Characterization of a Protease-resistant Domain of the Cytosolic Portion of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

NUCLEOTIDE- AND METAL-BINDING SITES*

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The detailed structure of membrane proteins is only known in a few cases. Among P-type ATPases, which are responsible for active cation transport, the best structure to date is only known to about 14 Å resolution, from cryo-electron microscopy; this is for Ca\(^{2+}\)-ATPase, which catalyzes uptake of Ca\(^{2+}\) from the cytosol to the endoplasmic or sarcoplasmic Ca\(^{2+}\)-storing compartment (1–8). However, large three-dimensional crystals of the protein are not available as yet, and the ATPase structure has been mainly deduced from electron microscopy studies of two-dimensional crystals (3–6), combined with studies by other techniques like chemical labeling, Förster-type resonance energy transfer, immunoreactivity of antibodies with various epitopes, site-directed mutagenesis, and proteolysis studies (reviewed in Refs. 7–9). By comparing structures of Ca\(^{2+}\)-ATPase with and without bound nucleotide, suggestions have been made concerning the likely location of the ATP-binding site in the cytoplasmic portion of this pump (10), but the actual structure of the ATP-binding site and the mechanism of ATP hydrolysis are still largely unknown. Nevertheless, it has been hypothesized that this region of the Ca\(^{2+}\)-ATPase might be organized like water-soluble phosphokinases, with a hinge transiently bringing an ATP-binding and a phosphate-accepting region in close apposition during the ATPase enzymatic cycle (Ref. 11 and reviewed in Refs. 8 and 12).

As a first step toward determining the structure of the entire ATPase, it would be useful to determine the structure of smaller fragments, provided that such fragments reflect the structure of the corresponding region in the intact protein. A few studies have paved the way in this direction, by trying to express in bacterial systems soluble fragments of ATPase with anticipated autonomous functional properties (13, 14). Proteolysis experiments can contribute to the success of such studies, since protease-resistant fragments probably constitute domains whose structural properties can be investigated with minimal risk of instability. Following our previous analysis of Ca\(^{2+}\)-ATPase proteolysis by proteinase K (15), we now report on the properties of closely related proteolytic fragments with molecular masses of 29/30 kDa, derived from the large cytosolic loop, which we show retain many of the properties of the corresponding region in intact Ca\(^{2+}\)-ATPase. In view of the stability and functional properties of these fragments, we suggest that such a soluble 30-kDa fragment of Ca\(^{2+}\)-ATPase could be a reasonable candidate for future three-dimensional structure determination. From our results we also infer possible structural information on the cytosolic portion of this pump (10), but the actual structure of the ATP-binding site and the mechanism of ATP hydrolysis are still largely unknown. Nevertheless, it has been hypothesized that this region of the Ca\(^{2+}\)-ATPase might be organized like water-soluble phosphokinases, with a hinge transiently bringing an ATP-binding and a phosphate-accepting region in close apposition during the ATPase enzymatic cycle (Ref. 11 and reviewed in Refs. 8 and 12).

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implications for the organization of the cytosolic portion of P-type ATPases.

**EXPERIMENTAL PROCEDURES**

Most of the methods used in this report have been described in detail previously, including the isolation of SR vesicles (16) and their FITC labeling (17), preparation of purified ATPase by deoxycholate extraction (18), proteolysis and analysis of the resultant peptides (15, 19, 20), handling of lanthanide ions (21), and general fluorescence methods, including the use of TNP-nucleotides (22). Fluorescence recordings were obtained with a SPEX fluorolog instrument; SDS-PAGE gels were digitized with a Bio-Rad camera and Molecular Analyst software. Total amino acid analysis after acid hydrolysis of the peptides was performed by F. Baleux, Unité de Chimie Organique, Département de Biochimie et Génétique Moléculaire, at Institut Pasteur, Paris. CD Spectra were run on a Jobin-Yvon CD6 spectrodichrograph and were analyzed with the Dichrosoft software.  

**Mass Spectrometry**—In this work, mass spectrometry was not performed after ElectroSpray Ionization (as in our previous work, see Ref. 15), but we used the matrix-assisted laser desorption ionization-time of flight technique (23). MALDI-TOF spectra of proteolytic peptides were obtained with a Voyager Elite Biospectrometry Workstation mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA). Each sample was first desalted and then loaded on the target by the dried droplet method; the matrix was 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Aldrich). Spectra were calibrated externally with a two-point calibration using the [M + H]± and [M + 2H]± ions from a protein standard, horse apomyoglobin (m/z = 16952.5 and 8476.8, respectively). The analysis was performed in the positive and linear modes, with an accelerating voltage of 25,000 V. Different extraction delays were used (150, 275, or 400 ms), and around 120 scans were averaged. Typically, the amount of peptide analyzed was 5–10 pmol.

MALDI-TOF spectra were run to determine the C termini of the soluble fragments produced by proteolysis (see Fig. 1) with various proteolytic enzymes. After proteolysis, complex were processed in one of two ways. In the case of Asp-N, the supernatant of Asp-N-treated SR was simply desalted by passing through a Sephadex G25M column equilibrated with 10 mM ammonium bicarbonate buffer at pH 8.1 and examined directly by mass spectrometry. Since the N terminus of the 30-kDa proteolytic peptide was already known from N-terminal sequencing, determination of its total mass by MALDI-TOF (to within 10 Da) allowed us to determine its C-terminal residue without ambiguity. In the case of elastase and proteinase K, which are proteolytic enzymes with relatively poor specificity (and also after use of Asp-N, for control), the peptides found in the supernatants of treated SR were further cleaved with trypsin (as shown for proteinase K in Fig. 5), and the smaller peptides generated in this way were then desalted by binding to a C18 reversed phase resin (Sep-Pak, Waters Corp., Milford, MA) and elution with 80% acetonitrile and 0.1% trifluoroacetic acid (v/v) in deionized water (24). In this case, mass spectrometry revealed two groups of peaks (see Fig. 6) in the 16–17-kDa range and in the 11–12-kDa range, respectively, corresponding to the N-terminal and C-terminal parts of the original peptides, respectively, and confirmed that these peptides had been cut by trypsin at a single site, Arg$^{205}$-$\text{Ala}^{206}$ (as shown for proteinase K in detail, see “Results”). The masses measured for the 16–17-kDa peptides confirmed the results of the previous N-terminal sequencing experiments. The masses measured for the 11–12-kDa, which corresponded to peptides starting at the trypsin cleavage site (Arg$^{205}$-$\text{Ala}^{206}$) and ending at the C termini of the original peptides, allowed to identify the latter unambiguously in all cases (again to within 10 Da).

**Binding of [γ-32P]ATP to Soluble Fragments, as Deduced from Ultrafiltration Experiments**—To measure directly equilibrium binding of ATP to the soluble p29/30 fragments resulting from Ca$^{2+}$-ATPase proteolysis, we took advantage of the ability of appropriate ultrafiltration membranes (Centricon 10 devices, Amicon) to concentrate these soluble peptides while allowing free passage of ATP. Samples for ultrafiltration (2 ml) were transferred to Centricon 10 tubes and centrifuged for 150 min at 5000 rpm in a Beckman JA-12 rotor. After centrifugation, the various samples were concentrated to about the same extent (about 30-fold), as indicated by measurements of protein concentrations. Protein-bound ATP versus free ATP was determined from the excess ATP in the concentrated sample over that present in the ultrafiltrate, after correction for the small “blank” value (a few %) found in experiments with protein-free samples. Control measurements with intact SR vesicles were included. Experiments were performed on samples containing various concentrations of ATP, to which a constant concentration of [γ-32P]ATP tracer was added, to measure competition between nonlabeled and labeled ligand (e.g. Ref. 65). $^3$HGlucose was also added as an inert tracer, and the concentrations of ATP were deduced from the ratios of $^3$H and $^3$P counts. The data were analyzed in terms of Michaelian binding, according to Equation 1.

$$[EL]/[L] = \frac{[E_{\text{tot}}]/[K_c]}{[E_{\text{tot}}]/[K_c] + \alpha} \quad \text{(Eq. 1)}$$

where [EL] and [L] are the concentrations of bound and free ligand (ATP), respectively; $[E_{\text{tot}}]$ and $K_c$ are the concentration of binding sites and the binding affinity, and $\alpha$ corresponds to a small fraction of nonspecific binding. Plotting the fraction of ATP bound (i.e. [EL]/[L]) as a function of free ATP ([L]), as in Fig. 4, thus permits us to directly estimate $K_c$ from the concentration of free ATP for which this fraction is reduced by half of its maximum value, of its concentration of ATP; this concentration is subsequently used for the estimation of the concentration of ATP-binding sites in the sample (EL). A full description of the method will be given elsewhere.

In the case of p29/30 peptides, a special problem arises because these peptides are prepared as the high speed supernatant of protease-treated SR vesicles; in such a protocol a small fraction (possibly a few percent) of the original membranes may escape pelleting, and these non-pelleted membranes may contain a small fraction of either residual intact ATPase or only partially cleaved ATPase (e.g. the membranous p83C peptide, which is able to bind ATP and be phosphorylated, see Ref. 15). Unfortunately, for estimation of a binding affinity, contamination of a sample (with an anticipated relatively poor affinity for its ligand) by a component of higher affinity is a real complication (e.g. see Ref. 66). To eliminate such contamination, we submitted the p29/30-containing supernatant to a preliminary step of centrifugation on an ultrafiltration membrane with a 100,000-dalton cut-off (2-ml samples were loaded onto Centricon 100 devices and centrifuged for 135 min at 2400 rpm in a Beckman JA-12 rotor); SDS-PAGE showed that contaminating SR membranes were retained by the Centricon membrane but that p29/30 peptides were recovered in the filtrate. These peptides were subsequently used for the ultrafiltration experiments with Centricon 10.

**Metal-binding Properties of Bis-Tris Buffer**—In some cases, for treatment of SR vesicles with proteinase K at pH 6.5, we replaced the Bis-Tris buffer of our original standard medium (15) by a Mops/NaOH buffer at the same pH. This is because we found that Bis-Tris buffer (but not Mops/NaOH buffer) binds Ca$^{2+}$ with an apparent dissociation constant around 25 mM at this pH, so that in the presence of 100 mM Bis-Tris and a certain amount of total Ca$^{2+}$, the free [Ca$^{2+}$] is about 5-fold lower than the total [Ca$^{2+}$]. Although this has no implication for the present results, it explains why, in our previous work (15), unexpectedly high concentrations of Ca$^{2+}$ were required to protect the ATPase transmembrane bundle from denaturation during proteinase K treatment.

**RESULTS**

**Protease-resistant Soluble Fragments of Ca$^{2+}$-ATPase**—The starting point for the present work was the observation that treatment of sarcoplasmic reticulum Ca$^{2+}$-ATPase with a variety of proteases generally leads to accumulation of soluble fragments of about 30 kDa, which are relatively resistant to further proteolysis. Fig. 1 shows an experiment in which SR vesicles were treated with elastase (El, left), proteinase K (pK, central lanes), or Asp-N (AspN, right). Aliquots of the treated samples were centrifuged, and peptides found in either the

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1 The abbreviations used are: SR, sarcoplasmic reticulum; Asp-N, endoproteinase Asp-N; PMSF, phenylmethylsulfonyl fluoride; FITC, fluoroisothiocyanate; Mops, 4-morpholinepropanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)aminotriis(hydroxymethyl) methane; Tricine, N-tris(hydroxymethyl)methylglycine; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Mops, NaOH buffer; Mops/NaOH buffer; Mops/NaOH buffer.

2 Available on-line at the following E-mail address: deleag@ibcp.fr

3 T. Menguy, S. Chenevais, F. Guillain, M. le Maire, P. Falson, and P. Champell, manuscript in preparation.
total sample (Tot), the pellet (Pel), or the supernatant (Sup) were separated by SDS-PAGE. In all three cases, irrespective of the proteolytic enzyme, the supernatant (lanes 4, 8, and 12) contains, in addition to the protease itself (asterisk), one or two major proteolytic fragment(s) of about 30 kDa (see arrows). In the case of Asp-N, a faint band at the position of intact ATPase also shows up in the supernatant, but this is presumably due to contamination of the protease with phospholipase. Treatment of SR vesicles with endoproteinase Glu-C protease also results in appearance of a 31-kDa fragment (not shown).

The fragments found in the supernatants were identified as peptides originating from the large cytosolic domain of Ca$^{2+}$-ATPase by immunoblotting with sequence-specific antibodies and N-terminal sequencing (15); mass spectrometry experiments (some of which will be described below in Fig. 6; see “Experimental Procedures”) were also performed for identification of C termini. The conclusion from all these experiments regarding the identification of the soluble peptide fragments is shown in Table I. It appears that the region surrounding the Arg$^{350}$-Ala$^{357}$ peptide bond, known to be highly reactive to trypsin, is not a major target for the other proteases. Cleavage by Asp-N is found to occur at locations just N-terminal of an aspartic residue (Ser$^{350}$-Asp$^{351}$ and Arg$^{615}$-Asp$^{616}$ bonds), and cleavage by endoproteinase Glu-C occurs after glutamic acid residues (e.g. at Glu$^{340}$-Thr$^{341}$), as expected. Cleavage by proteinase K does not appear to be particularly specific of certain amino acid residues (see also Ref. 15) but occurs at a few sites only, presumably because of the local peptide conformation. This also seems to apply to elastase. A remarkable finding is that all fragments more or less coincide, as they all start close to the ATPase phosphorylation site, Asp$^{351}$, and end close to Ser$^{410}$ in the Lys$^{505}$-Arg$^{515}$ region. This suggests the existence, between Asp$^{351}$ and Ser$^{410}$, of a region of relatively compact structure resistant to proteolysis, characteristic of a structural domain in the cytoplasmic portion of the ATPase. In the following sections, we mainly used peptides derived from proteinase K-treated SR vesicles (a mixture of “p29” and “p30” peptides, starting at Thr$^{357}$ and Ser$^{350}$ respectively, and ending after either Met$^{608}$ or Ser$^{610}$), to ask to what extent the isolated peptides retain properties resembling those of the polypeptide chain in the native structure. To do this, we studied nucleotide- and metal-binding properties of p29/30 fragments as well as their sensitivity to trypsin and reactivity toward FITC, and we
Characterized the secondary structure of these fragments as well as their hydrodynamic and spectroscopic properties.

Retention of Nucleotide- and TNP-nucleotide-binding Properties for p29/30 Fragments, as Deduced from TNP-nucleotide Fluorescence—To study the nucleotide-binding properties of these soluble Ca\(^{2+}\)-ATPase fragments, we took advantage of the fact that the fluorescence of a TNP-nucleotide is 3- or 4-fold higher when it is bound to Ca\(^{2+}\) than when it is free in solution (14, 25, 26). This is shown in panel A of Fig. 2, which shows control experiments with TNP-ATP and intact SR. Using excitation and emission wavelengths optimal for detection of TNP-ATP fluorescence (410 and 540 nm, respectively), addition of TNP-ATP to an SR-containing cuvette results in an increase in the intensity of the signal which is almost completely reversed when TNP-ATP is chased off the nucleotide-binding site by ADP addition. The difference between the residual signal intensity in the presence of both ATP and TNP-ATP and the signal intensity in the absence of both nucleotides corresponds to the fluorescence of free TNP-ATP. This can be seen in experiments in which TNP-ATP is added to an SR-free sample; in this case (see panel B experiment, in which the supernatant of a centrifuged SR suspension provides such a sample), the base-line signal before TNP-ATP addition is very low (because this signal is directly related to the amount of light scattered by the sample) and the small TNP-ATP-dependent signal is not altered upon ATP addition. Thus, the large TNP-ATP-dependent signal observed in the presence of SR vesicles, partially reversed by ATP, reveals TNP-ATP binding to the ATPase nucleotide site.

Panels C and D in Fig. 2 show the outcome of corresponding experiments performed on proteinase K-treated SR vesicles. With both total sample (panel C) and supernatant (panel D), ATP is still effective in reducing the signal observed in the presence of TNP-ATP, suggesting ATP-displaceable TNP-ATP binding to isolated p29/30 fragments. SDS-PAGE analysis of the various samples used in this experiment is shown on the right of Fig. 2, confirming degradation of Ca\(^{2+}\)-ATPase and formation of appreciable amounts of p29/30 after proteinase K treatment, as in Fig. 1. In complementary experiments, we found that both the TNP-ATP-dependent signal in the supernatant of proteinase K-treated samples and the magnitude of the ATP-induced drop in this signal (as in panel D) depend on the duration of the proteolysis period; they rise to a maximal value after about 30 min of proteolysis at pH 6.5 and subsequently decline, in close agreement with the amount of p29/30 fragments which can be estimated from SDS-PAGE gels (data not shown). In Fig. 2, the fact that changes in TNP-ATP fluorescence are smaller upon binding to non-centrifuged but proteinase K-treated ATPase (panel C) than upon binding to intact Ca\(^{2+}\)-ATPase (panel A) is probably due to a combination of factors, such as proteinase-induced modification of the environment of TNP-ATP in its site (resulting in reduced fluorescence enhancement upon binding), lower affinity of TNP-ATP for p29/30 than for Ca\(^{2+}\)-ATPase (see below), and slow conversion of p29/30 to smaller fragments (which no longer bind TNP-ATP). On the other hand, the fact that changes in TNP-ATP fluorescence are smaller upon binding to the supernatant of treated ATPase (panel D) than upon binding to non-centrifuged samples (panel C) is probably due in part to the fact that the membranous fraction still contains a few ATPase chains either completely intact or only partially cleaved by proteinase K, such as the membranous 83-kDa peptide which retains full reactivity toward ATP (15). In addition, p29/30 fragments may weakly bind to portions of the ATPase still attached to the membrane and be partially pelleted together with the membrane fraction (see lane 7 in Fig. 1).

Retention of Nucleotide and TNP-nucleotide-binding Properties for p29/30 Fragments, as Deduced from Intrinsic Fluorescence Measurements—In p29/30 fragments, one Trp residue, Trp\(^{552}\) (out of a total of 13 Trp residues in intact Ca\(^{2+}\)-ATPase) is present. Thus, it is also possible to study nucleotide binding to p29/30 by monitoring changes in intrinsic (Trp) fluorescence. TNP-ATP is known to quench Ca\(^{2+}\)-ATPase intrinsic fluorescence, both by a saturable mechanism due to Förster transfer from Trp\(^{552}\) to the TNP moiety of bound TNP-ATP (Ref. 27; \(R_0 = 24\) Å, see Ref. 22) and by a non-saturable quenching due to light absorption by the added nucleotide (an “inner filter” effect). These effects can be seen in panel A of Fig. 3, which illustrates a control experiment with intact Ca\(^{2+}\)-ATPase and TNP-ATP; in this case, there is a well defined break in the quenching curve (27), indicating high affinity binding of the TNP-nucleotide. When the experiment is repeated with p29/30 peptides (panel B of Fig. 3), there is also evidence for a saturable component in the quenching curve but with lower affinity (the apparent dissociation constant is a few \(\mu M\) for p29/30 peptides, compared with much less than 1 \(\mu M\) for intact SR, see also Refs. 14 and 25). The same result is obtained when TNP-ADP is added, instead of TNP-ATP (see below). Interpretation of saturable quenching in terms of binding to the nucleotide site is strengthened by the demonstration that this quenching is reversed when the TNP-nucleotide is chased off its binding site by addition of nucleotide. This is shown in panels C and D of Fig. 3, in which ADP was added to samples pretreated with TNP-ADP; both for control ATPase (panel C) and for p29/30 fragments (panel D), a high concentration of ADP does reverse the quenching resulting from previous addition of TNP-ADP. This reversal is partial, because although Förster quenching is reversed, the reduction in signal intensity due to inner filter...
peptides is shown in ATP, in the additional presence of 0.5 mM EDTA. The ordinate scale is from Trp fluorescence measurements.

Sample (ED) to chelate contaminating Ca$^{2+}$ and (treated for 30 min with 30 s) responsible for Ca$^{2+}$ long-term connection to the ATPase transmembrane domain reactivity.

Fluorescence of p29/30 fragments, since these fragments are not detected with the fluorescence of each sample in the absence of Ca$^{2+}$ and nucleotide. Taking dilution and inner filter effects into account in favor of p29 than after proteolysis under the previous conditions (compare SDS-PAGE results in panel E of Fig. 3 with those in panel E of Fig. 2), but the outcome of the above Trp fluorescence experiments was the same when proteolysis was performed in the pH 6.5 medium instead of the pH 8 sucrose medium (not shown). Note also that similar results were obtained with supernatants of SR vesicles that had been treated with elastase (as in Fig. 1) instead of proteinase K (data not shown). All these results provide unambiguous evidence for binding of both TNP-nucleotides and nucleotides themselves to 29/30 peptides, with a reduced but significant affinity.

![Fig. 3. Binding of nucleotides to p29/30 fragments, as deduced from Trp fluorescence measurements.](image)

Experiments were performed either (i) with the supernatant of proteinase K-treated vesicles (pK Sup, panels B and D) here, SR vesicles at 2 mg of protein/ml were treated for 30 min with 30 µg/ml proteinase K in a medium containing 300 mM sucrose, 1 mM Mg$^{2+}$, 0.01 mM Ca$^{2+}$, and 10 mM Tricine-Tris, at pH 8 and 20 °C, and 3-ml aliquots were centrifuged with a Beckman 45Ti rotor at 40,000 rpm, about 185,000 gav, for 90 min, or (ii) with control SR, incubated under the same conditions but in the absence of proteinase K (SR, panels A and C). SDS-PAGE analysis of the resulting peptides is shown in panel E on the right. Intrinsically fluorescence was detected with $\lambda_{ex}$ = 300 nm (bw = 2 nm), to minimize absorption by nucleotides, and with $\lambda_{em}$ = 330 nm (bw = 10 nm). The fluorescence level of p29/30 fragments was more than 10-fold lower than that of intact Ca$^{2+}$-ATPase; the data were normalized, 100% corresponding to the fluorescence of each sample in the absence of Ca$^{2+}$ and nucleotide.

In all cases, 100-µl protein aliquots were added to 1.9 ml of a medium containing 100 mM KCl and 50 mM Mops-Tris at pH 7 and 20 °C. Panels A and B, concentration dependence of fluorescence quenching by TNP-ATP, in the additional presence of 0.5 mM EDTA. The ordinate scale is logarithmic; dashed lines represent the inner filter component of quenching. Panels C and D, 2 µl of EDTA 0.5 x was first added to the sample (ED) to chelate contaminating Ca$^{2+}$ and (Mg$^{2+}$) contributed by the proteolysis medium; then, four additions of TNP-ADP were made, followed by several additions of ADP itself; panel C, additional of 0.4 µl of 10 mM TNP-ADP followed by additions of 50 µl of 200 mM ADP; panel D, additions of 1 µl of 10 mM TNP-ADP followed by additions of 50 µl of 200 mM ADP. The dotted lines show the “true” fluorescence levels in the presence of nucleotide taking dilution and inner filter effects into account.

Binding of [γ-32P]ATP to p29/30 Fragments, as Deduced from Ultrafiltration Experiments—For intact SR, binding of ADP or ATP in the absence of TNP-nucleotide can also be directly detected as a rise in ATPase intrinsic fluorescence (e.g. Ref. 30). However, when, in similar experiments, 1 mM ATP aliquots were sequentially added to p29/30 fragments, the resulting rise in fluorescence level was small; despite our efforts to reduce inner filter artifacts due to nucleotide absorbance as much as possible by excitation of Trp residues at 300 nm, this could only, after the first addition of ATP, compensate for the dilution and inner filter-induced artifacts, expected and indeed observed after subsequent ATP additions (data not shown). Evaluation with this method of the affinity of p29/30 fragments for ATP was therefore not possible.

Thus, to measure directly the equilibrium binding of [γ-32P]ATP to the soluble p29/30 peptides, we designed a protocol based on the opportunity provided by ultrafiltration mem-
residue was not determined quantitatively, hence the Edman degradation were compared for the labeled “p12-FITC” peptide and the unlabeled “p12” peptide (the ratio is plotted). The second alanine cleavage of either nonlabeled or heavily FITC-labeled p29/30 fragments (see text) were sequenced. PTH-amino acids found after each cycle of white dots Wratten 55 green filter; the before acid fixation and Coomassie Blue staining and illuminated with UV light (302 nm). Fluorescence (panel B) smaller peptides (designated as p17/18 and p12) are apparent (indicated), or again (iii) with proteinase K first, followed by trypsin (lane 6), Lanes 1 and 2, a sample similar to the one for lane 5, but prepared from unlabeled SR vesicles, was centrifuged. The supernatant was then incubated with 2 m FITC-labeled SR vesicles. Labeling was partial and corresponded to 1 nmol FITC/mg of protein. Lanes 4–6, the same labeled vesicles (2 mg/ml) were submitted to proteolysis, either (i) for 2 min with 125 µg/ml trypsin (lane 4); in panel A, fragments A and B are apparent, as well as A1, A2 superimposed with trypsin (Tr), and the trypsin inhibitor (Tr Inh), or (ii) for 30 min with 30 µg/ml proteinase K (lane 5, p29/30 peptides are indicated), or again (iii) with proteinase K first, followed by trypsin (lane 6). Lanes 1 and 2, a sample similar to the one for lane 5, but prepared from unlabeled SR vesicles, was centrifuged. The supernatant was then incubated with 2 µM FITC for 30 min and subsequently either (i) loaded onto lane 1, or (ii) submitted to trypsin treatment and then loaded onto lane 2, in the latter case, trypsin (Tr), trypsin inhibitor (Tr Inh), and three smaller peptides (designated as p17/18 and p12) are apparent (panel A). For fluorescence examination, the gel was deposited in a transparent box before acid fixation and Coomassie Blue staining and illuminated with UV light (302 nm). Fluorescence (panel B) was detected with a Kodak Wratten 55 green filter; the white dots are due to fluorescent defects in the plastic box. Panel C, the N termini of p12 peptides derived from trypsin cleavage of either nonlabeled or heavily FITC-labeled p29/30 fragments (see text) were sequenced. PTH-amino acids found after each cycle of Edman degradation were compared for the labeled “p12-FITC” peptide and the unlabeled “p12” peptide (the ratio is plotted). The second alanine residue was not determined quantitatively, hence the ×.

FIG. 5. Sensitivity to trypsin of the Arg605-Ala606 bond in p29/30 fragments, and reactivity of Lys515 toward FITC in these fragments. In this experiment, the same medium was used both for proteolysis with proteinase K and/or trypsin and for labeling with FITC; it contained 0.3 M sucrose, 1 mM Mg2+, 10 µM Ca2+, and 10 mM Tricine-Tris at pH 8 and 20 °C. After samples were run on a SDS-PAGE/Tricine gel, the gel was examined immediately for fluorescence (panel B) and subsequently stained with Coomassie Blue (Coom. blue) (panel A). Lane 7, molecular mass markers (see panel A); the large spot in lane 7 of panel B corresponds to the tracking dye in the migration front. Lane 3, FITC-labeled SR vesicles. Labeling was partial and corresponded to 1 nmol FITC/mg of protein. Lanes 4–6, the same labeled vesicles (2 mg/ml) were submitted to proteolysis, either (i) for 2 min with 125 µg/ml trypsin (lane 4); in panel A, fragments A and B are apparent, as well as A1, A2 superimposed with trypsin (Tr), and the trypsin inhibitor (Tr Inh), or (ii) for 30 min with 30 µg/ml proteinase K (lane 5, p29/30 peptides are indicated), or again (iii) with proteinase K first, followed by trypsin (lane 6). Lanes 1 and 2, a sample similar to the one for lane 5, but prepared from unlabeled SR vesicles, was centrifuged. The supernatant was then incubated with 2 µM FITC for 30 min and subsequently either (i) loaded onto lane 1, or (ii) submitted to trypsin treatment and then loaded onto lane 2, in the latter case, trypsin (Tr), trypsin inhibitor (Tr Inh), and three smaller peptides (designated as p17/18 and p12) are apparent (panel A). For fluorescence examination, the gel was deposited in a transparent box before acid fixation and Coomassie Blue staining and illuminated with UV light (302 nm). Fluorescence (panel B) was detected with a Kodak Wratten 55 green filter; the white dots are due to fluorescent defects in the plastic box. Panel C, the N termini of p12 peptides derived from trypsin cleavage of either nonlabeled or heavily FITC-labeled p29/30 fragments (see text) were sequenced. PTH-amino acids found after each cycle of Edman degradation were compared for the labeled “p12-FITC” peptide and the unlabeled “p12” peptide (the ratio is plotted). The second alanine residue was not determined quantitatively, hence the ×.

branes (Centricon 10 devices, Amicon) to concentrate these soluble peptides but let free [γ-32P]ATP flow through them; measurement of the amount of [γ-32P]ATP in the concentrate then corresponds to the sum of free ligand (as can be measured in the filtrate) and bound ligand (see “Experimental Procedures”), and including various amounts of nonradioactive ATP together with a constant amount of radioactive ATP tracer makes it possible to evidence competition between nonradioactive and radioactive ligands (e.g. Ref. 65). These experiments were conducted in the absence of Mg2+, and for comparative reasons included measurements with intact SR vesicles. The results of the experiments with intact SR (circles in Fig. 4), in which the membranes were loaded onto the Centricon 10 device at an initial concentration of 0.16 mg of protein/ml and were concentrated to 4.8 mg/ml (on average), are consistent with a Kd of 34 ± 11 µM, total number of sites 138 ± 32 (panel A), and Reactivity with FITC—After having demonstrated the ability of p29/30 fragments to bind nucleotides, we asked whether these fragments also retain two other properties characteristic of intact ATPase, i.e. strong susceptibility to trypsin of the Arg605-Ala606 peptide bond, and high reactivity toward FITC of Lys515. The results of experiments designed to explore these aspects are shown in Fig. 5. Panel A of this figure shows the result of Coomassie Blue staining of an SDS-PAGE/Tricine gel after separation of the fragments resulting from proteolysis of various samples with proteinase K, trypsin, or both. As a reference, intact Ca2+-ATPase and the associated Ca2+-binding proteins of SR vesicles are shown in lane 3. The well known bands A and B, as well
Mass spectrometry (MALDI-TOF) experiments were fully consistent with this conclusion and extended its validity to the fragments resulting from Asp-N and elastase treatment of Ca\textsuperscript{2+}-ATPase. The mass of the smaller peptide (17,227) corresponds closely to the theoretical value (17,225) expected for a peptide starting at Asp\textsuperscript{351} (the N-terminal residue of Asp-N fragments, as deduced from the sequencing experiments) and ending after Arg\textsuperscript{505}. The Arg\textsuperscript{505}-Ala\textsuperscript{506} bond is therefore highly reactive to trypsin in fragments resulting from Asp-N treatment of Ca\textsuperscript{2+}-ATPase. The mass of the smaller peptide then allows us to determine the C-terminal residue of the fragment without ambiguity, in this case Arg\textsuperscript{515} (as indicated in Table I). The same experiment was repeated with fragments resulting from Ca\textsuperscript{2+}-ATPase treatment with elastase (panel C); again two groups of peaks showed up, and the measured masses were consistent with the previously determined N-terminal residues and the existence of a unique tryptic cleavage site at the Arg\textsuperscript{505}-Ala\textsuperscript{506} bond (see figure legend). The same conclusion was also true for proteinase K fragments (panel D). Thus, all fragments retain an exquisite sensitivity to trypsin of their Arg\textsuperscript{505}-Ala\textsuperscript{506} peptide bond.
lanes, the supernatant of proteinase K-treated Ca\textsuperscript{2+}-ATPase was prepared from unlabeled SR vesicles, and p29/30 fragments were subsequently labeled by adding FITC directly to this supernatant, under less than stoichiometric conditions (to reduce unreacted FITC to a minimum); subsequently, as explained above, these fragments were treated with trypsin and processed for SDS-PAGE. Comparison of lanes 2 and 6 in Fig. 5B shows that FITC added to p29/30 only labels the p12 peptide and neither the p17 nor the p18 peptides, although the p12 peptide contains fewer lysine residues than the two other peptides (7 lysine residues compared with 12 or 13). Selectivity of labeling is thus demonstrated. In the second approach, the same protocol was used, but the unlabeled p29/30-containing supernatant was now either (i) reacted with an excess of FITC (16 \textmu M) first, followed by elimination of unreacted FITC (by passing through a Pharmacia PD10 column) and subsequent treatment with trypsin, or (ii) treated with trypsin in the absence of any FITC labeling. In both cases, this was followed by SDS-PAGE separation of the resulting peptides, electrotrans-fer onto polyvinylidene difluoride membranes, and N-terminal SDS-PAGE separation of the resulting peptides, electrotrans-fertreated with trypsin, or (ii) treated with trypsin in the ab-sence of any FITC labeling. In both cases, this was followed by SDS-PAGE separation of the resulting peptides, electrotrans-fer onto polyvinylidene difluoride membranes, and N-terminal sequencing of p12. In the peptide derived from the FITC-treated sample, we expected that sequencing would not be able to identify the FITC-modified residue. Indeed, the yield of the 10th residue (corresponding to Lys\textsuperscript{515}) was much lower in the FITC-treated sample (designated as p12-FITC) than in the non-reacted one (designated as p12) (see panel C in Fig. 5). This confirms that selective labeling of Lys\textsuperscript{515} has occurred in the p29/30 fragment, as in intact Ca\textsuperscript{2+}-ATPase, resulting in disappearance of at least close to 50% of Lys\textsuperscript{515} residues. The reason why the other half of the Lys\textsuperscript{515} residues appears not to have been modified was not investigated further. It is possible that labeling is less selective for p29/30 fragments than for ATPase; it is possible also that the labeling period in our experiments (30 min at pH 8 only) is simply not sufficient for 100% labeling of the proteolytic fragments, although it is sufficient for intact ATPase.

Hydrodynamic Properties of p29/30 Fragments, Secondary Structure, and Spectroscopic Characterization of p29/30 Fragments—The hydrodynamic properties of p29/30 fragments were examined by gel filtration on a TSK G3000 SWXL column (33). As shown in Fig. 7, a sharp major peak showed up in the eluant, together with minor smaller peptides. On the basis of column calibration with water-soluble proteins, the position of the major peak corresponds to a Stokes radius of 24 Å. Since \( R_{\text{min}} = \frac{z}{m} \) for a 29.5-kDa protein can be estimated to be 20.6 Å (using a partial specific volume of 0.745 cm\(^3\)/g), this Stokes radius corresponds to a frictional ratio of 1.17. This frictional ratio is compatible with an almost perfectly globular shape of the p29/30 fragments, i.e. a compact structure, consistent with the resistance of the fragments to most proteolytic enzymes.

The fact that p29/30 fragments have a well-organized structure was also checked by circular dichroism (CD) measurements. As shown in panel A of Fig. 8, the supernatants of SR vesicles treated with proteinase K for 15 and 30 min (dashed and solid lines, respectively) have CD spectra whose shapes are closely similar and whose amplitudes only slightly differ, consistent with almost complete conversion of ATPase to p29/30 fragments after 30 min. After 120 min proteolysis (dashed-dotted line), the slightly more pronounced trough at 205–207 nm is indicative of further hydrolysis of p29/30 to small disorganized peptides (random coils exhibit strongly negative ellipticities below 200 nm); however, this trend is much more apparent after an additional 10 h proteolysis at 37 °C (dotted line). After analysis of the total amino acid contents of the supernatant resulting from 30-min proteolysis, the measured ellipticity was converted to molar ellipticity: for this 30-min supernatant, the result, expressed in degrees cm\(^2\) dmol\(^{-1}\), is shown by the solid line in panel B of Fig. 8. Since such a supernatant sample in addition to p29/30 peptides contains the very small peptides derived from the 608/610–734/747 region (15), we also attempted to obtain purer p29/30 peptides by passing this supernatant over a Sephadex G25M column before measuring ellipticity and amino acid contents of the fraction eluted in the void volume; the result is shown by the dashed line in panel B, which, as expected, reveals a larger (negative) molar ellipticity at 222 nm (characteristic of \( \alpha \) helices) and a smaller relative contribution of the trough at 205–207 nm in the Sephadex eluate, compared with the initial supernatant. For comparison, the molar ellipticity of C12E8-solubilized de-oxycylate-extracted ATPase is also shown in panel B as a dashed-dotted line.

All these spectra were analyzed to estimate, according to various methods, the percentage of the various secondary structures present in p29/30 peptides. Depending on the method, as made possible by the “Dichroprot” software, 28 (±5%) of amino acids were estimated to be involved in \( \alpha \) helices in the p29/30-containing Sephadex eluate (compared with 43 (±5%) in C12E8-solubilized ATPase). According to some of these methods, e.g. the “variable selection method” (34, 35), the
proportion of amino acids involved in β-structures (either parallel or anti-parallel) was higher for p29/30 fragments than for the entire ATPase, 20 (±3)% compared with 14 (±3)% and β-turns represented 19 and 15%, respectively (again ±3%). Thus, an unambiguous outcome of these measurements is that the deconvolution analysis that a significant fraction of amino acids in p29/30 fragments are organized in defined secondary structures.

We finally performed a few experiments to characterize from a spectroscopic point of view the solubilized p29/30 subdomain. We first focused on intrinsic fluorescence. After proteinase K treatment, the fluorescence spectrum of non-centrifuged samples is equivalent to that of control ATPase, although slightly less intense (not shown). As shown in panel A of Fig. 9, normalized excitation spectra reveal that the Trp shoulder at 290 nm is less prominent for the p29/30-containing supernatant of such samples (dotted line) than for the total samples (solid lines); in addition, the emission spectrum of the p29/30-containing supernatant is red-shifted by about 6 nm compared with the fluorescence of the total sample, which probably indicates that the environment for Trp in p29/30 peptides is more polar than, on average, the environment of the other 12 Trp residues of Ca2+-ATPase; the latter residues in native ATPase are thought to reside in or close to the ATPase transmembrane sector (2).
We also examined the characteristics of the fluorescence of FITC, either free or bound to p29/30 fragments or intact Ca\(^{2+}\)-ATPase. Panel B in Fig. 9 shows that the excitation spectrum of FITC bound to intact Ca\(^{2+}\)-ATPase (solid line) is red-shifted by almost 10 nm with respect to that of free FITC (dotted line). This fact, together with the lower \(K_a\) observed for ATPase-bound FITC compared with free FITC (\(K_a\) of 5.8 instead of 6.4, see Ref. 36), indicates that bound FITC experiences an environment different from that for free FITC. Panel B also shows that the excitation spectrum of bound FITC after proteinase K treatment is an intermediate one, in both the non-centrifuged samples (data not shown) and the p29/30-containing supernatants (in Fig. 9B, the dashed line corresponds to a supernatant obtained after 30 min proteolysis). Complete disappearance of p29/30 fragments after extensive proteolysis results in an excitation spectrum similar to that of free FITC (dashed-dotted line). These results suggest the environment of bound FITC in p29/30 fragments produced by proteinase K treatment is somewhat different from its environment in intact Ca\(^{2+}\)-ATPase but is not changed to the extent that it would become completely exposed to the aqueous medium. This conclusion is strengthened by experiments in which solvent accessibility of FITC is examined with the quenching agent iodide (see inset to Fig. 9B); as previously found (36), FITC accessibility to iodide is higher for free FITC than for FITC bound to Ca\(^{2+}\)-ATPase (slopes for Stern-Volmer plots are 6.5 \(m^{-1}\) and 2.7 \(m^{-1}\), respectively), suggesting restricted accessibility to FITC, a suggestion supported by immunoreactivity data (37). In p29/30 fragments, this accessibility has an intermediate value, close to that of FITC in intact Ca\(^{2+}\)-ATPase (slopes for Stern-Volmer plots are 3.1 or 3.9 \(m^{-1}\) for supernatants after 15 or 45 min proteolysis, respectively; in the latter case, degradation of p29/30 fragments to much smaller peptides contributes to the larger slope). Thus, the environment of FITC in p29/30 fragments is still significantly protected from the aqueous solvent.

Finally, panel C in Fig. 9 shows that Trp\(^{552}\) and bound FITC are close enough in p29/30 fragments to allow fluorescence transfer from the indole moiety to the fluorescein moiety to occur. Since nothing is known about the relative orientations of these two fluorophores, we cannot, however, derive from the data a distance between these two moieties. For random relative orientation of the indole and fluorescein moieties, the modest transfer observed in Fig. 9C would imply a distance between the two fluorescent moieties larger than 25 or 30 Å, the Förster radius \(R_0\). Alternatively, the distance could be smaller if the two fluorescent moieties are positioned relative to each other in a relatively rigid way unfavorable for energy transfer.

Demonstration of Metal-binding Sites in p29/30—When the measurements of either TNP-ATP fluorescence or Trp fluorescence illustrated in Figs. 2 and 3 were repeated with p29/30 fragments in the presence of Mg\(^{2+}\) (5 mM), we found that the fluorescence responses due to nucleotide binding to the fragments were reduced, presumably because of a decreased binding affinity (data not shown). The same counterproductive effect of Mg\(^{2+}\) on TNP-ATP binding was previously noted by Moutin et al. (14) with the heterologously expressed isolated large cytoplasmic loop. Nevertheless, in our intrinsic fluorescence measurements, as those in Ref. 14, there was no direct evidence for binding of Mg\(^{2+}\) per se to the soluble p29/30 fragments; two additions of Mg\(^{2+}\), 5 mM each time, performed in the absence of nucleotide, had no effect on the intrinsic fluorescence level of p29/30 fragments (data not shown).

In contrast, by using lanthanide ions, we obtained direct evidence for metal binding to p29/30 in the absence of nucleotides. This was investigated by using Nd\(^{3+}\) ions and SR vesicles previously labeled with FITC at Lys\(^{515}\). Nd\(^{3+}\) has previously been shown to quench the fluorescence of bound FITC by up to 40%, even at high ionic strength (39, 40), probably because of efficient Förster transfer from the fluorescein moiety of bound FITC to an Nd\(^{3+}\) ion bound very close to it on the ATPase, at a distance of about 1 nm. The specificity of this interaction is suggested by the fact that Mg\(^{2+}\) competes with Nd\(^{3+}\), and it has been argued that the Nd\(^{3+}\) ion responsible for FITC quenching may be bound to a metal-binding subsite normally occupied by Mg\(^{2+}\) and located (because of the short FITC-Nd\(^{3+}\) distance) in the ATPase active cleft (39). Thus, in the experiment illustrated in Fig. 10, we examined quenching by Nd\(^{3+}\) of FITC fluorescence in FITC-labeled p29/30 fragments. These fragments were prepared by first labeling SR vesicles with FITC (17), followed by treatment with proteinase K and centrifugation. Increasing concentrations of Nd\(^{3+}\) were added either to non-centrifuged samples (closed symbols in Fig. 10) or to their supernatants (open symbols in Fig. 10).

When examined at excitation and emission wavelengths commonly used for free FITC (495 and 520 nm, respectively), total FITC fluorescence slightly varies during proteolysis (data not shown), presumably in part because of the small spectral change of bound FITC after proteolysis documented in Fig. 9B. When treated samples are centrifuged, the proportion of FITC fluorescence recovered in the supernatant increases with the proteolysis period, up to almost 100% (data not shown), in agreement with almost complete degradation of the region around Lys\(^{515}\) to soluble fragments. As proteolysis proceeds, the well-known sensitivity of covalently bound FITC to high affinity Ca\(^{2+}\) binding to Ca\(^{2+}\)-ATPase (41) progressively vanishes, again as expected (data not shown). Nevertheless, after 30 or 45 min proteolysis, quenching by Nd\(^{3+}\) is retained to a...
significant extent for proteolyzed samples as well as for their supernatants. Analysis of such quenching curves is best performed when the data are normalized, and residual fluorescence is expressed as percent of the initial fluorescence in the absence of Nd³⁺, as shown in Fig. 10. For 30–300 μM Nd³⁺, quenching by Nd³⁺ of FITC fluorescence in p29/30 fragments (triangles and diamonds) is definitely observed, although to a lesser extent than in Ca²⁺-ATPase (closed circles). Open and closed squares are two control experiments designed to confirm the insensitivity to Nd³⁺ of (i) FITC in solution (open squares) and (ii) FITC bound to the ATPase peptides resulting from extensive proteolysis (closed squares; in this case, proteolysis was performed as in the experiment corresponding to the dashed-dotted line in Fig. 9B). The dotted line illustrates a different control experiment showing that, as expected because of the lack of absorbance properties, La³⁺ does not quench fluorescence of FITC bound to intact Ca²⁺-ATPase. These data demonstrate that soluble p29/30 fragments retain some residual ability (although with reduced affinity, as in the case of nucleotide binding) to bind lanthanide ions relatively close to FITC bound at Lys⁵¹⁵.

**DISCUSSION**

In this report, we show that by using various proteolytic enzymes (Fig. 1), it is possible to prepare fragments of Ca²⁺-ATPase closely related to each other and all originating from the cytosolic portion of this membranous enzyme (Table I). According to hydrodynamic and circular dichroism analysis, these "p29/30" fragments have a compact globular shape with a relatively high content of secondary (α/β) structure (Figs. 7 and 8). They have a significant affinity toward nucleotides (Figs. 2–4) and also retain reactivity toward trypsin and FITC, like intact Ca²⁺-ATPase, and at the same sites (Figs. 5 and 6). All this suggests that these proteolytic fragments, which contain a major fraction of the ATPase cytosolic head, are released without loss of basic structural features from a region which, in the native tertiary structure, constitutes an autonomous structural domain.

The reasons why the properties of p29/30 fragments have not been recognized earlier are probably related to the fact that most previous proteolytic investigations on Ca²⁺-ATPase have been performed with trypsin. Studies with this enzyme have never resulted in production of fragments comparable to those described in the present work because trypsin has an extraordinary potency for cleavage of Ca²⁺-ATPase in the middle of the molecule, at Arg⁵⁰⁵-Ala⁵⁰⁶. We found no evidence for a particular sensitivity of the Arg⁵⁰⁵-Ala⁵⁰⁶ peptidic bond to proteases other than trypsin; thus, the high sensitivity to trypsin of this bond is probably due to a particular conformation of the Arg⁵⁰⁵-Ala⁵⁰⁶ peptidic bond, favorable for trypsinolysis, rather than to a strategic location of this bond at the boundary between two distinct domains of Ca²⁺-ATPase. It is remarkable that proteolysis fragments similar to our p29/30 fragments have in fact been already described for Na⁺-K⁺-ATPase and H⁺-K⁺-ATPase, after treatment with either trypsin (42) or other proteases (43–46).

Our extensive peptide sequencing and mass spectrometry data allow us to unambiguously conclude that p29/30 fragments start close to the phosphorylation domain, around residue Asp⁵³¹, and end in the 605–615 region, well in advance of the conserved region starting around residue 670 (the so-called "hinge" region). N-terminal cleavage sites are consistent with a rather superficial localization of the phosphorylatable Asp⁵³¹ residue, to which phosphorylating substrates must of course have access, in the region delimited by the CSD₃₀₃-KTGTLT motif. However, p29/30 fragments are probably not primary cleavage products, and the possibility therefore remains that accessibility to proteolytic attack is somewhat modified by previous cuts in the N-terminal and C-terminal regions of the ATPase (15). Concerning the C-terminal end in the 605–615 region, a region that has not been given much attention up to now, it is of interest that it is flanked by two conserved motifs, present in most eucaryotic P-type ATPases, D₆₀₃PPR and MIT₆₃₅GD. According to the Rost and Sander algorithm (47), the 608–616 region is surprisingly predicted to be α-helical and thus should be resistant to proteolytic enzymes; in contrast, the vulnerability of this region to proteolytic cleavage could indicate that this region forms a loop connecting p29/30 to the rest of the ATPase. It is worth noting also that according to the prediction in Ref. 2, 118–120 residues in the sequence corresponding to the isolated p29/30 fragments should be α-helical, i.e. more than 45%, whereas our CD measurements suggested a significantly smaller (about 28%) fraction of α-helical residues in these fragments.

Irrespective of the detailed conformation and location of the N-terminal and C-terminal boundaries of p29/30 fragments, it is clear from the present results that these fragments form a rather compact domain and that this domain is able to bind nucleotides with an affinity which is quite respectable but reduced compared with that of intact ATPase (Figs. 2–4). It is certainly not a surprise that a fragment lacking several highly conserved and functionally critical regions of the ATPase (including residues that obviously interact with the ATP-binding pocket like Arg⁶⁷⁸ and Lys⁶⁸⁴, see Ref. 12 for review) has a reduced affinity for its substrates. The important fact is the ability of p29/30 fragments to bind these ligands at all. Moutin et al. (14) previously concluded that the nucleotide-binding site was at least partially preserved in the ATPase peptide Lys⁵²⁹-Phe⁷⁴⁰ (prepared by heterologous expression in Escherichia coli), which corresponds to the entire "large cytosolic loop" of Ca²⁺-ATPase; our results show that the same conclusion is valid for our smaller p29/30 fragments, which only comprise Thr³⁵⁷/Ser³⁵⁰-Ser⁶¹⁰/Met⁶⁰⁸ peptides. The observation that the presence of Mg²⁺ does not favor ATP or ADP binding to p29/30 fragments is of interest. It is known that in intact Ca²⁺-ATPase, an enhancing effect of Mg²⁺ on the affinity of nucleotide binding is only observed for ATP but not for ADP (30); thus, such an enhancing effect of Mg²⁺ is not characteristic of the portion of the site that recognizes the adenine moiety. In Na⁺-K⁺-ATPase also, high affinity binding of ATP is not dependent on Mg²⁺. In addition, there is in Na⁺-K⁺-ATPase evidence for electrostatic interaction between the γ-phosphate of ATP and the phosphorylatable residue (48, 49); corresponding interactions will obviously be affected by proteolysis in our p30 or p29 peptides (starting at Ser⁶⁵⁰ and Thr⁶⁵⁷, respectively) since the region around the phosphorylation site (Asp⁵³¹) is either absent (p29) or presumably poorly structured (p30). Thus, the fact that MgATP does not appear to bind to p29/30 fragments (nor to bind to the entire large cytosolic loop, see Ref. 14) with an affinity higher than that of Mg²⁺-free ATP should not be taken as an objection against the nucleotide binding ability of p29/30 fragments, which we think is demonstrated here unambiguously. It is also worth pointing out that the nucleotide binding abilities of the proteolysis-derived p29/30 fragments (this work) and of the heterologously expressed large cytosolic loop (14) are of the same order of magnitude, despite the fact that the former fragments are 35–40% shorter than the latter (residues 350/357–608/610 compared with residues 329–740).

Although P-type ATPases lack the classical glycine-rich Walker sequence motifs found in water-soluble kinases (50), it has previously been suggested that like these kinases, the large cytosolic loop of Ca²⁺-ATPase comprises two distinct domains,
a phosphorylation domain and an ATP-binding domain (2). Mainly based on the existence of a tryptic split at Arg<sup>205</sup>-Ala<sup>206</sup> between the phosphorylation site (Asp<sup>351</sup>) and the ATP-protected FITC-binding site (Lys<sup>615</sup>), the Arg<sup>205</sup>-Ala<sup>206</sup> bond was originally thought to be involved in the separation between phosphorylation and ATP-binding subdomains. Subsequent work, however, indicated that residues located N-terminally of Arg<sup>205</sup> also play an important role in nucleotide binding (e.g. Ref. 51). Based on the present data, we speculate that in Ca<sup>2+</sup>-ATPase and probably also in other P-type ATPases, the ATP-binding region mainly consists of the p29/30 domain, with the phosphorylation site located at the N-terminal border of this domain. In this view, interaction with the rest of the molecule of the rather compact p29/30 domain would be made possible by flexibility of the regions connecting this domain to the rest of the molecule; on the one hand, the strategic location of the phosphorylation site at the N-terminal boundary of the domain would be consistent with the demand, from an energy-transducing enzyme, of conformational flexibility at the active site; on the other hand, the 605–615 region at the C-terminal boundary of the domain might constitute the real hinge with conformational flexibility. In this view, the conserved region starting around residue 670 (and whose previous description as a hinge was probably misleading)<sup>4</sup> would be part of a second lobe, perhaps not strictly necessary for nucleotide binding (see Ref. 52) but whose precise positioning could set the stage for a nucleophilic attack on the γ-phosphate of ATP and its transfer to the phosphorylatable Asp<sup>351</sup> residue; note that binding of Ca<sup>2+</sup> to the ATPase transport sites changes the precise positioning, with respect to the nucleotide-binding site, of Lys<sup>604</sup> in this putative second lobe (Refs. 53 and 54; see Ref. 12 for review).

With respect to the above considerations it should be pointed out that it is not possible to completely exclude the possibility (as considered, for instance, in Ref. 45) that in intact ATPase the ATP-binding site could consist of a groove formed between the p29/30 subdomain and the rest of the molecule (the latter would then again presumably include the conserved region starting around residue 670, see review in Ref. 12). In this case, however, the affinity for ATP binding on one side of an isolated p29/30 domain would probably exhibit a reduction of much more than 1 order of magnitude compared with intact ATPase, which was not the case (see Fig. 4). In addition, assuming that the FITC planar chromophore is located at the same place as the nucleotide base in intact ATPase, it would be difficult to understand why FITC bound to p29/30 fragments remains significantly shielded from the aqueous environment (Fig. 9B). Thus, a more likely hypothesis might be that a cavity in the p29/30 domain provides most of the ligands for binding the adenine moiety of nucleotides. The answer to the question of the exact nature of the ATP-binding site will hopefully be provided by x-ray crystallography analysis of this domain with or without ATP bound to it.

In addition to nucleotides, we have shown here that p29/30 fragments are able to bind Nd<sup>3+</sup> ions (Fig. 10); this observation deserves special comment. Although lanthanide binding to the ATPase Ca<sup>2+</sup>-transport sites has sometimes been advocated in the past, it has been demonstrated that these trivalent ions have an even higher affinity for other sites in the protein (21, 55–57), and the present study shows that some of these sites are located in the region of the ATPase cytosolic domain corre-

<sup>4</sup>Note also that in soluble kinases, the real hinge is located between the phosphorylation site and the nucleotide-binding site, not after both of them, as seemingly implied by the current description of Ca<sup>2+</sup>-ATPase where the so-called hinge region is rather far away toward the C-terminal end.
Soluble Domain of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase
Characterization of a Protease-resistant Domain of the Cytosolic Portion of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase: NUCLEOTIDE- AND METAL-BINDING SITES
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