initial model for the proposed membrane topography of the CTP which is depicted in Fig. 3. Several points are noteworthy. First, each of the five extramembranous hydrophilic domains displays a net positive charge which may serve to facilitate the funneling of citrate into the hydrophobic intramembranous portion of the CTP. Second, the CTP displays substantial asymmetry as indicated by the fact that the three interhelical loops facing one side of the membrane (i.e., Compartment A) are each approximately double the length of the loops facing the other side (i.e., Compartment B). Third, the amino and carboxy termini are both predicted to reside on the same side of the inner membrane. At present there is insufficient data to specify the matrix versus the intermembrane space compartments in our model.

Consideration of the sequence comparisons depicted in Fig. 2 together with the model for secondary structure depicted in Fig. 3 provides additional insight into CTP sequence conservation. For example, 37% of the CTP residues which are located within the six predicted transmembrane domains are either identical or conservatively substituted in at least four out of the five mitochondrial transporters that have been sequenced, whereas 32% of the extra-

membranous residues are similarly conserved. At the junctions between the hydrophobic and hydrophilic regions (defined as consisting of two residues adjacent to each side of the membrane border depicted in Fig. 3), 42% of the residues are conserved. Thus, while the highest conservation occurs at the hydrophobic–hydrophilic junctions (a point previously noted by Runswick et al., 1990), we suggest that significant conservation occurs throughout the CTP sequence. It is also noteworthy that the three large hydrophilic loops facing Compartment A show considerably greater conservation (i.e., 43%) than the two smaller hydrophilic loops facing Compartment B (i.e., 15%). Finally, it is especially interesting that one of these smaller loops contains two phosphorylation consensus sequences [i.e., residues 93–96 (casein kinase I) and residues 105–110 (protein kinase C)]. Since mitochondria are known to contain a variety of protein kinases (Bradford and Yeaman, 1986; Muller and Bandlow, 1987; Ferrari et al., 1990; Schwoch et al., 1990; Tuazon and Traugh, 1991), this raises the possibility that CTP function may be regulated by phosphorylation.

Investigations have been conducted to determine the number of sequences related to the CTP coding

Compartment A
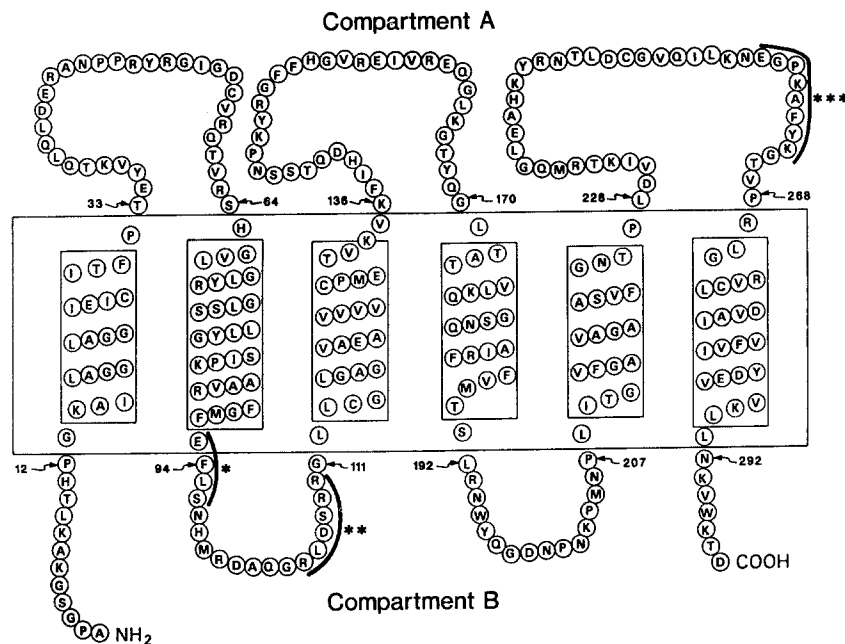
Fig. 3. Proposed model for the membrane topography of the mitochondrial citrate transport protein based on its hydropathy profile. The model depicts six putative transmembrane α-helical segments which are enclosed in boxes and are connected by five hydrophilic loops. Numbers refer to the location of a given residue within the CTP primary sequence. Compartments A and B refer to the two compartments adjacent to the inner mitochondrial membrane. * and ** denote phosphorylation consensus sequences (Kemp and Pearson, 1990) for casein kinase I and protein kinase C, respectively. *** denotes sequence that is highly conserved (i.e., six out of eight residues) with respect to the first zinc finger of nuclear receptors (Bouillaud *et al.*, 1992). Modified with permission from *J. Biol. Chem.* (Kaplan *et al.*, 1993).

region in both the rat and human genomes. In these studies, restriction digests of genomic DNA were analyzed by Southern blot employing a PCR-amplified partial CTP cDNA (encoding amino acids 10–203 of the CTP) as the hybridization probe. Our results indicate that under conditions of high stringency, the rat and human genomes contain between two to three and three to five sequences, respectively, that hybridize with the CTP cDNA probe. Thus, we can conclude that the human genome encodes a CTP which is homologous to the rat CTP. Additionally, our findings indicate that either multiple CTP genes exist (possibly including pseudogenes) or there is a single CTP gene in which the coding region is dispersed by introns that contain multiple restriction sites.

In summary, the information presented in this section provides clear-cut evidence that on the basis of structural considerations, the CTP belongs to the mitochondrial carrier family. The members of this family are likely to have originated from a common genetic predecessor and may utilize similar structural domains in the catalysis of substrate translocation.

ALTERATION OF MITOCHONDRIAL ANION TRANSPORTER FUNCTION IN DIABETES: A MODEL SYSTEM FOR STUDYING TRANSPORTER REGULATION

Investigations have been carried out to determine the effects of type 1 diabetes (i.e., insulin-dependent diabetes mellitus) on mitochondrial transporter function (Kaplan *et al.*, 1990b, 1991a). The underlying hypothesis for these studies was that the mitochondrial anion transporters are regulated in coordination with the metabolic pathways to which they either supply substrate or remove product. From this idea arose the following specific hypotheses. *Hypothesis 1*: Since the function of the liver mitochondrial CTP is essential for the supply of sufficient carbon precursor to the cytoplasm in order to support fatty acid and sterol biosyntheses (Greville, 1969; Watson and Lowenstein, 1970; Brunengraber and Lowenstein, 1973; Denton and Halestrap, 1979; Endemann *et al.*, 1982; Sugden and Williamson, 1982; Conover, 1987; Freed *et al.*, 1988), and since hepatic fatty acid and possibly

Table III. Levels of Functional Anion Transporters Extracted From Rat Liver Mitoplasts Isolated From Control and Diabetic Animals[a]

Transporter type	Specific transport activity	Percent increase	Total transport activity	Percent increase
Citrate transporter				
Control	3.79 ± 0.24 (13)		$226.78 \pm$ 18.41 (13)	
Diabetic	2.06 ± 0.13 (14)	-46	$125.41 \pm$ 7.69 (14)	-45
Pyruvate transporter				
Control	1.16 ± 0.09 (12)		$68.73 \pm$ 5.54 (12)	
Diabetic	2.31 ± 0.14 (12)	99	$139.91 \pm$ 9.34 (12)	104
Dicarboxylate transporter				
Control	1.27 ± 0.07 (7)		$74.96 \pm$ 4.30 (7)	
Diabetic	2.10 ± 0.15 (7)	65	$126.57 \pm$ 8.73 (7)	69
Phosphate transporter				
Control	75.23 ± 7.79 (6)		1637.84 ± 135.50 (6)	
Diabetic	88.28 ± 3.05 (6)	17	$1874.05 \pm$ 72.21 (6)	14

[a] Diabetic animals received 70 mg SZ (in citrate/phosphate buffer)/kg i.p. 3 weeks prior to the mitoplast preparation. Control animals received the buffer alone. Shortly before the mitoplast isolations, average blood glucose levels of 530 mg/dl and 132 mg/dl were determined with the diabetic and control animals, respectively. Transport values are means $\pm$ SE. Numbers in parentheses represent the number of repetitions of a given type of incubation. All diabetic values differ significantly from the controls (i.e., $P < 0.001$) except for the phosphate transporter where the differences are not statistically significant (i.e., $P > 0.10$). Specific activity measurements were: citrate transporter, BTC-sensitive citrate/citrate exchange (nmol/4 min/mg protein); pyruvate transporter, α-cyano-4-hydroxycinnamate-sensitve pyruvate uptake (nmol/6 min/mg protein;); dicarboxylate transporter, n-butylmalonate-sensitive malonate/malate exchange (nmol/min/mg protein); phosphate transporter, N-ethylmaleimide-sensitive phosphate/phosphate exchange (nmol/min/mg protein). Total activity values were determined by multiplying the specific activity by the total amount of protein in the extract. Modified with permission from *Arch. Biochem. Biophys* (Kaplan et al., 1990b).

sterol biosynthesis are decreased in SZ-induced diabetes (Elwood *et al.*, 1960; Foster and Siperstein, 1960; Burns and Elwood, 1969; Freed *et al.*, 1988), then based on the idea of coordinated regulation, we predict that the level of functional CTP is decreased. *Hypothesis 2*: Since the liver mitochondrial pyruvate and dicarboxylate transporters are essential components for pyruvate-supported hepatic gluconeogenesis (Meijer and Van Dam, 1974; Williamson, 1976; Pilkis, *et al.*, 1978, 1988), and since hepatic gluconeogenesis is elevated in SZ-induced diabetes (Exton *et al.*, 1972; Cohen, 1987), we predict that the functional levels of these two transporters are elevated in diabetes. Furthermore, the observations that: (i) the level of hepatic fatty acid oxidation and the resulting ketone body production is elevated in diabetes (Exton *et al.*, 1972; McGarry and Foster, 1979; Foster, 1984); and (ii) transport on the pyruvate carrier is a prime mechanism for the redistribution of ketone bodies from their site of production (i.e., the mitochondrial matrix) to the cytoplasm (Pande and Parvin, 1978; Halestrap *et al.*, 1980), lend further support to our prediction of an elevation in the functional level of the pyruvate transporter. *Hypothesis 3*: Since the mitochondrial phosphate transporter functions to supply inorganic phosphate to the mitochondrial oxidative phosphorylation apparatus (Fonyo, 1979; Meijer and Van Dam, 1981), and since oxidative

phosphorylation typically is unaltered in diabetes (Parks *et al.*, 1955; DiMarco and Hoppel, 1975; Rogers *et al.*, 1986), we predict that the level of functional phosphate transporter is not substantially altered by diabetes.

Our experimental strategy for these studies consisted of the extraction of each of the four mitochondrial carriers from mitoplasts obtained from control versus diabetic rats with Triton X-114 followed by the reconstitution of transporter function in liposomal systems using procedures that have previously been shown to result in the effective recovery of each of the four functional transporters (Kaplan and Pedersen, 1985; Kaplan *et al.*, 1986, 1989; Nalecz *et al.*, 1986). This approach offers important advantages relative to carrying out transport measurements in whole mitochondria isolated from control versus diabetic animals where a number of variables may alter the observed transport rates but nonetheless have little to do with alterations in the transport proteins *per se* [e.g., altered intramitochondrial substrate pools, pH gradient, lipid composition; see Kaplan *et al.*, (1990b) for a more detailed discussion]. The results we obtained are depicted in Table III and indicate that 3 weeks following the induction of diabetes with SZ we observed a decrease in the amount of functional citrate transporter that was extracted from diabetic animals relative to the level extracted from their

Fig. 4. Importance of mitochondrial anion transporters in the altered hepatic intermediary metabolism that characterizes experimental diabetes. This figure summarizes the effect of experimental diabetes on: (i) the levels of functional mitochondrial anion transporters which we have characterized; and (ii) the levels of associated metabolic pathways to which the mitochondrial transporters either supply substrate or remove product as documented by other investigators (see references listed in the text). Increases are denoted as (+) and decreases as (–). The denotation "ketone bodies" refers both to the levels of ketone bodies and the rate of fatty acid oxidation. Also, a question mark has been used to indicate the conflicting data as to whether or not diabetes in fact causes a decrease in sterol biosynthesis. Reproduced with permission from *Arch. Biochem. Biophys.* (Kaplan *et al.*, 1990b).

control counterparts (i.e., 46 and 45% decreases in the specific and total transport activities, respectively; diabetic vs. control). These decreases were highly significant ($P < 0.001$). In contrast, diabetes caused substantial increases in the amount of functional pyruvate (i.e., 99 and 104% increases in the specific and total transport activities, respectively; $P < 0.001$) and dicarboxylate (i.e., 65 and 69% increases in the specific and total transport activities, respectively; $P < 0.001$) carriers. Finally, no significant change was observed in the level of functional phosphate transporter that could be extracted from diabetic versus control mitoplasts. Thus all three hypotheses were confirmed.

The observation that SZ-induced diabetes diferentially affected transporter function in a predictable manner (i.e., the reconstitutable level of the citrate transporter decreased, whereas the levels of the pyruvate and dicarboxylate carriers increased and that of the phosphate transporter was unchanged) supports

the idea that the observed alterations are specific and do not reflect a more generalized nonspecific alteration in mitochondrial function. Additional experiments carried out with the citrate and pyruvate transporters indicated that no significant change occurred in the reconstitutable levels of these transporters at either 1.5 or 13 hours following the injection of SZ. These findings are of special interest since SZ is rapidly cleared from the blood (serum half-life of 15 min) (Schein *et al.*, 1973) and rapidly decomposes at physiological pH (Rerup, 1970), and thus rule out the possibility of a direct chemical effect of SZ on the liver mitochondrial citrate and pyruvate transporters. Figure 4 summarizes our findings and shows the putative functional linkage between the four mitochondrial transporters that we investigated and hepatic intermediary metabolism. This study represented the first in-depth investigation of mitochondrial transporter function in diabetes.

In a subsequent study (Kaplan *et al.*, 1991a), we

demonstrated that treatment of diabetic rats with insulin (i.e., 3 weeks after rats were made diabetic with SZ, they received daily injections of insulin for an additional 3 weeks) resulted in a reversal of the SZ-induced changes in citrate, pyruvate, and dicarboxylate transporter function. Thus, the diabetes-induced alterations in transporter function were a consequence of the insulin deficiency that characterizes SZ-induced diabetes. To our knowledge, these investigations provided the first clear-cut evidence that insulin participates in the regulation of the functional levels of liver mitochondrial anion transport proteins. In a final series of studies (Kaplan et al., 1991b) we determined the effect of type 2 diabetes (i.e., non-insulin-dependent diabetes mellitus; adult-onset diabetes) on the functional levels of the mitochondrial anion transporters. We observed that experimental type 2 diabetes did not cause significant changes in the extractable and reconstitutable specific (and total) transport activities of the citrate, pyruvate, and dicarboxylate transporters. The observation that in this model of diabetes, where insulin levels are not profoundly deficient (Bonner-Weir et al., 1981; Giroix et al., 1983; Schaffer et al., 1987), transporter function is unaltered, in combination with our earlier finding that in type 1 diabetes, where a profound insulinopenia exists (Dulin and Soret, 1977), transporter function is altered, lends further support to our conclusion that insulin plays an important role in the regulation of the mitochondrial anion carriers.

In concluding this section, we point out that it is our contention that diabetes provides an excellent opportunity for studying the molecular mechanisms by which mitochondrial transport proteins are regulated. The ability to chemically induce a pathology that gives rise to specific and unique changes in the functioning of several transporters, which can be reversed by insulin therapy, provides a physiologically/pathologically relevant experimental system which we believe will allow us to unravel the molecular bases for mitochondrial transporter regulation.

PERSPECTIVES AND FUTURE DIRECTIONS

During the last 5 years, significant progress has been made in development of the tools which will enable a detailed molecular understanding of the structure, the substrate translocation mechanism, and the regulation of the CTP. Thus, several labor-

atories (Bisaccia et al., 1989; Kaplan et al., 1990a; Glerum et al., 1990) have purified the CTP in a form that is reconstitutively active and displays the functional properties characteristic of the complete native tricarboxylate transport system of rat liver mitochondria. Chemical modification studies have resulted in the identification of essential types of residues within the CTP and are progressing toward the development of active-site directed probes. Cloning and sequencing studies (Kaplan et al., 1993) have resulted in the assignment of the CTP (i.e., the 32.6-kDa protein) to the mitochondrial anion carrier family on the basis of sequence conservation and structural considerations, and have permitted the development of an initial model for the secondary structure of this carrier protein. Finally, the documentation of changes in the function of the CTP and other mitochondrial transporters in an important disease (i.e., diabetes), which can be reversed by hormone supplementation (Kaplan et al., 1990b, 1991a), provides an experimental system which should enable elucidation of the molecular mechanisms by which transporter function is regulated.

With this recent progress as a foundation, the field in many ways is now poised to address the most interesting questions regarding the CTP. In our view, several of the more important areas which are amenable to study in the near-term are as follows. First, experiments using site-specific polyclonal antibodies, in situ proteolysis, and vectorial chemical labeling should enable either confirmation or revision of our initial model for the proposed topography of the CTP within the inner mitochondrial membrane. Clearly, the development of an accurate, experimentally verified model for CTP topography will prove invaluable in elucidating the transport mechanism. Second, a combination of chemical modification (with active site-directed probes) and both random, as well as site-specific, mutagenesis of the CTP should provide important information concerning the roles of specific amino acid residues and structural domains in the chemical mechanism by which CTP catalyzes substrate translocation. The mutagenesis approach could be carried out using a yeast expression system in which the chromosomal copy of the CTP gene has been deleted. In fact, we have recently initiated studies aimed at development of such an expression system. Related to this, information about the amino acid sequence of the yeast CTP would provide important insight as to which CTP residues are conserved between species and are thus

more likely to be of mechanistic importance. Third, the issues as to whether there is more than one gene encoding the CTP and whether there is more than one expressed isoform are important and need to be resolved. With respect to the latter point, conclusive evidence is needed establishing whether or not the 38–39-kDa citrate carrier protein that has been isolated from rat liver by the Azzi laboratory (Glerum et al., 1990) originates from the mitochondrial inner membrane. Fourth, the isolation of a cDNA that encodes the CTP will now enable exploration of the structure, organization, and ultimately the regulation of the CTP gene(s). Finally, employing diabetes and insulin supplementation as a physiologically/pathologically relevant means of manipulating mitochondrial transporter function, one can now determine whether transporter regulation involves alterations in the amount of transport protein and/or in the intrinsic properties of a given transporter per se. The latter might arise as a consequence of the covalent modification of a transporter or the expression of different transporter isoforms.

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