The Reversible Antiport-Uniport Conversion of the Phosphate Carrier from Yeast Mitochondria Depends on the Presence of a Single Cysteine*

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Wild type and mutant phosphate carriers (PIC) from Saccharomyces cerevisiae mitochondria were expressed in Escherichia coli as inclusion bodies, solubilized, purified, and optimally reconstituted into liposomal membranes. This PIC can function as coupled antiport (P\textsubscript{i}/P\textsubscript{2} antiport and P\textsubscript{i}/Pi\textsubscript{2} net transport, i.e., P\textsubscript{i}/OH\textsuperscript{-}/Pi\textsubscript{2} antiport) and uncoupled uniport (mercuric chloride-induced P\textsubscript{i} efflux). The basic kinetic properties of these three transport modes were analyzed. The kinetic properties closely resemble those of the reconstituted PIC from beef heart mitochondria. A competitive inhibitor of phosphate transport by the PIC, phosphonoformic acid, was used to establish functional overlap between the three physiological transport modes and the induced efflux mode. Replacement mutants were used to relate the reversible switch from antiport to uniport to a specific residue of the carrier. There are only three cysteines in the yeast PIC. They are at positions 28, 134, and 300 and were replaced by serine, both individually and in combinations. Cysteine 300 near the C-terminal loop and cysteine 134 located within the third transmembrane segment are accessible to bulky hydrophilic reagents from the cytosolic side, whereas cysteine 28 within the first transmembrane segment is not. None of the three cysteines is relevant to the two antiport modes. Cysteine 134 was identified to be the major target of bulky SH reagents, that lead to complete inactivation of the physiological transport modes. The reversible conversion between coupled antiport and uncoupled uniport of the PIC depends on the presence of one single cysteine (cysteine 28) in the PIC monomer, i.e., two cysteines in the functionally active dimer. The consequences of this result with respect to a functional model of the carrier protein are discussed.

The mitochondrial phosphate carrier (PIC)\textsuperscript{1} catalyzes transport of inorganic phosphate into the mitochondrial matrix where the phosphate is utilized for phosphorylating ADP to ATP (LaNoue and Schoolwerth, 1984; Wohlrab, 1986; Wehrle and Pedersen, 1989; Krämer and Palmieri, 1989, 1992). The function of the PIC was described as P\textsubscript{i}/H\textsuperscript{+} symport, respectively, P\textsubscript{i}/OH\textsuperscript{-} antiport. The primary structure of the beef heart PIC was elucidated by protein (Aquila et al., 1987) and DNA sequencing (Runswick et al., 1987), and the PIC gene was cloned and sequenced from Saccharomyces cerevisiae (Phelps and Wohlrab, 1991). The PIC is a typical member of the structural family of mitochondrial carriers with six transmembrane segments (Aquila et al., 1985; Kuan and Saier, 1993). Recently the yeast PIC has been expressed as inclusion bodies in Escherichia coli (Murakami et al., 1993; Wohlrab and Briggs, 1994). Procedures have been described to solubilize the PIC from inclusion bodies in a functionally active state (Wohlrab and Briggs, 1994).

The function of the PIC was studied after purification from various kinds of mitochondria (for reviews see Wohlrab (1986), Wehrle and Pedersen (1989), and Krämer and Palmieri (1989)) and reconstitution into proteoliposomes (Wohlrab, 1980; De Pinto et al., 1982; Wehrle and Pedersen, 1982). PIC catalyzes both homologous P\textsubscript{i}/P\textsubscript{i} as well as heterologous P\textsubscript{i}/OH\textsuperscript{-} antiport with high activity (Wohlrab and Flowers, 1982; Stappen and Krämer, 1993, 1994). Transport kinetics using biaxial initial velocity studies identify the PIC as a member of the mitochondrial carrier family also in functional terms. Its mechanism is of the simultaneous (sequential) type, involving a ternary complex in transport catalysis that requires the binding of two ligands at the same time (Stappen and Krämer, 1994). An additional property that places the PIC into this functional family is its ability to switch to uniport (efflux) activity after chemical modification with some mercurial reagents (Stappen and Krämer, 1993). It was previously shown that the aspartate/glutamate carrier (Dierks et al., 1990a), the ADP/ATP carrier (Dierks et al., 1990b), and the carnitine carrier (Indiveri et al., 1991) can reversibly be converted by mercurial reagents from coupled antiport to uncoupled uniport, a function that comprises both carrier-like and channel-like properties. An analysis of this conversion, specifically with the aspartate/glutamate carrier, identified the involvement of at least two cysteines and was interpreted to reveal an intrinsic preformed channel structure as a common element in those carriers (Dierks et al., 1990b). These kinds of structural domains had already been postulated on the basis of functional considerations (Klingenberg, 1981) as well as of transport experiments (Brustovetsky and Klingenberg, 1996).

The aim of the present work was to relate the reversible switch from coupled antiport to uncoupled uniport to a specific residue of the carrier protein by using replacement mutagenesis of the yeast PIC expressed in E. coli. This technique has already been used to identify a number of other residues, which are important for the carrier’s physiological function with respect to...
spect to substrate recognition and inhibitor interaction (Wohlrab and Briggs, 1994; Phelps et al., 1996). We demonstrate now that the conversion between antiport and uniport of the PIC exclusively depends on the presence of cysteine 28 in the PIC monomer, i.e. two cysteine 28 residues in the functionally active dimer.

**EXPERIMENTAL PROCEDURES**

**Materials and Their Sources—**[^32P]Phosphate was obtained from Amersham-Buchler. Sigma supplied Triton X-114, mersalyl acid, pCMBS, DTT, phosphonoformic acid, HEPES, PIPEs, and turkey egg yolk phosphatidylcholine. Dowex 2-X10 and SLS were from Fluka, Bio–Beads SM-2 from Bio-Rad, Sephadex G-75 from Pharmacia, and pyridoxal phosphate and HgCl₂ from Merck. All SH reagents (HgCl₂, pCMBS, mersalyl acid) were prepared from frozen stock solutions and were diluted with water or the respective gel filtration buffer. Pyridoxal phosphate was dissolved in 1 mM imidazole (pH 6.5). All other chemicals were of analytical grade.

**Generation of Mutants, Expression, Isolation, and Purification of the PIC**—The gene coding for the PIC was cloned from a yeast genomic library as described earlier (Phelps and Wohlrab, 1991) and the PIC was expressed in the *E. coli* strain BL21 (DE3) (Murakami et al., 1993; Wohlrab and Briggs, 1994). Mutants were generated as described earlier (Phelps and Wohlrab, 1993; Wohlrab and Briggs, 1994). The expression strain BL21 (DE3) was transformed with the plasmid pNYHM131 either coding for the wild type PIC or a mutant. A total of 1 liter of 2YT medium (plus 100 mg of carbenicillin) was inoculated with an overnight colony of transformed BL21 (DE3) and grown to an OD₆₀₀ of 0.6 (about 5 h) under vigorous shaking at 37 °C. PIC expression was initiated by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside plus 100 μg of carbenicillin. Growth was continued for 3 h, cells were harvested and stored at −20 °C, not longer than 48 h before continuing the procedure. All the following steps were carried out at 0–4 °C. The pellet from 125 ml of culture was suspended in TE (10 mM Tris base, 0.1 mM EDTA, 1 mM DTT, pH 7.0) and passed twice through a French pressure cell, followed by 10 min of centrifugation at 12,000 × g. The pellet was homogenized with 10 ml of TE and centrifuged at 12,000 × g for 3 min, and the pellet was stored at −70 °C. Isolation of the PIC from inclusion bodies was carried out applying a previously described procedure for the oxoglutarate carrier (Fierronte et al., 1993) with some modifications. The pellet was washed three times in TE-buffer containing 2% Triton X-114, followed by centrifugation at 12,000 × g for 2.5 min. The pellet was resuspended in 50 mM KCl, 20 mM HEPES, 20 mM potassium Pi, pH 6.5, and 20 μl of protein solution were mixed with HEPES/potassium Pi buffer containing 1.67% SLS, followed by addition of 800 μl of water. The resulting solution was used for reconstitution. Fig. 1 shows a silver-stained SDS-PAGE of the PIC after purification.

**Reconstitution Procedure—**The solubilized PIC was reconstituted into preformed liposomes by the Amberlite method as described for the bovine heart PIC (Stappen and Krämer, 1990), including addition of Triton X-114 in excess over SLS. The reconstitution procedure was modified with respect to the phospholipid/protein and phospholipid/detergent ratio. Optimal transport activity was obtained at a phospholipid concentration of 16 mg/ml, a phospholipid/protein ratio of 140 mg/mg, and a detergent/phospholipid ratio of 0.62 mg/mg. This means that 70 μl of Triton X-114 (10%, w/v), 112 μl of liposomes (10% EYPC (w/v) in 50 mM KCl, 20 mM HEPES, 20 mM potassium Pi, pH 6.5), and 20 μl of protein solution were mixed with HEPES/potassium Pi buffer (50 mM HEPES, 30 mM potassium Pi, pH 6.5) to yield a final volume of 700 μl. A detergent/Amberlite ratio of 12 mg/g and 15 column passages were used for detergent removal. The amount of PIC recovered in the proteoliposomes after reconstitution was found to vary between 27 and 43% of the protein in the SLS-solubilized fraction.

**Measurement of Transport Activity—**The reconstituted transport activities (P/Pₐntiport, P net transport, and P efflux) were determined by measuring the flux of [32P]Pi. The applied methods resembled those described previously for the analysis of the aspartate/glutamate carrier (Dierks and Krämer, 1988; Dierks et al., 1990a). In most experiments, antiport activity was determined by the forward exchange procedure. The assay was started by adding labeled phosphate to the proteoliposomes containing unlabeled substrate inside and the increase in internal label was followed. In some experiments, the P/Pₐntiport mode was measured by the backward exchange method, which is similar to the procedure used for determination of the net transport mode (P/Pₐntiport) or the HgCl₂-induced efflux mode. For this, the internal pool was prelabeled by incubating the proteoliposomes with [32P]Pi of high specific radioactivity at 21 °C for 10 min. The external substrate (and label) was then removed by size exclusion chromatography on Sephadex G-75 columns at 4 °C. Since the PIC also catalyzes net transport, the use of this method depends on the availability of reversible inhibition during chromatography. Routinely, 200 μM mersalyl acid was used for this purpose. After removal of external phosphate, transport was started by adding 10 mM DTT (net transport) or 1 mM DTT together with external phosphate (P/P net transport, antiport). The merscurial-induced uniport was assayed by adding 0.5 mM HgCl₂ to proteoliposomes after the size exclusion chromatography. After the desired period of time carrier-mediated transport was stopped by adding 25 mM pyridoxal phosphate with or without 10 mM DTT. After application of the stop mix, each sample was passed through an anion exchange column (Dowex 1-X10, Cl⁻ form) to remove the external label. Further details were as described previously (Dierks and Krämer, 1988; Dierks et al., 1990a).

For PIC mutants lacking Cys-134, an alternative inhibition technique had to be developed since these mutants cannot be inhibited by mersalyl. In this case, the internal pH of the proteoliposomes was adjusted to pH 6.8 during fermentation by high internal buffer (HEPES, 50 mM). The external pH in the size exclusion chromatography was set to 5.5 (PIPEs, 5 mM). Due to the H⁺-coupled transport mechanism, the pH gradient prevented loss of internal substrate. Before starting net transport, the external pH was adjusted to 6.8 by adding 50 mM HEPES, pH 7.0. The data of Table I indicate that retention of internal phosphate by this method was comparable with the generally applied procedure involving inhibitor addition.

**Forward exchange rates were determined by fitting the time course of isotope equilibration to a single exponential**$y = A(1 - e^{-kt}) + B$, leading to the first order rate constant $k$ (in min$^{-1}$). The specific activity ($μmol/min · mgₚᵢᵣ$) was calculated from $k$ (min$^{-1}$), the final value of isotope equilibration (dpn), the specific radioactivity (dmp/mmol), the volume of the proteoliposome fraction (ml), and the protein concentration ($μg/ml$) as published previously (Dierks and Krämer, 1988). Backward exchange rates were calculated by the equation $\nu' = k \cdot S_{in} \cdot V_{in}$ in which $k$ (min$^{-1}$) is the apparent first order rate constant calculated by...
TABLE I
Comparison of reversible inhibition of phosphate efflux by mersalyl or by an inverse pH gradient during size exclusion chromatography of proteoliposomes

The first order rate constants for two transport modes of the reconstituted PIC are given (for conversion see "Experimental Procedures"). In this experiment the wild type protein was used as a control for the following studies using mutants in which Cys134 was replaced. The values are means of two experiments.

| Experiment          | Rate constant of phosphate transport after mersalyl addition | Rate constant of phosphate transport after inverse pH gradient |
|---------------------|-------------------------------------------------------------|-------------------------------------------------------------|
| Control             | 0.01 min⁻¹                                                   | 0.065 min⁻¹                                                 |
| P/OH⁻ antiport      | 0.73 min⁻¹                                                   | 0.60 min⁻¹                                                  |
| HgCl₂-induced efflux| 0.56 min⁻¹                                                   | 0.41 min⁻¹                                                  |

* Control means either without removal of mersalyl by addition of DTE or without application of a pH shift from 5.3 to 6.8, respectively.

Comparison of Beef Heart and Yeast PIC—So far, for functional characterization of the mitochondrial PIC, mainly the protein from beef heart or pig heart was studied after isolation using Triton X-100 or X-114 (Wohlrab, 1986; Krämer and Palmieri, 1989). A detailed functional analysis after reconstitution by chromatography on Amberlite was carried out with the beef heart protein (Stappen and Krämer, 1993, 1994). In the present work, we use a different protein (yeast instead of beef heart PIC), a different source (bacterial inclusion bodies instead of integral mitochondria), a different detergent (SLS instead of Triton X-114), and an altered reconstitution procedure (optimized for SLS-solubilized protein). Consequently, it was essential to compare the properties of the yeast PIC isolated from E. coli inclusion bodies with the data previously obtained in experiments using the beef heart protein.

Since for the PIC, in contrast to the ADP/ATP carrier, no side-specific inhibitors are available, the orientation of the carrier protein in proteoliposomes was previously established by analyzing side-specific substrate interaction (Stappen and Krämer, 1993). Such a kinetic analysis of the P₁/P₁⁻ antiport of the yeast PIC, both for the wild type and the Cys-28 → Ser mutant as representative examples for the recombinant proteins, is shown in Fig. 2. For the P₁/P₁⁻ and the P₁/OH⁻ antiport (net transport), the Kₘ values (transport affinity) at both sides of the proteoliposomes are shown for the wild type and two mutants (Table II). The following conclusions could be drawn. (i) Only one single kinetic component is observed for interaction of phosphate at the inside and the outside, respectively. (ii) The Kₘ values for phosphate in the wild type and the Cys-28 → Ser mutant are identical. (iii) A comparison shows that the corresponding values of the beef heart and yeast PIC for interaction with phosphate at the two sides of the protein are more or less identical. (iv) These data also prove that the PIC of beef heart and yeast are oriented in the same direction after reconstitution, i.e. right side out (Stappen and Krämer, 1993). These results indicate that the methods previously derived for the PIC from beef heart mitochondria can be applied to the reconstituted yeast carrier protein.

The reconstituted beef heart PIC was shown to catalyze three different functions, i.e. homologous P₁/P₁⁻ antiport, heterologous P₁/OH⁻ antiport (net transport), as well as substrate-unspecific uniport after treatment with HgCl₂ (Stappen and Krämer, 1993). The same was observed for the PIC in intact yeast mitochondria (Stappen, 1994), as well as for the wild type yeast PIC isolated from inclusion bodies after expression in E. coli (Table II, see also Fig. 5). The transport affinities (Kₘ) turned out to be very similar, whereas the Vₘₐₓ values were in general higher for the yeast PIC. Since the efficiency of reconstitution (the share of successfully incorporated protein) was not quantitated in these experiments in detail, the same-
what different $V_{\text{max}}$ values do not necessarily mean that the molecular activity of the PIC from the different sources is in fact different. Irrespective of this restriction, it is interesting to note that the ratio of $P_i^- / P_i^-$ antiport to $P_i^+ / \text{OH}^-$ antiport (net transport) is also very similar for the two proteins (Stappen and Krämer, 1994).

Characterization of Uniport Activity of the Yeast PIC Expressed in E. coli Cells—To unequivocally correlate the observed uniport activity to the reconstituted yeast PIC and to define its properties in the mutants studied, we used various inhibitors. The unspecific inhibitors pyridoxal phosphate and mersalyl acid were previously described as effective reagents stopping transport (Stappen and Krämer, 1993). The data in Table III show that pyridoxal phosphate works in all cases. Mersalyl acid, on the other hand, is ineffective when applied to mutants in which Cys-134 has been replaced by Ser. Consequently, we had to develop another method for reversibly inhibiting net transport during size exclusion chromatography, namely application of an inverse pH gradient (see “Experimental Procedures”). The SH reagent pCMBS also reacts with Cys-134 and/or Cys-300 (Table III), as shown above for mersalyl. Besides interacting with Cys-28, HgCl$_2$ blocks the transport reaction, at least in part, by reacting also with Cys-134 and/or Cys-300.

The availability of phosphonoformic acid (PFA) (Kempson, 1988), originally discovered to block the kidney phosphate carrier, prompted us to characterize the properties of the mercuration-induced phosphate efflux (Fig. 3) by addition of 5 mM phosphate $(\bullet)$, 5 mM PFA $(\triangle)$, and 5 mM mersalyl $(\uparrow)$. B, reciprocal plot of the dependence of $P_i/^P_i$ activity on the concentration of external phosphate $(\bullet)$, no inhibitor added; $(\uparrow)$, 5 mM PFA added).

The transport rates, however, are still significant, i.e. more than 20% of that of the wild type. Most importantly, the ratio of $P_i^- / P_i^-$ antiport to $P_i^+ / \text{OH}^-$ antiport (net transport) remained more or less constant, irrespective of the altered absolute values of the two physiological transport modes.

Whereas both the homologous and the heterologous antiport activities were retained in the PIC proteins, this was not the case with the merccuric chloride-induced uniport (Fig. 4). This induced phosphate efflux catalyzed by the wild type as well as the Cys-28 → Ser and Cys-300 → Ser mutant proteins is shown in Fig. 5. The relative efflux rate by the Cys-300 → Ser mutant

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**Table III**

Inhibition of various mutants of the reconstituted yeast mitochondrial phosphate carrier by various inhibitors

| Mutant strain | Transport mode | Relative transport activity$^a$ after addition of reagent |
|---------------|---------------|----------------------------------------------------------|
|               |               | PLP (20 mM) | pCMBS (0.3 mM) | Mersalyl (0.3 mM) | HgCl$_2$ (0.5 mM) | PFA (5 mM) |
| Wild type     | $P_i^- / P_i^-$ AP | $<3$ | 5 | $<5$ | --$^b$ | 50$^c$ |
|               | $P_i^+ / \text{OH}^-$ AP | $<3$ | 3 | $<5$ | --$^b$ | 160 |
|               | Ind. efflux | 105 | 105$^d$ | 48 | |
| C28S          | $P_i^- / P_i^-$ AP | 3 | 6 | 6 | 29 | |
| C134S         | $P_i^- / P_i^-$ AP | $<3$ | 45 | 36 | |
| C300S         | $P_i^- / P_i^-$ AP | $<3$ | 7 | 5 | |
| C134S/C300S   | $P_i^- / P_i^-$ AP | $<3$ | 89 | |
| C28S/C134S/C300S | $P_i^- / P_i^-$ AP | $<3$ | 76 | |

$^a$ Data as $k$ values in % of control ($P_i^- / P_i^-$ antiport without added inhibitor).

$^b$ Not measurable since HgCl$_2$ induces efflux under these conditions.

$^c$ At 2 mM external phosphate.

$^d$ In the presence of mersalyl (efflux conditions).
For uniport (efflux) induction 0.5 mM HgCl\(_2\) was used. All values are means of at least three determinations; the bars indicate the standard deviation. The P\(_i\)/P\(_i\)\(^-\) antiport activity of the wild type was set to 100%, and this measurement was included in all other experiments for determination of transport activities to provide an appropriate basis for the normalization procedure. The value of the three transport activities were compared on the basis of the derived first order rate constants (k) of the respective transport modes (see “Experimental Procedures”). Basic phosphate efflux from proteoliposomes containing inhibited wild type PIC was <2% of the mercurial-induced efflux activity.

is similar to that of the wild type, while the Cys-28 \rightarrow Ser mutant, even when treated with high mercurial concentrations, is not able to undergo the functional switch from coupled antiport to uncoupled uniport (efflux). The induced uniport activities of all PICs are compared in Fig. 4. In all mutants in which Cys-28 was replaced by Ser, independent of a replacement of the other two cysteine residues, Cys-234 and Cys-300, the functional switch to uniport could not be induced.

**DISCUSSION**

The mitochondrial PIC can reversibly be switched from coupled antiport to uncoupled uniport. We have used replacement mutants of the yeast PIC expressed in *E. coli* to relate this switch to specific residues of this protein. The involvement of cysteine residues in this switch can most appropriately be studied with the yeast PIC, since 1) it has only three cysteines *versus* eight in the beef heart PIC used in earlier studies, and 2) its preparation is facilitated with *E. coli* inclusion bodies. We have shown that the yeast PIC, isolated and purified from inclusion bodies, solubilized by SLS and reconstituted into liposomes resembles the beef heart PIC in all the relevant functional aspects.

The mercuric chloride-induced uniport has now been definitively identified with the reconstituted PIC. Contaminating mitochondrial channel proteins cannot be responsible for this activity since the PICs are heterologously expressed. Furthermore, we found that PFA, a competitive inhibitor of P\(_i\)/P\(_i\)\(^-\) antiport, effectively inhibits also the induced uniport mode. Since PFA stimulates P\(_i\)/OH\(^-\) (P\(_i\)\(^-\) net transport), we conclude that it is a transport substrate of the PIC. PFA is not available in labeled form, and thus its true substrate properties could not be directly investigated. Nevertheless, its effect on the mercuric chloride-induced P\(_i\)\(^-\) efflux can be taken as a further indication that the external binding site of the reconstituted PIC has retained its properties. This site, as shown earlier, is the cytosolic site of the PIC (Stappen and Krämer, 1993).

It has been shown for many other carrier proteins that cysteines are not essential for basic transport functions (van Iwaarden *et al.*, 1991, 1992). The present results permit us to characterize the functional significance of the yeast PIC cysteines (Fig. 6). Cys-300 does not seem to be relevant for transport activity or for uniport induction. We did, however, detect a 50% reduction of transport after its replacement by serine. No additional changes were observed in combination with the Cys-134 \rightarrow Ser replacement. Cys-134 is responsible for inhibition of all transport modes by mersalyl acid and also by pCMBS. A comparison with published effects on the PIC (Stappen and Krämer, 1993) makes it likely that Cys-134 is also responsible for inhibition by 5,5-dithiobis(2-nitrobenzoic acid). Although a reaction of mersalyl with Cys-300 seems to be responsible for a slight reduction in antiport activity by the Cys-134 \rightarrow Ser mutant relative to the Cys-134 \rightarrow Ser/Cys-300 \rightarrow Ser and the Cys-28 \rightarrow Ser/Cys-300 \rightarrow Ser/Cys-300 \rightarrow Ser mutants, it is obvious that the major target of mersalyl is Cys-134. The reactivities of the cysteines indicate that Cys-134 (and presumably also Cys-300) is accessible to large, hydrophilic ligands, whereas Cys-28 is accessible only to the small Hg\(^{2+}\) ion. This was documented by the observation that mercurials other than HgCl\(_2\) did not affect the reaction of Hg\(^{2+}\) with Cys-28. Whereas the accessibility of Cys-300 from the external (cytosolic) side seems obvious, this is not so for Cys-134. Our results suggest that Cys-134 is in an aqueous environment with connected to the cytosolic side of the protein, whereas Cys-28 is not. Interestingly, Cys-28 has previously been found to be the target for inhibition of phosphate transport by oxygen (Phelps and Wohlrab, 1993). In a recent paper, furthermore, His-32, Glu-126, and Glu-137 were found to be essential for a coupled phosphate/H\(^+\) pathway in the PIC (Phelps *et al.*, 1996). In fact, these residues line up at the same side of helices A and C, in which Cys-28 and Cys-134 are located. It may be questioned whether a reaction of HgCl\(_2\) with other residues besides cysteine should be considered. This has in fact been analyzed in detail with respect to the aspartate/glutamate carrier, where a functionally significant reaction was found to be confined to cysteine residues (Dierks *et al.*, 1990a, 1990b). In any case, the specific action on Cys-28 and Cys-134 of the PIC was proven here by the absence of these effects in the mutants lacking these cysteines.

Most interesting, however, is the observation that the reversible switch from the physiological transport modes to the unphysiological and mercuric chloride-induced uniport depends on the presence of Cys-28. This functional shift from antiport to uniport, which is correlated with the appearance of some channel-type functions, has been observed in several mitochondrial carriers, namely the aspartate/glutamate, the ADP/ATP (Dierks *et al.*, 1990a; Dierks *et al.*, 1990b), and the carnitine carrier (Indiveri *et al.*, 1991). The present results, however, for
the first time correlate a specific residue with this phenomenon. The observation that mitochondrial carriers retain their original activation energy of transport after this functional switch was interpreted to indicate that a similar conformational change occurs during solute transfer both in the antiport and the mercuric chloride-induced uniport (Herick and Krämer, 1995). Modification of Cys-28 by HgCl₂ yields a loss of specificity of ligand interaction at the internal PIC binding site. No such loss of the external binding site is observed (Dierks et al., 1990b; Stappen and Krämer, 1993). In conclusion, our findings suggest that Cys-28 may possess a gating function on the matrix side of the PIC.

There is evidence from a large number of investigations that the structural basis for differences in the mechanisms of coupling among antiport, symport and uniport in secondary systems and differences between carrier and channel-type of functions may be very subtle (Nikaido and Saier, 1992; Krämer, 1994). Thus, for example, a single amino acid replacement in the bacterial lactose carrier (Lac-permease) shifts its transport from a coupled to an uncoupled mechanism (Eelkema et al., 1991; King and Wilson, 1990; Kaback, 1992). Furthermore, a number of studies using electrophysiological techniques provide evidence for channel properties in carrier proteins, e.g. neurotransmitter transporters (Cammack and Schwartz, 1996; DeFelice and Blakely, 1996), the chloroplast triose phosphate carrier (Schwarz et al., 1994) as well as the mitochondrial ADP/ATP carrier (Tikhonova et al., 1994; Brustovetsky and Klingenberg, 1996). This concept is particularly attractive in view of the fact that conditions are known where large pores appear in the inner mitochondrial membrane (permeability transition pore, “megachannel”) (Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996). Taken together, the distinction between “carrier-type” and “channel-type” of transport mechanisms seems to become minimal, when analyzed in terms of functional elements in both types of solute transport systems (Nikaido and Saier, 1992; Krämer, 1994; DeFelice and Blakely, 1996).

The data presented here prove that the reversible shift between the coupled and the uncoupled transport mode of the mitochondrial PIC only depends on the modification of a single cysteine. It has to be pointed out, however, that mitochondrial carrier proteins are functional dimers, i.e. there are two cysteines at position 28. Interestingly, earlier investigations of the aspartate/glutamate carrier indicated that the modification of two cysteine residues is necessary for the reversible shift from antiport to uniport (Dierks et al., 1990a; Dierks et al., 1990b). Although the primary structure of the aspartate/glutamate carrier is not yet known and thus the two cysteines have not been identified, it is obvious that, on the basis of the apparently common mechanism of this antiport/uniport conversion in mitochondrial carriers, the two results are related. Consequently, studying the functional involvement of the two cysteines lo-
cated in the two homologous monomers of mitochondrial carrier proteins may be an interesting clue for understanding the crosstalk of the individual subunits during transport catalysis.

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