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Homodimeric Intrinsic Membrane Proteins. Identification and Modulation of Interactions between Mitochondrial Transporter (Carrier) Subunits

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Abstract

Transporter (carrier) proteins of the inner mitochondrial membrane link metabolic pathways within the matrix and the cytosol with transport/exchange of metabolites and inorganic ions. Their strict control of these fluxes is required for oxidative phosphorylation. Understanding the ternary complex transport mechanism with which most of these transporters function requires an accounting of the number and interactions of their subunits. The phosphate transporter (PTP, *Mir1p*) subunit readily forms homodimers with intersubunit affinities changeable by mutations. Cys28, likely at the subunit interface, is a site for mutations yielding transport inhibition or a channel-like transport mode. Such mutations yield a small increase or decrease in affinity between the subunits. The PTP inhibitor N-ethylmaleimide decreases subunit affinity by a small amount. PTP mutations that yield the highest (40%) and the lowest (2%) liposome incorporation efficiencies (LIE) are clustered near Cys28. Such mutant subunits show the lowest and highest subunit affinities respectively. The oxaloacetate transporter (*Oac1p*) subunit has an almost 2-fold lower affinity than the PTP subunit. The *Oac1p*, dicarboxylate (*Dic1p*) and PTP transporter subunits form heterodimers with even lower affinities. These results form a firm basis for detailed studies to establish the effect of subunit affinities on transport mode and activity and for the identification of the mechanism that prevents formation of heterodimers that surely will negatively impact oxidative phosphorylation and ATP levels with serious consequences for the cell.

Keywords

Mitochondria; Carrier; Transporter; Reconstitution; Dimer; Affinity

Introduction

Complexes of same proteins occur for water-soluble proteins. Each subunit of hemoglobin, for example, has the prosthetic group (heme) essential for oxygen binding. Yet it is the tetramer ($\alpha\beta$)₂ that provides characteristics essential for the physiologically relevant oxygen transport. Membrane proteins often also have functions that require them to exist as homomultimers in the membrane, e.g. four same subunits line the K⁺ channel [1]. Three same subunits of the Na

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+/-betaine symporter BetP (each subunit with its own transmembrane transport path) interact and couple their transport cycles to achieve highly effective osmoregulated uptake of glycine betaine [2]. It is not yet clear why such coupling is necessary. With other membrane proteins the purpose of their homooligomeric structure is also not clear. In aquaporin channels [3] and ClC chloride channels [4] the atoms of a single protomer form water and ion conduction pathways yet these transport proteins form tetramers and dimers, respectively. The human voltage-dependent proton channel Hv1 contains only a voltage-sensor domain, lacking a separate pore domain. This proton channel exists as homodimer within the membrane and the reason for this dimeric structure is not clear [5].

Many mitochondrial transport (carrier) proteins appear to function as homodimers [6]. Inhibitors of these transporters seem effective at one mole per two moles of transporter subunit [7;8]. Purified and reconstitutively-active transporters are homodimers [9;10;11]. Such homodimer as functional entity has been challenged [12]. Quantitation of the transporter subunits in the mitochondrial membrane or in purified preparations is challenging since the mitochondrial membrane contains many other similar proteins and these subunits when purified are complexed with a mass of detergent equal to their own. Nevertheless PTP subunits interact in the reconstituted mixture of inhibited and uninhibited subunits since the transport activity is lower than expected [13]. Kinetic studies of many of these mitochondrial transporters support a ternary complex transport mechanism with simultaneously accessible internal and external binding sites [14;15]. Such a mechanism can more readily be accommodated by a dimeric transporter. The only high resolution structure of a member of this transporter family is that of a single subunit of the bovine heart ADP-ATP carrier complexed with the high affinity inhibitor carboxyatractyloside (CAT) [16]. It consists of a funnel lined by six transmembrane helices, i.e. possibly a self-sufficient substrate path through the membrane [17;18]. Another structure of this bovine transporter suggests two potential subunit interfaces, one of which includes a cardiolipin molecule [19]. Other indirect evidence suggests that this transporter from yeast could function as a monomer [20].

Results in this communication show that the purified and reconstitutively transport competent subunit of PTP readily forms dimers. Such homodimer formation has been assayed with subunits whose mutations affect liposome incorporation efficiency (LIE), mode of transport or that have been reacted with a covalently bound transport inhibitor. In addition, heterodimer and homodimer formation has been characterized with subunits of other members of this transporter family.

Materials and Methods

Construction of Expression Vectors

Single site mutants were constructed by introducing mutations into the yeast PTP bacterial expression vector [21] using the QuikChange Site-Directed mutagenesis kit (Stratagene). To some of the subunits five His residues were added to the C-terminal His as affinity tag (Htag). Mutagenesis primers (PAGE purified) and sequencing primers (salt free) were obtained from Operon. The whole PTP gene of the prepared plasmids (Qiagen midiprep kit) was sequenced (Sequen). Genes of the dicarboxylate transporter DIC1 and the oxaloacetate transporter OAC1 were obtained as FLEXGene clones (ScCD00024031 (DIC1, YLR348C) and ScCD00023645 (OAC1, YKL120W)) from the Harvard Institute of Proteomics. They were cloned into the pDEST14 bacterial expression vector using Gateway Technology (Invitrogen). A Shine-Dalgarno consensus sequence (GAAGGAG) was cloned into the DIC1 expression plasmid 9 bases upstream of the transporter gene initiation codon ATG. The same construct was prepared for OAC1 except the Shine-Dalgarno sequence used was CAAGGAG.

Protein expression

The transporter subunits were expressed in E.coli BL21 (D3), purified, and the inclusion bodies pellets stored as small aliquots at -80°C as described [21].

Dimer Formation Assay

The dimer formation assay is part of the optimized standard procedure used for reconstituting transport activity of yeast transporter proteins expressed as bacterial inclusion bodies and using the nonionic detergent octaethyleneglycol mono-n-dodecylether (C_{12}E_8) (Anatrace) [13]. The inclusion bodies pellet [21] was solubilized with 1.18% sodium sarkosyl (Fluka) in TE (10mM Tris.HCl, 0.2mM EDTA, pH7.0) to dissociate the subunits and to minimize protein unfolding. The protein suspension was thoroughly vortexed for seven minutes at room temperature. This was carried out with the Htag subunits and the subunits without Htag (nHtag) [22]. Htag subunits show normal reconstituted phosphate transport activity [13]. The two different subunit solutions were mixed in the desired ratio, diluted to 0.42% sodium sarkosyl and submitted to 160,000xg centrifugation (50K rpm, 50Ti Beckman Coulter rotor) for 50min at 4°C . The supernatant (50K supernatant) was applied to a Bio-Gel P-6 DG (Bio-Rad) gel filtration column (4cm $\times$ 4mm) that had been equilibrated with 2% C_{12}E_8 in MSB (25mM PIPES, 300mM NaCl, pH7.0) [13]. This detergent exchange step was followed by an equilibration with Talon affinity beads (Clontech) for 60 minutes at 4°C on a labquake rotation shaker. The beads were washed with 2% C_{12}E_8 and 10mM imidazole in MSB medium and the transporter subunits eluted with the same medium and 200mM imidazole. The protein fractions were analyzed with high resolution SDS PAGE [23] and the gel stained with Coomassie Blue. Typical total subunit protein concentration was 0.25 mg/ml in the first dimer mixture after inclusion bodies solubilization with sarkosyl. Subunit protein concentrations were determined from a series of human carbonic anhydrase concentrations (to cover the wide and not completely linear O.D. range) on the same SDS PAGE gel such that staining time with Coomassie Blue (overnight) and destaining time (60min) was the same for the transporter subunits and the reference protein. The gels were dried between cellophane sheets and optically scanned. The protein concentrations were quantitated with the Scion Image program.

Liposome incorporation efficiency (LIE)

The LIE is the percentage of subunit protein in the reconstituted liposomes compared to the amount of protein in the protein / lipid mixture before detergent removal to form proteoliposomes [24]. Protein were quantitated by optically scanning the silver stained SDS PAGE gels.

Results

PTP Subunit Homodimer Formation

Fig. 1 shows various protein fractions of the subunit interaction assay. Since the nHtag subunit competes with the Htag subunit to achieve a nHtag:Htag dimer, a higher concentration of the nHtag subunit has to be present in the 50K supernatant to yield only nHtag:Htag dimers in the Talon eluate. Fig. 1B shows that the nHtag subunit does not bind to the Talon beads. Fig. 2 shows the ratio of subunits in the 50K supernatant compared to that in the Talon beads eluate. While the nHtag subunit must bind to the Htag subunit to be present in the Talon beads eluate, Htag subunits can bind to the Talon beads either as Htag subunit monomer or dimer. Fig. 2 shows that to have 50% of the wild type Htag subunits that elute from the Talon beads complexed with a nHtag subunit, the ratio of nHtag subunits to Htag subunits in the 50K supernatant must be about 3.2 (see also Fig. 4). The ratio of the two subunits in the Talon beads eluate increases to a value somewhat larger than the expected 1.0 as the subunit ratio in the 50K supernatant is increased. Such binding probably is due to low affinity sites between the

subunits and is unlikely to be physiologically relevant. Experiments were carried out to assay sarkosyl's ability to selectively eliminate this apparent low affinity binding. As expected, sarkosyl cannot selectively suppress such low affinity binding without also affecting the high affinity binding between the subunits. Such low affinity (low specificity) binding can likely be eliminated by phospholipids or protein(s) intrinsic to the inner membrane.

Dimer Formation of Mutant PTP Subunits with Extreme LIE

It has been demonstrated that single mutations can have a dramatic impact on PTP's LIE, i.e. the percentage of transporter subunits in the reconstitution mix that incorporates into the liposomes used to assay transport activity [25]. It was hypothesized that such mutations yield homodimers of a range of affinities between subunits. If such mutations indeed do affect the affinity between the subunits, then they could be part of a new and novel strategy for investigating the effect of membrane protein interactions on protein function. The LIE of the wild type PTP subunit is about 15%. Almost all the PTP subunits constructed with a single mutation (110 constructs [24]) and that were characterized were replacements with Ala. Some however had conservative replacements. Two mutants (Glu95Gln, Arg159Ala) with lowest LIE (about 2%) and one (Ser285Ala) with highest LIE (about 40%) were assayed for subunit affinities. The mutations of these three mutants as well as the mutations of another six mutants with clearly the highest LIE and four with the lowest LIE [24] are shown in Figure 3.

Fig. 2 and Fig. 4 show that the affinities between PTP subunits with the highest LIE mutation Ser285Ala is much lower than between the wild type PTP subunits. But the two mutations with the lowest LIE (Glu95Gln, Arg159Ala) increase the affinity between the subunits. When the yeast PTP sequence is threaded (<http://bioinf.cs.ucl.ac.uk/psipred/>) upon the high resolution bovine ADP-ATP carrier / CAT complex structure [19] Ser285 locates to the region between matrix helices h12 and h34. This region of the protein is part of the likely region of contact between the transporter subunits (Fig. 3) [19;26]. Another region of contact that includes cardiolipin has been suggested [19]. Cardiolipin is not essential for the physiological competence of the yeast ADP-ATP carrier but is important for its function and stability [27]. The bovine ADP-ATP carrier has three cardiolipin high affinity binding sites and its reconstituted activity depends on cardiolipin [28]. There is no evidence that yeast PTP has a function- or structure-relevant role for cardiolipin. Five of the other mutations that yield PTP subunits with the highest LIE are also at hydroxyl amino acid sites (Thr79, Ser84, Ser145, Ser293, Thr297); the sixth (His311) is its C-terminal amino acid. These six mutation sites are clustered in the region of the transporter subunit structure between helices h12 and h34, i.e. the likely transporter subunit region of contact. PTP's Thr297 maps onto the ADP-ATP carrier's Phe296 and is thus beyond the C-terminal Ile293 of the subunit's high resolution structure [16]. PTP's Ser293 maps onto the ADP-ATP carrier's penultimate C-terminal residue Glu292. The C-terminal Ile293 is located in the subunit structure region between h12 and h34 (Fig. 3).

The seven mutants with highest LIE have all near normal phosphate transport activities except for the Thr79Ala mutant. Thr79 is part of a motif of hydroxyl / basic residues that line the same side of transmembrane helix H2. A disruption of this motif is deleterious to transporter activity [24] and as suggested here also to the affinity between subunits. The lack of effect on transport activity by the decrease in intersubunit affinity caused by the mutation S285A (and probably the other five mutations) may have two reasons. First the transport activity as measured could have as major component the channel-like activity mode [29] and second the subunits of the dimeric transporter in the membrane, surrounded by phospholipids, may still be sufficiently close in this instance to permit the subunit / subunit interactions required for transport.

Dimer Formation by PTP Cys28 Mutants

Most mitochondrial transporters catalyze heterologous metabolite exchange like aspartate / glutamate (antiport) or homologous P_i^- / P_i^- exchange (antiport) or P_i^- and H^+ cotransport (symport) as catalyzed by PTP. PTP upon exposure to mercurials switches to a transport mode of unidirectional and somewhat substrate unspecific transport, i.e. net phosphate flux (no proton gradient (symport); no transmembrane P_i^- (antiport)). $HgCl_2$ induces this shift by reacting only with Cys28 [29]. A transport-inhibiting disulfide, reversible with dithiothreitol (DTT), can form between the two Cys28 of the homodimer [26]. Replacement of both Cys28 with Lys (or Glu) (mercurial-like effect) should induce a channel-like transport mode. Replacing one Cys28 with Lys and the other with Glu (simulating the disulfide effect with a salt bridge) should block transport. This indeed is observed (unpublished observation). Do these mutations alter the affinity between the subunits? Fig. 4 shows that the affinities are altered even though the changes are small. Transport thus appears to require a dynamic interaction between the two H1 transmembrane helices. Locking them into relative positions blocks transport and pushing them apart induces channel-like activity, i.e. the transport cycles of the two subunits are uncoupled from each other. This model appears to require only a mutation like Cys28Lys in each subunit with a resulting small decrease in subunit affinity.

Interactions between Subunits of Other Mitochondrial Transporters

Evidence based on native gel electrophoresis suggests that during biosynthesis the yeast PTP forms homodimers as the subunits are inserted into the membrane (stage V) [30]. Step(s) must exist in this biosynthetic process that guarantees the formation of homodimers. Are sequence similarities between members of the mitochondrial transport protein family [31] conserved such that substantial affinities between subunits of different transporters exist? If so then the biosynthetic steps have to control the composition of the dimers.

The OAC1 transporter catalyzes the proton cotransport or the exchange of the anions malonate, oxaloacetate, sulfate and thiosulfate [32]. The DIC1 transporter catalyzes the exchange of malonate, malate, succinate and phosphate [33]. Both are of interest here because DIC1 catalyzes P_i^- / P_i^- exchange like PTP and OAC1 catalyzes H^+ and anion cotransport like PTP and the subunits of both transporters have been reconstituted from bacterial inclusion bodies. Fig. 4 shows that the subunits from the different transporters do have affinity for each other yet they are lower than those of the PTP subunits. Fig. 4 also shows that heterodimers between PTP and either DIC1 or OAC1 subunits show low affinity for each other, underscoring the significance of the dimer composition control steps during biosynthesis.

The residues replaced in the single mutation sites of the seven highest LIE PTP mutants (Fig. 3) are hydroxyl amino acids (except for the C-terminal His311). Residues occupying these locations in the Oac1p subunit (corresponding PTP residues in parentheses) are Ala90 (Thr79), Ile95 (Ser84), Ser158 (Ser145), Thr299 (Ser285), Gln308 (Ser293), and Leu312 (Thr297). The equivalent His311 of PTP is beyond the C-terminal of the threader-aligned Oac1p sequence. If PTP's six hydroxyl residues have a direct / indirect role in subunit affinity, then it is not unreasonable for the Oac1p subunits with only two hydroxyl residues to have a lower affinity.

Discussion

We have documented that the PTP subunit, optimized for reconstitution with the detergent $C_{12}E_8$, forms homodimers during steps that lead to reconstituted transport (Fig. 1 and Fig. 2). The affinity between the subunits can be increased or decreased with single site mutations that yield LIEs much lower or higher, respectively, than that of the wild type PTP subunit (Fig. 4). The specific transport activities of the six PTP mutants with the lower affinities (highest LIE) are on the average 1.7 fold higher than the six mutants with the higher affinities (lowest LIE).

At this time there is no obvious explanation for this observation. The symport transport assay used does not exclude the possibility that part may be channel-like / H^+ gradient-independent P_i^- transport. This mode of transport has been suggested to arise as helices H1 of the homodimer are somewhat pushed apart, i.e. upon reacting Cys28 with $HgCl_2$ [29] or by replacing Cys28 with a Lys or a Glu. Such mutations do lower the affinity (Fig. 4).

Reconstitution experiments of the type that first suggested interacting transporter subunits as functional entity [13] have to be interpreted with caution, i.e. high affinity subunits (due to mutation or inhibitor) may not readily form heterodimers. Fig. 4 however shows that the NEM-PTP subunit affinity barely differs from that of the unreacted PTP and thus the conclusion of the experiment are valid. Such an experiment was recently used to demonstrate a lack of subunit interaction in the functional yeast ADP-ATP carrier (AAC2) [20]. One of the subunits had all four of its Cys replaced causing likely a change in its affinity and introducing uncertainty into the interpretation of the results.

Very little data is available on residues at the subunit interface [6]. Cys28 of yeast PTP is likely located at the subunit interface. Residues that upon replacement yield extremes in LIE do cluster near Cys28 (Fig. 3). These novel results present an opportunity to probe the relationship between subunit affinities, transport activity and mode of transport. If such LIE-changing mutations are tolerated by the yeast biosynthetic machinery then some of them might act as dominant lethals since channel-like P_i transport would be detrimental to oxidative phosphorylation.

Our results demonstrate for the first time that subunits of different transporters can have rather big differences in their affinity for each other. And this suggests that among the 34 transporters of *S.cerevisiae* or the possibly 60 of *H.sapiens* subunit affinities could range from very high to none. Our results also demonstrate that dimers can form between subunits of different transporters. Such heterodimers could pose a serious challenge for the cell since it is critically important to meticulously control, coordinate, and couple the fluxes of charged metabolites across the mitochondrial inner membrane to satisfy the requirements of the mitochondrial matrix and to preserve the proton motive force essential for the chemiosmotic mechanism of oxidative phosphorylation. It will be of interest to detail the biosynthetic steps that prevent formation of heterodimeric transporters.

Abbreviations

CAT	carboxyatractyloside
PTP	phosphate transport protein
LIE	liposome incorporation efficiency
Htag	C-terminal six histidine tag
nHtag	no C-terminal six histidine tag
NEM	N-ethylmaleimide

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Figure 1.

SDS PAGE gel of PTP subunit preparations used to assay dimer formation. (A)(1) wild type subunits, (2) wild type subunits (Htag), (3,4) subunits with Htag and without Htag (nHtag) in 50K supernatant (two different concentrations), (5,6) subunit mixture after gel filtration (two different concentrations), (7) nHtag subunits not bound to Talon beads, (8,9) subunits eluted from Talon beads (two different protein concentrations). (B) wild type PTP nHtag subunits in all lanes, (2) before 50K centrifugation, (3,4) in 50K supernatant, (5,6) after gel filtration, (7) not bound to Talon beads, (8,9) eluted from Talon beads (two different volumes of fraction).

Figure 2.

Interactions of PTP subunits. Ratio of nHtag subunits to Htag subunits in the 50K supernatant and in the Talon beads eluate. Wild type (●), Ser285Ala mutant (□), Glu95Gln mutant (△), Arg159Ala mutant (◇) subunits.

Figure 3.

Location of single mutations with large impact on PTP subunit dimer formation. The PTP sequence was projected upon the bovine ADP-ATP carrier subunit / CAT complex structure (Protein Data Bank 2C3E with Cn3D) (<http://www.ncbi.nlm.nih.gov/>). (A) View from the matrix and (B) view from the plane of the membrane. The short helices h12 (yellow), h34, and h56 are indicated. The loop (yellow) centered in the narrow part of the funnel-like structure is not present in the PTP sequence. Mutation sites (yellow) yielding lowest LIE have purple and blue (assayed) labels and highest LIE have red and green (assayed) labels.

Figure 4.

Affinities of various transporter subunits. PTP mutants (1) Glu95Gln, (2) Arg159Ala, (3) wild type, (4) wild type inhibited with N-ethylmaleimide, (5) Ser285Ala, (7) Cys28Glu, (8) Cys28Lys, (9) Cys28Glu / Cys28Lys. (11) Oac1p, (12) Oac1p / wild type PTP, (13) Dic1p / wild type PTP. A forward slash (/) indicates that two different subunits form the dimer. Oac1p is the subunit of the oxaloacetate transporter and Dic1p is the subunit of the dicarboxylate carrier.