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Interaction of phenylisothiocyanates with the mitochondrial phosphate carrier. I. Covalent modification and inhibition of phosphate transport

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The effects of phenylisothiocyanate (PITC) and of the polar analogue *p*-sulfophenylisothiocyanate (*p*-sulfoPITC) on the phosphate carrier of bovine heart mitochondria have been investigated. Incubation of mitochondria with the two phenylisothiocyanates leads to inhibition of the phosphate carrier protein. The inhibition of phosphate transport by PITC is unaffected by the addition of dithioerythritol (DTE) or by variation of the pH. The inhibition by *p*-sulfoPITC is in part removed by DTE; the remaining inactivation of the phosphate carrier, which can be attributed to the reaction with NH_2 groups, is temperature and pH-dependent. Inhibition of phosphate transport by both *p*-sulfoPITC and PITC depends on the time of incubation and the concentration of the inhibitor. Preincubation with mersalyl protects the carrier protein against the inactivation by *p*-sulfoPITC but not against PITC. Other SH reagents tested do not show any protective effect. It can thus be concluded that two types of lysine residues are essential for the activity of the phosphate carrier. Lysine(s) of the former type are located at the surface of the membrane and are topologically related to the functional SH groups of the protein. Lysine residue(s) of the latter type are buried in the hydrophobic phase of the membrane.

Introduction

The inorganic phosphate required for oxidative phosphorylation is taken up by the mitochondria via a transport system of the inner mitochondrial membrane known as the phosphate carrier. This carrier, which catalyzes a phosphate/H^+ symport, has been purified and its activity reconstituted in liposomes [1–3]. Recently, the amino acid sequence of the phosphate carrier has been determined by both amino acid analysis [4] and DNA sequencing [5]. By comparison of the primary structure of the phosphate carrier, the ADP/ATP carrier and the uncoupling protein, it was suggested that these three carrier proteins form a family of proteins, probably originating from a common ancestor [4,5].

Although these results have greatly improved our understanding of the structure of the phosphate carrier, the mechanism of transport remains still unknown. One approach for obtaining more information about the molecular mechanism of a carrier system is the investigation of the role of

Abbreviations: PITC, phenylisothiocyanate; *p*-sulfoPITC, *p*-sulfophenylisothiocyanate; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecylsulfate.

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essential functional groups of the transport protein. In the case of the phosphate carrier, so far SH groups represent the only well-defined functional groups (for reviews, see Refs. 6–8). Since phosphate is an anion, it is likely that positively charged amino acids such as arginine or lysine may play an important role in binding and translocation of the substrate anion phosphate catalyzed by the phosphate carrier.

In this paper we show that two lysine-specific reagents, *p*-sulfophenylisothiocyanate and phenylisothiocyanate, are irreversible inhibitors of the mitochondrial phosphate carrier. Our data further show that amino groups important for the transport activity are topologically related to the SH groups of the phosphate carrier known to be essential in the transport mechanism.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) and Dowex AG-1-X8 (100–200 mesh) were purchased from Bio Rad, ^{32}P-phosphoric acid from Radiochemical Centre (Amersham, U.K.), egg yolk phospholipids (Lecithin from eggs) and PITC from Fluka, cardiolipin from Avanti-Polar Lipids, Triton X-114, mersalyl and *p*-chloromercurybenzenesulfonate from Sigma, *p*-sulfoPITC from Aldrich and dithioerythritol from Serva.

Modification of mitochondria with p-sulfoPITC and PITC. Bovine heart mitochondria were prepared as described in Ref. 9 and stored at −70 °C. Thawed mitochondria were washed twice by centrifugation in a medium containing 0.25 M sucrose, 20 mM Hepes, 2 mM EGTA (pH 7.5) (here called SHE medium). Mitochondria were then resuspended in the same medium at a protein concentration of 20 mg/ml and incubated with either *p*-sulfoPITC or PITC under the conditions specified in the legends of the tables and the figures.

After 10 min, the reaction was stopped by adding DTE (unless otherwise indicated) to a final concentration of 10 mM, and the mitochondria were washed twice at pH 8.5 in the SHE medium supplemented with 10 mM DTE (unless otherwise indicated) and once at pH 6.5 in 20 mM KCl, 2 mM EGTA and 20 mM KH_2PO_4 buffer. Incubation of mitochondria with *p*-sulfoPITC and PITC at pH 9.0 was carried out in a medium containing 0.25 M sucrose, 20 mM borate and 2 mM EGTA (pH 9.0) (SBE medium).

Protection by sulfhydryl reagents of the modification of mitochondria with p-sulfoPITC and PITC. Bovine heart mitochondria (20 mg protein/ml) were incubated for 10 min in SHE (pH 7.5) or SBE (pH 9.0) medium with 100 nmol/mg protein of the sulfhydryl reagents indicated in the legends to the Tables III and IV; then, *p*-sulfoPITC or PITC (2 mM) was added for the modification reaction. After further 10 min the reaction was stopped by adding 10 mM DTE. Washing of mitochondria was carried out as described above.

Isolation of the phosphate transport protein. Isolation was performed as described previously [3], except that bovine heart mitochondria were solubilized by 2.5% Triton X-114 at a final concentration of 20 mg protein/ml and in the presence of 4 mg/ml cardiolipin. The SDS gel electrophoresis of the purified fraction consisted of one protein band with an apparent M_r of 33000, previously identified as the phosphate carrier [3]. Only some preparations were contaminated to a small extent by a protein with an apparent M_r of 31500 corresponding to the 2-oxoglutarate carrier [10].

Incorporation of the phosphate carrier into liposomes. Liposomes were prepared as described previously [3] by sonication of 100 mg/ml egg yolk phospholipids in a buffer containing 50 mM KCl, 20 mM KH_2PO_4, 20 mM Hepes and 2 mM EGTA (pH 6.5). The phosphate carrier protein was incorporated into liposomes by the freeze-thaw sonication method [10,11]. 1.0 ml liposomes were mixed with 50 μl hydroxyapatite eluate. After 2 min at 0 °C the mixture was frozen in liquid nitrogen, thawed in a water bath at 10–15 °C and pulse-sonicated (0.3 s sonication,0.7 s intermission) for 6.0 s at 0 °C. The proteoliposomes were diluted with 350 μl of sonication buffer and distributed in Eppendorf cups (320 μl each).

Assay of phosphate transport in liposomes. After 4 min incubation at 25 °C, the proteoliposomes were used for transport measurements by the inhibitor stop method [12]. The phosphate-phosphate exchange was initiated by adding carrier free ^{32}P-phosphate (150000–200000 cpm) and terminated after 1 min by the addition of 2 mM

N-ethylmaleimide, a well known inhibitor of the phosphate carrier. In control samples *N*-ethylmaleimide was added 1 min before the labelled phosphate. To remove the external radioactivity each sample was applied to a Dowex AG1-X8 column, chloride form (0.5 × 4 cm equilibrated with 170 mM sucrose). The liposomes eluted with 1.2 ml 170 mM sucrose were collected and counted. The activity of the phosphate-phosphate exchange was calculated by subtracting the control values from the experimental samples. The *N*-ethylmaleimide-insensitive radioactivity associated to the control samples was always less than 12% with respect to the *N*-ethylmaleimide-sensitive phosphate-phosphate exchange and was not affected at all by *p*-sulfoPITC and by PITC.

Other methods. Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [13]. The stacking gel contained 5% polyacrylamide and the separation gel contained 17.5% acrylamide and an acrylamide-to-bisacrylamide ratio of 150. Staining was performed by the silver nitrate method [14]. Protein was determined by the Lowry method modified for the presence of Triton [15].

Results

PITC and the polar analogue *p*-sulfoPITC were chosen as reagents for the identification of essential lysine residues of the phosphate carrier. While the hydrophobic PITC is expected to modify preferably lysine residues within the core of the membrane [16], the polar *p*-sulfoPITC will, instead, interact with lysines located at the surface of the membrane.

In the experiment reported in Table I, mitochondria were incubated with 2 mM *p*-sulfoPITC or 2 mM PITC at pH 7.5 and 9, respectively. After washing the mitochondria, the phosphate carrier was solubilized, purified and tested for transport activity in the reconstituted system. Both PITC and *p*-sulfoPITC cause a substantial decrease of the phosphate–phosphate exchange activity either at pH 7.5 or at pH 9.0. Since isothiocyanates react with nucleophiles in their unprotonated form [17] and may therefore react also with cysteines, it was necessary to check

TABLE I

EFFECT OF DTE ON THE INHIBITION OF PHOSPHATE TRANSPORT CAUSED BY *p*-SULFOPITC OR PITC

The mitochondria, incubated for 10 min with 2 mM *p*-sulfoPITC or 2 mM PITC at 0 °C and at the pH values indicated, were washed at pH 8.5 in the presence or absence of DTE before solubilization. The activity was measured in liposomes reconstituted with purified phosphate carrier. In the control samples at pH 7.5 the specific phosphate-phosphate exchange activity was 39.7 μmol phosphate/min per mg protein.

Incubation of mitochondria	Washing at pH 8.5	Residual activity (%)	
		pH 7.5	pH 9.0
Control	–	100	103
Control	DTE	98	106
p-SulfoPITC	–	32	23
p-SulfoPITC	DTE	80	54
PITC	–	52	41
PITC	DTE	62	57

whether the inhibition can be accounted for by an interaction of *p*-sulfoPITC and PITC with SH groups. This was done by including an excess of DTE in the washing medium at pH 8.5, since these conditions reverse the binding of these inhibitors with SH groups, but not with NH_2 groups. The results show that DTE restores the activity decreased by *p*-sulfoPITC only partially. The remaining inhibition, which can be attributed to the reaction with NH_2 groups, is more evident at pH 9.0 than at pH 7.5, in agreement with the knowledge that *p*-sulfoPITC reacts with unprotonated NH_2 groups. In the case of PITC, the extent of reactivation by DTE is very small at both pH's. Since PITC is relatively hydrophobic, it will mainly interact with lysines of the phosphate carrier which are located in the hydrophobic part of the protein, where cysteines are supposed to be protonated and therefore not reactive. The results obtained would suggest the presence of lysines located both on the surface and in the membrane core, which are important for the function of the phosphate carrier from mitochondria.

The effect of temperature on the inhibition of phosphate transport by *p*-sulfoPITC and by PITC is shown in Table II. The mitochondria were treated with *p*-sulfoPITC or PITC at 0 °C and 25 °C, respectively, either at pH 7.5 or at pH 9. The medium used for washing the mitochondria

before the solubilization and isolation of the phosphate carrier was always supplemented with DTE in order to remove any possible reaction of the *p*-sulfoPITC and PITC with cysteines. When the modification was carried out at 0 °C and pH 7.5, *p*-sulfoPITC causes a slight inhibition of the reconstituted phosphate transport activity, in agreement with the data reported in Table I. When, on the other hand, the mitochondria were labeled at 25 °C at the same pH, *p*-sulfoPITC causes a strong inhibition of phosphate transport. Also at pH 9.0, the DTE-insensitive inhibition of the purified and reconstituted phosphate carrier by *p*-sulfoPITC was significantly increased when the labeling was carried out at 25 °C as compared to 0 °C. These results indicate that the rate of lysine modification by *p*-sulfoPITC is markedly temperature-dependent, whereas the inhibition of phosphate transport caused by PITC either at pH 7.5 or at pH 9.0 is not affected by a change in the temperature from 0 °C to 25 °C.

For optimization of the DTE-insensitive modification of lysine residues of the phosphate carrier, the influence of incubation time and inhibitor concentration was investigated in more detail.

Fig. 1 shows the time dependence of the reaction of mitochondria with *p*-sulfoPITC at pH 7.5 and 9.0 when carried out at 25 °C. It can be seen that, even at 25 °C, the DTE-insensitive inhibition of the phosphate carrier by *p*-sulfoPITC is strongly dependent on the time of the incubation at both pH's. The rate of inactivation of the phosphate carrier, however, is much higher at pH 9.0 than at pH 7.5. Thus half maximal inhibition of the reconstituted phosphate–phosphate exchange activity is achieved after 5 min at pH 7.5 and after 0.5 min at pH 9.0. The DTE-insensitive inhibition of phosphate transport by PITC is also time-dependent (not shown). In this case, however, due to the hydrophobic nature of PITC, it is questionable whether the reaction is stopped rapidly enough by the addition of DTE.

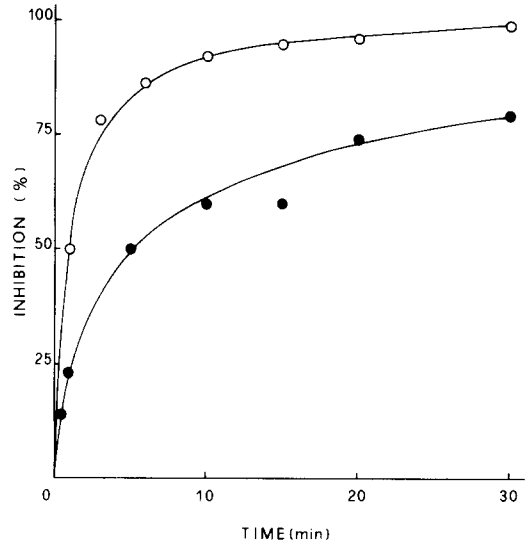

Fig. 1. Time-course of the inhibition of phosphate transport by *p*-sulfoPITC. Mitochondria were incubated at 25 °C with 2 mM *p*-sulfoPITC for the times indicated in the figure at pH 7.5 (●) or 9.0 (○). Washing of the mitochondria at pH 8.5 before solubilization was carried out in the presence of DTE. In the control samples the specific phosphate–phosphate exchange activity was 39.7 (at pH 7.5) and 43.0 (at pH 9.0) μmol phosphate/min per mg protein, respectively.

TABLE II

EFFECT OF TEMPERATURE ON THE INHIBITION OF PHOSPHATE TRANSPORT BY *p*-SULFOPITC AND BY PITC

The mitochondria, incubated for 10 min with 2 mM *p*-sulfoPITC or PITC at 0 °C or at 25 °C and at the pH values indicated, were washed at pH 8.5 in the presence of DTE before solubilization. The activity was measured in liposomes reconstituted with purified phosphate carrier. In the control samples at 0 °C and pH 7.5 the specific phosphate-phosphate exchange activity was 42.0 μmol phosphate/min per mg protein.

Incubation of mitochondria	Residual activity (%)			
	pH 7.5		pH 9.0	
	0 °C	25 °C	0 °C	25 °C
Control	100	97	101	104
p-SulfoPITC	75	27	55	10
PITC	60	53	67	61

The dependence of the inhibition of phosphate transport on the concentration of *p*-sulfoPITC and PITC, respectively, is shown in Fig. 2. Obviously, the polar *p*-sulfoPITC is more effective than the apolar PITC. Thus 50% DTE-insensitive inhibition of phosphate transport at pH 7.5 is obtained with 1.2 mM *p*-sulfoPITC and with 4.0 mM PITC. At pH 9.0 the concentration necessary for 50% inhibition is 0.6 mM (*p*-sulfoPITC) and 3.8 mM (PITC) (not shown).

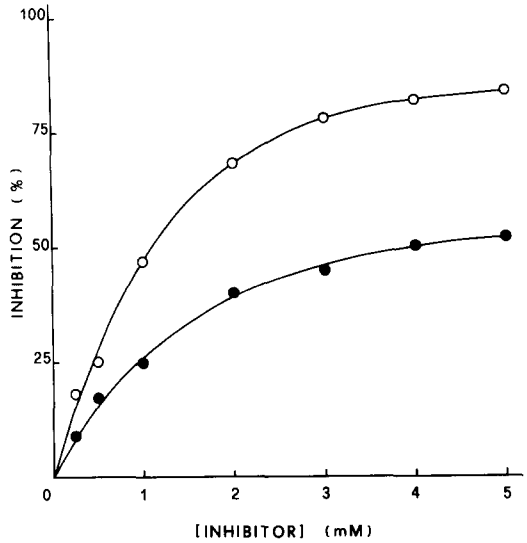

Fig. 2. Concentration dependence of the inhibition of phosphate transport by *p*-sulfoPITC and PITC. Mitochondria were incubated with the indicated concentrations of *p*-sulfoPITC (○) or PITC (●) at 25 °C and at pH 7.5. After 10 min they were washed at pH 8.5 in the presence of DTE before solubilization. In the control samples the specific phosphate-phosphate exchange activity was 43.0 μmol phosphate/min per mg protein.

Since isothiocyanates react with lysines in their unprotonated form, it is important to ascertain whether the pH of the incubation medium influences the observed inhibition in the expected manner. Since lysines located in the lipid core of the membrane are supposed to be always unprotonated whereas those near the membrane surface should undergo protonation and deprotonation according to the pH of the external medium, it has to be expected that the pH of the incubation buffer may influence only the reactivity of those residues which are located at the surface of the membrane. In the experiment reported in Fig. 3, mitochondria were labelled at different pH values either with 2 mM *p*-sulfoPITC or with the same concentration of PITC. The reaction was stopped by adding DTE; the washing procedure was carried out as described in Materials and Methods. The results show that the inhibition by PITC, on the one hand, is only slightly affected by changing the pH. This is in agreement with the idea that the hydrophobic PITC modifies lysine residues which are within the core of the membrane. In the case of *p*-sulfoPITC, on the other hand, the pH of the incubation medium has a pronounced effect upon the inhibition of phosphate transport. This is consistent with the suggestion that *p*-sulfoPITC preferably reacts with residues located at the surface of the membrane. These residues show a protonation–deprotonation equilibrium which is shifted towards the unprotonated form at higher pH values.

It has to be taken into account that, besides lysines, *p*-sulfoPITC also reacts with SH groups of the phosphate carrier (see Table I). It was

TABLE III

PROTECTION BY MERSALYL AGAINST THE *p*-SULFOPITC INHIBITION OF PHOSPHATE TRANSPORT

Mitochondria were incubated for 10 min in the presence or absence of mersalyl (100 nmol/mg protein) at the temperatures and the pH values indicated. Following this incubation, 2 mM *p*-sulfoPITC was added where indicated. The total incubation time was 20 min. The mitochondria were washed at pH 8.5 in the presence or absence of DTE as indicated in the Table. In the control samples at 0 °C and pH 7.5 the specific phosphate-phosphate exchange activity was 37.3 μmol phosphate/min per mg protein.

Incubation of mitochondria	Washing at pH 8.5	Residual activity (%)			
		pH 7.5		pH 9.0	
		0 °C	25 °C	0 °C	25 °C
Control	–	100	100	104	103
Control	DTE	98	105	105	106
Mersalyl	–	11	6	18	5
Mersalyl	DTE	90	88	106	90
p-SulfoPITC	–	34	9	20	2
p-SulfoPITC	DTE	83	33	54	12
Mersalyl + *p*-sulfoPITC	–	9	2	15	6
Mersalyl + *p*-sulfoPITC	DTE	98	90	94	93

therefore important to investigate whether the cysteines modified by *p*-sulfoPITC are the same as those which react with mersalyl, a well-known reversible SH-blocking reagent of phosphate transport [7]. To this end the mitochondria were preincubated with mersalyl before adding *p*-sulfoPITC. After 10 min incubation in the presence of both inhibitors the reaction was stopped with DTE and the mitochondria were washed as described in Materials and Methods. The results of this kind of experiments are shown in Table III. When the mitochondria were treated with mersalyl alone the transport of phosphate was strongly inhibited; however, after addition of DTE the carrier activity was fully recovered. When *p*-sulfoPITC was used, DTE was not able to restore the activity completely. When, however, the mitochondria were preincubated with mersalyl, then treated with *p*-sulfoPITC and finally with DTE, the activity is nearly completely restored under all conditions. In subsequent experiments using lower concentrations of mersalyl it was proven that the extent of transport inhibition by mersalyl always correlates with the extent of protection against the action of *p*-sulfoPITC (not shown). Although mersalyl is known to react exclusively with cysteine residues, it can be envisaged that the lysine residue(s) modified by *p*-sulfoPITC are either sensitive to conformational changes occurring upon binding of mersalyl, or sterically hindered with respect to reaction with *p*-sulfoPITC when mersalyl is bound to its target cysteines.

In order to find out whether sulfhydryl reagents other than mersalyl were also able to protect the phosphate carrier protein against inhibition by *p*-sulfoPITC, mitochondria were treated with *p*-sulfoPITC in the presence of different SH reagents. The results of these experiments, reported in Table IV, clearly show that, whereas mersalyl completely protects the phosphate carrier against the inhibition by *p*-sulfoPITC, *p*-chloromercurybenzenesulphonic acid and mercury chloride do not show any protective effect. Table IV also demonstrates that the inhibition of the phosphate carrier by PITC is not affected by the preincubation of the mitochondria with any SH reagent including mersalyl.

TABLE IV

EFFECT OF SULFHYDRYL REAGENTS ON THE *p*-SULFOPITC AND PITC INHIBITION OF PHOSPHATE TRANSPORT

Mitochondria were incubated for 10 min at pH 9.0 in the presence or absence of 100 nmol/mg protein of the SH reagent mersalyl, *p*-chloromercurybenzenesulfonate or $HgCl_2$. Following this incubation, 2 mM *p*-sulfoPITC or PITC was added where indicated. The total incubation time was 20 min. The temperature was 0 °C. The washing of the mitochondria at pH 8.5 was carried out in the presence or absence of DTE as indicated in the Table. In the control samples without DTE the specific phosphate–phosphate exchange activity was 40.0 μmol phosphate/min per mg protein.

Incubation of mitochondria	Residual activity (%)	
	without DTE	with DTE
Control	100	103
Mersalyl	7	100
p-Chloromercurybenzenesulfonate	11	92
$HgCl_2$	0	100
p-SulfoPITC	24	55
Mersalyl + *p*-sulfoPITC	4	90
p-Chloromercurybenzenesulfonate + *p*-sulfoPITC	11	56
$HgCl_2$ + *p*-sulfoPITC	8	52
PITC	44	55
Mersalyl + PITC	10	53
p-Chloromercurybenzenesulfonate + PITC	7	58
$HgCl_2$ + PITC	4	52

Discussion

Phenylisothiocyanate and the polar analogue *p*-sulfophenylisothiocyanate were found to be effective inhibitors of phosphate transport by the mitochondrial phosphate carrier.

Isothiocyanates react with unprotonated nucleophiles. Thus it can be rationalized that the hydrophobic PITC interacts only with lysines, since in the hydrophobic moiety of the membrane cysteines are present in the protonated form. The polar non penetrant *p*-sulfoPITC instead can react both with unprotonated cysteines and with lysines exposed to the aqueous phase. When mitochondria were incubated at 0 °C and pH 7.5 with *p*-sulfoPITC (Table I) the phosphate transport was strongly inhibited. The inhibition, however, was largely removed after washing the

mitochondria with DTE at alkaline pH, conditions which lead to hydrolysis of the derivative formed between isothiocyanates and sulfhydryl groups. This inhibition was not surprising, since it is well documented that sulfhydryl reagents are powerful inhibitors of phosphate transport. However, a considerable proportion of the inhibition of phosphate transport by *p*-sulfoPITC, even at 0 °C and pH 7.5, cannot be accounted for by reaction of the inhibitor with SH groups and can therefore be attributed to its interaction with lysines.

In order to improve the discrimination between the interaction of *p*-sulfoPITC with lysines on the one hand, and with cysteines on the other, the experimental conditions had to be modified. By rising the temperature of the incubation medium from 0 °C to 25 °C we observed a significant increase in the reactivity of the lysine residues (Table II). Since the covalent derivative formed between isothiocyanates and amino groups is not cleaved by washing with DTE at pH 8.5, we were able to evaluate separately the effect of lysines modification on phosphate transport. The data of Figs. 1 and 2 demonstrate that the DTE-insensitive inhibitions of phosphate transport by *p*-sulfoPITC and PITC are concentration- and time-dependent. The specific modification of lysine residues was also confirmed by labeling the mitochondria at different pH values, either with *p*-sulfoPITC or PITC (Fig. 3). The inhibition due to interaction with *p*-sulfoPITC proved to be pH-dependent as can be expected, since the reagent interacts with lysine(s) located at the surface of the membrane. At physiological pH these residue(s) are mainly positively charged and hence weakly reactive. At higher pH values the unprotonated form is predominant leading to a stronger reactivity. The inhibition by PITC, on the other hand, is virtually not affected by changing the pH, since this apolar reagent interacts with the buried lysines of the hydrophobic phase of the membrane, which should be independent from the bulk-phase pH. All together our results demonstrate the involvement of lysine residues in the function of the phosphate carrier. This conclusion is consistent with the early observation of Tyler [18] who showed that formaldehyde is able to inhibit the entry of phosphate into the mitochondria. Since PITC and *p*-sulfoPITC seem

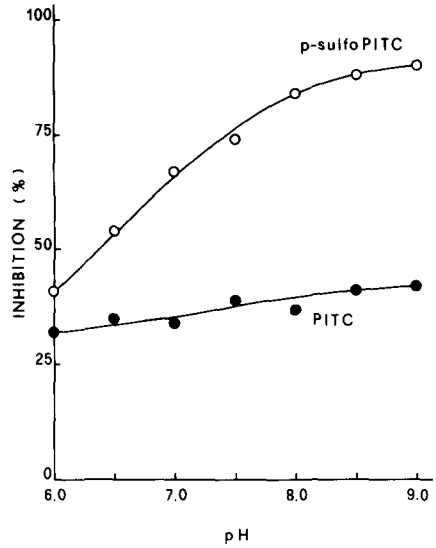

Fig. 3. pH dependence of the inhibition of phosphate transport by *p*-sulfoPITC and PITC. Mitochondria were incubated at 25 °C with 2 mM *p*-sulfoPITC (○) or PITC (●) for 10 min at the indicated pH values. SBE medium was used for the incubation at pH 9.0 and SHE medium for the incubation at the other pH values. Washing of the mitochondria at pH 8.5 was carried out in the presence of DTE before solubilization. In the control samples the specific phosphate-phosphate exchange activity was 44.3 μmol phosphate/min per mg protein. The data are from a representative experiment. Similar results were obtained in four different experiments.

to interact with different lysines, we suggest the presence of at least two types of such essential lysine residues in the phosphate carrier, one type located in the hydrophobic moiety of the protein and the other at the surface of the protein. Both kinds of lysines seem to be important for the transport of phosphate because their modification leads to the inhibition of the phosphate carrier.

Since SH groups have been shown earlier to be important for the proper function of the phosphate carrier [7], we investigated the possible relationship between essential amino and sulfhydryl groups of the phosphate carrier. This was done by preincubating the mitochondria with mersalyl before the reaction with *p*-sulfoPITC. It is interesting that mersalyl protects the phosphate carrier against the DTE-insensitive inhibition caused by *p*-sulfoPITC. This result indicates that, after binding of mersalyl to SH groups in the phosphate carrier, the lysine residue(s) which can be modified by *p*-sulfoPITC are no longer available to the

isothiocyanate inhibitor. This can in principle be explained by steric hindrance and/or a conformational change brought about by mersalyl. In order to discriminate between these two explanations, we have studied the effect of *p*-chloromercurybenzenesulphonic acid and mercury chloride, both well-known SH-blocking inhibitors of the phosphate carrier. These two reagents are considerably smaller than mersalyl. As shown in Table IV, both reagents failed to protect the carrier from *p*-sulfoPITC inhibition. However, mercury chloride, *p*-chloromercurybenzenesulphonic acid and mersalyl are supposed to interact with the same cysteines. Thus, the most likely explanation of these results is that the bound mersalyl sterically hinders the interaction of *p*-sulfoPITC with closely related amino groups of the phosphate carrier. Kolbe and Wohlrab [19] have shown that cysteine 42 is the *N*-ethylmaleimide binding site. It may therefore be speculated that *p*-sulfoPITC modifies lysine 41. It is also interesting to point out that the protective effect of mersalyl is not observed when mitochondria are labelled with PITC. This finding indicates that the essential lysine residues of the hydrophobic mojety of the phosphate carrier are not topologically related to the functional SH groups of the protein, although they are important for the transport of phosphate.

The question arises now whether the lysine residues modified by *p*-sulfoPITC are located at the substrate-binding site of the phosphate carrier. Although a definite answer can of course not be given, there are indications that this is not the case. Ligeti and Fonyo have recently shown that the SH groups of the phosphate carrier reacting with mersalyl are not involved in substrate binding [20]. Our present data indicate that these SH groups are topologically related to the lysine residues modified by *p*-sulfoPITC. Furthermore, we have found that the presence of phosphate during the incubation of mitochondria with *p*-sulfoPITC does not protect the phosphate carrier against inhibition (not shown). On the basis of the primary structure of the phosphate carrier which has recently been published [4,5], we may be able to locate the particular lysine residues modified by *p*-sulfoPITC and by PITC within the amino acid sequence of the protein. Experiments in this respect are currently in progress in our laboratory.

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