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Differences in the susceptibility of various cation transport ATPases to vanadate-catalyzed photocleavage

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Illumination of sarcoplasmic reticulum vesicles by ultraviolet light in the presence of 1 mM vanadate causes photocleavage of the Ca^{2+}-ATPase into two fragments (Vegh et al. (1990) Biochim. Biophys. Acta 1023, 168–183). In the absence of Ca^{2+} the photocleavage occurs in the N-terminal half of the molecule near the phosphate acceptor Asp-351. In the presence of 2 mM Ca^{2+} the photocleavage shifts to the C-terminal half of the ATPase, near the FITC binding site (Lys-515). About half of the Ca^{2+}-ATPase was cleaved rapidly, accompanied by nearly complete, irreversible loss of ATPase activity when illuminated in the presence of 2 mM $CaCl_2$; further cleavage of the enzyme was slow and affected primarily the C-terminal fragment produced in the presence of Ca^{2+}. Solubilization of the Ca^{2+}-ATPase with $C_{12}E_8$ did not affect the site of photocleavage in either conformation. The vanadate-induced Ca^{2+}-ATPase crystals were disrupted during photocleavage, while the binding of anti-ATPase antibodies directed against the phosphorylation site (PR-8) and against the FITC binding region (PR-11) was enhanced. The bovine kidney Na^+,K^+-ATPase was insensitive to photocleavage under conditions where about half the Ca^{2+}-ATPase was fragmented. The slight cleavage of the pig gastric H^+,K^+-ATPase after prolonged illumination produced fragments that are distinct from the fragments of the Ca^{2+}-ATPase.

Introduction

Vanadate-sensitized photocleavage of the polypeptide chain was observed within the last few years in several enzymes that interact with phosphate compounds. Among these are dynein [1–13], myosin [14–18], ribulose-1,5-diphosphate carboxylase/oxygenase [19], adenylate kinase [20], phosphofructokinase [21], myosin light chain kinase [22] and the Ca^{2+}-ATPase of sarcoplasmic reticulum [23].

The mechanism of photocleavage is essentially unknown, but in the case of myosin [16] and ribulose-1,5-diphosphate carboxylase/oxygenase [19] serine residues were identified as the target of photomodification. The site of the vanadate catalyzed photocleavage in the Ca^{2+}-ATPase of sarcoplasmic reticulum is influenced by the conformation of the enzyme [23]. Depending on the Ca^{2+} concentration of the medium during photocleavage, distinct pairs of peptides are formed. Based on their relationship to the tryptic peptides of the Ca^{2+}-ATPase and their reactivity with monoclonal and polyclonal anti-ATPase antibodies the site of photocleavage was located in the N-terminal half of the ATPase molecules in the absence of Ca^{2+} and in the C-terminal half when the Ca^{2+} concentration was raised above 10^{-5} M. Since both cleavage sites appear to be located in or near the active site of the Ca^{2+}-ATPase the photocleavage may offer new information about the structure of the ATP binding site.

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Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, the Ca^{2+},Mg^{2+}-activated ATPase of sarcoplasmic reticulum (EC 3.6.1.38); SDS, sodium dodecylsulfate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DOC, deoxycholic acid; $C_{12}E_8$, octaethyleneglycol dodecyl ether; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ELISA, enzyme-linked immunoadsorbent assay; FITC, fluorescein 5'-isothiocyanate.

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In this study the photomodification of the Ca^{2+}-ATPase is further analyzed and contrasted with the behavior of Na^+,K^+-ATPase and H^+,K^+-ATPase.

Experimental procedures

Materials

Dog kidney Na^+,K^+-ATPase was provided by Dr. Joseph D. Robinson, Department of Pharmacology, State University of New York, Health Science Center, Syracuse, NY 13210; hog gastric H^+,K^+-ATPase was from Dr. Edd C. Rabon, University of California, Los Angeles, Ion Transport Facility, UCLA School of Medicine, Los Angeles, CA 90073. Antibodies PR-8 and PR-11 were supplied by Dr. Paul M. Rowe, Laboratory of Neurochemistry, Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD 20892.

Other chemicals. Sodium vanadate (meta) and sodium thiosulfate were purchased from Fisher Scientific Co., Rochester, NY 14624; ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), Tris, disodium-ATP, phospho*enol*pyruvate, NADH, pyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), sodium deoxycholate, anti-rabbit IgG conjugated with horseradish peroxidase, bisacrylamide, ammonium persulfate, Tris-maleate, *o*-phenylenediamine-3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane (Bistris), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, sodium salt (Tes), DL-dithiothreitol (DTT) and Tween 20 were obtained from Sigma Chemical Co., St. Louis, MO 63178; *N,N,N',N'*-tetramethylethylenediamine (TEMED) from Eastman Organic Chemicals, Rochester, NY 14650; Ca^{2+} ionophore A23187 and $C_{12}E_8$ from Calbiochem-Behring Diagnostics, La Jolla, CA 92037; acrylamide, Coomassie brilliant blue R-250 and β-mercaptoethanol were from Bio-Rad, Inc., Richmond, CA 94804; sodium dodecylsulfate (SDS) (purified by crystallization for sequencing experiments) from Polysciences, Inc., Washington, PA 18975; uranium diacetate oxide and silver nitrate were supplied by Alfa Products, Thiakol Ventman Division, Danvers, MA 01923; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) from Grand Island Biological Co., Grand Island, NY 14072. All other chemicals were of analytical grade.

Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were isolated from predominantly white rabbit skeletal muscles essentially as described by Nakamura et al. [24]. The preparations were frozen in liquid nitrogen and stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate buffer (pH 7.0), at a protein concentration of 30–40 mg/ml in polyethylene containers at −70 °C. In several experiments the sarcoplasmic reticulum preparations were further purified by partial extraction with deoxycholate as described by Meissner et al. [25].

Preparation of H^+,K^+-ATPase enriched membrane fraction

A purified membrane fraction enriched in H^+,K^+-ATPase was provided Dr. E. Rabon, University of California, Los Angeles, Ion Transport Facility, UCLA School of Medicine, Los Angeles, CA 90073. The preparation was isolated from hog gastric mucosa as described by Chang et al. [29].

Purification of Na^+,K^+-ATPase

The purified Na^+,K^+-ATPase was provided by Dr. Joseph D. Robinson, Dept. of Pharmacology, State University of New York, Health Science Center, Syracuse, NY 13210. The preparation was obtained from dog kidney medullae as described by Jørgensen [30].

Preparation of 'monovanadate' stock solution

Stock solutions of 50 mM vanadate were prepared by boiling Na_3VO_4 at pH 10 in water for 15 min. The pH was adjusted to 7.4 by stepwise addition of HCl and the solution was again boiled for 10 min to minimize their decavanadate content [23,26,27,28].

Vanadate-mediated photosensitized cleavage

The conditions used for photocleavage were different from those employed in our earlier experiments [23]. For illumination the samples were transferred into a quartz cuvette with 1 mm lightpath and 430 μl volume (Markson Science, Inc., Del Mar, CA 92014, U.S.A.). The light source for photocleavage was a high-pressure mercury/xenon lamp (USH-5086A) from Ushio, Inc., Japan. The light was filtered by a UV filter (Corning 7-60) and a 10 cm water heat filter. The UV filter had less than 1% transmittance below 320 nm and above 380 nm, transmitting an effective irradiation wavelength of 366 nm (mercury line). The irradiation time was 0–2 h at a distance of 60 cm at room temperature. As a result of these changes the rate of photocleavage was increased nearly 10-fold compared with the rates seen earlier [23] and the aggregation of the Ca^{2+}-ATPase was essentially eliminated without change in the size and properties of the cleavage products of the Ca^{2+}-ATPase.

ATPase assay

ATPase activities were measured by a coupled enzyme system of pyruvate kinase and lactate dehydrogenase [31,32]. The measurements were made at 25 °C in a medium of 0.1 M KCl, 20 mM Tris-HCl, 5 mM

$MgCl_2$, 0.45 mM $CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 0.42 mM phosphoenol pyruvate, 0.42 mM NADH, 7.5 IU/ml pyruvate kinase, 18 IU/ml lactate dehydrogenase, 1 μM A23187 at pH 7.4, and sarcoplasmic reticulum proteins at a final concentration of 5 μg/ml. The change of absorbance was measured at 340 nm using a Perkin-Elmer Lambda 3-B dual wavelength spectrophotometer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The samples were dissolved in a solution of 5% SDS, 10 mM Tris-HCl (pH 8.0), 1% β-mercaptoethanol, and 10% glycerol to a final protein concentration of 1 mg/ml; 50–70-μl aliquots were applied for electrophoresis on 6–18% gradient gels, essentially according to Laemmli [33]. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) were used as molecular weight markers (Pharmacia, Piscataway, NJ 08854, U.S.A.). Proteins were stained either with Coomassie blue or with silver.

For silver staining the gels were incubated for 60 min in 50% methanol, 12% acetic acid and 0.0185% formaldehyde followed by rinsing with 50% methanol three times. The gels were soaked for 1 min in 0.8 mM sodium thiosulfonate; after washing three times with distilled H_2O they were incubated for 20 min in a solution of 0.2% $AgNO_3$ and 0.028% formaldehyde, followed by washing with distilled H_2O three times. The bands were developed in a solution of 0.57 M Na_2CO_3, 0.02% formaldehyde and 0.016 mM sodium thiosulfonate. After the bands became visible the gel was washed two times with distilled H_2O and the reaction was terminated by 50% methanol and 12% acetic acid. The gels were photographed immediately after the staining was completed.

Enzyme-linked immunoabsorbent assay (ELISA)

Sarcoplasmic reticulum proteins (0.03, 0.1, 0.3 and 1 μg) were immobilized in polyvinyl chloride microtiter wells. After incubation with PR-8 and PR-11 antipeptide antibodies to the Ca^{2+}-ATPase the bound antibodies were determined by anti-rabbit antibody horseradish peroxidase (HRP) conjugate (1 : 1000 dilution). The absorbance of the colored product of *o*-phenylenediamine was measured at 405 nm as described by us earlier [34].

Crystallization of Ca^{2+}-ATPase and electron microscopy

The two-dimensional Ca^{2+}-ATPase crystals were induced in sarcoplasmic reticulum membranes by incubation in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA and 5 mM monovanadate [35–39]. The protein concentration was 2 mg/ml. The samples, which were illuminated in the presence of 0.1 mM $CaCl_2$ (VC cleavage), were supplemented before crystallization with 5 mM EGTA to lower the Ca^{2+} concentration below approx. 10^{-8} M, since Ca^{2+} destabilizes the E_2V crystals of Ca^{2+}-ATPase [37]. The crystal formation becomes extensive already after 24 h at 2 °C. After 24 h, aliquots were negatively stained with 1% uranyl acetate and viewed in a Siemens Elmiskop I electron microscope at 60 kV accelerating voltage.

NH_2-terminal amino acid sequence

To obtain the NH_2-terminal amino acid sequence of the products of photocleavage the fragments of purified Ca^{2+}-ATPase were separated by SDS-polyacrylamide gel electrophoresis either according to Laemmli [33] or in a multi-zone gel electrophoresis system at neutral pH [40,41] as modified by Moos et al. [42]. The proteins were electroblotted onto poly(vinylidene difluoride) (PVDF) microporous transfer membrane (Immobilon) (produced by Millipore, Bedford, MA 01730) and stained with Coomassie blue as described by Matsudaira [43]. The corresponding bands were cut out with a razor and stored at −20 °C until N-terminal sequence analysis.

Preparation of antibodies

The PR-8 and PR-11 antibodies directed against the phosphorylation site and the FITC binding site of Na^+,K^+-ATPase, respectively, were prepared according to Rowe et al. [44] and were kindly provided by Dr. Paul M. Rowe.

Results and Discussion

Illumination of sarcoplasmic reticulum membranes by ultraviolet light in the presence of 1 mM vanadate causes rapid, conformationally specific photocleavage of the Ca^{2+}-ATPase into two fragments [23]. When the Ca^{2+} concentration of the medium was kept below 10^{-8} with EGTA, the photocleavage occurred at the V cleavage site near the phosphate acceptor Asp-351, producing fragments with approximate molecular masses of 87 kDa (V_1) and 22 kDa (V_2), respectively (Fig. 1A, left panel). In the presence of 2 mM Ca^{2+} the vanadate-catalyzed photocleavage occurred at the VC cleavage site located in the nucleotide binding domain, near the FITC binding site on Lys-515, and fragments of 71 kDa (VC_1) and 38 kDa (VC_2) were obtained (Fig. 1A, right panel). Detailed evidence for the location of cleavage sites was presented earlier [23]. The rate of photocleavage of Ca^{2+}-ATPase was similar in media containing Tris, Hepes or Mops buffers (not shown); under our conditions the inhibition of the photocleavage by Tris [18] was not observed.

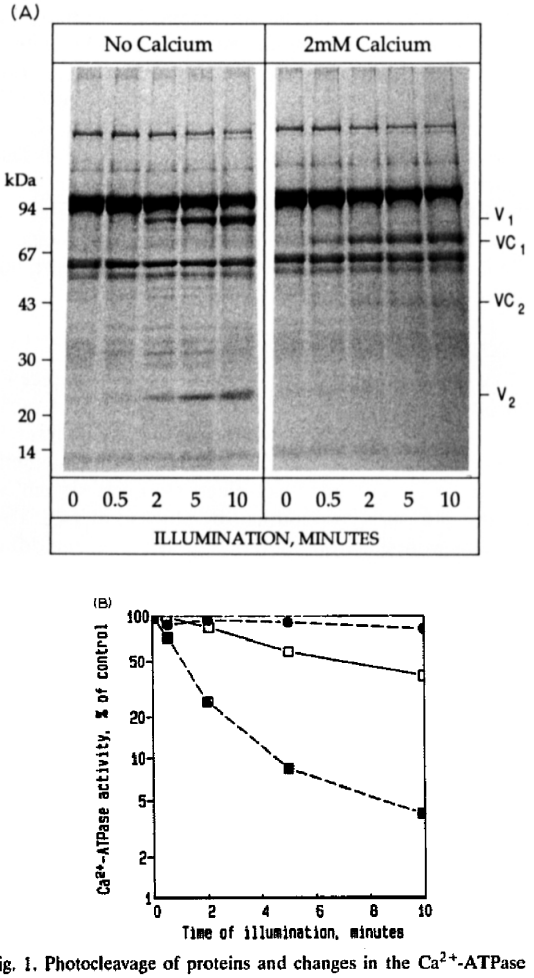

Fig. 1. Photocleavage of proteins and changes in the Ca^{2+}-ATPase activity during ultraviolet illumination of sarcoplasmic reticulum in the presence of vanadate. Sarcoplasmic reticulum vesicles (2 mg protein per ml) were illuminated by ultraviolet light for 0, 0.5, 2, 5 and 10 min, as described under Methods. The standard medium contained 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM monovanadate, and either 0.5 mM EGTA or 2 mM $CaCl_2$ as indicated below. After illumination, aliquots were taken for measurement of ATPase activity and to check photocleavage by gel electrophoresis. Panel A shows the cleavage pattern of Ca^{2+}-ATPase after 0, 0.5, 2, 5, and 10 min of illumination in the presence of 1 mM vanadate and either no Ca^{2+} or 2 mM Ca^{2+}. The band at ≈109 kDa is the Ca^2 ATPase. The principal cleavage fragments are V_1 (87 kDa) and V_2 (22 kDa) in the absence of calcium and VC_1 (71 kDa) and VC_2 (38 kDa) in the presence of 2 mM calcium. In panel B the ATPase activities of the illuminated samples shown under A are expressed as percent of control values on a logarithmic scale. The ATPase activity of control samples taken at time zero without illumination was 3.8 μmol mg^{-1} min^{-1}. Symbols: ●------●, no vanadate, 2 mM $CaCl_2$; □——□, 1 mM vanadate, 0.5 mM EGTA and no Ca^{2+}; ■------■, 1 mM vanadate, 2 mM $CaCl_2$.

The photocleavage was accompanied by irreversible loss of Ca^{2+}-stimulated ATP hydrolysis, that was particularly pronounced in the presence of Ca^{2+} (Fig. 1B). The extent of inhibition was approx. 95% after illumination for 10 min in the presence of 2 mM Ca^{2+}, but only approx. 50% in the absence of Ca^{2+}; under both conditions about half of the ATPase molecules underwent photocleavage. Therefore the massive inhibition of ATPase activity in the presence of Ca^{2+} is probably due to some vanadate-sensitized chemical modification of the Ca^{2+}-ATPase that does not result in photocleavage. Illumination of the enzyme in the absence of vanadate caused neither photocleavage nor loss of ATPase activity.

The vanadate-catalyzed photocleavage was restricted to the Ca^{2+}-ATPase and other proteins of sarcoplasmic reticulum were not affected noticeably. Particular attention was given to the phosphorylase (94 kDa), in view of its similar mobility to the Ca^{2+}-ATPase (110 kDa) on SDS-polyacrylamide gels. There were usually only trace amounts of phosphorylase in our sarcoplasmic reticulum preparations. Furthermore, in control experiments no significant photocleavage of purified rabbit skeletal muscle phosphorylase was observed under conditions where about half the Ca^{2+}-ATPase underwent degradation (not shown). Since all products of photocleavage reacted with anti-ATPase antibodies [23], phosphorylase does not contribute to the formation of V and VC type cleavage fragments.

The photocleavage of Ca^{2+}-ATPase reached a maximum after 1 h of illumination, when about half of the Ca^{2+}-ATPase molecules was split (Fig. 2); longer illumination up to 2 h produced no significant change in the density of the 110 kDa band of the Ca^{2+}-ATPase. The V_1 and V_2 fragments produced in the absence of Ca^{2+} (Fig. 2, left panel) and the VC_1 fragment produced in the presence of Ca^{2+} (Fig. 2, right panel) also remained unaffected by continued illumination between 1 and 2 h. The VC_2 fragment produced during the first few minutes of photolysis (Fig. 1) was replaced during continued illumination by a family of bands ranging in size between 38 and 30 kDa (Fig. 2, right panel).

The arrest of the photocleavage after about half of the ATPase was degraded is not due to reduction of vanadium (V) to vanadium (IV) or to other changes in the medium, since repeated resuspension of the sarcoplasmic reticulum vesicles into fresh media after 20 and 40 min of illumination did not produce more extensive photocleavage; furthermore, the high-speed supernatant obtained after 20 min of illumination in the presence of sarcoplasmic reticulum vesicles, was as effective as fresh medium in promoting the cleavage of Ca^{2+}-ATPase when mixed with fresh sarcoplasmic reticulum vesicles. Therefore about half of the Ca^{2+}-ATPase was either resistant or became insensitive to photocleavage whether or not Ca^{2+} was present in the system. As solubilization of the sarcoplasmic reticulum in $C_{12}E_8$ (2 mg/ml) did not significantly influence

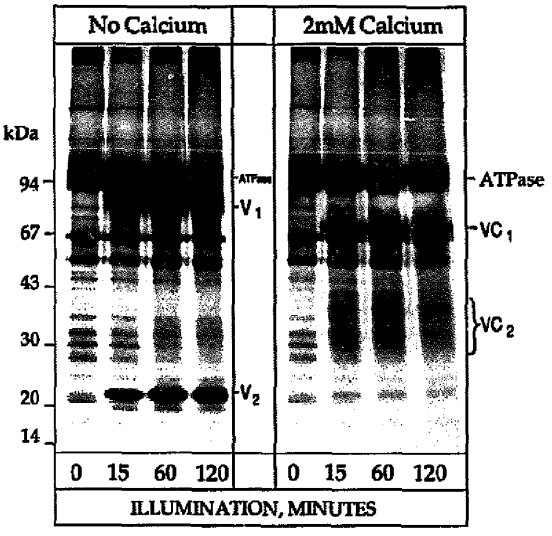

Fig. 2. The effect of prolonged illumination on the photocleavage of the Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles (2 mg protein/ml) were illuminated from 0 to 120 min with high-pressure mercury/xenon lamp, as described under Methods, in a medium of 0.1 M KCl, 20 mM Hepes-K^+ (pH 7.0), 5 mM $MgCl_2$, 1 mM monovanadate and either 1 mM EGTA (no calcium) or 2 mM $CaCl_2$. Aliquots containing 25 μg protein were applied for SDS-polyacrylamide gel electrophoresis. The procedure used for silver staining of the protein bands was described under Methods.

either the pattern or the extent of photocleavage (Fig. 3, left panel), the insensitivity of the remaining ATPase molecules cannot be attributed to the presence of inside-out vesicles, in which the cytoplasmic domain of the Ca^{2+}-ATPase would be buried in the vesicle interior. However, the dimeric association of Ca^{2+}-ATPase is known to persist at the detergent : protein ratios used in these experiments [45] and may mask the cleavage sites.

Vanadate may also induce chemical modification that could render some ATPase molecules insensitive to photocleavage. The reasons for the incomplete cleavage are being investigated.

Effect of photocleavage on the reaction of antibodies with the Ca^{2+}-ATPase

The V cleavage site is close to the phosphate acceptor Asp-351, while the VC cleavage site is not far in the primary sequence from Lys-515 that serves as acceptor for covalent labeling with FITC [23]. Since FITC labeling selectively inhibits the reactions catalyzed by ATP [46,47], both regions of the molecule affected by the photocleavage are probable components of the active site of the Ca^{2+}-ATPase.

The structural consequences of photocleavage at these two sites were explored using antibodies directed against synthetic peptides representative of the structures around Asp-351 and Lys-515, respectively (Table I). The polyclonal antibody PR-8 was generated by a 13-residue peptide containing the 370–382 region of the *Torpedo* electric organ Na^+,K^+-ATPase [44]. This is a highly conserved region of cation transport ATPases and it is almost entirely identical with the sequence at 345–357 in the Ca^{2+}-ATPase (Table I). The PR-11 antibody was produced against a peptide having the same sequence as the 505–517 region of the Na^+,K^+-ATPase [44]; PR-11 is expected to crossreact with the analogous 513–525 sequence in the Ca^{2+}-ATPase (Table I).

The PR-8 antibody binds with high affinity to the Ca^{2+}-ATPase both in the absence and in the presence of Ca^{2+}; the accessibility of the binding site of PR-8 increased after photocleavage of the Ca^{2+}-ATPase (Table II). This increase is quite significant considering that photocleavage affected only approximately one-half of the ATPase molecules.

TABLE I

Comparison of the amino acid sequences of the phosphorylation sites and the FITC binding sites of Na^+,K^+-ATPase and Ca^{2+}-ATPase

The top line of the table shows the sequence of the synthetic peptide that was used to develop antiserum PR-8 in rabbit [44]. This sequence corresponds to amino acids 370–382 in the *Torpedo* Na^+,K^+-ATPase, that contains the phosphate acceptor aspartyl residue (D376). Therefore this sequence is expected to be part of the active site of Na^+,K^+-ATPase. The analogous highly conserved sequence of the Ca^{2+}-ATPase of rabbit skeletal muscle sarcoplasmic reticulum is shown on the second line (residues 345–357). The bottom half of the table shows the FITC binding region of the Na^+,K^+-ATPase (amino acids 505–517) and the corresponding sequence of the Ca^{2+}-ATPase (amino acids 513–525). The Lys-507 in Na^+,K^+-ATPase and Lys-515 in Ca^{2+}-ATPase can be selectively labeled with fluorescein 5'-isothiocyanate. The synthetic peptide containing this sequence of the Na^+,K^+-ATPase was used to develop antiserum PR-11 in rabbit.

Antibody	Target peptide				Site
PR-8	370	T S T I C S D K T G T L T	382	Na^+,K^+-ATPase	Phosphorylation
	345	T S V I C S D K Y G T L T	357	Ca^{2+}-ATPase	
PR-11	505	V M K G A P E R I L D R C	517	Na^+,K^+-ATPase	FITC binding
	513	F V K G A P E G V I D R C	525	Ca^{2+}-ATPase	

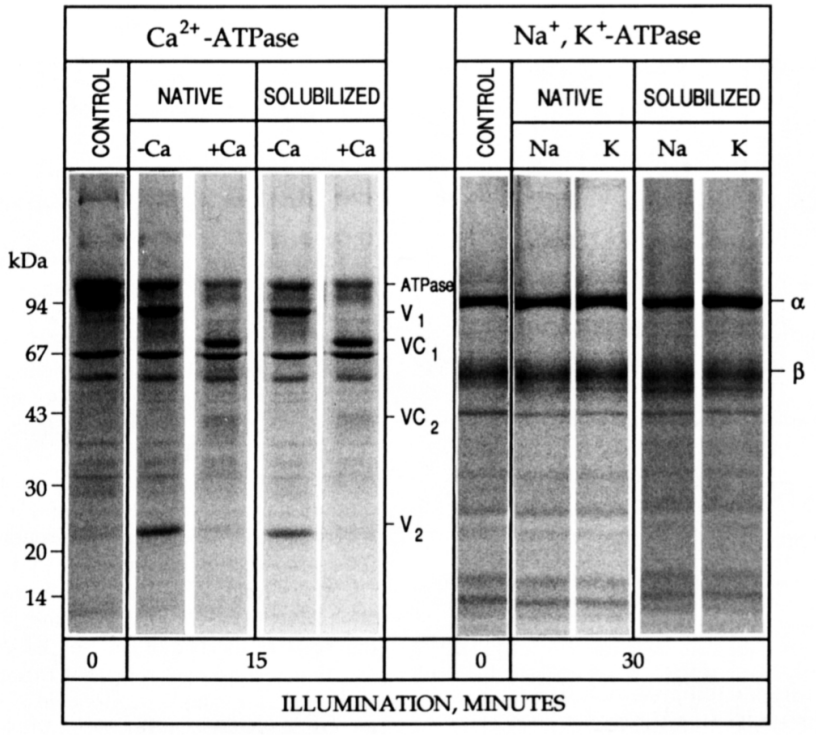

Fig. 3. Illumination of membranous and detergent-solubilized Ca^{2+}-ATPase and Na^+,K^+-ATPase preparations by ultraviolet light in the presence of vanadate. Rabbit skeletal muscle sarcoplasmic reticulum preparations were illuminated for 15 minutes as described in Fig. 2, either in the standard medium (native) or in a medium containing 2 mg $C_{12}E_8$/mg protein to solubilize the Ca^{2+}-ATPase (solubilized). Dog kidney Na^+,K^+-ATPase (2 mg protein/ml) was suspended in 20 mM Hepes-Na^+ (pH 7.0), 5 mM $MgCl_2$, 0.5 mM EGTA-Na^+, and 1 mM monovanadate (Na medium) or in 20 mM Hepes-K^+ (pH 7.0), 5 mM $MgCl_2$, 0.5 mM EGTA-K^+, and 1 mM monovanadate (K medium), either in the absence of detergent (native) or in the presence of 2 mg $C_{12}E_8$/mg protein (solubilized). Samples were illuminated for 30 min. Control samples were kept in the dark. Aliquots containing 80 μg protein were taken for electrophoresis.

The PR-11 antibody binds only weakly to the Ca^{2+}-ATPase in the presence of Ca^{2+} and little binding was observed in the presence of EGTA (Table II). The photocleavage products obtained after illumination had significantly enhanced binding capacity for PR-11 both in the absence and in the presence of Ca^{2+}.

The structural change that is responsible for the opening of the binding sites for PR-8 and PR-11 during photocleavage, may also contribute to the loss of ATPase activity and to the disruption of Ca^{2+}-ATPase crystals (see below).

It is noteworthy that prior labeling with FITC inhib-

Fig. 4. The effect of V and VC type photocleavage on the vanadate-induced two-dimensional crystallization of Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles (2 mg protein/ml) were suspended in a medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and either 0.5 mM EGTA (A–D) or 0.1 mM $CaCl_2$ (E–H). Samples B–D and F–H also contained 1 mM monovanadate. The effect of illumination on the crystallization of Ca^{2+}-ATPase was tested as follows: Control samples without (A) and with calcium (E) were illuminated for 15 min to test the effect of illumination in the absence of vanadate. Samples B and F served as non-illuminated controls in the presence of 1 mM monovanadate. The crystallization of Ca^{2+}-ATPase was induced by the addition of 5 mM monovanadate and 0.5 mM EGTA as described under Methods. All control samples (A, B, E, F) showed good crystallization of the Ca^{2+}-ATPase, indicating that illumination in the absence of vanadate, or incubation without illumination in the presence of vanadate did not affect the ability of the Ca^{2+}-ATPase to crystallize. Samples C and G were illuminated in the presence of vanadate to produce either the V type photocleavage in the absence of Ca^{2+} (C), or the VC type photocleavage in the presence of Ca^{2+} (G). The crystallization of the Ca^{2+}-ATPase in the presence of 5 mM vanadate and 0.5–5 mM EGTA was inhibited by photocleavage in both samples. In samples D and H the Ca^{2+}-ATPase was crystallized before illumination in the presence of 5 mM monovanadate and either 0.5 mM EGTA (D) or 5 mM EGTA and 0.1 mM $CaCl_2$ (H). The Ca^{2+}-ATPase crystals were well developed in both systems (see panels B and F, respectively). The subsequent illumination for 15 min produced only V type fragments in both cases, with complete destruction of the preformed Ca^{2+}-ATPase crystals. The pattern of photocleavage was tested in all samples by SDS-polyacrylamide gel electrophoresis.

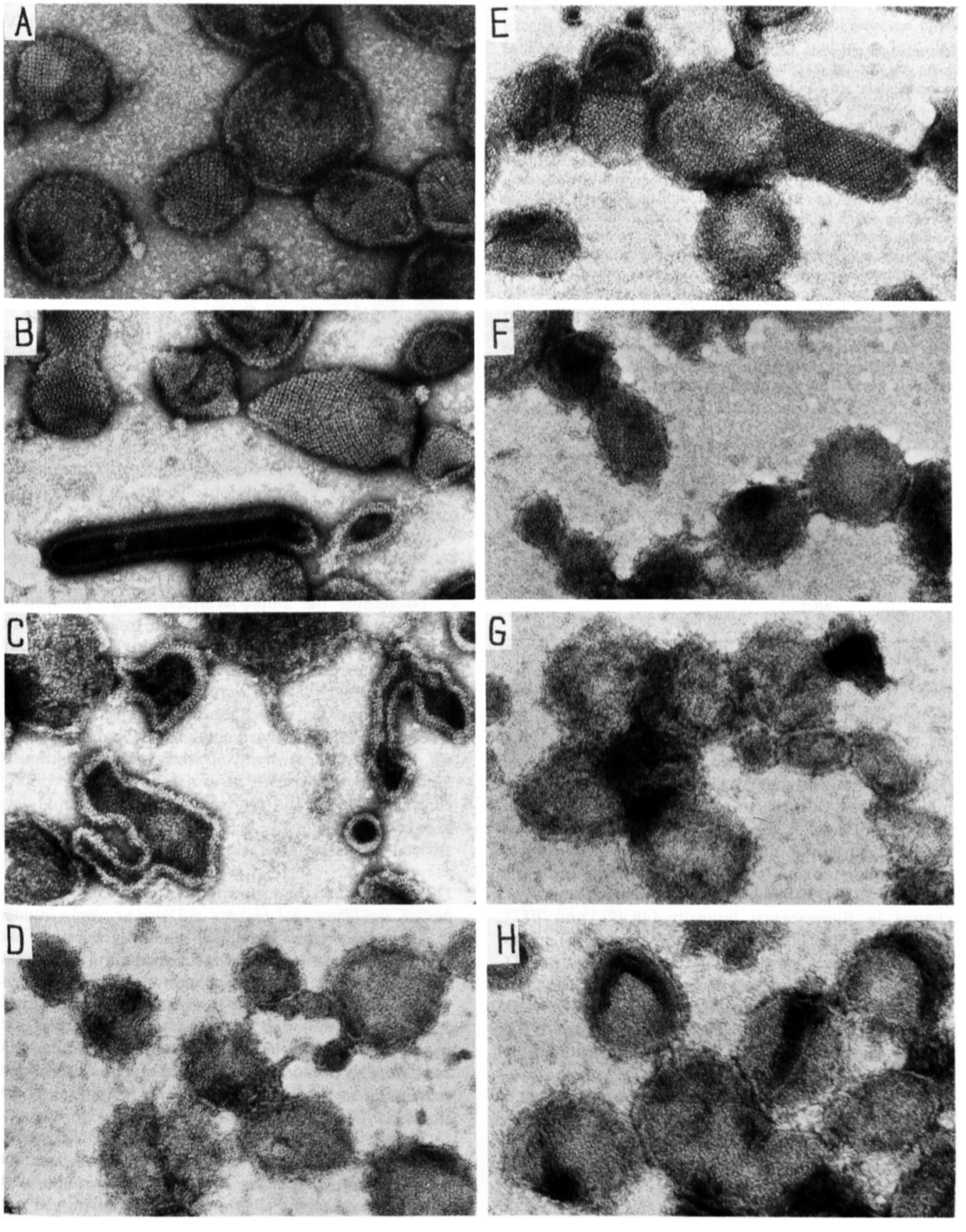
A
E
B
F
C
G
D
H

TABLE II

The effect of V and VC type photocleavage on the binding of PR-8 and PR-11 antibodies to the sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles (2 mg protein/ml) were suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM monovanadate and either 0.1 mM $CaCl_2$ (+Ca) or 0.5 mM EGTA (−Ca). Aliquots were illuminated for 15 min. The V and VC type cleavage was ascertained by SDS-polyacrylamide gel electrophoresis. The non-illuminated samples remained uncleaved (not shown). For measurement of antibody binding the antibody solutions (1:10 dilution) were preincubated for 1 h at 2 °C, either with control sarcoplasmic reticulum (no illumination) or with sarcoplasmic reticulum after illumination (1 mg protein/ml); the concentration of the unbound antibody in the medium was determined by centrifugation of the samples at 10000× *g* for 1 h and analysis of the antibody content of the supernatant solution, using sarcoplasmic reticulum vesicles adsorbed to microtiter wells as described under Methods. The absorbance of the products of peroxidase reaction was measured at 405 nm, in a Titertek microtitration plate photometer. The difference in absorbance between samples incubated without sarcoplasmic reticulum and the samples obtained after preincubation with sarcoplasmic reticulum was expressed as percent of the total absorbance.

Antibody	Sample	Fraction of Ab bound to native SR (%)	
		+Ca	−Ca
PR-8	no illumination	63	70
	after illumintion	77	90
PR-11	no illumination	13	1
	after illumination	45	23

ited the photocleavage of the Ca^{2+}-ATPase even during prolonged (1–2 h) illumination both in the presence and absence of calcium; the formation of the V_2 fragment was particularly reduced (not shown). The inhibition was not due to absorption of exciting light, since fluorescein at identical concentration produced no significant inhibition. The monovanadate-induced conformation change of the Ca^{2+}-ATPase is readily observed in FITC-labeled ATPase preparations by a change in fluorescence intensity [48,49], and FITC clearly does not interfere with the binding of monovanadate to the Ca^{2+}-ATPase [26–28]. However, FITC inhibited the binding of decavanadate to the ATP binding site [26–28], suggesting that oligomeric vanadate species with binding characteristics similar to that of ATP may be involved in the photocleavage [23].

The effect of photocleavage on the crystallization of Ca^{2+}-ATPase

Two-dimensional membrane crystals of the Ca^{2+}-ATPase develop upon incubation of sarcoplasmic reticulum vesicles with 5 mM Na_3VO_4 in the absence of Ca^{2+} [35–39].

The crystallization of the Ca^{2+}-ATPase was completely inhibited by prior photocleavage either in the absence or presence of Ca^{2+} (Figs. 4C and 4G); the V type photocleavage also disrupted the preformed Ca^{2+}-ATPase crystals (Figs. 4D and 4H). The complete inhibition of crystallization caused by the photocleavage of only approximately half of the Ca^{2+}-ATPase molecules is consistent with the hypothesis that cleaved and intact ATPase molecules interact with each other in Ca^{2+}-ATPase oligomers, but are unable to crystallize. In control experiments the crystallization of the Ca^{2+}-ATPase was not affected by prior illumination in the absence of vanadate (Figs. 4A and 4E), compared with non-illuminated control samples incubated under otherwise identical conditions in the presence of 1 mM vanadate (Figs. 4B and 4F).

It is noteworthy that the tryptic hydrolysis of the Ca^{2+}-ATPase at the T_1 cleavage site (Arg-505) did not interfere with crystallization, while cleavage at the T_2 site (Arg-198) prevented the formation of Ca^{2+}-ATPase crystals [36,38]. Since the T_2 tryptic cleavage site is close to the site of V type cleavage observed in the absence of Ca^{2+} [23], the loss of ATPase activity in the two cases may be related to similar changes in the structure of the Ca^{2+}-ATPase.

Attempts at amino acid sequencing of the photocleavage products

To identify the amino acid(s) that are involved in the photomodification the cleavage fragments of the Ca^{2+}-ATPase were separated according to Moos et al. [42], transferred onto Immobilon sheets, stained with Coomassie blue [43] and were processed for amino acid sequence analysis in collaboration with Dr. John H. Collins and Alicia Wawrzynow at the Dept. of Biological Chemistry, University of Maryland in Baltimore. Similarly to the case of myosin [14,16,18], the photocleavage products did not yield identifiable amino acid sequences, presumably because the photocleavage destroyed the N-terminal amino acid.

Differences in the susceptibility of Ca^{2+}-ATPase, Na^+,K^+-ATPase and H^+,K^+-ATPase to photocleavage

There is a close structural similarity between the Ca^{2+}-ATPase of sarcoplasmic reticulum [50,51], the Na^+,K^+-ATPase of plasma membrane [52,53], and the H^+,K^+-ATPase of gastric mucosa [54,55]. All three ATPases are inhibited by vanadate. Therefore it was expected that all three ATPases will show similar susceptibility to photocleavage. Based on the experiments performed so far (Figs. 3 and 5), this is apparently not the case.

In a Ca^{2+}-free medium containing either Na^+ or K^+ as the principal cation, the α and β subunits of the purified Na^+,K^+-ATPase of dog kidney medulla were completely resistant to photocleavage during 30 min

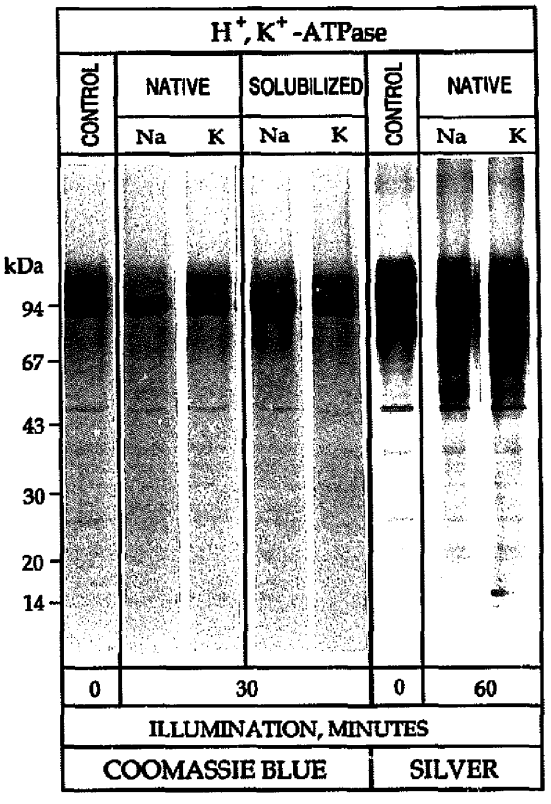

Fig. 5. Illumination of membranous and solubilized H^+,K^+-ATPase by ultraviolet light in the presence of vanadate. Vesicles containing H^+,K^+-ATPase (2 mg protein/ml) were suspended in 0.1 M KCl, 20 mM Hepes-K^+ (pH 7.0), 1 mM $MgCl_2$, and 1 mM monovanadate (K-medium) or in 0.2 M NaCl, 20 mM Hepes-Na^+ (pH 7.0), 1 mM $MgCl_2$, and 1 mM monovanadate (Na-medium), either in the absence (native) or in the presence of 2 mg $C_{12}E_8$ per mg protein. The control samples were kept in the dark, while the other samples were illuminated for 30–60 min. The Coomassie blue stained gels used for electrophoresis contained 50 μg, and the silver stained gels 25 μg protein per sample. Electrophoresis was carried out according to Laemmli [33] as described under Methods.

illumination, both in the absence of detergents and in the presence of 2 mg $C_{12}E_8$ per mg protein (Fig. 3, right panel). Under similar conditions about half of the Ca^{2+}-ATPase was cleaved after only 15 min on illumination (Fig. 3, left panel).

The 100 kDa band of the H^+,K^+-ATPase was somewhat reduced in density after 30 min illumination in either Na^+ or K^+ medium (Fig. 5); the formation of small amounts of cleavage products, ranging in molecular size between 100 kDa and approx. 50 kDa was detected by silver staining of the gels (Fig. 5), but in contrast to the Ca^{2+}-ATPase, no unique fragments were obtained that could be related to specific cleavage of the H^+,K^+-ATPase at a single cleavage site.

Solubilization of H^+,K^+-ATPase by 2 mg $C_{12}E_8$/mg protein had no noticeable effect on the pattern or extent of photocleavage.

These observations suggest that the photocleavage of the Ca^{2+}-ATPase by vanadate is associated with a unique structural feature that is not shared by the Na^+,K^+-ATPase, and the H^+,K^+-ATPase, although all three enzymes were partially inactivated during illumination in the presence of vanadate.

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