A gene encoding the mitochondrial dicarboxylate transport protein (DTP) has been identified for the first time from any organism. Our strategy involved overexpression of putative mitochondrial transporter genes, selected based on analysis of the yeast genome, followed by purification and functional reconstitution of the resulting protein products. The DTP gene from the yeast *Saccharomyces cerevisiae* encodes a 298-residue basic protein which, in common with other mitochondrial anion transporters of known sequence and function, displays the mitochondrial transporter signature motif, three homologous 100-amino acid sequence domains, and six predicted membrane-spanning regions. The product of this gene has been abundantly expressed in *Escherichia coli* where it accumulates in inclusion bodies. Upon solubilization of the overexpressed DTP from isolated inclusion bodies with Sarkosyl, 28 mg of DTP was obtained per liter of *E. coli* culture at a purity of 75%. The purified, overexpressed DTP was then reconstituted in phospholipid vesicles where both its kinetic properties (i.e. $K_m = 1.55 \text{mM}$ and $V_{max} = 3.0 \text{ pmol/min/mg protein}$) and its substrate specificity were determined. The intraliposomal substrates malonate, malate, succinate, and phosphate effectively supported $[1^C] \text{malonate}$ uptake, whereas other anions tested did not. External substrate competition studies revealed a similar specificity profile. Inhibitor studies indicated that the reconstituted transporter was sensitive to inhibition by $n\text{-butyramalonate}$, $p\text{-chloromercuribenzoate}$, mersalyl, and to a lesser extent pyridoxal 5'phosphate but was insensitive to $N\text{-ethylmaleimide}$ and selective inhibitors of other mitochondrial anion transporters. In combination, the above findings indicate that the identified gene encodes a mitochondrial transport protein which upon overexpression and reconstitution displays functional properties that are virtually identical to those of the native mitochondrial dicarboxylate transport system.

In conclusion, the present investigation has resulted in identification of a gene encoding the mitochondrial DTP and thus eliminates a major impediment to molecular studies with this metabolically important transporter. Based on both structural and functional considerations, the yeast DTP is assignable to the mitochondrial carrier family. Additionally, the development of a procedure that enables the expression and isolation of large quantities of functional DTP provides the foundation for comprehensive investigations into the structure/function relationships within this transporter via site-directed mutagenesis, as well as for the initiation of crystallization trials.

The dicarboxylate transport protein (i.e. DTP) from mammalian mitochondria catalyzes an electroneutral exchange across the inner mitochondrial membrane of dicarboxylates (e.g. malonate, malate, succinate) for inorganic phosphate and certain sulfur-containing compounds (e.g. sulfite, sulfate, thiosulfate) (1–5). It is inhibited by substrate analogues (e.g. n-butyramalonate) (2, 3, 6) as well as by certain sulfhydryl reagents (e.g. mersalyl and pCMB but not $N\text{-ethylmaleimide}$) (2, 7–10). The DTP plays an important role in hepatic gluconeogenesis, urea synthesis, and sulfur metabolism (11, 12). Moreover, its function, which is elevated in type 1 diabetes (13), can be normalized with insulin therapy, thereby suggesting a role for insulin in DTP regulation (14). Yeast mitochondria also contain a DTP with properties generally similar to those observed with the higher eukaryotic transporter (15–18).

Due to its importance in intermediary metabolism, the DTP has been intensively investigated. Thus, it has been purified from both higher eukaryotic (19–22) and yeast (23) mitochondria and kinetically characterized in isolated mitochondria (1, 2, 9, 16, 17) as well as following functional reconstitution of the purified protein (22–25). Despite this progress, prior to the present investigation, no information has been available concerning the sequence of either the DTP or its gene, primarily due to the difficulty in obtaining a sufficient quantity of the purified protein. This major impediment has prevented detailed molecular studies with this carrier.

In the present paper, we report the identification of the gene encoding the yeast mitochondrial DTP. Our strategy involved overexpression of putative mitochondrial transporter genes identified from analysis of the yeast genome, followed by purification and functional reconstitution of the resulting protein products. This approach provides, for the first time from any source, information on the deduced primary structure of a mitochondrial DTP. Our results permit the assignment of the DTP to the mitochondrial carrier family based on both structural and functional considerations. Finally, these studies provide the tools (i.e. the DTP gene and abundant quantities of its purified, functional protein product) which will enable com-

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**Identification of a Novel Gene Encoding the Yeast Mitochondrial Dicarboxylate Transport Protein via Overexpression, Purification, and Characterization of Its Protein Product**

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David Kakhnashvili†, June A. Mayor¶, David A. Gremsé§, Yan Xu‡, and Ronald S. Kaplan‡‡

From the Departments of †Pharmacology and ¶Pediatrics, College of Medicine, University of South Alabama, Mobile, Alabama 36688

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*The abbreviations used are: DTP, dicarboxylate transport protein; GCG, Genetics Computer Group; PAGE, polyacrylamide gel electrophoresis; pCMB, p-chloromercuribenzoic acid; PCR, polymerase chain reaction.*
Identification and Overexpression of the DTP Gene

RESULTS

Identification, Overexpression, and Purification of the Yeast Gene Encoding the Mitochondrial Dicarboxylate Transport Protein—Identification of the yeast gene encoding the mitochondrial DTP was accomplished utilizing the following strategy. BLAST and TFastA comparisons of the yeast mitochondrial citrate transporter sequence (GenBank accession number: U17503) were performed against the GenBank data base. Yeast protein sequences identified via these searches were then examined to determine whether they (i) constituted basic proteins of approximately 300 amino acids in length; (ii) contained the mitochondrial transporter signature sequence; (iii) consisted of six putative membrane-spanning domains (based on hydropathy analysis); and (iv) contained three homologous sequence domains of approximately 100 residues in length that are related to each other and to the sequence domains present in other mitochondrial transporters of known function (based on dot matrix analysis). Approximately 30 protein sequences, including six with known function, were identified based on the above parameters. Putative mitochondrial transporter sequences of unknown function were then prioritized for overexpression based upon how closely they matched all of the above criteria.

We then proceeded to overexpress and test the function of a gene product as follows. First, a given sequence was amplified by PCR, cloned into the pET-21a (+) plasmid, transformed into a bacterial expression host, and overexpressed. Second, the overexpressed protein product was solubilized from an isolated inclusion body fraction, incorporated into phospholipid vesicles, and assayed for dicarboxylate transport activity. Utilizing this approach, we attempted to overexpress gene products that encode putative mitochondrial transporters of unknown function (i.e. accession numbers: Z25485, X92441, U18590, X90518, S44213, X87371, and U19028). We achieved overexpression with five of the seven genes (i.e. X90518 and X87371 did not overexpress). Upon reconstitution of a given overexpressed protein in a liposomal system, only the product

prehensive studies into the molecular mechanism of this transporter.

EXPERIMENTAL PROCEDURES

Construction of the Expression Plasmid, Bacterial Overexpression, and Subsequent Isolation of the Yeast DTP—The gene encoding the yeast mitochondrial DTP corresponds to the reverse complement of nucleotides 34,460–35,356 of GenBank accession number U19026. The DTP gene plus an additional 79 downstream nucleotides (i.e. nucleotides 34, 381–34, 450) was amplified from total Saccharomyces cerevisiae genomic DNA (Novagen; strain S288C) via PCR. Amplifications employed a forward primer (corresponding to nucleotides 35, 356–35, 334) that contained an engineered NdeI site and a reverse primer (corresponding to nucleotides 34, 381–4, 400) that contained an engineered BamHI site and were conducted as previously detailed (26), except that an annealing temperature of 59 °C was employed. The resulting single 1-kilobase pair amplification product was directionally cloned into pET-21a (+) plasmid DNA (Novagen) essentially as described previously (26). NovaBlue-competent cells (Novagen) were transformed with the DTP DNA construct following the manufacturer’s instructions. Transformants were screened for inserts via (i) direct colony PCR and (ii) restriction digestion of plasmid DNA purified with the Wizard Plus Mini-preps DNA purification system (Promega). Positive plasmids were then used to transform BL21(DE3)-competent cells (Novagen), the expression host, and transformants were screened for inserts as described above. The sequence of the cloned DTP gene was determined in its entirety as described previously (26). A consensus sequence was defined based on data obtained from both strands of the insert and indicated that the open reading frame of the DTP gene (i.e. nucleotides 34, 340–35, 356 of GenBank accession number U19026) had been amplified and subcloned with fidelity.

Bacterial overexpression, isolation of the resulting inclusion bodies, and extraction of the DTP from the inclusion bodies with 1.2% Sarkosyl were performed essentially as described previously in detail for the yeast mitochondrial citrate transport protein (26) except that the sonication time was extended (i.e. a total of 11–13 cycles of 30 s of sonication) followed by 30 s of cooling/cycle.

Functional Reconstitution of the Overexpressed Dicarboxylate Transport Protein—The overexpressed, isolated DTP was reconstituted into preformed asolectin vesicles via the freeze-thaw-sonication method. Briefly, asolectin vesicles were prepared in buffer A (120 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 7.4) (19). Solubilized DTP (5–15 μg) (approximately 14–42 μg of protein) was added to 525 μl of asolectin vesicles, 150 mM Hepes, 80–90 μl of buffer B (10 mM Tris, 0.1 mM EDTA, pH 7.0, 1 mM dithioerythritol), 97 μl of buffer C (10 mM potassium phosphate, 20 mM KCl, 1 mM EDTA, pH 7.2), 31.5 μl of 605.53 mM malate (or, where indicated, alternative intraliposomal substrate; final concentration = 20 mM) and 43 μl of 10% (w/v) Triton X-114, and then frozen in liquid nitrogen. Immediately prior to assay, a given sample was thawed, indicated on ice, and extraliposomal substrate was removed (13). The resulting proteoliposomes were assayed for transport as described below.

Measurement of Reconstituted pCMB-sensitive Malonate Uptake—Transport incubations were carried out at room temperature (i.e. 23.0 ± 0.5 °C), unless otherwise indicated, as follows. Proteoliposomes (90.2 μl) were preincubated with 10.82 μl of either 2.8 μM pCMB (control) or deionized water (experimental) for 20 s. Transport was then initiated by the addition of 9.02 μl of 20 mM [14C]malonate (DuPont NEN; specific radioactivity approximately 2.3–5.5 μCi/mmol; final concentration in reaction mix 1.6 μM). 1 min later, 90 μl of the reaction mix was removed and diluted with 480 μl of ice-cold buffer A containing 0.3 mM PCMB (final concentration). Aliquots (80 μl) of the diluted mixture were placed onto pre-scan Sephadex columns, and intraliposomal [14C]malonate was separated from external label (13, 19) and then quantified by liquid scintillation counting. The pCMB-sensitive malonate uptake rate was calculated by subtracting the control value from the experimental value. These differences were compared to the PCMB-sensitive malonate transport rate observed in the absence of competing anion.

The inhibitor studies were conducted as follows. Proteoliposomes (75.0 μl) were preincubated with 9.0 μl of either deionized water (experimental), 2.8 μM PCMB (control reaction), or one of the inhibitors listed in Table III for 1 min. Transport was then triggered by the addition of 7.5 μl of 3.66 mM [14C]malonate (final concentration = 0.3 mM). 1 min later, transport was quenched by diluting 75 μl of the reaction mixture with 400 μl of ice-cold buffer A containing 0.3 mM PCMB (final concentration). Intraliposomal radiolabeled malonate was then separated from external label and quantified as described above. Percent inhibition was calculated by 1) subtracting the control transport rate from the rate observed in the presence of a given agent; 2) determining the ratio of this difference to the uninhibited pCMB-sensitive malonate transport rate; and 3) the use of the formula (1 − ratio) × 100.

The kinetic parameters of the reconstituted, overexpressed DTP were determined as follows. Proteoliposomes (75.0 μl) were tempered at 30 °C for approximately 3 min and were then preincubated with 9.0 μl of either 2.8 mM pCMB (control) or deionized water (experimental) for 30 s. Transport was subsequently triggered by the addition of 7.5 μl of various concentrations of [14C]malonate (final concentration = 0.25–2.0 μCi/mmol; specific radioactivity 1.4–2.9 × 105 cpm/mmol). Following a 10-s transport period (i.e. initial rate), 75 μl of the reaction mix was removed and placed into 400 μl of ice-cold buffer A containing 0.3 mM PCMB (final concentration). The intraliposomal radiolabel was then separated and quantified as described above. The pCMB-sensitive transport rate was calculated by subtracting the control value from the experimental value obtained at each substrate concentration tested.
Identification and Overexpression of the DTP Gene

The overexpressed yeast mitochondrial DTP was solubilized from inclusion bodies with Sarkosyl and incorporated into phospholipid vesicles in the presence of 20 mM of one of the substrates listed below. Transport reactions were triggered by the addition of 1.6 mM [14C]malonate and carried out for 1 min as described under “Experimental Procedures.” Data represent means of three to four incubations ± S.E.

### Table I

| Internal substrate | Malonate uptake ($\mu$mol/min/mg protein) |
|--------------------|------------------------------------------|
| None               | 71 ± 9                                   |
| Malonate           | 421 ± 53                                 |
| Malate             | 413 ± 37                                 |
| Succinate          | 331 ± 27                                 |
| Phosphate          | 289 ± 36                                 |
| α-Ketoglutarate    | 85 ± 5                                   |
| Citrate            | 62 ± 5                                   |
| Isocitrate         | 59 ± 4                                   |
| Phosphoenolpyruvate| 65 ± 2                                   |
| Pyruvate           | 50 ± 2                                   |
| ADP                | 78 ± 1                                   |

The specificity of the reconstituted, overexpressed DTP for external substrate was examined by measuring the ability of a high concentration (i.e. 20 mM) of unlabeled potential substrate to inhibit the [14C]malonate/malate exchange. As depicted in Table II, malonate, malate, phosphate, and succinate effectively inhibited radiolabeled malonate uptake, whereas phosphoenolpyruvate, citrate, isocitrate, α-ketoglutarate, pyruvate, and ADP did not. Thus, the overexpressed DTP displays the same specificity for external substrate as that observed with native DTP after purification and reconstitution in liposomes (20, 21, 23).

The sensitivity of the DTP to a variety of inhibitors was tested. Table III indicates that n-butylmalonate, a specific inhibitor of the mitochondrial DTP in yeast (15–18), as well as in higher eukaryotes (2, 3, 6), substantially inhibited malonate/malate exchange. In contrast, phenylsuccinate, 1,2,3-benzenetricarboxylate, and α-cyano-4-hydroxycinnamate, which selectively inhibit the α-ketoglutarate (28), citrate (29), and pyruvate (30) transporters, respectively, did not significantly inhibit DTP function. The sulfhydryl reagents pCMB (see “Experimental Procedures”) and mersalyl caused complete inhibition, whereas DTP function was unaffected by N-ethylmaleimide. Additionally, the lysine-selective agent pyridoxal 5'-phosphate partially inhibited DTP function. These findings are in complete agreement with data obtained with the native DTP in isolated mitochondria (2, 7–10), as well as with purified and reconstituted DTP (19–23), the sole exception being that the

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**Fig. 1.** SDS-PAGE profile of the expression of the yeast mitochondrial dicarboxylate transport protein in *E. coli*. Proteins were separated in a 4.5% polyacrylamide stacking gel followed by a 14–20% linear gradient gel (27). Lanes 1 and 8, 1.5 μl of Bio-Rad SDS-PAGE low range molecular weight standards. Lanes 2 and 5, 17 μg of *E. coli* cell protein harvested either 2 h following induction with isopropyl-β-D-galactoside (lane 2) or immediately prior to the induction (lane 5). Lanes 3 and 6, 2.7 μl of the inclusion body fraction originating from cells harvested either 2 h following induction (lane 3) or immediately prior to induction (lane 6). Lanes 4 and 7, 2.7 μl of the Sarkosyl-solubilized inclusion body fraction originating from cells harvested either 2 h following induction (lane 4) or immediately prior to induction (lane 7).

of U19028 displayed substantial dicarboxylate transport activity (note: S44213 displayed a very low dicarboxylate activity that was slightly over background (i.e. approximately 3% of the value obtained with U19028); the other expressed proteins did not catalyze any detectable dicarboxylate transport).

DTP overexpression is demonstrated by the SDS-PAGE profile, depicted in Fig. 1, of sequential steps in the procedure. Lane 2 demonstrates that 2 h following induction with isopropylthio-β-D-galactoside, harvested cells contained a prominent protein band with an apparent molecular mass of 32 kDa and that this band represents approximately 24% of total cellular protein, based on scanning densitometric analysis. Lane 3 shows that isolated inclusion bodies primarily contain the 32-kDa band. This protein is then effectively solubilized with the detergent Sarkosyl (lane 4), and its purity is approximately 75%. Lanes 5–7 indicate that immediately prior to induction, the 32-kDa band is absent from the harvested cell, the inclusion body, and the Sarkosyl-solubilized inclusion body fractions. In summary, the procedure described above yields 28 mg (average of several preparations) of the 32-kDa protein band per liter of *Escherichia coli* culture at a purity of 75%. Functional Characterization of the Overexpressed, Purified Yeast Dicarboxylate Transport Protein—To characterize the function of the abundantly expressed 32-kDa protein, we incorporated the Sarkosyl-solubilized inclusion body fraction (i.e. Fig. 1, lane 4) into phospholipid vesicles and measured the ability of the resulting proteoliposomes to catalyze pCMB-sensitive malonate/malate exchange (i.e. a defining reaction of the mitochondrial DTP). We observed that upon reconstitution, the Sarkosyl-solubilized inclusion body fraction catalyzed a pCMB-sensitive malonate/malate exchange with a specific activity of 879 ± 30 nmol/min/mg protein (reactions conducted at 30 °C). Importantly, incorporation into liposomes of the Sarkosyl-solubilized inclusion body fraction obtained from cells harvested immediately prior to induction, which did not contain the 32-kDa band (i.e. Fig. 1, lane 7), did not yield any detectable pCMB-sensitive malonate/malate exchange.

**TABLE I**

| Internal substrate | Malonate uptake ($\mu$mol/min/mg protein) |
|--------------------|------------------------------------------|
| None               | 71 ± 9                                   |
| Malonate           | 421 ± 53                                 |
| Malate             | 413 ± 37                                 |
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| α-Ketoglutarate    | 85 ± 5                                   |
| Citrate            | 62 ± 5                                   |
| Isocitrate         | 59 ± 4                                   |
| Phosphoenolpyruvate| 65 ± 2                                   |
| Pyruvate           | 50 ± 2                                   |
| ADP                | 78 ± 1                                   |

Lineweaver-Burk analysis (correlation coefficient = 0.994; data not shown). These values compare quite favorably with those reported for DTP purified from isolated yeast mitochondria (i.e. $K_m = 2$ mM; $V_{max} = 1.5 \mu$mol/min/mg; succinate/malate exchange) (23).

The specificity of the reconstituted, overexpressed DTP for external substrate was examined by measuring the ability of a high concentration (i.e. 20 mM) of unlabeled potential substrate to inhibit the [14C]malonate/malate exchange. As depicted in Table II, malonate, malate, phosphate, and succinate effectively inhibited radiolabeled malonate uptake, whereas phosphoenolpyruvate, citrate, isocitrate, α-ketoglutarate, pyruvate, and ADP did not. Thus, the overexpressed DTP displays the same specificity for external substrate as that observed with native DTP after purification and reconstitution in liposomes (20, 21, 23).

The sensitivity of the DTP to a variety of inhibitors was tested. Table III indicates that n-butylmalonate, a specific inhibitor of the mitochondrial DTP in yeast (15–18), as well as in higher eukaryotes (2, 3, 6), substantially inhibited malonate/malate exchange. In contrast, phenylsuccinate, 1,2,3-benzenetricarboxylate, and α-cyano-4-hydroxycinnamate, which selectively inhibit the α-ketoglutarate (28), citrate (29), and pyruvate (30) transporters, respectively, did not significantly inhibit DTP function. The sulfhydryl reagents pCMB (see “Experimental Procedures”) and mersalyl caused complete inhibition, whereas DTP function was unaffected by N-ethylmaleimide. Additionally, the lysine-selective agent pyridoxal 5'-phosphate partially inhibited DTP function. These findings are in complete agreement with data obtained with the native DTP in isolated mitochondria (2, 7–10), as well as with purified and reconstituted DTP (19–23), the sole exception being that the
overexpressed DTP is insensitive to phenylsuccinate. It is of interest to note that Lancar-Benba et al. (23) reported that phenylsuccinate caused a 57% inhibition of the purified native yeast DTP. The likely reason for this variation is the use of malonate as the transportable substrate in our studies (based on the fact that the DTP displays a higher affinity and greater specificity for this substrate (1, 19) rather than succinate.

Analysis of the Amino Acid Sequence of the Mitochondrial Dicarboxylate Transport Protein—The nucleotide sequence of the amplified, cloned DTP gene was determined (GenBank accession number U79459) and is identical to the sequence of a region of chromosome XII (GenBank accession number U19028). A detailed analysis of the deduced amino acid sequence of the overexpressed DTP was performed. The yeast mitochondrial DTP is comprised of 298 amino acids with a calculated mass of 32,991 Da. This molecular mass value is similar to the range of values (i.e. 32–35 kDa) for other mitochondrial transport proteins of known sequence from both higher and lower eukaryotes (for review see Refs. 26, 31). In common with other mitochondrial anion transport proteins, the DTP is quite basic displaying an isoelectric point of 10.5 and a net charge of +14.8 (pH 7.0). The polarity of the DTP is 40%, a value which is rather high for an integral membrane protein but which typifies the mitochondrial anion transporters (31).

As depicted in Fig. 2, dot matrix comparisons of the DTP sequence against itself at both moderate (A, window of 30 and stringency of 33) and high (B, window of 30 and stringency of 17) stringencies using the GCG programs. The hydropathy profile (C) was calculated by the method of Kyte and Doolittle (50) using the GCG PepPlot program and a window of 11 residues. The horizontal line at −0.4 indicates the average hydropathy of 84 sequenced soluble proteins (50). I–VI represent potential membrane-spanning α-helices.

Table II
The substrate specificity of the reconstituted dicarboxylate transport protein

| Competing anion | Activity remaining (%) | Inhibition of pCMB-sensitive malonate/malate exchange | % | S.E. |
|-----------------|------------------------|-----------------------------------------------------|----|-----|
| Malonate        | 14 ± 3                 | 62 ± 1                                               |    |     |
| Malate          | 15 ± 3                 | 62 ± 1                                               |    |     |
| Phosphate       | 23 ± 4                 | 62 ± 1                                               |    |     |
| Succinate       | 26 ± 1                 | 101 ± 7                                              |    |     |
| Phosphoenolpyruvate | 94 ± 4             | 102 ± 6                                              |    |     |
| Citrate         | 101 ± 7                | 102 ± 6                                              |    |     |
| Isocitrate      | 102 ± 6                | 102 ± 6                                              |    |     |
| ω-Ketoglutarate | 102 ± 5                | 104 ± 4                                              |    |     |
| Pyruvate        | 104 ± 4                | 108 ± 6                                              |    |     |

Table III
The effect of mitochondrial anion transporter inhibitors on the activity of the overexpressed, reconstituted dicarboxylate transport protein

The overexpressed DTP was incorporated into phospholipid vesicles as described in Table II. Proteoliposomes were preincubated with a given inhibitor for 1 min. Transport was then triggered by the addition of 0.3 mM [14C]malonate and quenched 1 min later with pCMB. In the absence of inhibitor, the pCMB-sensitive malonate uptake rate was 547 ± 28 nmol/min/mg protein. Data depicted below represent means of three incubations ± S.E.

| Inhibitor                  | Concentration | Inhibition of pCMB-sensitive malonate/malate exchange | % | S.E. |
|----------------------------|---------------|-----------------------------------------------------|----|-----|
| n-Butylmalonate            | 20            | 62 ± 1                                               |    |     |
| Phenylsuccinate            | 20            | 62 ± 1                                               |    |     |
| 1,2,3-Benzeneetricarboxylate | 20            | 62 ± 1                                               |    |     |
| α-Cyano-4-hydroxycinnamate | 0.3          | 62 ± 1                                               |    |     |
| Mersalyl                   | 0.3          | 101 ± 1                                              |    |     |
| N-Ethylmaleimide           | 1.0          | 62 ± 1                                               |    |     |
| Pyridoxal 5'-phosphate     | 10           | 49 ± 1                                               |    |     |

Fig. 2. Dot plot self-comparisons and hydropathy profile of the yeast mitochondrial dicarboxylate transport protein. The yeast DTP sequence was compared with itself at moderate (A, window of 30 and stringency of 33) and high (B, window of 30 and stringency of 17) stringencies using the GCG programs. The hydropathy profile (C) was calculated by the method of Kyte and Doolittle (50) using the GCG PepPlot program and a window of 11 residues. The horizontal line at −0.4 indicates the average hydropathy of 84 sequenced soluble proteins (50). I–VI represent potential membrane-spanning α-helices.

A search of the Swiss-Prot and the GenBank data bases was carried out in order to identify other sequences that may be
closely related to the DTP. The DTP sequence displayed the greatest similarity to DNA sequences encoding a protein of unknown function from a *Drosophila melanogaster* library (i.e. 43.0% identity over a 142-amino acid overlap; accession number L49195), the mitochondrial α-ketoglutarate carrier (e.g. the *Caenorhabditis elegans* mitochondrial transporter displays 40.4% identity over a 272-amino acid overlap; accession number X76114), and the mitochondrial uncoupling protein (e.g. the rat uncoupling protein displays 33.7% identity over a 272-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633). Lesser, but nonetheless significant, similarity was observed between the DTP and the yeast mitochondrial FAD (36.5% identity over a 74-amino acid overlap; accession number P40464), ADP/ATP 2 (26.8% identity over a 181-amino acid overlap; accession number P04633).

**DISCUSSION**

The present investigation has resulted in the first identification of a gene encoding the mitochondrial DTP from any organism. This represents a significant advance in that it (i) defines the primary structure of this metabolically important carrier, thereby enabling both a detailed analysis of the properties of this sequence, as well as a comparison to other mitochondrial transporter sequences; (ii) overcomes the chief impediment to investigating DTP function at the molecular level; and (iii) validates our approach to identifying mitochondrial transporter genes via overexpression and functional reconstitution of their protein products. In addition, the ability to obtain abundant quantities of purified, functional transporter now enables a variety of structure/function studies which here-tofore were not possible.

Our conclusion that the identified gene encodes the complete mitochondrial dicarboxylate transport system is based on several findings. First, the reconstituted DTP maintains a strict requirement for intraliposomal counteranion and thus catalyzes an obligatory exchange reaction (Table I). Malonate, malate, succinate, and phosphate support this exchange, whereas α-ketoglutarate, citrate, isocitrate, phosphoenolpyruvate, pyruvate, and ADP do not. These characteristics are identical to those observed with the native transporter following functional reconstitution of the purified protein (20, 21, 23). Second, the overexpressed DTP displays the same external substrate specificity profile as the internal profile described above, a property that is also characteristic of the native carrier in isolated mitochondria (1, 9) and the purified transporter (19, 20, 22, 23). Furthermore, neither α-ketoglutarate nor citrate can serve as substrates for the reconstituted transporter. This, coupled with the observation that certain dicarboxylates and phosphate are excellent substrates, clearly distinguishes the overexpressed DTP from the α-ketoglutarate and citrate transporters. Third, kinetic analysis of the reconstituted, overexpressed DTP demonstrates the functional competency of the overexpressed transporter and yields potassium and maximum values that compare quite favorably to those obtained with the purified yeast mitochondrial DTP (23).

Fourth, the overexpressed transporter can be inhibited by *n*-butyraldehyde (the classical inhibitor of the native DTP (2, 3, 6–18)) as well as by sulfhydryl reagents such as PCMB and mersalyl but is insensitive to *N*-ethylmaleimide. Thus, the overexpressed DTP displays an inhibitor sensitivity profile which is quite similar to that of the native carrier. Based on the above criteria, we conclude that the identified gene encodes the complete dicarboxylate transport system, which has been overexpressed in a form that retains its native functional properties.

Another significant point pertains to the evidence that the identified gene encodes a functional transporter. This evidence is 2-fold. First, based on the functional criteria described above, the overexpressed protein provides properties identical to those of the native transporter following functional reconstitution, the purified protein (20, 21, 23).

**FIG. 3. Alignment of the dicarboxylate transporter sequence with four other yeast mitochondrial transporter sequences.** The sequences of the yeast mitochondrial dicarboxylate transporter (abbreviated as DIC in this figure), phosphate carrier (PHOS), FAD transporter, ADP/ATP translocase (i.e. AAc transportor (CIT), corresponding to accession numbers U79459, J05302, L41168, J04021, and U17503, respectively, were aligned using the GCG PileUp program. The vertical order of the sequences was adjusted in order to place the dicarboxylate transporter sequence in the top row. A consensus sequence was derived based on the criteria that a given residue is either identical or conservatively substituted (indicated by an *asterisk*) in at least four out of the five sequences. Conserved residues were assigned based on the MDM* subtable (from Ref. 51). **Numbering** refers to the dicarboxylate transporter sequence.
Identification and Overexpression of the DTP Gene

overexpressed DTP is assigneable to the mitochondrial carrier family.

Several additional points regarding the strategy employed in this study should be noted. First, the fact that this approach has enabled identification of the transport functions encoded by two different yeast genes (i.e. the DTP and the mitochondrial citrate transport protein) demonstrates its widespread utility in determining the functions encoded by putative mitochondrial transporter genes from the fully sequenced yeast genome and possibly from the genomes of other organisms as well. Second, since several mitochondrial transporters have now been successfully overexpressed in E. coli, we believe that this approach will prove generally applicable with most of the mitochondrial anion transport proteins. Finally, the power of this strategy is further substantiated by the observation that purification of the DTP from isolated yeast mitochondria yielded 30 μg of protein (23). In contrast, overexpression results in an amount of protein that is 3 orders of magnitude greater.

In conclusion, identification of the yeast gene encoding the mitochondrial DTP, coupled with the overexpression and purification of its functional protein product, enables an array of structural studies to commence. These include the systematic and comprehensive use of site-directed mutagenesis to elucidate the structure/function relationships within the DTP, as well as the initiation of crystallization trials with this metabolically important transporter.

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