Interaction of 4-Azido-2-Nitrophenyl Phosphate, a Pseudosubstrate, with the Sarcoplasmic Reticulum Ca-ATPase

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ABSTRACT: In the dark, 4-azido-2-nitrophenyl phosphate (ANPP) is a phosphate analog which behaves like a simple energy-rich phosphate donor for the sarcoplasmic reticulum (SR) Ca-ATPase. Like *p*-nitrophenyl phosphate (pNPP), ANPP is hydrolyzed by the enzyme only in the presence of calcium and magnesium ($K_{0.5}$ and V_{max} are 0.3 mM and 60 nmol mg^{-1} min^{-1}, respectively). After photoirradiation in the absence of magnesium, SR Ca-ATPase is specifically labeled by [^{32}P]ANPP in the presence and in the absence of calcium. The presence of nucleotide in the medium provides some protection against photolabeling but less than phosphorylation by inorganic phosphate. The maximal stoichiometry of covalently bound ANPP extrapolates to 0.8 mol/mol of Ca-ATPase in the absence of magnesium. Autoradiography of a sodium dodecyl sulfate–polyacrylamide gel, after controlled trypsin digestion of the photolabeled protein, reveals that the radioactivity is incorporated in the B subfragment. Three radioactive polypeptides with approximate molecular masses of 55, 25, and 6 kDa are obtained depending on the digestion conditions. N-Terminal sequence analysis of the 50- and 25-kDa peptides reveals the same sequence beginning at Ala-506, whereas two different sequence beginning at Ala-506 and Phe-584 are observed in the 6-kDa peptides.

The Ca-transporting ATPase of sarcoplasmic reticulum is an integral membrane protein the cDNA clone of which was obtained some years ago (MacLennan et al., 1985). Its amino acid sequence combined with low-resolution structural data make possible secondary structure prediction (Stokes, 1991; Green & Stokes, 1992) and suggest three main domains: an ATP binding and phosphorylation cytoplasmic domain, a stalk region, and a possible calcium site within a transmembrane domain. The precise structure of the catalytic site is still unknown, although structure predictions have been proposed (Taylor & Green, 1989). Some amino acids of the catalytic site have been labeled with various fluorescent probes (Mitchinson et al., 1982; Yamashita & Kawakita, 1987; Kawakita & Yamashita, 1987; Wakabayashi et al., 1990; Stefanova et al., 1992, 1993) and substrate analogs (Yamamoto et al., 1988, 1989; McIntosh et al., 1992; Inesi et al., 1992; Lacapère et al., 1993). Some other amino acids have also been reported to be cross-linked in a nucleotide-protectable manner (Yamasaki et al., 1990; McIntosh, 1992).

The calcium transport is usually coupled with the hydrolysis of an ATP molecule, but simple energy-rich phosphate donors such as acetyl phosphate (DeMeis & Hasselbach, 1971; Bodley & Jencks, 1987), *p*-nitrophenyl phosphate (Inesi, 1971; Nakamura & Tonomura, 1978), or dinitrophenyl phosphate (Rossi et al., 1979; Hasselbach, 1988) are substrates of the calcium ATPase. They are called pseudosubstrates and their hydrolysis results in the pumping of calcium into the vesicle.

4-Azido-2-nitrophenyl phosphate (ANPP)[1] is a phosphate analog which competes with P_i for the binding to mitochondrial (Lauquin et al., 1980), *Escherichia coli* (Pougeois et al., 1983a) and chloroplast (Pougeois et al., 1983b) F_1-ATPase in the dark. It has been previously mentioned that ANPP is hydrolyzed by the sarcoplasmic reticulum Ca-ATPase under the standard hydrolysis conditions (Pougeois & Lauquin, 1985). Therefore, ANPP also belongs to the pseudosubstrate family. Its structure is very close to those of pNPP and dNPP, but it contains an azide group which permits covalent binding of the molecule to the protein after photoirradiation (Garin et al., 1989).

We present here a study of the interaction of ANPP with the SR Ca-ATPase; we show that in the dark it is a pseudosubstrate, calcium- and magnesium-dependent, with low affinity when compared with the usual substrate such as ATP but with similar affinity when compared to other pseudosubstrates. Upon photoirradiation, we show that [^{32}P]-ANPP specifically binds to the Ca-ATPase. After controlled tryptic digestion, we characterized the region of the protein which is photolabeled by [^{32}P]ANPP. The structure of the catalytic site of the Ca-ATPase is discussed on the basis of these results and other previous structural studies.

MATERIALS AND METHODS

SR vesicles were prepared and protein concentration was determined as described in Champeil et al. (1985).

The synthesis of [^{32}P]ANPP was performed as described in Garin et al. (1989).

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[1] Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; ANPP, 4-azido-2-nitrophenyl phosphate; $8N_3$-ADP, 8-azidoadenosine 5′-diphosphate; AP_3PL, adenosine triphosphopyridoxal; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TNP-$8N_3$-ATP, 8-azido-2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TNP-$2N_3$-ATP, 2-azido-2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PTH, phenylthiohydantoin; FITC, fluorescein isothiocyanate; (biotinyl-s^{6}ITP)$_2$, disulfide of the 3′(2′)-*O*-biotinylthioinosine 5′-triphosphate; PVDF, poly(vinylidene difluoride).

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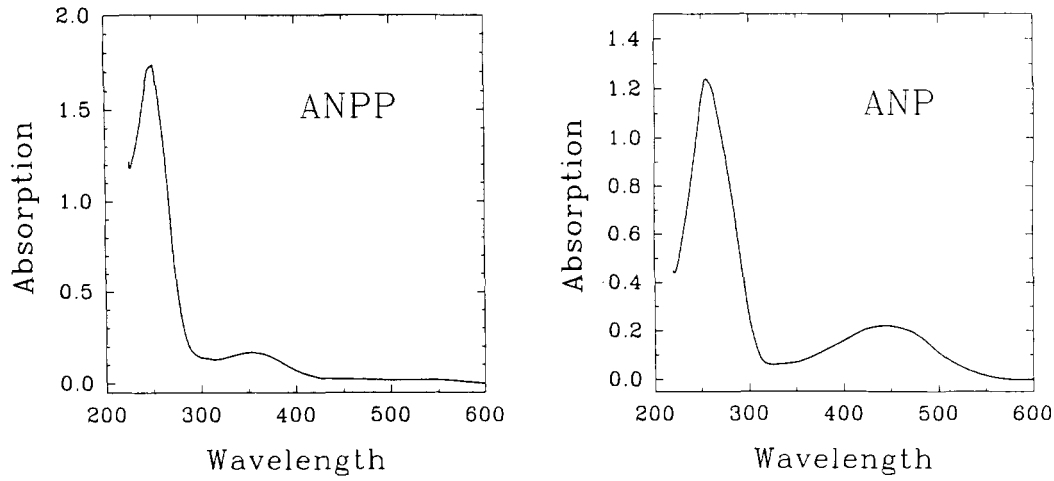

FIGURE 1: Absorption spectra of ANPP and ANP. (Left) Solution of ANPP (0.1 mM) in 50 mM MOPS-KOH, pH 7.0, and 100 mM KCl. (Right) Solution of ANP (0.067 mM) in the same buffer.

The ANPPase activity was measured spectrophotometrically following the time dependence of ANP liberation at 450 nm with E_{450} = 3300 M^{-1} cm^{-1} since ANP and ANPP exhibit different absorption spectra. UV spectra with maxima at 250 and 350 nm were observed with a solution of ANPP in 50 mM MOPS-KOH buffer (pH 7.0) whereas the maxima were at 256 and 444 nm (Figure 1) with a solution of ANP in the same buffer. The extinction coefficients of ANP were obtained from a spectrum of a solution (50 mM MOPS-KOH, pH 7.0) of an accurately weighed sample of dried ANP (E_{256} = 18 500 M^{-1} cm^{-1} and E_{444} = 3300 M^{-1} cm^{-1}). The concentration of ANPP was determined from the amount of P_i released from complete alkaline hydrolysis as described in Lauquin et al. (1980). The extinction coefficients of ANPP in our medium (pH 7.0) were then determined from its spectrum (E_{250} = 17 500 M^{-1} cm^{-1} and E_{350} = 1400 M^{-1} cm^{-1}).

The pNPPase activity was measured spectrophotometrically by following the time dependence of pNP liberation at 410 nm with E_{410} = 6880 M^{-1} cm^{-1} (Rossi et al., 1979).

The level of phosphoenzyme intermediate obtained with [^{32}P]ANPP was determined by quenching the reaction with perchloric acid followed by washing the denaturated protein on a Millipore filter (1 μm), which was subsequently counted in a liquid scintillation counter.

Photoaffinity labeling of Ca-ATPase was obtained by incubating the SRV with the [^{32}P]ANPP for a few minutes in darkness and subjecting it to 30-s photoirradiation (Lauquin et al., 1980), using a Xe XB 100 lamp (1000 W). A glass plate was placed between the light source and the SR preparation to eliminate short-wavelength UV light (Garin et al., 1989).

When measuring stoichiometries of covalently bound [^{32}P]-ANPP, the photolabeled Ca-ATPase was recovered in the excluded fraction obtained by elution–centrifugation with Sephadex G50 column equilibrated with 100 mM Tris-HCl, pH 7.5 (Penefski, 1977). The protein concentration was estimated by the method of Bradford (1976), and radioactivity was measured by liquid scintillation.

Gel electrophoresis was carried out using SDS–polyacrylamide slab gels in a Laemmli buffer system or in a Bicine–Tris buffer (see below). Standards used were (a) 200-kDa myosin, (b) 97-kDa phosphorylase *b*, (c) 69-kDa bovine serum albumin, (d) 46-kDa ovalbumin, and (e) 30-kDa carbonic anhydrase.

Tryptic digestion of SR membranes was carried out at room temperature as follows: photolabeled Ca-ATPase (1–2 mg/mL) obtained by elution–centrifugation (as described above) was digested at different ratios of trypsin/SR protein, either 1/100 or 1/20 or 1/10 (w/w), for 2–3 min in 100 mM MOPS, pH 7.0, and 2 mM $CaCl_2$. The reaction was stopped by adding soybean trypsin inhibitor in twice the amount of trypsin.

Small peptides generated by the digestion were analyzed by SDS–polyacrylamide gel electrophoresis using Bicine and sulfate as trailing and leading ions, respectively, and Bistris and Tris as counterions in the stacking and separating phase, respectively (Wiltfang et al., 1991). Peptides were then electroblotted onto PVDF Pro-Blott membranes (Applied Biosystems) using 10 mM Caps (pH 11.0) with 20% methanol as transfer buffer. The transfer was carried out using a Bio-Rad miniblot chamber at 4 °C for 45 min at 90 V. After autoradiography, bands of the Pro-blott membrane corresponding to photolabeled peptides were excised and placed in a vertical cross-flow reaction cartridge (Blott cartridge, Applied Biosystems).

Amino acid sequence analysis was performed on an automated Applied Biosystems sequanator (477A) equipped with an on-line PTH-amino acid analyzer (Model 120A). The instruments were operated using the program recommended by the manufacturer.

For acidolytic cleavage of the photolabeled 6-kDa peptide, the radioactive band of the Bicine–Tris gel was excised, and incubated for 36 h in 70% formic acid. The band was then washed three times with 100 mM Tris-HCl (pH 7.5) and analyzed using a new Bicine–Tris gel.

RESULTS

ANPP Is a Pseudosubstrate for Ca-ATPase. Figure 2 shows typical records of continuous absorption changes observed at 450 nm and produced by ANP liberation following ANPP cleavage by the SR Ca-ATPase (see Materials and Methods for more details). Figure 2A shows that ANPPase activity is calcium-dependent since SRV incubated in the presence of EGTA does not exhibit any absorption changes whereas calcium addition induces absorption change (first arrow in Figure 2A). Further addition of EGTA stops any further changes (second arrow). ANPP is therefore a substrate for a calcium-activated enzyme, similar to ATP and not to P_i, the reverse substrate for a calcium-depleted enzyme. Figure 2B shows that ANPPase activity also requires magnesium as

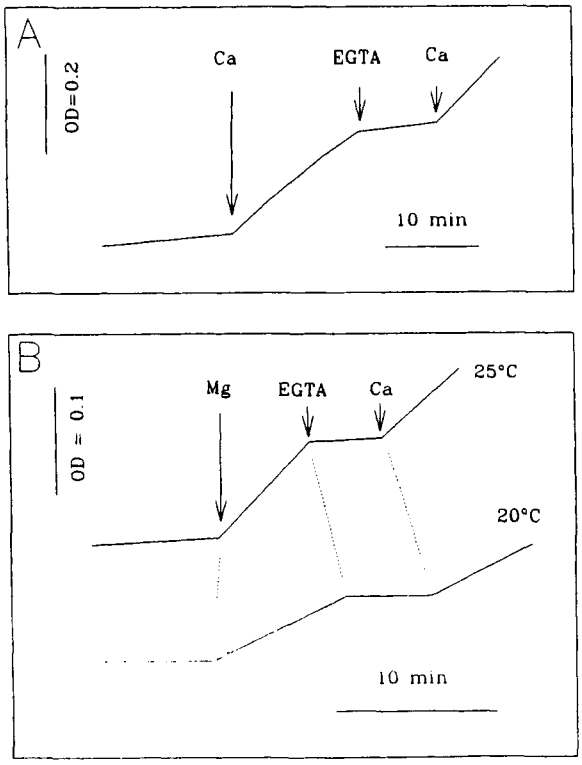

FIGURE 2: Calcium and magnesium dependence of ANPPase activity of sarcoplasmic reticulum preparations. Medium was adjusted to pH 7.0 by 50 mM MOPS-KOH in the presence of 100 mM KCl. (A) SRV (0.5 mg/mL) were added followed by 0.3 mM ANPP, 0.2 mM EGTA, and 10 mM $MgCl_2$. At the first arrow 0.3 mM $CaCl_2$ was added, at the second arrow 0.2 mM EGTA, and at the third 0.2 mM $CaCl_2$. (B) SRV (0.5 mg/mL) were added followed by 0.2 mM ANPP. At the first arrow 10 mM $MgCl_2$ was added, at the second arrow 0.2 mM EGTA, and at the third 0.3 mM $CaCl_2$. Experiments were performed at 20 and 25 °C for lower and upper traces, respectively.

cosubstrate, similarly to ATP (Vianna, 1975), to pseudosubstrates such as pNPP (Chaves-Ribeiro et al., 1980), and even to P_i (Lacapère et al., 1981). In the presence of contaminating calcium (enough to saturate the calcium transport sites at this pH) magnesium addition (first arrow) induces an absorption change which remains calcium-dependent as indicated by the effect of EGTA addition (second arrow). ANPPase activity is also temperature-dependent as shown by the difference between the two traces in Figure 2B. A decrease of 5 °C induces a decrease of the rate by a factor of 1.8.

Comparison of Figure 2, panels A and B, shows that ANPPase activity depends on ANPP concentration, since a change of ANPP concentration from 0.3 to 0.2 mM decreases the rate of ANP liberation from 27 to 22 nmol (mg of protein)$^{-1}$ min^{-1}. We have measured a complete concentration dependence of this ANPPase activity as shown in Figure 3 (left). The maximal rate obtained by extrapolation to infinite concentration of substrate is 62.5 nmol (mg of protein)$^{-1}$ min^{-1}. The pNPPase activity measured under similar conditions is shown by the dotted line in Figure 3 (left). The maximal rates of pNPPase and ANPPase activity are very close, whereas apparent K_m values are different, 1 and 0.3 mM, respectively. We have also checked the magnesium concentration dependence of this ANPPase activity, Figure 3 (right) exhibits a bell-shaped curve also observed with other substrates such as pNPP (inset of Figure 3, right), acetyl phosphate (DeMeis & Hasselbach, 1971), or ATP (DeMeis & DeMello, 1973). The activation observed with low concentrations of magnesium could be attributed to the formation of the MgANPP complex, the true substrate compared with free ANPP, as previously suggested for pNPP (Chaves-Ribeiro et al., 1980) and dNPP (Hasselbach, 1988). The reasons for the inhibition by high concentration of magnesium are not so clear. It could be that magnesium forms a ternary complex with ANPP (Mg_2ANPP) that does not bind, and/or magnesium alone can bind to an inhibitory site or compete with calcium [similarly to ATP; see Lacapère et al. (1990)]. We also cannot exclude that MgANPP has a low solubility constant.

Photoaffinity Labeling of Ca-ATPase by [^{32}P]ANPP. In the dark and in the presence of 50 μM [^{32}P]ANPP, 10 mM $MgCl_2$, and 0.1 mM $CaCl_2$, we have measured a small amount of acid-stable covalent phosphoenzyme. Upon photoirradiation, a very similar value of covalently bound [^{32}P]ANPP was obtained under the same condition. On omitting magnesium from the medium, we were able to measure the stoichiometries of [^{32}P]ANPP covalently bound upon photoirradiation without contamination by the phosphoenzyme. Figure 4 shows the ANPP concentration dependence of the covalently bound [^{32}P]ANPP. The apparent K_d (0.2 mM) deduced is consistent with the K_m measured in Figure 3 (left). The extrapolated maximal stoichiometry of covalently bound [^{32}P]ANPP (4 nmol/mg) is lower than the stoichiometry expected for a saturated site (5–6 nmol/mg; Lacapère & Guillain, 1993). This difference might be explained by a yield of 60–80% photolabeling, a value close to that measured previously with F_1-ATPase (Lauquin et al., 1980).

The [^{32}P]ANPP binding to sarcoplasmic reticulum proteins is documented by gel electrophoresis and autoradiography (Figure 5). In the absence of photoirradiation no labeling was observed (data not shown). Due to the possible formation of a long-lived, chemically reactive intermediates, we have checked that no labeling was observed when the proteins were added after the photoirradiation. Upon photoirradiation both in the presence and in the absence of calcium (panels A and B in Figure 5, respectively), the major photolabeling is on the Ca-ATPase (lanes 1 and 4 in panel A and lanes 1 and 5 in panel B). Additional photolabeled proteins are also observed, among which the one on top of the gel, with a very high molecular weight, may be the ryanodine receptor. In the middle of the gel, two additional photolabeled proteins are observed; one of them, with an apparent molecular mass of about 40 kDa, might be a protein that has previously been observed by other authors (Campbell & MacLennan, 1983). In the presence of calcium, ATP and ADP addition to the medium gave approximately 30–40% protection against [^{32}P]-ANPP photoincorporation, whereas photolabeling is conserved in the presence of adenosine (compare autoradiography in lanes 4, 5, and 6 corresponding to gel presented in lanes 1, 2, and 3 in panel A of Figure 5). This difference can be explained by a difference in the affinity of the Ca-ATPase for these compounds (Lacapère et al., 1990). Similar partial protection by nucleotides has been previously observed with the same substrate for different proteins (Pougeois et al., 1983a,b), whereas P_i addition prior to photoirradiation protected efficiently against photolabeling. We performed photolabeling under conditions where P_i is a substrate for phosphorylation of the Ca-ATPase, i.e., in the absence of calcium. Panel B of Figure 5 shows that addition of P_i alone slightly protected against photolabeling (lanes 2 and 6), whereas simultaneous addition of magnesium and P_i protected efficiently against photolabeling (lanes 4 and 8). We concluded that P_i was a more efficient protector than ADP against photolabeling, in agreement with previous studies of the F_1-ATPase. In our case, it is also known that ATP, nonhydrolyzable ATP analogs,

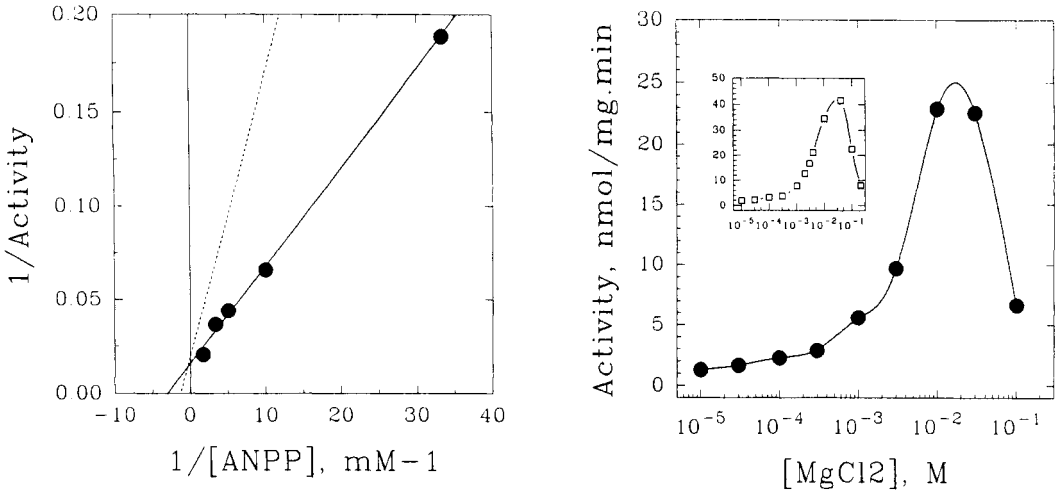

FIGURE 3: ANPP and magnesium concentration dependence of ANPPase activity. The medium was 50 mM MOPS-KOH, pH 7.0 at 25 °C, 100 mM KCl, and 0.5 mg/mL SRV in the presence of either 5 mM $MgCl_2$ and various ANPP concentrations (left) or 0.2 mM ANPP and various magnesium concentrations (right). Dotted line in left panel is the pNPPase activity measured under the same conditions as for ANPP. Inset in right panel is the magnesium concentration dependence of pNPPase activity measured in the presence of 1 mM pNPP.

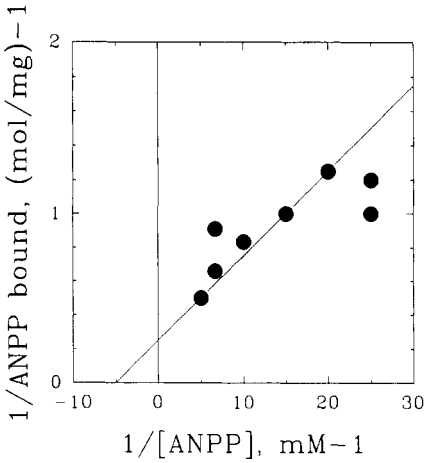

FIGURE 4: Binding of [^{32}P]ANPP to SR vesicles. SRV (1 mg/mL) were incubated in the dark for 2 min in the presence of various ANPP concentrations in a medium containing 100 mM MOPS-KOH (pH 7.0) and 0.1 mM $CaCl_2$. After 30 s of photoirradiation, SRV-bound and free ANPP were separated by elution–centrifugation with a Sephadex G50 column (as described under Materials and Methods).

or TNP-ATP can bind to phosphorylated enzyme and behave as an activator (Champeil & Guillain, 1986; Bishop et al., 1987; Seebregts & McIntosh, 1989; Lacapère & Guillain, 1993). Thus, the Ca-ATPase can accommodate the simultaneous presence of nucleotide and phosphate within the catalytic site. Accordingly, the low protection against the photolabeling by ANPP observed in the presence of ADP can be due to the concomitant and nonexclusive binding of ANPP and ADP.

Identification of Photolabeled Peptide. After photolabeling of Ca-ATPase with [^{32}P]ANPP in the presence of calcium, the enzyme was subjected to partial tryptic cleavage (Figure 6). Panel A shows SDS–PAGE of undigested protein (lane 2) and after tryptic cleavage performed at a 1/100 or 1/20 ratio (lanes 3 and 4, respectively). Corresponding autoradiography shows that the radioactivity is localized in the Ca-ATPase B tryptic fragment after mild digestion (lane 6) and on a smaller peptide of about 25 kDa after stronger digestion (lane 7). The same pattern of photolabeled peptides has been observed for SDS–PAGE and corresponding autoradiography starting with Ca-ATPase photolabeled in the presence of EGTA (data not shown), meaning that the same domain of the protein is labeled by ANPP under both conditions (Ca and EGTA). Panel B shows the autoradiography of a Bicine–Tris gel (see Material and Methods) loaded with photolabeled Ca-ATPase digested by trypsin at a 1/100 or 1/10 ratio (lanes 1 and 2, respectively). In this case the low molecular weight peptides are better separated and we have observed photolabeled peptides of 25 and 6 kDa. We electroblotted these peptides and performed amino acid sequence analysis (see Materials and Methods). The N-terminal sequences of the different labeled peptides are shown in Table 1. We derived the C-terminal residue on the basis of the estimated molecular weight and by looking for the Arg and Lys amino acids within the primary sequence since trypsin cleaves proteins forming mainly peptides with Arg and Lys termini.

The N-terminal sequence analysis of the 6-kDa electrophoresis band reveals the presence of two peptides beginning at Ala-506 and Phe-584 (see Table 1). They are both part of the larger peptide of 25 kDa beginning at Ala-506. Therefore, we cannot determine which one of the two smaller peptides is photolabeled. Thus, we performed acidolytic digestion (Asp-|-Pro cleavage) of the 6-kDa band in order to get smaller peptides (see Material and Methods). After such treatment, the autoradiography of a Bicine–Tris gel showed no displacement of the photolabeled band (data not shown) compared to that prior to digestion. This result is in favor of the photolabeling of the Ala-506 peptide, which does not contain any -Asp-Pro-.

DISCUSSION

The preceeding data indicate that, in the dark, ANPP is a substrate for the SR Ca-ATPase, in contrast to F_1-ATPase, as previously mentioned by Pougeois and Lauquin (1985), since it exhibits a calcium-activated hydrolysis (Figure 2). It should be recalled that occupation of the specific transport sites by calcium triggers the phosphorylation of the Ca-ATPase by ATP, whereas the calcium-deprived enzyme reacts with inorganic phosphate in the reverse direction of ATP synthesis. ANPP also requires the presence of magnesium to be hydrolyzed, but a high concentration of magnesium inhibits ANPP hydrolysis (Figure 3) similarly to ATP (De Meis & De Mello, 1973). The ANPP maximal rate of hydrolysis is slow [62 nmol (mg of protein)$^{-1}$ min^{-1}] compared to ATP [about 1–3 μmol (mg of protein)$^{-1}$ min^{-1}] but is similar to other pseudosubstrates such as pNPP or dNPP (Rossi et al.,

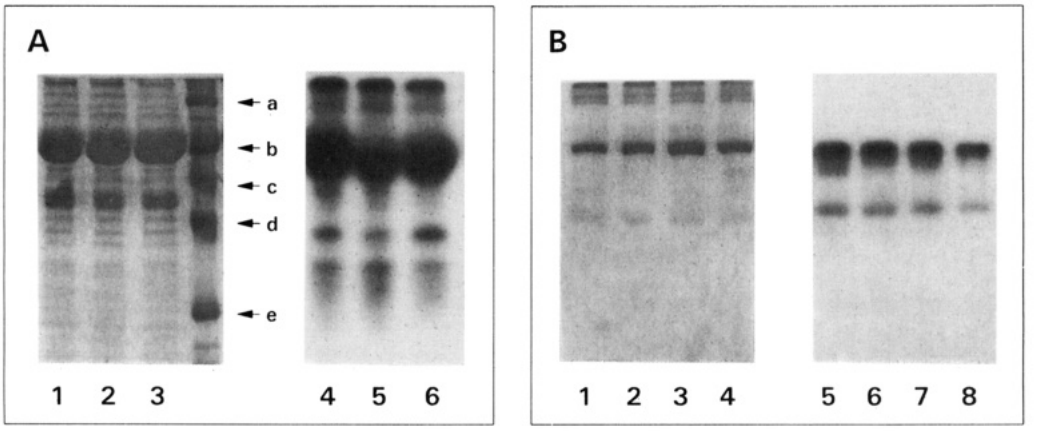

FIGURE 5: Photoaffinity labeling of SR vesicles with [^{32}P]ANPP. SDS–PAGE of SR proteins with the corresponding autoradiography after photoirradiation in the presence of calcium (panel A) or in the absence of calcium (panel B). Panel A, Laemmli gel followed by autoradiography: the photoirradiation medium was 100 mM MOPS–Tris, pH 7.0, 0.1 mM $CaCl_2$, 0.065 mM [^{32}P]ANPP, and 1 mg/mL SRV without (lanes 1 and 4) or with 2 mM ADP or 2 mM adenosine (lanes 2 and 5 or 3 and 6, respectively). Panel B, Bicine–Tris gel followed by autoradiography: medium was 100 mM Mes–Tris, pH 6.0, 2 mM EGTA, 0.065 mM [^{32}P]ANPP, and 1 mg/mL SRV without (lanes 1 and 5) or with 20 mM P_i (lanes 2 and 6), 20 mM $MgCl_2$ (lanes 3 and 7), and 20 mM P_i and 20 mM $MgCl_2$ (lanes 4 and 8). MW standards are described under Materials and Methods.

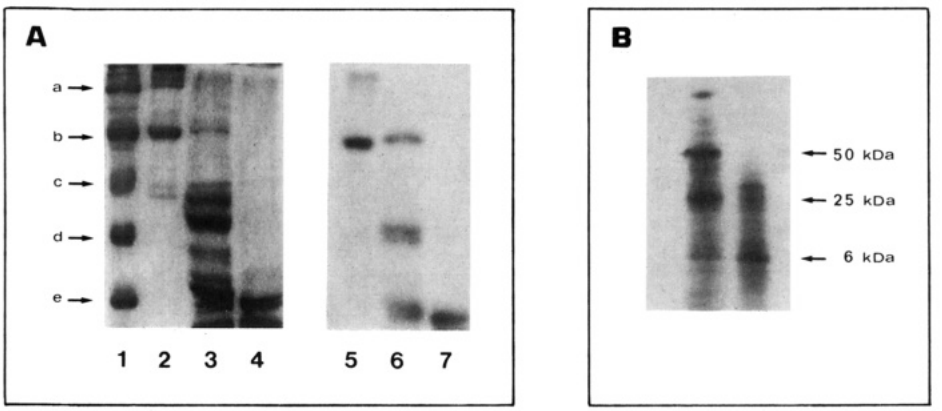

FIGURE 6: Tryptic cleavage of SRV photolabeled with [^{32}P]ANPP. Panel A, Laemmli gels with the corresponding autoradiography of Ca-ATPase membranes before tryptic cleavage (lanes 2 and 5) or after tryptic cleavage (lanes 3 and 6 or 4 and 7) at 1/100 or 1/20 trypsin/SRV, respectively. Panel B, autoradiography of Bicine–Tris gel of Ca-ATPase after tryptic cleavage (lanes 1 and 2) at 1/100 and 1/10 trypsin/SRV, respectively.

Table 1: Peptides Isolated by Gel Electrophoresis[a]

estimated molecular mass	sequenced N-terminus	estimated C-terminus
55kDa	AAVGNKMF... (506)	...NYLEG (994)
25kDa	AAVGNKMF... (506)	..(K)..(KK)..K....(R) (686, 712, 728, 751)
6kDa	AAVGNKMF... (506)	...K (550)
	FMEYETDL... (584)	...RR (638)

[a] The first eight amino acids of the peptides were obtained by N-terminal sequencing (see Materials and Methods). The amounts of the 55-, 25-, and 6-kDa peptides sequenced were 20, 15, and 10 and 5 pmol, picomoles respectively.

1979). The apparent K_m for ANPP is much lower (0.3 mM) compared to ATP (0.1 μM) but slightly higher than pNPP (1 mM). It should be mentioned that a change in affinity has already been observed between pNPP and dNPP (Rossi et al., 1979), two pseudosubstrates which have chemical formulas differing only by one nitroxide. In the case of ATP, the presence of ribose and adenosine rings might help in the positioning of the phosphate and facilitate the phosphorylation.

In this paper, we show that [^{32}P]ANPP photoirradiated in the presence of SR vesicles binds covalently to the Ca-ATPase and that ANPP concentration dependence of covalently bound ANPP extrapolates to about 0.8 mol of ANPP/mol of protein (Figure 4). Photoirradiation of SR vesicles in the presence of [^{32}P]ANPP leads to selective photolabeling of Ca-ATPase, although some other minor labeled proteins are also observed on autoradiography of electrophoresis gels (Figure 5). This is not surprising since our preparation contains other proteins sensitive to ATP such as kinases or ryanodine receptor. Similar photolabeling of the Ca-ATPase has been observed in the presence and in the absence of calcium, although ANPP is hydrolyzed only in the presence of calcium. This is consistent with previous data showing that, at pH 7.0, ATP binds both in the presence and in the absence of calcium with similar affinities (Lacapère & Guillain, 1993), whereas ATP is hydrolyzed only in the presence of calcium. Although ANPP is a substrate like ATP, it behaves like a phosphate analog and binds to the phosphorylation site. This is shown by the differences observed between nucleotides and P_i protection against [^{32}P]ANPP photoincorporation (Figure 5). We think that the low protection observed in the presence of nucleotides

can be due to the concomitant binding of ANPP and nucleotides within the catalytic site, whereas the phosphorylation of the enzyme by P_i prevents ANPP binding to the phosphorylation site.

After partial tryptic digestion, similar patterns of photolabeled peptides have been observed on gel electrophoresis independently of the initial conditions of labeling, i.e., in the presence of calcium or in the presence of EGTA. We observed that the ANPP binding site is localized on the B fragment (beginning at Ala-506), whereas it has been previously shown that the phosphorylation site was located on Asp-351 within the A fragment (Allen & Green, 1976). After more pronounced digestion we were able to identify two smaller peptides (see Table 1), but we were unable to clearly distinguish between them. However, these two peptides, beginning at Ala-506 and Phe-584, are both part of crucial areas of the protein. The first one contains Thr-532 and Thr-533, which have been labeled by $8N_3$-ADP (Lacapère et al., 1993). The second one overlaps with a larger peptide recently cross-linked with the phosphorylation site (Gutowski-Eckel et al., 1993), and an analogous region of this peptide has also been labeled by $2N_3$-ATP within H^+-ATPase (Davis et al., 1990). Thus these two regions are probably close to the adenosine part of the nucleotide but also not far from the phosphorylation site since the distance between the phosphate and the nitrene group of ANPP is close to 5 Å (see Figure 7, top). Vilsen et al. (1991) have also suggested, from site-specific mutagenesis experiments, that distant domains within the primary sequence are part of the catalytic site, the hinge domain having a role in the binding and transfer of phosphate. Their results are in agreement with labeling experiments showing the covalent modification of Lys-684 in the presence of calcium by AP_3-PL, an ATP analog modified on the γ-phosphate (Yamamoto et al., 1988). It should also be mentioned that glutaraldehyde cross-linked two distant amino acids within the primary sequence, Arg-678 and Lys-492 (McIntosh, 1992), and blocked the phosphorylation by P_i. Moreover, in the presence of EGTA, AP_3PL covalently modified Lys-492 (Yamamoto et al., 1989).

Lys-492 has also been labeled by both TNP-$8N_3$-ATP and TNP-$2N_3$-ATP (McIntosh et al., 1992; Inesi et al., 1992). It should also be mentioned that although these ATP analogs, modified on the ribose, bind with good affinity, they are very slow substrates for phosphorylation (McIntosh & Woolley, 1993), suggesting that TNP-ATP could bind differently within the ATP binding site. The crucial role of the hydroxyl groups in the ribose ring of ATP has been clearly demonstrated since elimination of these groups leaves a very poor substrate for the SR Ca-ATPase (Anderson & Murphy, 1983; Coan et al., 1993). Furthermore, the substitution of the hydroxyl groups by several constituents leads to substrates with very different affinities, good affinity for TNP-ATP (Dupont et al., 1982), similar affinity for arylazido-ATP (Carvalho-Alves et al., 1985), or poor affinity for benzoyl-ATP (Cable & Briggs, 1984) and spin-labeled ATP (Oliveira et al., 1988). Among the different ATP analogs modified on the ribose ring, it has been shown that A1 fragment was labeled by oxidized ATP (Mignaco et al., 1990; Hohenegger & Makinose, 1992), whereas a mixture of 72% A and 28% B fragments was labeled by benzoyl-ATP (Cable & Briggs, 1984). In the latter case, the photoreactive link is constituted by a 6–7-Å long "arm" (Mahmood & Yount, 1984), thus giving a possible explanation for the labeling of the two fragments A and B. The ribose group is therefore probably close to amino acids belonging to both the A and B fragments. This is also the case for the

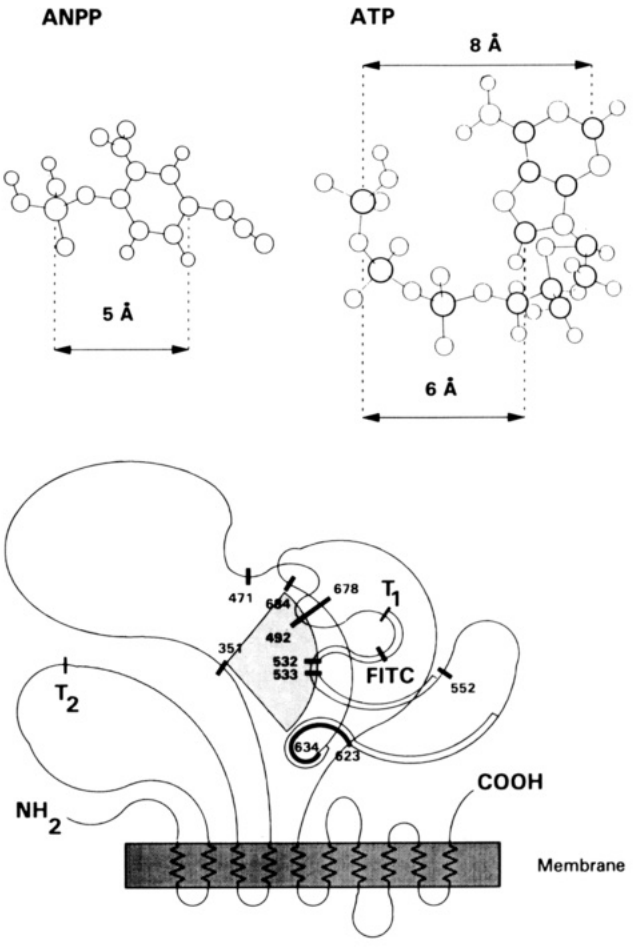

FIGURE 7: Structures of ANPP and ATP (A) and two-dimensional representation of the Ca-ATPase (B). Panel A shows computerized models of ANPP and ATP with the distances separating the reactive phosphate and the carbon groups ($2N_3$ and $8N_3$). Panel B shows a two-dimensional representation of the possible sites of ANPP binding (double-lined portions) and other residues modified by affinity probes or cross-linked. The general scheme is taken from MacLennan et al. (1985). Asp-351 is phosphorylated by both ATP and P_i (Allen & Green, 1976) and is also cross-linked to residues Ile-625 and Met-700 (Gutowski-Eckel et al., 1993). Cys-471 is modified with the disulfide of the biotinyl-ITP (Kison et al., 1989). Lys-492 is modified with TNP-$8N_3$-ATP and TNP-$2N_3$-ATP (McIntosh et al., 1992; Inesi et al., 1992) and with pyridoxal-ATP in the presence of EGTA (Yamamoto et al., 1989) but also cross-linked with Arg-678 by glutaraldehyde (McIntosh, 1992). Lys-515 is modified by FITC (Mitchinson et al., 1982). Thr-532 and Thr-533 are modified by $8N_3$-ADP (Lacapère et al., 1993). Trp-522 is the only tryptophan localized out of the membrane portion. Residues 623–634 represent the equivalent residues (560–566) of the yeast plasma membrane H^+-ATPase modified with $2N_3$-ATP (Davis et al., 1990). Lys-684 is modified with pyridoxal-ATP in the presence of calcium and EGTA (Yamamoto et al., 1988). T1 and T2 represent the first and second tryptic cleavage. The gray portion connects the different regions or modified amino acids which are at a short distance from the phosphorylated Asp-351.

terminal phosphate group of ATP, since ATP molecules modified on the terminal phosphate, such as imidazolidate or carbodiimide ATP (Bill et al., 1988; Murphy, 1990), inactivate the Ca-ATPase by forming an intramolecular cross-link between the A1 and B fragments (Murphy, 1990; Gutowski-Eckel et al., 1993).

The question raised is how can we explain all these results, especially the possible labeling of the same region of the protein by ANPP and $8N_3$-ADP or $2N_3$-ATP. If ATP has an extended shape, the terminal phosphate and the adenine ring are more than 12 Å apart, whereas in a U-shape (Andre et al., 1990), the distance is reduced to 6–9 Å for the C_8 and C_2, respectively

(see Figure 7, top). Thus, ANPP and azidonucleotides with a U-shape structure and in the *anti* conformation would have similar distances between the phosphate and nitrene groups and therefore would be able to label the same region of the protein. The A and B fragments are probably "intimately mixed" to constitute the catalytic site, but we do not have yet sufficient data to construct a precise structure taking into account the distance between the different labeled amino acids based on a possible folding of the ATP molecule. A simple model of the catalytic site can be drawn to assemble the different labeling sites obtained by covalent modification or cross-linking (Figure 7, bottom). A more detailed structure would require the knowledge of the complete structure of the nucleotide bound to the catalytic site as well as the amino acids close to the ribose ring. It should be noted that NMR studies on ATP bound to the Ca-ATPase catalytic site have shown that ATP has an *anti* conformation (Clore et al., 1982). Other NMR studies on $Co(NH_3)_4ATP$ bound to Na,K-ATPase have shown that the nucleotide adopts a bent conformation, in which the triphosphate chain lies nearly parallel to the adenine moiety (McD.Stewart et al., 1989).

Finally, we anticipate that external distances between covalently bound probes obtained by fluorescence energy transfer measurements (Bigelow & Inesi, 1991), three-dimensional reconstruction obtained by electron microscopy diffraction (Toyoshima et al., 1993), and localization of surface-exposed regions obtained by antibodies (Tunwell et al., 1991; Mata et al. 1992) coupled with internal structure of the catalytic site (as previously discussed) will help to specify the folding of the chain and lead soon to a more detailed molecular structure.

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