The Phosphate Carrier from Yeast Mitochondria

DIMERIZATION IS A PREREQUISITE FOR FUNCTION*

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Wild type phosphate carrier (PIC) from Saccharomyces cerevisiae and recombinant PIC proteins with different C-terminal extensions were expressed in Escherichia coli as inclusion bodies. From these, PIC was isolated with the detergent sodium lauroyl sarcosinate in a form, partially monomeric and unfolded. This PIC associates to stable dimers after exchanging the detergent to the polyoxyethylene detergent $C_{12}E_8$ and dialysis. Combining two differently tagged monomers of PIC and following this with affinity chromatography yields defined homo- and heterodimeric forms of PIC, which are all fully active after reconstitution. As a member of the mitochondrial carrier family PIC is supposed to function as a homodimer. We investigated its dimeric nature in the functionally active state after reconstitution. When reconstituting PIC monomers a sigmoidal dependence of transport activity on the amount of inserted protein is observed, whereas insertion of PIC dimers leads to a linear dependence. Heterodimeric PIC constructs consisting of both an active and an inactivated subunit do not catalyze phosphate transport. In contrast, reconstitution of a mixture of active and inactive monomeric subunits led to partially active carrier. These experiments prove (i) that PIC does not function in monomeric form, (ii) that PIC dimers are stable both in the solubilized state and after membrane insertion, and (iii) that transport catalyzed by PIC dimers involves functional cross-talk between the two monomers.

The mitochondrial phosphate carrier (PIC)\(^1\) or phosphate transport protein (PTP) catalyzes transport of phosphate into the mitochondrial matrix where the phosphate is utilized for oxidative phosphorylation (1–6). The primary structure of the beef heart PIC was elucidated by protein (7) and DNA/protein sequencing (8) and the PIC gene was cloned and sequenced from Saccharomyces cerevisiae (9). The yeast PIC has been expressed as inclusion bodies in Escherichia coli (10, 11). Methods have been described to solubilize mitochondrial carriers from inclusion bodies including PIC in a functionally active state (11–13).

PIC is a typical member of the structural family of mitochondrial carriers with subunits of six transmembrane segments and a molecular mass of 32 kDa (14, 15). There are several lines of evidence that mitochondrial carriers do not function as monomers but form dimers in the functional state. The first and still one of the most convincing indications up to now was the observation of a binding stoichiometry of one molecule of the tightly binding ligand carboxyatractylate to two monomeric units of the ADP/ATP carrier (16). An even lower binding stoichiometry was observed for the ligands ADP and ATP (17), which in experiments with fluorescent nucleotide analogs led to the suggestion of a tetrameric functional unit of the ADP/ATP carrier (18). By using cross-linking and analytical ultracentrifuge techniques it was shown that the ADP/ATP carrier as well as the mitochondrial uncoupling protein, at least in the solubilized state, forms a homodimer (19, 20). In recent experiments the formation of an intermolecular disulfide bridge between the monomers of the ADP/ATP carrier provided further evidence for the dimeric state of this mitochondrial carrier (21). Studies with PIC in mitochondria demonstrated the requirement of less than one NEM per subunit of PIC (22) and in reconstituted proteoliposomes that a disulfide between Cys-28 of the two monomers reversibly blocks transport (23). Besides these findings, it has been argued also on a theoretical point of view that a dimeric state is favorable for carrier function (24). It is noteworthy that there is a further reason for the acceptance of the dimeric nature of mitochondrial carriers, namely the “consensus minimal unit” of about 12 transmembrane segments which holds true for many carrier proteins (25–27).

The oligomeric state of secondary carriers has been investigated in a number of cases. Evidence has been provided for both the monomeric and the dimeric form of the E. coli lactose permease to be functional (28). However, the dominating evidence suggests that lactose permease is functional as a monomer with 12 transmembrane segments (28). The situation is not better understood for other well studied carriers. Mammalian facilitative sugar carriers, i.e. unceptors (GLUT-family) and Na−-coupled symporters (SGLT-family), were found to function both as monomers and oligomers, and cooperative interactions were suggested as a regulatory mechanism (29–31). There is experimental evidence that also the erythrocyte anion transporter (band 3 protein) may exist as a mixture of dimers and tetramers (32–34), but evidence for a monomeric function of this protein has also been provided (35). Recently, by coexpression and co-reconstitution of functional and non-functional monomers of the small secondary carrier EmrE, the oligomeric state of this protein has been demonstrated (36). However, several of the methods used to prove oligomeric associations in these proteins may be questioned (37), and con-

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† The abbreviations used are: PIC, phosphate carrier; $C_{12}E_8$, octyl polyoxyethylene; $C_{4}E_{6}$, dodecyl octaoxyethylene; DTT, dithiothreitol; NEM, N-ethylmaleimide; SLS, sodium lauroyl sarcosinate; PIPES, 1,4-piperazinediethanesulfonic acid; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.
The supernatant was discarded. Finally the pellet was solubilized in TE buffer containing 0.1 m M EDTA, 1 m M DTT, adjusted to pH 7.0 with HCl) and stored at 20 °C. All the following steps were carried out on ice (11).

EXPERIMENTAL PROCEDURES

Materials—Enzymes were of analytical grade. All sulfhydryl reagents used were prepared freshly. The reagents were diluted with water or the respective gel filtration buffer. Pyridoxal phosphate in high concentrations was dissolved in 1% imidazole (pH 6.5).

Generation of Tagged Carrier Proteins—Cloning of DNA and subsequent transformation steps were carried out using standard techniques (38, 39). The 3' part of the mir gene coding for the phosphate carrier was amplified by polymerase chain reaction using two oligonucleotide primers annealing upstream (5'-GAGTCTGGTTTGGC-3') of the KpnI site and downstream of the 3' part thereby including the FLAG tag (tags underlined), respectively, and a BamHI restriction site downstream from the stop codon. Polymerase chain reaction (30 s 94 °C, 30 s 50 °C, 60 s 72 °C, 30 cycles) was carried out using Taq polymerase (Boehringer, Mannheim) and a Thermocycler (Perkin-Elmer). Plasmid pNYHM131 (13, 40) was used as template. The polymerase chain reaction products were cut with BamHI and KpnI and cloned into the plasmid pUC18 for sequencing. Sequencing was carried out using a Pharmacia (Freiburg, Germany) A.L.F. DNA sequencer and the AutoRead sequencing kit (Pharmacia, Freiburg, Germany) as recommended by the supplier. Appropriate fragments were subsequently cloned into plasmid pNYHM131 via BamHI and HindIII sites. All cloning steps were carried out in the E. coli strain DH5α. The expression of the different proteins was carried out in E. coli strain BL21 (DE3) as described below.

Isolation and Purification of the PIC—Expression strain BL21 (DE3) carrying plasmids coding for the wild type PIC or a mutant, respectively, was transformed. A total of 1 liter of 2YT medium (plus 100 μg of carbenicillin) was inoculated with a fresh overnight colony of transformed BL21 (DE3) and grown to an OD600 of 0.6 (about 5 h) under constant aeration. The color reaction was initiated by adding freshly prepared 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT reagent) solution. The reaction was stopped after 10 min by adding 10 mM EDTA.

Reconstitution Procedure—PIC was reconstituted into pre-formed phospholipid vesicles by using the ambrilamide method (42). This method was modified (13) with regard to the applied phospholipid/protein and phospholipid/phosphoprotein ratio. Maximum exchange rates were obtained with a phospholipid concentration of 16 mg/ml, a phospholipid/protein ratio of 6.25 μg/mg, and a detergent/phospholipid ratio of 0.62 mg/ml. This means that 70 μl of Triton X-114 (10%, v/v), 112 μl of preformized liposomes (10% of egg yolk phospholipids in 50 mM KC1, 20 mM HEPES, 20 mM EDTA, pH 6.5), 20 μl of protein solution; HEPES (pH 6.5, final concentration 50 mM) and phosphate (final concentration 30 mM) were added up to 700 μl. A detergent/ammonium ratio of 12 mg/g in combination with 15 column passages was used to remove the detergent (43).

Measurement of Transport Activity and Calculation of Rates—The methods were identical to those described for the analysis of the aspartate/glutamate carrier (44, 45) and PIC (13, 46). Transport activity was determined using forward exchange experiments (44). The assay was started by adding labeled substrate. The time course of isotope equilibration was fitted to the data points according to a single exponential function (ln y = -kt + b) which delivered the apparent first order constant k (min⁻¹). The specific activity (μmol/min · mg of protein) was calculated from k (min⁻¹), from the final value of the isotope equilibration (dpm), the specific radioactivity (dpm/nmol), the volume of the proteoliposome fraction (ml), and the protein concentration (μg/ml) as published previously (44).

RESULTS

Construction of Different Monomers—In order to be able to monitor the formation of defined dimeric proteins, the individual monomers and the different types of dimers must be experimentally distinguishable. In the solubilized state mitochondrial carriers are assumed to be dimers of identical monomers. PIC monomers had thus to be rendered different artificially. For this purpose, we constructed monomers of the wild type...
PIC was solubilized in SLS and dialyzed in the presence of different detergents as described under “Experimental Procedures.” The protein recovery is normalized to the protein concentration before dialysis. Transport activity was determined as homologous phosphate/phosphate exchange in forward exchange experiments.

Reconstitution of Solubilized PIC from Different Association States—We did not succeed in directly reconstituting PIC solubilized from inclusion bodies in SLS as the only detergent, however, after the simple addition of appropriate other detergents, reconstitution of the SLS-solubilized PIC was successful (see “Experimental Procedures and Table II”). For proper formation of dimers and for optimum reconstitution, we tried several detergents for replacing SLS as the solubilizing agent, some of which are listed in Table I. When considering both protein recovery after the dialysis step, in which protein is lost due to aggregation, as well as the specific transport activity obtained after reconstitution of the dried protein, the polyoxyethylene detergent C12E8 proved to be most favorable. Consequently, this detergent was used in all further experiments. The various constructs and combinations were all active when reconstituted from preparations in which C12E8 was used as detergent (cf. Fig. 3 and Table II).

Detailed studies on the mitochondrial ADP/ATP carrier and uncoupling protein using analytical ultracentrifugation techniques have shown that these mitochondrial carrier proteins exist as dimers after solubilization with Triton X-100 (19, 20). Mitochondrial carriers used for these studies were isolated from intact mitochondria. The presence of monomers of these transport proteins has only been demonstrated in SDS gels. It is not known, however, under which conditions these dimers can be formed. The experimental strategy used in the present study could be applied due to the fact that we found that PIC was in a monomeric state when solubilized from E. coli inclusion bodies using SLS. This was established by several kinds of experiments.

By gel electrophoresis using SLS as detergent instead of SDS, we showed that PIC, when solubilized from inclusion bodies, is in monomeric form (about 30 KDa), just like the protein in SDS gels (Fig. 1). Although addition of the polyoxyethylene detergent C12E8 significantly improved the reconstitution of PIC, it did not change its behavior in the non-denaturing SLS gel. After dialysis and detergent exchange to C12E8, the apparent molecular weight in SLS was significantly increased, although not as much as would have been expected for a dimer. Besides the observation of an increased apparent molecular weight in SLS gels, however, the oligomeric (dimeric) state of PIC after dialysis was proven by the fact that stable homo- and heterodimers could be formed under these conditions (see below). It was not possible to reconstitute PIC in functionally active form from the SLS-solubilized state directly (experiments not shown). This, however, was achieved by using SLS-solubilized protein after addition of nonionic detergents (see below). The conformational state of PIC in SLS micelles after solubilization from inclusion bodies was investigated using attenuated total reflection Fourier transform infrared spectroscopy. This lead to an estimation of the α-helix content which is clearly too low (experiments not shown) when compared with results obtained for the purified ADP/ATP carrier by CD spectroscopy (47). A further experimental indication for PIC being in an unfolded state when solubilized in SLS is the drastically changed accessibility of cysteines to the alkylating reagent NEM in comparison to PIC in functionally active form (see below). It is noteworthy, that by cross-linking studies applying intermolecular disulfide bridges it was found, that the ADP/ATP carrier is not in the correctly folded state even when solubilized in Triton X-100 (21).

An interesting reconstitution experiment corroborated our findings of SLS-solubilized PIC being in the monomeric state as obtained by gel electrophoresis. In Fig. 2, the observed absolute transport activity of the reconstituted PIC protein is correlated to the amount of PIC incorporated into a constant amount of phospholipid. A linear dependence at low protein/phospholipid ratios for this kind of experiment has been found for many mitochondrial carriers (5, 6) and other secondary transporters, too (48). This is also true for PIC, when isolated from intact mitochondria (Fig. 2). The same result holds for PIC isolated from inclusion bodies when the detergent SLS has been exchanged for the polyoxyethylene detergent C12E8. Interestingly, PIC solubilized from inclusion bodies by SLS in the presence of added C12E8, i.e. before removal of the detergent SLS, showed a completely different pattern. At low PIC/phospholipid ratios, the inserted PIC protein was not active in

| Detergent          | Recovery % | Transport activity μmol/min·mg protein |
|--------------------|------------|----------------------------------------|
| Triton X-114       | 60 ± 15    | 144 ± 18                               |
| Triton X-100       | 45 ± 8     | 108 ± 21                               |
| C12E8              | 12 ± 5     | 36 ± 8                                 |
| C12E8              | 82 ± 10    | 180 ± 25                               |
| Dodecyl maltoside  | 74 ± 12    | 54 ± 16                                |

| PIC construct      | Detergent | Transport activity μmol/min·mg protein |
|--------------------|-----------|----------------------------------------|
| [PIC]              | SLS + C12E8 | 124 ± 13                             |
| [His-PIC]          | SLS + C12E8 | 110 ± 16                             |
| [FLAG-PIC]         | SLS + C12E8 | 95 ± 10                              |
| [His-PIC]2         | C12E8      | 180 ± 25                             |
| [FLAG-PIC]2        | C12E8      | 159 ± 18                             |
| [His-PIC/FLAG-PIC] | C12E8      | 168 ± 14                             |

**TABLE I**

Protein recovery and phosphate transport activity after reconstitution of the wild type PIC from different detergents

**TABLE II**

Transport activity of different PIC constructs

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**Fig. 1. Gel electrophoresis of PIC in different states of aggregation.** A, non-denaturing conditions in SLS (12% gel with 10% glycerol, 0.1% SLS, Coomassie staining). B, denaturing SDS-gel electrophoresis (12% SDS-polyacrylamide gel electrophoresis, Coomassie staining). First and fifth lanes, molecular weight standards; second lanes, PIC after dialysis in C12E8; third lanes, PIC in SLS with added C12E8; fourth lanes, PIC in SLS.
phosphate transport, only after a significant amount of PIC has been added was a sharp (sigmoidal) rise of the reconstituted carrier activity observed. In order to be sure that the sigmoidal dependence was not caused by an improper insertion of PIC into the proteoliposomes at low protein/lipid ratios, we applied the proteoliposomes containing His-PIC to polyvinylidene difluoride membranes and subjected the samples to Western blotting. The inset in Fig. 2 demonstrates that PIC was inserted properly into the proteoliposomes.

Construction and Reconstitution of Heterodimers—For the experimental strategy used it was necessary to obtain a pure preparation of PIC heterodimers. The first prerequisite, the controlled formation of dimers in solution, was already described above. Another prerequisite is a sufficient stability of the dimers formed. Experiments which proved the stability of the dimeric constructs will be described at the end of “Results.” The third prerequisite, finally, is the ability to experimentally select particular heterodimers from the set of different forms of PIC obtained after solublization and dimer formation. When starting with the two differently tagged monomers, after dialysis we obtain a mixture of both types of homodimers ([His-PIC]2 and [FLAG-PIC]2), the desired heterodimer ([His-PIC/FLAG-PIC]), and in addition presumably residual amounts of not correctly folded and assembled monomers ([His-PIC] and [FLAG-PIC]). In order to select the heterodimer from this mixture, we applied two consecutive affinity columns (Fig. 3). In the first step, the starting mixture was applied to a Ni-NTA affinity column. The monomer [FLAG-PIC] and the homodimer [FLAG-PIC]2 was not bound but was directly eluted, as was proven by Western blotting for the two tags (experiment not shown). The bound species which all carry the His tag ([His-PIC], [His-PIC]2, and [His-PIC/FLAG-PIC]) were then eluted by imidazole buffer. After concentration and exchange of buffer this eluate was applied to a FLAG-antibody affinity column, to which the heterodimer [His-PIC/FLAG-PIC] was bound as the only protein species. After elution the protein was reconstituted and analyzed kinetically. As a control, also the other species, i.e. homodimers formed and the eluates from the different columns, were reconstituted and analyzed for phosphate transport (data not shown). In Fig. 4 the kinetics of reconstituted PIC proteins from different steps of purification is shown.

1. Expression of differently tagged monomers
2. Solubilization in sodium laurol sarcosinate
3. Mix of monomers and exchange of detergent (C₁₂E₄)
4. Ni-NTA affinity chromatography
5a. Pass through of column
5b. Eluate with imidazole buffer
6. Anti-FLAG affinity chromatography
7a. Pass through of column
7b. Eluate with FLAG peptide

FIG. 3. Schematic drawing of the procedure to isolate defined heterodimers. PIC constructs with two different molecular tags, namely His-tag and FLAG-tag, respectively, were expressed in E. coli. After solubilization of the inclusion bodies by the detergent SLS, PIC was found to be in monomeric form. The monomers were mixed and the detergent SLS was exchanged for C₁₂E₄, which led to a mixture of different monomeric, homo- and heterodimeric forms. This mixture was applied to Ni-NTA-agarose columns and the imidazole eluate contains only constructs with His-tags. After a second FLAG-antibody column, the pure heterodimer is in the FLAG-peptide eluate.
Both the specific activity, resembling the functionality of the protein, and the shape of the kinetics, being correlated with the size and integrity of the proteoliposomes (49), was found to be very similar for the different preparations. Table II summarizes the phosphate transport activity of the relevant PIC constructs, whether starting from monomeric or from different dimeric forms.

It should be mentioned that the use of the Ni-NTA column was only possible after exchanging the detergent SLS for non-ionic detergents, otherwise binding of His-tagged PIC to the affinity columns was significantly reduced. Although the procedure as described here could successfully be used to isolate pure heterodimers, there was a problem with protein stability. The consecutive application of solubilized PIC to two different columns with intermediate dialysis steps led to inactivation and precipitation and therefore to a significant loss of protein, i.e. the final yield of active heterodimer was low. Based on the results described so far, we therefore developed a modification of this method, which makes possible the use of one single column only, at least for particular experiments. For this purpose, the two different monomeric PIC constructs were mixed not in an 1/1 ratio but in a ratio of 1/7 ([His-PIC] and [FLAG-PIC], respectively). On the one hand, by this strategy the content of the desired [His-PIC]/[FLAG-PIC] in the mixture applied to the Ni-NTA column was reduced from 50% in case of a 1/1 mixture to 22% after mixing in a 1/7 ratio. On the other hand, after application of the Ni-NTA column the relative content of the heterodimer in the eluate is more than 93%, i.e. the contribution of the contaminating [His-PIC], homodimer is only about 6% and thus the second FLAG antibody column could be neglected for qualitative experiments in which we were interested in a high specific activity of the heterodimer.

The Heterodimeric PIC in the Phospholipid Membrane—We have used the monomeric and dimeric PIC constructs so far to define the state of aggregation and the reconstitutibility of different forms of the carrier. The major aim of this work, however, to prove whether PIC when inserted into the bilayer membrane is functioning in the dimeric form only, cannot be achieved by this approach, since all kinds of dimers used were similarly active. A discrimination of the actual state of PIC in the membrane requires (i) the construction of a dimer from two different monomers which are characterized by a clearly different state of activity each and (ii) the analysis of the functional properties of this construct after reconstitution into the membrane.

For this purpose, we applied chemical modification by NEM. In contrast to PIC from beef heart, it has been shown for intact PIC from S. cerevisiae that NEM is unable to block its function, since the NEM-sensitive cysteine at position 42, as present in the beef heart PIC, is lacking in the yeast carrier (50). This is of course true for PIC isolated from inclusion bodies, too, when NEM is applied to the reconstituted protein, i.e. when PIC is in the dimeric form and in the native state of conformation (Table III) (11). The activity of S. cerevisiae PIC, however, was completely blocked by NEM when the alkylating reagent was applied to the SLS-solubilized protein, i.e. before reconstitution. Obviously, this is due to the fact that the protein is in the monomeric state and (partially) unfolded under these conditions (see above) which makes at least one of the three available cysteines of PIC from yeast accessible for modification.

Reconstitution of monomers treated with NEM, as well as reconstitution of homodimers constructed from NEM-treated monomers not surprisingly led to proteoliposomes which were completely inactive in phosphate transport (Table III). When we reconstituted dimers consisting of one active and one inactive, NEM-treated monomer each, the heterodimeric state of which was proven by application of different molecular tags as described above, the membrane-inserted complexes were completely inactive, too. This result is a clear indication for the fact that only the dimer can be the active form of PIC in the membrane and not the monomer.

There are, however, two possible objections against this interpretation. First, it may be argued that NEM-treated PIC proteins are per se able to inactivate other basically active monomers in some kind of trans-effect. A related argument is based on the possibility that NEM might be carried over during the isolation, which then would lead to inactivation of the added active PIC monomers which were not NEM treated. These arguments were ruled out by control experiments in which NEM-inactivated monomers ([His-PIC(NEM)]) and active monomers ([FLAG-PIC]) were mixed and reconstituted directly, i.e. without forming and separating defined heterodimers. The data of Table III prove that the dimers, which in this case must have been formed by association of [FLAG-PIC] monomers to homodimers, are perfectly active, despite the presence of NEM-inactivated [His-PIC (NEM)] monomers in

![Fig. 4. Phosphate transport kinetics of selected PIC constructs.](image-url)
Transport activity of different PIC constructs after modification with 2 mM NEM

The PIC constructs were solubilized in different detergents and are therefore either in monomeric (SLS + C12E8) or dimeric form (C12E8). NEM incubation was carried out at exactly the same conditions (NEM- and phosphate concentration, pH, temperature, duration of incubation). The different PIC constructs were reconstituted as described under “Experimental Procedures,” the heterodimeric constructs were isolated after an incubation was carried out at exactly the same conditions (NEM- and phosphate concentration, pH, temperature, duration of incubation). The different PIC proteins were expressed in inclusion bodies, solublized with SLS, and reconstituted after addition of C12E8. For reconstitution, unmodified and NEM-treated PIC was mixed in different ratios. In each series of experiments, the measured transport activity of the assay in with unmodified PIC only was used was set to 100%. Circles, mixture of wild type PIC (unmodified and NEM-treated, respectively); squares, mixture of [HIS]-PIC(NEM)] and [FLAG-PIC]. The dotted line represents a direct correlation of activity to the share of active monomer, the full line relates transport activity to the calculated value of active dimer for both series of experiments.

| PIC construct | Detergent | Transport activity (μmol/min/mg protein) |
|---------------|-----------|----------------------------------------|
| [PIC]         | SLS + C12E8 | 124 ± 13                               |
| [His-PIC]     | SLS + C12E8 | 110 ± 16                               |
| [PIC]         | C12E8     | 180 ± 25                               |
| [His-PIC/FLAG-PIC] | C12E8 | 153 ± 24                               |
| [His-PIC(NEM)/FLAG-PIC] | C12E8 | <1                                     |
| [His-PIC(NEM/FLAG-PIC)] | C12E8 | <1                                     |
| [His-PIC(NEM)] + [FLAG-PIC] | C12E8 | 69 ± 15                                |

DISCUSSION

The definition of the state of aggregation of membrane-embedded carrier proteins was the topic of numerous studies.
involving techniques of cross-linking, electron microscopy, electrophoresis, chromatography, rotational diffusion, ultracentrifugation, radiation inactivation, as well as reconstitution titration. As a matter of fact, the state of aggregation is not only a question of correct numbers. It is highly relevant for the functional models of solute carriers whether carrier proteins act as monomers, dimers, or higher aggregates. In view of the fact that the three-dimensional structure of not a single solute carrier protein is available so far, solving this question becomes even more interesting. The most advanced structural analysis of a solute carrier correlating the spatial arrangement of particular amino acid residues with carrier function, the lactose permease of *E. coli* (51), offers an interesting view on the hypothetical substrate translocation pathway. Nevertheless, even in this case the correct arrangement of transmembrane segments as well as the true state of aggregation is not yet completely clear.

The major conclusion reached from reconstituting defined constructs of PIC are that the dimer is functionally active, whereas the monomer is not. Particularly striking was the finding that reconstitution of the monomeric form of PIC resulted in a strongly sigmoidal titration curve, indicating that the first monomers inserted into liposomes are not able to function in phosphate transport. We interpret the sigmoidal shape of the titration curve by the obvious assumption that more than one functionally active monomer must be present in one liposome, in order to be able to form a transport-active complex. It should be noted that the result of the experiment on reconstituting partially inactivated heterodimers does not exclude higher states of aggregation. On the basis of experiments with the mitochondrial ADP/ATP carrier using fluorescent nucleotide analogs, in fact a tetrameric state of the functional unit of this carrier has been suggested (18). However, the observed shape of the titration curves of reconstitution argues against the presence of higher aggregates being essential for transport function. If this would be the case, a sigmoidal shape of the dependence of activity on the amount of added carrier protein would have been expected also for the insertion of dimers into liposomes.

A further conclusion can be drawn from the experiment on reconstitution of heterodimers of both active and inactive subunits. The fact that this construct is inactive not only proves the stability of the dimeric form. It also indicates that monomers do not function by themselves within the complex, i.e., that there is some kind of cross-talk between the two subunits. It may be assumed that only one pathway for phosphate exists within the dimer, and that the inactivation of one subunit is sufficient to render the whole complex inactive. An alternative explanation would be the existence of two independent pathways through the two PIC subunits, the function of each of which depends on the integrity of the corresponding pathway in some kind of mutual interaction. It should be noted that previous kinetic studies both on the mitochondrial aspartate/glutamate and phosphate carrier, respectively, were the basis for defining of the family of mitochondrial carriers with a common kinetic mechanism, namely a simultaneous bisubstrate kinetics (5, 6, 52, 53). This analysis argued for the presence of two substrate pathways in mitochondrial carrier proteins. An experimental basis to decide this question on a molecular level seems to be at hand now by using more sophisticated versions of heterodimers created by methods described in the present publication.

Finally, a further conclusion can be drawn. For a correct interpretation it was necessary to prove that the dimeric constructs were stable in the course of the experiments. Whereas the stability of the dimeric forms in the solubilized state, at least in the time range of a few hours, was already proven by the successful application of the strategy for selective isolation, this was not equally simple for the membrane-inserted state. By comparing the activity of defined heterodimers consisting of two active monomers on the one hand, and of both an active and an inactive monomer on the other, we showed that PIC dimers are stable at least within a time range of 22 h. Thus, beside the fact that the dimeric form is essential for function, it has, to our knowledge, been shown for the first time here that there is no dynamic equilibrium between monomers and dimers of mitochondrial carriers in the membrane-inserted state.

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