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Photoaffinity labeling of the mitochondrial phosphate carrier by 4-azido-2-nitrophenyl phosphate

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The effect of 4-azido-2-nitrophenyl phosphate (ANPP), a photoreactive analogue of phosphate, on the phosphate carrier of pig-heart mitochondria has been investigated. In the dark, ANPP inhibits the transport of phosphate in a competitive manner with a K_i of 3.2 mM. Upon photoirradiation with visible light, [^{32}P]ANPP binds covalently to the phosphate carrier and the inhibition becomes irreversible. Both the inhibition of phosphate transport and the incorporation of [^{32}P]ANPP into the phosphate carrier depend on the concentration of the inhibitor and the pH of the medium. Incubation of the mitochondria with phosphate during illumination in the presence of ANPP protects the carrier against inactivation and decreases the amount of radioactivity which is found to be associated with the purified protein. By extrapolation it is calculated that at 100% inactivation of the phosphate carrier 0.35 mol of reagent are bound per mol of 33 kDa carrier protein. It is concluded that ANPP can be used for photoaffinity labeling of the mitochondrial phosphate carrier at the substrate-binding site.

Introduction

The synthesis of ATP during oxidative phosphorylation requires uptake of ADP and phosphate into the mitochondria. The translocation of phosphate across the inner mitochondrial membrane is catalyzed by a specific transport system known as the phosphate carrier (for a review, see Refs. 1–3). This protein has been purified from heart mitochondria and reconstituted in liposomes [4–6]. The amino-terminal part of the protein has been sequenced [7–8] and cysteine at position 42 has been identified as the binding site of the inhibitor *N*-ethylmaleimide [7].

Nothing, however, is known so far about the substrate-binding site of the phosphate carrier. Indeed, it has not even been established whether the particular –SH group responsible for the inhibition by *N*-ethylmaleimide is located at or near the substrate-binding site, although it has been reported that the presence of phosphate decreases to some extent the reactivity of SH-blocking reagents with the phosphate carrier [9–11].

In order to have a tool to characterize the phosphate-binding site, the reactivity of the phosphate carrier with the photoreactive substrate analogue ANPP has been investigated. ANPP had been introduced by Lauquin et al. [12] to label the phosphate-binding site of the F_1-ATPase. By photoirradiation of submitochondrial particles with [^{32}P]ANPP, these authors showed that also a 30

Abbreviations: ANPP, 4-azido-2-nitrophenylphosphate; ANP, 4-azido-2-nitrophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulphate.

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kDa protein is labeled by ANPP. This was tentatively suggested to be the phosphate carrier. In this paper we show that ANPP inhibits the phosphate carrier. The inhibition becomes irreversible in the presence of light. Phosphate protects the carrier against inactivation and labeling by [^{32}P]ANPP. Complete inhibition of the phosphate carrier is accomplished when 0.35 mol ANPP are bound per mol of phosphate carrier.

Materials and Methods

Materials. ANP and ANPP were synthesized from 4-amino-2-nitrophenol (Aldrich) as described by Lauquin et al. [12]. [^{32}P]ANPP was synthetized by the same method using [^{32}P]phosphate. Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad. [^{32}P] phosphate was obtained from the Radiochemical Centre (Amersham, U.K.), egg yolk phospholipids from Fluka, cardiolipin from Avanti-Polar Lipids, Triton X-114 and *N*-ethylmaleimide from Serva. Mitochondrial phospholipids were isolated by the procedure described in Ref. 13. All the other reagents were of the highest purity commercially available.

Incubation with ANPP. Pig-heart mitochondria were prepared as described in Ref. 14 and stored at −70°C. For the incubation with ANPP, thawed mitochondria were washed twice in 250 mM sucrose/10 mM Tris-HCl (pH 6.8)/1 mM EGTA/1 mM NaF, resuspended in the same medium at a concentration of 10 mg protein/ml and then incubated with the indicated concentrations of ANPP at 0°C in the dark. After 30 min, aliquots were transferred into glass tubes and illuminated with a 250 W Osram Halogen lamp for 15 min at 0°C as described in Ref. 12, while the rest was kept in the dark. To prevent cleavage of ANPP, NaF was included in the incubation buffer to inhibit potential phosphatase activity [12,15]. In control experiments it was shown that NaF has no effect on the phosphate carrier activity. After the incubation, the mitochondria treated with ANPP with or without illumination were washed twice in 20 mM K H_2PO_4/20 mM KCl/2 mM EGTA (pH 6.5). In the experiments where the transport of phosphate was measured by the swelling technique, freshly prepared mitochondria were incubated with ANPP and ANPP-treated mitochondria were directly transferred into the assay mixture.

Isolation of the phosphate carrier. It was performed as described previously [6], except that the mitochondria were solubilized by 2% Triton X-114 at a final concentration of 15 mg protein/ml.

Incorporation of the phosphate carrier into liposomes. Liposomes were prepared as described previously [6] by sonication of a mixture of 80 mg/ml egg yolk phospholipids and 20 mg/ml mitochondrial phospholipids in a buffer containing 50 mM KCl/20 mM Hepes/1 mM EDTA (pH 6.5). In the experiments where the phosphate-phosphate exchange was measured, 20 mM phosphate was also included in the sonication buffer.

The phosphate carrier protein was incorporated into liposomes by the freeze-thaw-sonication procedure [16,17]. 1 ml liposomes was mixed with 50 μl mitochondrial extract or hydroxyapatite eluate. After 2 min at 0°C the mixture was frozen in liquid N_2, thawed in a water bath at 15°C and then pulse-sonicated (0.3 s sonication, 0.7 s intermission) for 6.0 s at 0°C.

Assay of phosphate transport. In mitochondria the transport of phosphate was assayed by monitoring the rate of mitochondrial swelling, i.e., by recording the decrease in absorbance at 546 nm of the mitochondrial suspension in 100 mM ammonium phosphate [18,19] with an Eppendorf photometer model 1101 M. Alternatively, phosphate transport in intact mitochondria was assayed by measuring the uptake of [^{32}P]phosphate by the inhibitor stop method [20]. Mitochondria (2 mg/ml) were incubated at 0°C in 100 mM KCl/20 mM Tris-HCl (pH 6.5)/1 mM EGTA/1 μg per ml rotenone/20 mM butylmalonate (to inhibit the dicarboxylate carrier which is also able to transport phosphate). The uptake was started by adding 0.5 mM [^{32}P]phosphate and stopped 5 s later with 1 mM mersalyl. After rapid centrifugation of the mitochondria, the radioactivity was counted and the amount of substrate incorporated into the matrix space was calculated as described [20].

In order to measure phosphate transport in proteoliposomes, proteoliposomes prepared in the presence or in the absence of 20 mM phosphate were distributed in Eppendorf cups (160 μl each) and equilibrated at 25°C. After 4 min they were

used for exchange or uptake measurements, respectively, essentially as described previously [6]. Transport was initiated by adding carrier free [^{32}P]phosphate (exchange) or the indicated concentrations of [^{32}P]phosphate (uptake) and was stopped after 1 min by the addition of 2 mM *N*-ethylmaleimide. In control samples *N*-ethylmaleimide was added 30 s before the labeled substrate. 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 phosphate uptake or phosphate-phosphate exchange was calculated by subtracting the control values from the experimental samples.

SDS-gel electrophoresis. Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [21]. The stacking gel contained 5% polyacrylamide and the separation gel contained 17.5% acrylamide and an acrylamide-to-bisacrylamide ratio of 150. Staining with Coomassie blue R 250 and destaining were carried out according to Downer et al. [22]. Autoradiographies were performed making use of Kodak X-OMAT S films. Gel slices 1 mm thick were digested by 0.6 ml 15% H_2O_2 at 70°C for 8 h [23], were mixed with 4 ml of scintillation mixture (Maxi-fluor Baker, The Netherlands) and counted.

Other methods. Protein was determined by the Lowry method modified as described in Ref. 24 and adapted to the presence of Triton [25]. To measure the binding of [^{32}P]ANPP to the phosphate carrier, preparations of purified carrier obtained from [^{32}P]ANPP-treated mitochondria were delipidated [26] and assayed for radioactivity. Alternatively, the [^{32}P]ANPP binding was measured by dissolving [23] the protein bands, obtained from SDS gels, corresponding to the purified phosphate carrier and counting the radioactivity.

Results

Inhibition of the phosphate carrier by ANPP without illumination

In preliminary experiments the effect of ANPP on the transport of phosphate was tested in intact mitochondria without illumination. It was found that ANPP inhibits the uptake of [^{32}P]phosphate catalyzed by the phosphate carrier, causing 50% inhibition at a concentration of approx. 4 mM.

In order to analyze the inhibition of the phosphate carrier caused by ANPP in the dark, the influence of this inhibitor on the rate of phosphate transport must be determined. Since the rate of phosphate uptake in mitochondria is very high and cannot be measured accurately without the use of rather sophisticated apparatus [20,27], we have studied the effect of ANPP on phosphate transport in reconstituted liposomes. The reciprocal plot presented in Fig. 1 shows that ANPP inhibits the transport of phosphate in a competitive manner with respect to the substrate. The K_i can be determined to be 3.2 mM. In contrast, under the same experimental conditions, ANP, the non phosphorylated analogue of ANPP, shows no effect on phosphate transport in reconstituted liposomes (not shown).

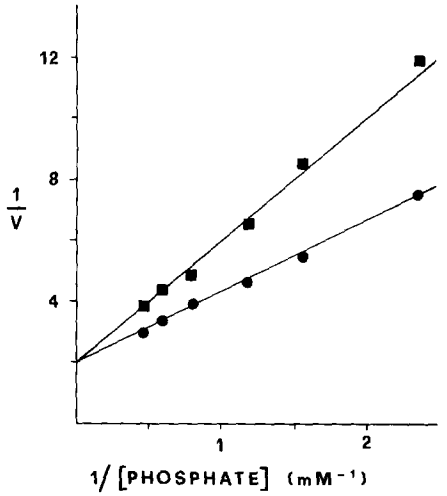

Fig. 1. Competitive inhibition of phosphate transport by ANPP in the dark. The mitochondria were solubilized with Triton X-114 and the extract was incorporated into liposomes. The activity of phosphate transport was measured in reconstituted liposomes. The reaction mixture contained the indicated concentrations of [^{32}P]phosphate and, where indicated, 2.8 mM ANPP added together with the labeled substrate. Reaction time: 1 min; temperature: 25°C. *V* is expressed in μmol/min per mg protein. ■, with ANPP; ●, without ANPP. ANPP has no effect on the radioactivity associated to control samples, i.e., with the stop inhibitor added before the labeled substrate.

Inhibition of the phosphate carrier by ANPP upon illumination

As an azido compound, ANPP can react with proteins after photoactivation and formation of a nitrene intermediate. Thus the effect of ANPP on the transport of phosphate in intact mitochondria was investigated upon illumination. As a control, ANP was also tested. Pig heart mitochondria were incubated in the dark at 0°C in the presence of 1.4 mM ANPP or ANP and in the absence of phosphate. After 30 min an aliquot of the incubation was subjected to photoirradiation for 15 min at 0°C, while the rest was kept in the dark. Both portions were then tested for phosphate-transport activity by the swelling technique, i.e., in the presence of a very high concentration of phosphate. The results illustrated in Fig. 2A show that both ANPP and ANP when used in the dark do not have any effect on the swelling of mitochondria in the presence of 100 mM ammonium phosphate. When, on the other hand, mitochondria were illuminated in the presence of the azido compounds, ANPP strongly inhibits the rate of swelling, whereas ANP still has no effect (Fig. 2B). The striking difference between the effect of ANPP on the mitochondrial swelling in 100 mM phosphate under illumination and its lack of effect in the dark demonstrates the formation of an irreversible modification of the phosphate carrier by ANPP during irradiation. Furthermore, the failure of ANP to inhibit the phosphate carrier even in the presence of light indicates that the phosphate group and not the hydrophobic part of ANPP is important for the interaction between the inhibitor molecule and the phosphate carrier.

Fig. 3 illustrates the dependence of the light-induced ANPP inhibition of the phosphate carrier on the concentration of the inhibitor. In these experiments mitochondria were illuminated in the presence of ANPP. After washing of the mitochondria in the presence of an excess of phosphate, the membranes were solubilized with Triton X-114 and the activity of the reconstituted phosphate transport was measured in liposomes. The results show that the inhibition of the phosphate-phosphate exchange activity increases with increasing concentrations of ANPP (Fig. 3), reaching a value of 76% at 1.6 mM ANPP. As a control, Fig. 3 shows that the activity of the phosphate carrier is not influenced by ANPP without photoirradiation, in agreement with the finding that the inhibition by ANPP in the dark is

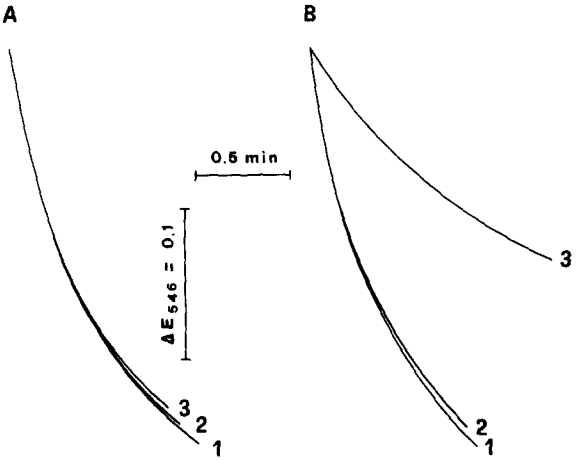

Fig. 2. The effect of ANPP and ANP, in the dark and upon illumination, on turbidity changes corresponding to swelling of mitochondria in ammonium phosphate. Freshly prepared mitochondria were incubated with 1.4 mM ANPP (curves 3) or ANP (curves 2) with and without illumination. 0.5 mg mitochondrial protein were transferred to cuvettes containing 0.5 ml of 100 mM ammonium phosphate, 10 mM Tris-HCl, 1 μg rotenone (pH 7.4). Temperature: 25°C. (A) without illumination; (B) with illumination. Curves 1, controls without addition of ANPP or ANP.

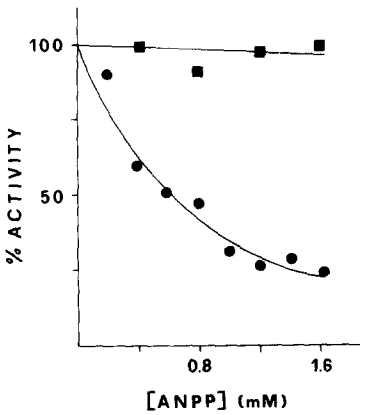

Fig. 3. Concentration dependence of the inhibition of the phosphate-phosphate exchange activity by ANPP in the dark and upon illumination. Mitochondria incubated with the indicated concentrations of ANPP with (●) or without (■) illumination were washed in the presence of an excess of phosphate before solubilization. The activity was measured in liposomes reconstituted with Triton X-114 mitochondrial extracts.

reversible (Fig. 1 and 2A). It should also be pointed out that photoirradiation of the mitochondria alone, i.e., in the absence of ANPP, does not affect the phosphate carrier (not shown).

It is known that the binding affinity of phosphate for the phosphate carrier increases on lowering the pH [28]. It was therefore interesting to investigate also the pH dependence of the light-induced inhibition of the phosphate carrier by ANPP. For this purpose, mitochondria were illuminated in the presence of 0.3 mM ANPP at different pH values. After washing and solubilization of the mitochondria, phosphate-transport activity was measured at the same pH of 6.5 in liposomes reconstituted with the different mitochondrial extracts. It was found that the inhibition of the phosphate carrier by ANPP in the light increases substantially on lowering the pH from 7.4 to 6.4 (data not shown). In a typical experiment, the residual activity of the phosphate carrier treated with 0.3 mM ANPP was 73% at pH 7.0 and only 32% at pH 6.4. Correspondingly, the amount of [^{32}P]ANPP bound to the purified phosphate carrier was increased from 0.14 to 0.29 mol/mol of carrier protein.

Protein labeling by [^{32}P]ANPP

In order to identify the mitochondrial proteins labeled by ANPP, the SDS-extract, the Triton X-114 extract and the purified phosphate carrier obtained from [^{32}P]ANPP photolabeled mitochondria were subjected to SDS-polyacrylamide gel electrophoresis. The radioactive-labeled proteins were then identified by autoradiography. Lanes 4 of Fig. 4A and B show that [^{32}P]ANPP is bound to the purified phosphate carrier which corresponds to a single band of an apparent molecular weight of 33 kDa. Among the other protein bands present in the SDS or Triton X-114 extracts of mitochondria only two are substantially labeled (Fig. 4, lanes 2 and 3). On the basis of their staining intensity and their molecular weight, they can be identified as the ADP/ATP carrier (30 kDa) and the β-subunit of the F_1-ATPase complex (50 kDa). The labeling of the latter by [^{32}P]ANPP has been reported by Lauquin et al. [12].

On the basis of the competition experiments it can be assumed that ANPP binds to the phosphate carrier at the substrate-binding site. Therefore, it was necessary to test whether phosphate correspondingly protects the phosphate carrier from inactivation and labeling by [^{32}P]ANPP. Fig. 5 shows that, in fact, the light-induced inhibition of the phosphate-transport activity by 0.8 mM ANPP is progressively suppressed by the presence of increasing concentrations of phosphate during photoirradiation. A nearly complete protection is achieved in the presence of 10 mM phosphate. The specificity of the protection of the phosphate carrier against ANPP inactivation was investigated by testing the effect of several anions. As shown in Fig. 5, sulphate up to 20 mM has no protective effect. Similarly ADP, malate, citrate and 2-oxoglutarate have virtually no effect on the inhibition of the phosphate carrier by ANPP (not shown). In contrast, arsenate, which is known to be a substrate of the phosphate carrier in mitochondria [29], exhibits a significant protection, although lower than that exerted by phosphate. Under the conditions of Fig. 5, the inhibition of the phosphate-transport activity caused by 0.8 mM ANPP is reduced to 23% by 20 mM arsenate.

The observed protection by phosphate against inhibition by ANPP is accompanied by a decreased

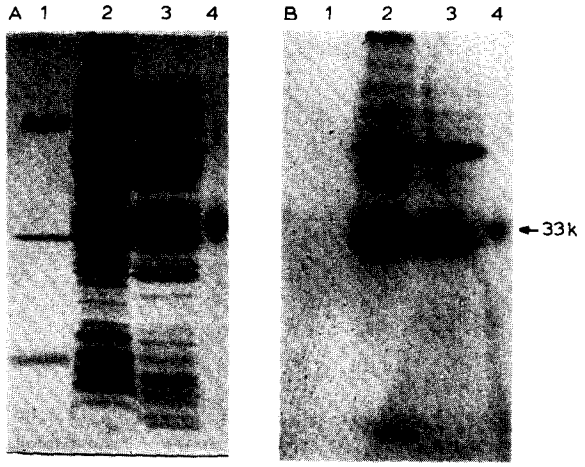

Fig. 4. Labeling of the phosphate carrier by [^{32}P]ANPP. SDS-polyacrylamide gel electrophoresis (A) and autoradiography (B) of the SDS extract (lanes 2), the Triton X-114 extract (lanes 3) and the purified phosphate carrier (lanes 4) obtained from 1 mM [^{32}P]ANPP photolabeled mitochondria. Lanes 1, marker proteins (bovine serum albumin, carbonic anhydrase, cytochrome *c*).

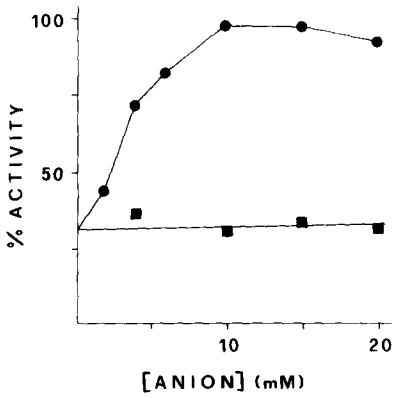

Fig. 5. Effect of phosphate and sulphate on the light-induced inhibition of the phosphate carrier by ANPP. The indicated concentrations of phosphate (●) or sulphate (■) were present during the incubation of the mitochondria with 0.8 mM ANPP in the light. The activity was measured as in Fig. 3.

labeling of the carrier. Thus the radioactivity associated with the phosphate carrier purified from [^{32}P]ANPP photolabeled mitochondria is much less in the presence of phosphate during photoirradiation than in its absence (Fig. 6).

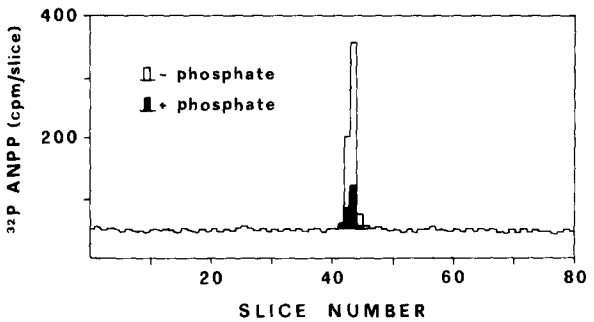

Fig. 6. [^{32}P]ANPP labeling of the phosphate carrier in the presence and absence of phosphate. The mitochondria were illuminated in the presence of 1 mM [^{32}P]ANPP with and without 20 mM phosphate. The phosphate carrier was purified and subjected to SDS gel electrophoresis. The distribution of radioactivity was determined after staining, destaining and slicing the gels. The histograms show [^{32}P]incorporation in the presence and absence of phosphate. For fractions 1–41 and 47–80 only the values of radioactivity obtained in the absence of phosphate are reported, since those in the presence of phosphate are not significantly different. Fractions 42–45 corresponding to an M_r of 33000 are the only ones which were stained by Coomassie blue.

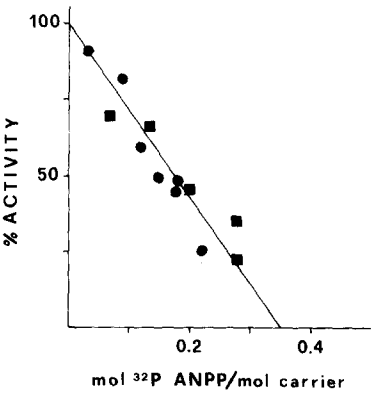

Fig. 7. Inhibition of the phosphate-phosphate exchange activity as a function of the amount of [^{32}P]ANPP bound to the phosphate carrier. Mitochondria were photolabeled by different concentrations of [^{32}P]ANPP. After purification of the phosphate carrier the activity was measured in proteoliposomes. The amount of [^{32}P]ANPP bound (mol/mol protein) was determined by counting the radioactivity of the purified carrier preparations after delipidation (●) or gel electrophoresis (■) and assuming a molecular weight of the carrier of 33 kDa.

Stoichiometry of [^{32}P]ANPP binding

The stoichiometry of ANPP binding to the phosphate carrier was investigated by incubating the mitochondria with increasing concentrations of [^{32}P]ANPP in the presence of light. After washing of the mitochondria, the phosphate carrier was solubilized and purified. Both the amount of [^{32}P]ANPP bound to the protein and the transport activity in the reconstituted system were determined. The results are reported in Fig. 7. If the data correlating binding and extent of inhibition are extrapolated to 100% inhibition a maximal binding of 0.35 mol of [^{32}P]ANPP per mol of 33 kDa phosphate carrier protein is obtained.

Discussion

It has been demonstrated in this paper that ANPP can be used as photoaffinity label for the phosphate carrier of mitochondria. When mitochondria are incubated with ANPP and then illuminated, the phosphate carrier is irreversibly modified. It is interesting to note that ANPP is able to inhibit the transport of phosphate in mitochondria and in proteoliposomes even without illumination. The inhibition is correlated with the amount of inhibitor used. Almost 80% inhibition

is achieved when mitochondria are treated with 1.6 mM ANPP in the light (Fig. 3). Since azido derivatives show an unspecific reactivity towards aminoacid side-chains, it was important to see whether the observed inhibition was the result of a random modification of the carrier by the azido moiety of the label. The results obtained by incubating the mitochondria with ANP, the non-phosphorylated analogue of ANPP, both in the dark and in the light, clearly demonstrate that the presence of the phosphate group is an essential requirement for the label molecule to inhibit the transport of phosphate. Thus we can suggest that ANPP reacts at the phosphate-binding site of the phosphate carrier. This suggestion is supported by the kinetic data of Fig. 1. In fact when ANPP is incubated in the dark with the reconstituted phosphate carrier the label inhibits the transport of phosphate in a competitive manner. This can easily explain why ANPP, if used in the dark, shows no inhibition when the uptake of phosphate is measured by the swelling technique. Swelling of mitochondria is in fact measured in the presence of 100 mM phosphate, whose high concentration is able to remove ANPP inhibition. Since ANPP behaves as a competitive inhibitor, one should expect that phosphate is also able to protect the phosphate carrier against the inactivation caused by ANPP in the light. Indeed we have found that the transport of phosphate is not inhibited when the mitochondria are illuminated with ANPP in the presence of at least 10 mM phosphate (Fig. 5). The protective effect of phosphate shows again that ANPP reacts with the phosphate carrier at the phosphate-binding site. Moreover, among several other anions tested, only arsenate, which is also a substrate for the phosphate carrier [29], is able to prevent the inhibition of phosphate transport by ANPP. It is interesting to note that the inhibition of phosphate transport by ANPP is pH-dependent. At lower pH values the label shows a stronger inhibitory effect. This result could be explained by the increased affinity of the phosphate group at lower pH, since it has been shown that the K_m of phosphate for the phosphate carrier decreases on lowering the pH of the medium [28]. In this respect it is possible to suggest that positive charges are important for the recognition of the substrate at the substrate-binding site of the protein.

In order to ascertain how many molecules of the reagent become bound to the phosphate carrier at maximal inhibition of transport, ^{32}P-radioactive ANPP has been used. Incubation of the mitochondria with [^{32}P]ANPP in the presence of light results in the labeling of the phosphate carrier and, in addition, of the ADP/ATP translocator and the β-subunit of the F_1-ATPase complex (Fig. 4). The latter result is in agreement with the finding of Lauquin et al., who used ANPP to label the phosphate-binding site of the F_1-ATPase [12,30]. When the labeling is carried out in the presence of phosphate the amount of [^{32}P]ANPP bound to the phosphate carrier is drastically reduced (Fig. 6). Phosphate also protects the β-subunit of the ATPase and, less efficiently, the ADP/ATP translocator against labeling (results not shown). Conversely, ADP shows a protective effect on the ADP/ATP carrier and the F_1-ATPase complex, but not on the phosphate carrier. Correlation of the [^{32}P]ANPP incorporation into the purified phosphate carrier with the activity inhibition indicates that at 100% inhibition only 0.35 mol of ANPP are bound per mol of 33 kDa carrier protein. This result could be explained by assuming that the phosphate carrier exists in an oligomeric structure formed by two identical subunits. However, the amount of ANPP bound per protein may be underestimated for at least two reasons: (a) the phosphate group of the label may be partly hydrolyzed as reported by Lauquin et al. [12], and (b) some molecules of the carrier may not have reacted with ANPP. A stoichiometry of 1 : 1 can therefore not be excluded by the present results. In conclusion, we have shown that ANPP is a suitable label to characterize the phosphate-binding site of the mitochondrial phosphate carrier. We are currently investigating the location and the modified aminoacid(s) in the primary structure of the phosphate carrier.

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