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Phosphate transport protein of rat heart mitochondria: location of its SH-groups and exploration of their environment

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(1) The properties of the SH groups of the phosphate transport protein of rat heart mitochondria were investigated on the basis of inhibition caused by SH reagents under different conditions. (2) The essential thiol groups are located near the external surface, as they are accessible to impermeable reagents from the external space. (3) The environment of the sulfhydryl groups influences their reactivity, as alteration of the external pH affects adversely their reactions with ionizable and non-ionizable SH reagents. (4) Intramitochondrial pH exerts a transmembrane effect: alkalinization augments and acidification diminishes the reaction rate of the sulfhydryl groups on the opposite surface of the membrane. (5) Changes of the concentration of the transported substrate occurring exclusively in the extramitochondrial space do not influence the reactivity of the essential SH groups. (6) It is concluded that in transport studies the phosphate transport protein of heart and liver mitochondria show basic similarity. It is suggested that the amino-acid sequence around the NEM-reactive cysteine (i.e., Lys-41 – Cys-42 – Arg-43) does not participate in substrate binding.

Introduction

The functional properties of the mitochondrial phosphate transport protein and the location and accessibility of its essential SH group(s) in situ have been investigated mainly in liver mitochondria [1–9].

Phosphate transport protein has recently been purified from heart mitochondria and its amino-acid sequence has been determined both by amino-acid analysis and from the DNA sequence [10–12]. The only NEM-reactive cysteine (out of a total of 6 or 8), Cys-42, lies between Lys-41 and Arg-43. The vicinity of the two basic amino acids raised the possibility that this region could participate in the formation of the substrate binding site [10].

The models proposed for the transmembrane arrangement of the protein [11,12] could allow the correlation of data of transport studies with the molecular structure of the protein. However, as indicated above, amino-acid sequence analysis was carried out in phosphate transport protein of bovine heart mitochondria and data on phosphate transport and on the essential SH group(s) were obtained in rat liver mitochondria. The two proteins were shown to be different in their electrophoretic mobility, peptide maps arising after cyanogen bromide treatment, their immunological reactions [13] and their amino-acid composition [14]. Phosphate transport protein of liver mitochondria contains more acidic but less basic residues than that from heart, the total difference being about 30 residues [14]. In contrast to the profound organ differences, species differences appeared to be minor [13].

The aim of the present study was to investigate the properties of the essential sulfhydryl groups of phosphate transport protein of rat heart mitochondria in situ in order to obtain data relevant to the established amino-acid sequence and to compare heart and liver protein.

Materials and Methods

Materials

DTNB, NEM, mersalyl and valinomycin were purchased from Serva, DTT from Calbiochem, rotenone from K and K Laboratories (Plainview, NY). [^{3}H]

Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ME, 2-mercaptoethanol; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediaminedehydrochloride.

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Acetate and [^{14}C]methylamine were synthesized by the Centre d'Etudes Nucleaires de Saclay (Gif sur Yvette, France). All the other reagents were of highest purity commercially available.

Preparation of mitochondria

Rat heart mitochondria were prepared as described in Ref. 15. Protein content was determined by the biuret method using bovine serum albumin as standard.

Measurement of phosphate transport

Most data on the phosphate transport protein of liver mitochondria issue from experiments where phosphate transport was coupled to cation movements and the light-scattering changes occurring due to the osmotic water movement were followed [1,3,16]. Heart mitochondria swell rather poorly, thus the optical approaches cannot be applied. As in our experiments the phosphate content of the mitochondria was very low or the absence of extramitochondrial phosphate was required, steady-state phosphate exchange, as described in Ref. 17, could not be measured. Therefore, the activity of the phosphate transport protein was assessed on the basis of net phosphate uptake into respiring mitochondria coupled to the accumulation of either Sr^{2+} or (in the presence of valinomycin) K^+.

Mitochondria (1–2 mg protein per ml) were incubated in the following medium: 10 mM Tris-HCl/3 mM $MgCl_2$/5 mM *n*-butylmalonate/1 μM rotenone/ 244 mM sucrose (pH between 6.8 and 7.5). After 1 min incubation the required inhibitors were added followed by 10 mM Tris-ascorbate, 200 μM TMPD and approx. 100 nmol $Sr(NO_3)_2$ per mg protein. In some experiments, phosphate was present in the incubation medium; in other experiments, it was only added after the inhibitory agents. The concentration of phosphate and the timing of its addition is indicated in the legends to the figures. In Figs. 1–3 the incubation medium was supplemented by radiolabelled phosphate (approx. 0.1 μCi per ml).

Phosphate uptake was terminated after 60 s by rapid centrifugation at 12000 rpm for 90 s in a Janetzki TH-12 bench centrifuge. The supernatant was discarded, the tube rinsed with chilled 0.25 M sucrose and wiped dry. The pellet was extracted by 1 ml 7% ice-cold perchloric acid and 0.8 ml protein-free extract was used for phosphate determination according to Ref. 18. Alternatively, radioactivity of the extract was measured on the basis of the Cerenkov effect in a Beckman LS-250 liquid scintillation spectrometer.

In the experiment in which the initial phosphate content of mitochondria was determined (Table I), at the end of the preincubation period, phosphate movements were stopped by an excess of mersalyl and the suspension was layered on the top of 7 ml ice-cold 0.5 M sucrose (containing 10 mM Tris-HCl and 30 μM mersalyl) and spun for 2 min in a Janetzki K-24 centrifuge at 15000 $\times$ *g*. Thereafter, the tubes were handled as described above.

In the experiment of Fig. 4A, phosphate uptake was followed indirectly by measuring the concomitant K^+ uptake in the presence of valinomycin. The medium contained 0.5 mM KCl and ion movement was initiated by 250 ng valinomycin per mg protein. The apparatus consisted of a K^+-sensitive membrane electrode attached to a pH meter and a potentiometric recorder, produced by Radelkis, Hungary.

Measurement of intramitochondrial pH

Intramitochondrial pH was calculated on the basis of the distribution of [^{3}H]acetate and [^{14}C]methylamine as described by Nicholls [19]. Mitochondria (1.5 mg per ml) were incubated in 1 ml of the above medium supplemented with 0.25 μCi/ml [^{14}C]methylamine and 1.5 μCi/ml [^{3}H]acetate. After incubation for 1 min in the presence of the substances indicated in Table I, mitochondria were spun as detailed above. The pellet was dissolved in 100 μl concentrated formic acid and the radioactivity was measured in a Beckman LS-250 liquid scintillation spectrometer.

Results

Location of the essential SH groups and the effect of the external pH on their reaction

The suggestion that the essential sulfhydryl groups of phosphate transport protein in rat liver mitochondria are located near the external surface is based on the fact that phosphate transport could be blocked by various impermeable SH reagents [2,3,5,7,9]. The fact that reversible SH reagents (mersalyl, DTNB) protect the protein against the irreversible action of the agent NEM suggests that all three compounds react with the same thiol group(s). Therefore, we compared the effect of the permeable non-ionizing reagent NEM with the action of the impermeable and ionizable substances mersalyl and DTNB in rat heart mitochondria. The results of Figs. 1–3 demonstrate that all the three applied reagents inhibited phosphate transport completely. The effective concentration of NEM corresponded to that reported previously for liver mitochondria. In contrast, inhibition of phosphate uptake was attained in heart mitochondria only by considerably higher concentration of mersalyl (approx. 2-fold) and DTNB (5–6-fold) than in liver mitochondria. Thus, the essential SH groups of the phosphate transport protein of heart mitochondria are accessible from the external space similarly to those in liver, but the lower sensitivity towards hydrophilic reagents points to differences in their environment.

The influence of the vicinity on the reactions of the thiol groups of phosphate transport protein was examined by varying the extramitochondrial pH. In the

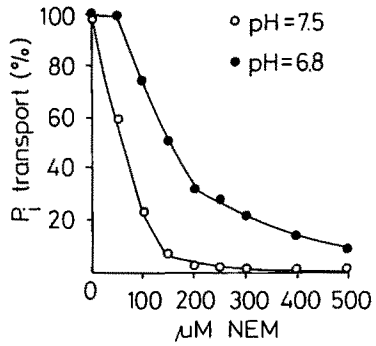

Fig. 1. Inhibition of phosphate uptake into rat heart mitochondria by NEM at pH 7.5 (○) and 6.8 (●). Phosphate (1 mM, specific activity 300 d.p.m./nmol) was present before the SH reagent. Protein concentration was 1 mg/ml. The action of the indicated NEM concentrations was stopped after 60 s by a 2-fold excess of 2-mercaptoethanol and phosphate uptake was initiated 60 s later by the addition of respiratory substrate (10 mM Tris-ascorbate plus 200 μM TMPD) and Sr^{2+} (120 nmol $Sr(NO_3)_2$ per mg protein). Points of the graph represent the mean of duplicate determinations. The control rates of phosphate transport (100% value) at pH 7.5 were 70 nmol·min^{-1}·mg^{-1} protein and at pH 6.8, 75 nmol·min^{-1}·mg^{-1} protein.

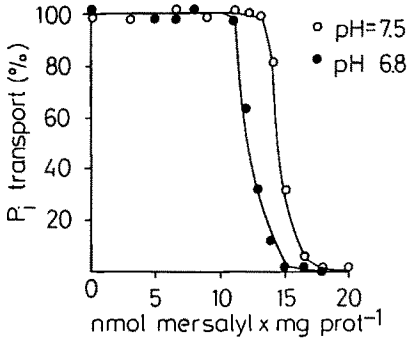

Fig. 2. Inhibition of phosphate uptake into rat heart mitochondria by mersalyl at pH 7.5 (○) and 6.8 (●). The experimental conditions were the same as detailed for Fig. 1. Mersalyl was allowed to react for 30 s. One nmol mersalyl per mg protein corresponds to 1 μM concentration. The control rates of phosphate transport (100% value) at pH 7.5 were 120 nmol·min^{-1}·mg^{-1} protein, and at pH 6.8, 105 nmol·min^{-1}·mg^{-1} protein.

case of simple thiol compounds (like cysteine, 2-mercaptoethanol, etc.), alkalinization of the solution increases the reaction rate both with NEM or DTNB [2,7]. The essential sulfhydryl groups of the phosphate transport protein behave similarly only if tested with NEM. In the experiment shown in Fig. 1, 50% inhibition of phosphate transport was achieved by 150 μM NEM at pH 6.8, contrasted to 60 μM at pH 7.5. In five identical experiments the increase of the 50% inhibitory concentration at pH 6.8 as compared to pH 7.5 varied between 2-fold and 5-fold. These results corresponded well to results obtained with liver mitochondria using the same transport system (data not shown).

Opposite dependence on external pH was observed when the ionizable, non-penetrant reagents mersalyl or DTNB were used (Figs. 2 and 3). At pH 6.8 the addition of up to 11 nmol mersalyl per mg protein did not inhibit phosphate transport, and 15 nmol per mg caused complete inhibition, the half-maximal effect occurring at 12 nmol per mg. At pH 7.5, even 13 nmol mersalyl per mg protein was without any effect and 50% inhibition was achieved only by 14.5 nmol per mg. In liver mitochondria changes of similar magnitude have been reported.

The reaction with DTNB resembles that with mersalyl. A shift of the external pH from 6.8 to 7.5 increased the concentration required for 50% inhibition from 80 μM to 170 μM (Fig. 3).

Accordingly, the strategic SH groups of the phosphate transport protein are located also in heart mitochondria at or close to the outer surface of the inner membrane and they behave similarly to those of liver mitochondria: in both cases, alkaline pH of the extramitochondrial space favours their reaction with NEM but hinders their reaction with mersalyl or DTNB.

The transmembrane effect of the internal pH on the reaction of the essential sulfhydryl groups

In liver mitochondria a transmembrane pH effect was reported: variations of the intramitochondrial pH caused alterations of the reactivity of the SH groups on the opposing side of the membrane. It was proposed that the changes of reactivity reflected a modification of the protein conformation related to the transport function [4,7]. It was thus crucial to decide whether the same phenomenon can be observed also in heart mitochondria.

Intramitochondrial pH and phosphate content were measured in parallel under various conditions and correlated to the reactivity of the essential sulfhydryl groups. Thiol groups of phosphate transport protein in situ were allowed to react with a given concentration of

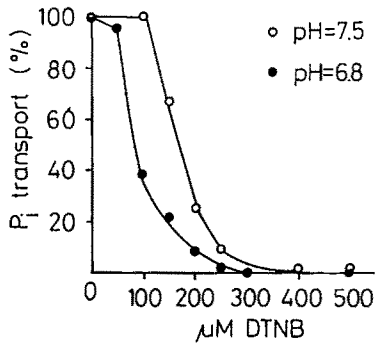

Fig. 3. Inhibition of phosphate uptake into rat heart mitochondria by DTNB at pH 7.5 (○) and 6.8 (●). The experimental conditions were the same as detailed for Fig. 1. The action of the indicated concentrations of DTNB was stopped after 60 s by a 3-fold excess of 2-mercaptoethanol and phosphate uptake was initiated after a further 60 s. The control rates of phosphate transport (100% value) at pH 7.5 were 125 nmol·min^{-1}·mg^{-1} protein, and at pH 6.8, 90 nmol·min^{-1}·mg^{-1} protein.

TABLE I

Phosphate content and internal pH of rat heart mitochondria incubated under various conditions

The two parameters were measured in parallel as described in Materials and Methods. The external pH was 7.4. The values represent the mean of three parallel determinations which differed by less than 5%. One representative experiment out of three similar ones is reported.

Preincubation in the presence of	P_i content (nmol/mg protein)	pH_{int}
–	7.6	7.77
Phosphate (5 mM)	9.7	7.41
Valinomycin (250 ng/mg) + KCl (10 mM) + ascorbate (10 mM) + TMPD (200 μM)	12.2	8.77
Nigericin (500 ng/mg)	4.7	7.18
Nigericin (500 ng/mg) + phosphate (5 mM)	4.7	7.18

SH reagent for a definite period and the rate of reaction was assessed on the basis of transport inhibition brought about by this treatment.

As shown in Table I, non-respiring heart mitochondria suspended in sucrose-based medium maintain an internal pH more alkaline than the suspending medium and contain a moderate amount of phosphate. When incubation is carried out in the presence of 5 mM phosphate, the phosphate content increases and the alkalinity of the internal space disappears. Parallel to these changes, the sensitivity of the transport process for the irreversible SH reagent NEM decreases. In the experiment shown in Fig. 4A, 50% inhibition was achieved by approx. 90 μM NEM in the presence of 5 mM phosphate, instead of 60 μM in its absence. On the other hand, preincubation of mitochondria with respiratory substrate, valinomycin and K^+ but without phosphate increased the internal pH by 1.0 unit and the phosphate content by 4.6 nmol per mg. Under these conditions, NEM proved to be considerably more effective: 20 μM was sufficient for 50% inhibition (Fig. 4A). It should be noted that phosphate content was increased by both treatments but changes of the internal pH were opposite. Alterations of the reactivity of the SH groups parallelled changes of the pH and not that of the phosphate content.

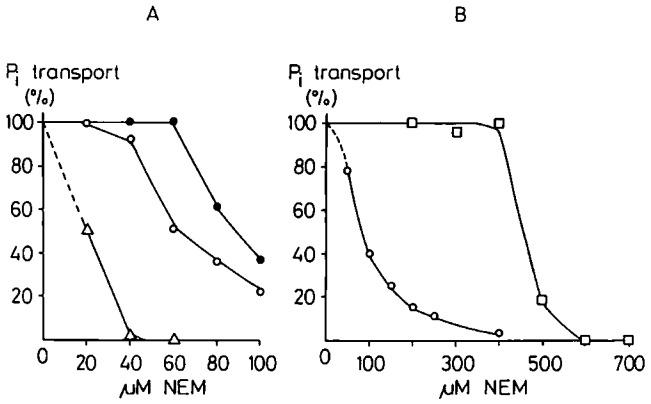

Fig. 4. Alterations of the reactivity of the SH groups of phosphate transport protein. Intramitochondrial pH was manipulated in (A) by the addition of phosphate (5 mM) (●) or valinomycin (250 ng/mg) plus respiratory substrate in the absence of phosphate (△) or in (B) by nigericin (500 ng/mg) (□); (○) control samples without any pretreatment. The extramitochondrial pH was 7.4. The action of the indicated concentrations of NEM was stopped after 60 s by a 2-fold excess of 2-mercaptoethanol and the reaction mixture was supplemented to contain both phosphate (5 mM), respiratory substrate and valinomycin (in (A)) or Sr^{2+} (in (B)). Phosphate uptake was followed in (A) indirectly on the basis of K^+ movements and in (B) directly by chemical determination (as detailed in Materials and Methods). The control rates of phosphate transport (100% value) in (A) were 150 nmol·min^{-1}·mg^{-1} protein, and in (B) 65.2 nmol·min^{-1}·mg^{-1} protein.

These experiments demonstrate that heart mitochondria are similar to liver mitochondria: intramitochondrial acidification decreased, whereas alkalinization increased the reactivity of thiol groups on the opposing surface of phosphate transport protein.

Does substrate binding change the reactivity of the essential SH group(s)?

The presence of the substrate of an enzyme or a transport protein was shown to change the reaction between the amino-acid residues participating in the formation of the binding site and the appropriate group-specific reagents. In the case of phosphate transport protein the simplest way of testing this point would be to allow the protein to react with SH reagents in the presence of different concentrations of inorganic phosphate. The results detailed in the previous section indicate that alteration of the external phosphate concentration can give no information about any direct effect on the reactivity of the SH groups because of the concomitant internal pH change.

Phosphate uptake and the consequent internal acidification could be prevented in liver mitochondria by nigericin [8]. In K^+-free medium this ionophore catalyzes the exchange of internal K^+ for external H^+ and thus creates a pH gradient which not only effectively opposed the inward movement of phosphate, but also discharged part of the endogenous phosphate. In heart mitochondria the pretreatment with nigericin resulted in a drop of the intramitochondrial pH by almost 0.6 units and a significant decrease of the phosphate content (Table I). In line with the internal acidification, the sensitivity for NEM also decreased: 450 μM NEM was required for 50% inhibition of phosphate transport (Fig. 4B). As expected, in the presence of nigericin, neither internal pH nor phosphate content depended on the presence or absence of phosphate in the extramitochondrial space.

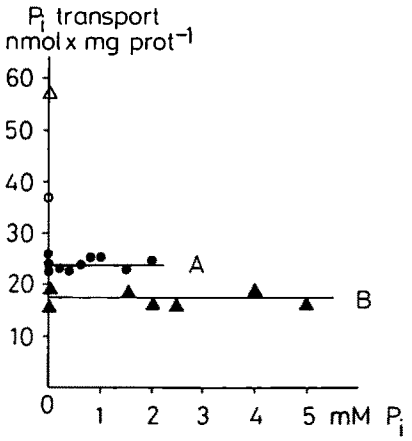

Fig. 5. Inhibition of phosphate transport protein by NEM in the presence of different concentrations of phosphate. NEM (500 μM) was allowed to react with mitochondria in the presence of nigericin (250 ng/mg) and the indicated concentrations of phosphate at pH 7.4. After 60 s the reaction was stopped by a 5-fold excess of 2-mercaptoethanol; phosphate was supplemented up to 5 mM and phosphate uptake was triggered by the addition of respiratory substrate plus Sr^{2+}. Phosphate uptake was determined by the chemical method. All the other conditions were as described in Materials and Methods. (●,▲) samples inhibited by NEM, (○,△) control samples in the absence of NEM. Experiments A (●) and B (▲) were carried out on two different mitochondrial preparations.

Consequently, nigericin pretreatment provided the suitable experimental condition to determine the direct effect of extramitochondrial phosphate concentration changes occurring in the proximity of the investigated SH groups. Fig. 5 demonstrates the results of two separate experiments in which phosphate transport protein was partially inhibited by NEM in the presence of various concentrations of inorganic phosphate. No tendency to any change in sensitivity could be revealed in either experiment. As the K_m of the transport process lies around 1.6 mM, the protective effect of the substrate should be apparent in this concentration range.

Discussion

In spite of the differences of the immunological reactions, peptide mapping and amino-acid composition, phosphate transport protein of heart mitochondria proved to be similar to that of liver mitochondria in several aspects.

(1) The sulfhydryl groups essential for the transport function are accessible from the external space. This observation is in accord with both models proposed on the basis of the amino-acid sequence [11,12], where Cys-42 falls between two helical transmembrane segments, facing the external compartment.

(2) Intramitochondrial pH exerts a transmembrane effect: it modifies the steric position and consequently the reaction rate of the thiol groups of phosphate transport protein located near the opposite surface of the membrane. The reported variations of phosphate transport inhibition are several times larger than could be the effect of a decrease of the reagent concentration due to its binding to SH groups not belonging to the phosphate transport protein [20]. Thus, we maintain our suggestion that the observed alterations in the reactivity of the essential SH groups of the protein reflect conformational changes of the protein itself. As this conformational change represents a basic similarity of the liver and heart protein molecules, we propose it to be an inherent property of the translocation process.

(3) The environment of Cys-42 does not participate in phosphate binding because the transported substrate, phosphate, does not protect the essential thiol groups against SH reagents. This feature could be tested only in that conformation of the protein where the SH groups are in the least accessible position (namely, in the presence of nigericin). However the fact that the minimal amount of internal phosphate can still be exchanged for external radiolabelled phosphate proves that the substrate has access to its binding site also in this state.

The vicinity of the reacting SH group is negatively charged as ionization of mersalyl and DTNB (anionic reagents) slows down their rate of reaction. It is highly unlikely that this negative environment could participate in $H_2PO_4^-$ ion binding.

We propose that the essential cysteine(s) have a 'permissive' role, in that their free state would be a prerequisite for the transport-related conformational change to occur and their blocked state would sterically interfere with this molecular motion.

At this stage of speculation recent findings concerning another H^+-coupled transport process, the lactose carrier of *E. coli*, should be recalled. In the case of this protein, blocking of Cys-148 resulted in the complete inhibition of the transport process. However, replacement of Cys-148 for Ser or for Gly by means of site-directed mutagenesis proved that the presence of cysteine at that position was not an absolute requirement for the transport function [21].

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