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The effect of dicyclohexylcarbodiimide and cyclopiazonic acid on the difference FTIR spectra of sarcoplasmic reticulum induced by photolysis of caged-ATP and caged-Ca^{2+}

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The photochemical release of Ca^{2+} from caged-Ca^{2+} in the absence of ATP, and the release of ATP from caged-ATP in the presence of Ca^{2+} induce characteristic difference FTIR spectra on rabbit sarcoplasmic reticulum that are related to the formation of Ca_2E_1 and E ~ P intermediates of the Ca^{2+}-ATPase, respectively. Dicyclohexylcarbodiimide (10 nmol/mg protein) abolished both the Ca^{2+}-and ATP-induced difference FTIR spectra parallel with inhibition of ATPase activity. Cyclopiazonic acid (50 nmol/mg protein) inhibited the Ca^{2+}-induced difference spectrum measured in the absence of ATP, but had no significant effect on the ATP-induced difference spectrum measured in the presence of 1 mM Ca^{2+}. The dog kidney Na^+,K^+-ATPase did not give significant difference spectrum after photolysis of caged-ATP in Ca^{2+}-free media containing 90 mM Na^+ and 10 mM K^+, with or without ouabain. We propose that both the Ca^{2+} and the ATP-induced difference FTIR spectra of the Ca^{2+}-ATPase reflect the occupancy of the high-affinity Ca^{2+} transport site of the enzyme.

Introduction

Light-induced release of ATP [1,2] or Ca^{2+} [3] from their caged derivatives induce characteristic changes in the difference FTIR spectrum of sarcoplasmic reticulum. The principal changes seen after Ca^{2+} release consist of an increase in absorbance at 1663 and 1676 cm^{-1} and a decrease in absorbance at 1653 cm^{-1}; these changes are related to the formation of Ca_2E_1 enzyme intermediate [3]. The difference spectra produced by the photolysis of caged-ATP show an increase in absorbance at 1676, 1650 and 1624 cm^{-1} and a decrease in absorbance at 1662 and 1640 cm^{-1} associated with the phosphorylation of the Ca^{2+}-ATPase (E ~ P) and the translocation of calcium [1,2]. The difference bands induced either by ATP or Ca^{2+} are relatively narrow, suggesting that they originate from changes in side chain vibrations, but definitive assignment of the structural change cannot be made. Interaction of Ca^{2+} with carboxylate groups may be responsible for some of the changes.

In this report we explore the effects of dicyclohexylcarbodiimide and cyclopiazonic acid on the Ca^{2+}- and ATP-induced difference spectra of the Ca^{2+}-ATPase. Dicyclohexylcarbodiimide and its fluorescent derivatives are potent inhibitors of the Ca^{2+}-ATPase when reacted in the absence of Ca^{2+}; Ca^{2+} at μmolar concentration provides nearly complete protection against the inhibition [4,5]. Based on these observations it was

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Abbreviations: caged-Ca^{2+}, Ca^{2+} complex of Nitr-5; Nitr-5, 1,2-amino-5-[1-hydroxy-1-(2-nitro-4,5-methylene dioxyphenyl)methyl]-phenoxyl-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrasodium salt; caged-ATP, adenosine 5'-triphosphate P^3-1-(2-nitrophenyl ethyl ester) disodium salt tetrahydrate; Ca^{2+}-ATPase, Ca^{2+},Mg^{2+}-stimulated ATPase of sarcoplasmic reticulum (EC 3.6.1.38); CPA, cyclopiazonic acid; DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; FTIR, Fourier transform infrared; Mops, 3-(*N*-morpholino)propane-sulfonic acid; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid.

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suggested that DCCD reacts with carboxylate groups at the high-affinity Ca^{2+} binding site of the Ca^{2+}-ATPase [4–6].

Cyclopiazenic acid is a specific inhibitor of ATP hydrolysis by the Ca^{2+}-ATPase of sarcoplasmic reticulum [7–9], but has no significant effect on the hydrolysis of ATP by the kidney Na^+,K^+-ATPase, gastric H^+,K^+-ATPase, the mitochondrial F_1-ATPase, the Ca^{2+}-ATPase of erythrocytes or the Mg^{2+}-ATPase of skeletal muscle T-tubules and surface membranes [8]. CPA was suggested to inhibit the binding of Ca^{2+} to the Ca^{2+}-ATPase [9] and to interfere with the Ca^{2+}-induced conformational changes related to Ca^{2+} transport [8]. ATP protects the enzyme in a competitive manner from inhibition by CPA [8].

In this report the effects of DCCD and CPA on the ATP and Ca^{2+}-induced FTIR difference spectra of the sarcoplasmic reticulum Ca^{2+}-ATPase are described, and the effects of ATP on the FTIR spectrum of sarcoplasmic reticulum Ca^{2+}-ATPase and the kidney Na^+,K^+-ATPase are compared.

Experimental procedures

Materials

Adenosine 5'-triphosphate, dithiothreitol, EGTA, imidazole, magnesium chloride, 2-mercaptoethanol, glutathione, K-Mops, cyclopiazonic acid, bovine serum albumin, and Trizma base were obtained from Sigma Chemical Co., St. Louis, MO, calcium chloride from Baker Chemical Co., Phillipsburg, NJ, and A23187 from Behring Diagnostics, La Jolla, CA. Sodium chloride, potassium chloride and sodium vanadate were the products of Fisher Scientific Co., Fairlawn, NJ. Caged-ATP, Nitr-5, and ouabain were obtained from Calbiochem, La Jolla, CA, dicyclohexylcarbodiimide from Eastman Organic Co., Rochester, NY, and Hepes from Grand Island Biological Co., Grand Island, NY.

Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were isolated from rabbit muscles according to Nakamura et al. [10]. 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. The protein concentration was determined according to Lowry et al. [11].

Reaction of sarcoplasmic reticulum with dicyclohexylcarbodiimide and with cyclopiazonic acid

For reaction with DCCD sarcoplasmic reticulum vesicles were thawed and suspended in a medium containing 100 mM KCl, 50 mM K-Mops (pH 6.2), 20 μg A23187/ml, 10 nmol DCCD/mg microsomal protein, and 1 mM EGTA at a protein concentration of ≅ 1 mg/ml. Control samples also contained 1.5 mM $CaCl_2$. After incubation for 60 min at 2–4°C the samples were centrifuged at 80000 × *g* for 50 min and assayed for ATPase activity.

The reaction of sarcoplasmic reticulum vesicles with cyclopiazonic acid was carried out as described by Seidler et al. [8] in a ^2H_2O medium of 0.1 M KCl, 10 mM imidazole (p^2H 7.4) and 0.5 mM $MgCl_2$ at 2°C at CPA concentrations of 12.5–150 nmol/mg protein for 0.5–24 h. CPA was added from a 0.2 M stock solution in dimethylsulfoxide. The final concentration of dimethylsulfoxide at the highest concentration of CPA was 3% (v/v); control experiments run with 3% DMSO without CPA indicated no significant effect either on the ATPase activity or on the FTIR spectra.

Preparation of samples for FTIR measurements

For FTIR measurements the native or DCCD-treated sarcoplasmic reticulum vesicles were suspended in ^2H_2O buffer containing 100 mM KCl, 0.5 mM $MgCl_2$, 10 mM imidazole (p^2H 7.4) and centrifuged for 40 min at 80000 × *g*. The pellet was resuspended in the same medium and the centrifugation was repeated. The final sediment was suspended in ^2H_2O buffer at a protein concentration of ≅ 20–30 mg/ml. The total calcium concentration was determined with a Perkin-Elmer atomic absorption spectrometer (Model 3030), and the total calcium concentration was adjusted to ≅ 0.3 mM–0.6 mM. Further additions were made as described in the figure legends.

The CPA-treated samples and the corresponding control samples containing only DMSO were supplemented either with caged-ATP (1–2.5 mM) or with caged-Ca^{2+} (1–2.5 mM) and used directly for FTIR measurements.

Measurement of the ATPase activity

The ATPase activity was measured either by the coupled enzyme assay as described earlier [12], or by measuring the liberation of inorganic phosphate.

Sarcoplasmic reticulum vesicles (≅ 0.1 mg protein/ml) were incubated at 20°C in a medium containing 100 mM KCl, 50 mM K-Mops (pH 6.2), 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM $CaCl_2$. The reaction was stopped with 2% TCA and after centrifugation in a clinical centrifuge, aliquots of the supernatant were assayed for P_i according to Fiske-SubbaRow [13].

Isolation of Na^+,K^+-ATPase

Na^+,K^+-ATPase was extracted from dog kidney and purified according to Jørgensen [14]. The final pellet was suspended in ^2H_2O buffer containing 20 mM Hepes (p^2H 7.6), 0.1 mM EGTA, 1 mM $MgCl_2$ and

either 10 mM KCl and 90 mM NaCl, or only 100 mM KCl. After centrifugation at 270000 × *g* for 4 h the pellet was suspended in the same buffer. Further additions were made as described in the figure legends.

FTIR measurements

FTIR spectra were analyzed on a Mattson Cygnus 100 spectrometer using MCT detector and temperature controlled cells (Model TFC-M25; Harrick Scientific Corp., Ossining, NY) equipped with barium fluoride windows and 50 μm spacers. The FTIR spectrometer was modified for photolysis of caged-Ca^{2+} by opening an extra window on the housing and inserting a germanium mirror into the lightpath, that reflected the 360 nm light onto the sample cell. A 500 W high pressure mercury xenon arc lamp (type USH 5085A) operated with an Oriel type 8530 power supply in a type 66011 housing was used as light source for photolysis. The light beam after passing through a water heat filter and a Corion 5840 (CS 7-60) ultraviolet absorbing filter was focused with quartz lenses on the sample cell.

Routinely 64 scans were collected, coadded, zero-filled once and apodized with triangular function. The resolution was 4 cm^{-1}. The spectra were recorded before and during or after 1 min illumination with light of predominantly 360 nm wavelength. The difference absorbance (ΔA) was calculated according to

$$\Delta A = -\log I_{\text{after}}/I_{\text{before}}$$

where I_{after} and I_{before} correspond, respectively, to the spectra taken after and before UV illumination. The spectra presented in this report represent the averages of 4–32 difference spectra taken on individual samples under identical experimental conditions.

Results and Discussion

The effect of DCCD on the difference spectrum of sarcoplasmic reticulum induced by photolysis of caged-Ca^{2+}

Photolysis of caged-Ca^{2+} in a suspension of sarcoplasmic reticulum vesicles produces the difference FTIR spectrum shown in Fig. 1 (line 1). The difference spectrum is characterized by positive bands at 1676, 1663 and a negative band at 1653 cm^{-1}, that are related to the formation of the Ca_2 E_1 intermediate of the Ca^{2+}-ATPase [3]. There are also bands at 1633, 1599–1600, 1519, 1509, 1496, 1487, 1339 and 1264

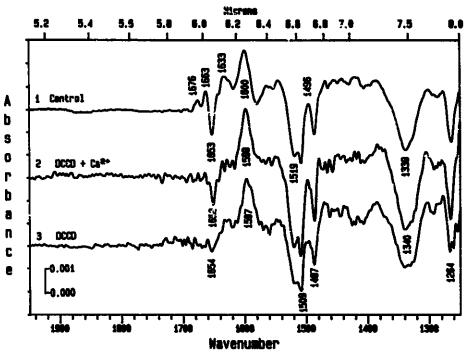

Fig. 1. Effect of DCCD treatment on the difference FTIR spectra of sarcoplasmic reticulum induced by Ca^{2+} release from Nitr-5-Ca^{2+} complex. (Trace 1) Control sarcoplasmic reticulum. Native sarcoplasmic reticulum vesicles (40 mg protein/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.4), 0.5 mM $MgCl_2$, 1.5 mM Nitr-5, 3 mM DTT and ≅ 0.32 mM total Ca^{2+}. FTIR spectra were taken before and after illumination with 360 nm light for 1 min. The spectra represent the average of six different spectra (after minus before illumination). (Trace 2) Sarcoplasmic reticulum treated with DCCD in the presence of Ca^{2+}. The conditions were similar to those in trace 1, except that the sarcoplasmic reticulum vesicles were incubated with 10 nmol DCCD/mg SR for 60 min at 2–4°C in a medium containing 0.41–0.83 mM total Ca^{2+} before illumination. The protein concentration was 27 mg/ml. 16 difference spectra were averaged. (Trace 3) Sarcoplasmic reticulum treated with DCCD in the absence of Ca^{2+}. The conditions were similar to those in trace 1 except that the sarcoplasmic reticulum vesicles were incubated prior to illumination with 10 nmol DCCD/mg SR for 60 min in a Ca^{2+}-free medium containing 1 mM EGTA. The protein concentration was 24 mg protein/ml. After incubation with DCCD the total Ca^{2+} concentration was adjusted to 0.27–0.58 mM. 16 difference spectra were averaged. For other details see Experimental procedures.

TABLE I

The effect of DCCD on the ATPase activity

The reaction of sarcoplasmic reticulum with DCCD (10 nmol/mg protein) was carried out at 2°C in a medium of 0.1 M KCl, 50 mM K-Mops (pH 6.2), 20 μg/ml A23187 and 1.0 mM EGTA. To DCCD control samples 1.5 mM $CaCl_2$ was also added. After 60 min the samples were diluted with ^2H_2O medium containing 0.1 M KCl, 0.5 mM $MgCl_2$ and 10 mM imidazole (p^2H 7.4) and processed for FTIR measurements and for measurement of ATPase activity, as described under Methods. The Ca^{2+} concentration of the samples was determined by atomic absorption spectrometry and adjusted to the level described in the legend to Fig. 1.

Additions	ATPase activity (μmol mg^{-1} min^{-1})	% inhibition
None (control)	1.14 ± 0.03	0
DCCD + 1 mM EGTA	0.28 ± 0.06	86
DCCD + 1.5 mM Ca^{2+}	1.04 ± 0.01	8

cm^{-1} that are due to the photolysis products of caged-Ca^{2+} [3].

Reaction of sarcoplasmic reticulum vesicles with 10 nmol DCCD/mg protein at 2°C for 1 h in the presence of 1.0 mM EGTA caused 86% inhibition of ATPase activity (Table I); in control samples treated with 10 nmol DCCD/mg protein under the same conditions but in the presence of 1.5 mM Ca^{2+} the ATPase activity was only slightly reduced ($\cong 8\%$) compared with DCCD-free control samples.

DCCD treatment of sarcoplasmic reticulum in a Ca^{2+}-free medium nearly completely abolished the difference absorbance induced by photolysis of caged-Ca^{2+} at 1676, 1663, and 1653 cm^{-1} (Fig. 1, line 3 versus line 1). The difference absorbance bands at 1633, 1597–1600, 1519, 1509, 1339–1340 and at 1264 cm^{-1} are caused by the photolysis of caged-Ca^{2+} and remained unaffected by DCCD treatment of sarcoplasmic reticulum (Fig. 1, lines 1 and 3). After DCCD treatment in the presence of 0.5 mM excess Ca^{2+} (Fig. 1, line 2), the 1676 and 1663 difference bands were abolished, but the negative band at 1652 cm^{-1} was only slightly reduced in amplitude.

These observations suggest that DCCD may react with several groups in the Ca^{2+}-ATPase, but the protection of ATPase activity by Ca^{2+} is associated with a group that gives rise to the negative band at 1652 cm^{-1}. We propose that the absorption at $\cong 1652$ cm^{-1} is attributable to the C=O vibration of a carboxylate

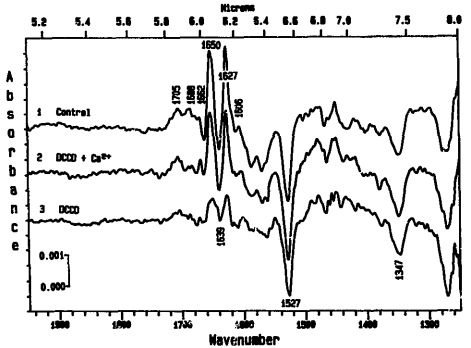

Fig. 2. Effect of treatment with DCCD on the difference FTIR spectra of sarcoplasmic reticulum induced by release of ATP from caged-ATP in the presence of Ca^{2+}. (Trace 1) Control sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.4), 5 mM $MgCl_2$, 1.3 mM total Ca^{2+}, 1.5 mM caged-ATP, 3 mM DTT, 50 μM A23187. FTIR spectra were taken before and during illumination with 360 nm light for 1 min at 6°C. (Trace 2) Sarcoplasmic reticulum treated with DCCD in the presence of Ca^{2+}. The conditions were similar to those in trace 1 except that the sarcoplasmic reticulum vesicles were incubated prior to illumination with 10 nmol DCCD/mg SR protein for 60 min at 2–4°C in medium containing 0.41–0.83 mM total Ca^{2+} The protein concentration was 21 mg protein/ml. 24 difference spectra were averaged. (Trace 3) Sarcoplasmic reticulum treated with DCCD in the absence of Ca^{2+}. The conditions were similar to those in trace 1 except that the sarcoplasmic reticulum vesicles were incubated prior to illumination with 10 nmol DCCD/mg SR for 60 min at 2–4°C in a Ca^{2+}-free medium containing 1 mM EGTA. The protein concentration was 18 mg protein/ml. 24 difference spectra were analyzed.

group and the negative band at this position after photolysis of caged-Ca^{2+} arises from the reaction of the protein carboxylate ion with calcium.

The effect of DCCD on the difference FTIR spectrum of sarcoplasmic reticulum induced by photolysis of caged-ATP

Photolysis of caged-ATP in the presence of Ca^{2+} produces the difference spectrum shown in Fig. 2 (line 1). The spectrum is characterized by positive bands at 1705, 1688, 1650–1651, 1626–1627, and by negative bands at 1662 and 1637–1639 cm^{-1}; these were attributed by Barth and his colleagues [1,2] to the formation of the E ~ P intermediate of the Ca^{2+}-ATPase and the associated reactions. All these bands are markedly reduced in intensity after reaction of the Ca^{2+}-ATPase with DCCD in the absence of Ca^{2+} (Fig. 2, line 3) with inhibition of ATPase activity (Table I), but are only moderately affected when the reaction with DCCD was performed in the presence of Ca^{2+} (Fig. 2, line 2) that protected the ATPase activity (Table I). The intensity of bands related to the photolysis products of caged-ATP (1525–1527, 1347 and 1264 cm^{-1}) is essentially identical in the three samples (Fig. 2, lines 1–3).

As the formation of the E ~ P intermediate from ATP requires the binding of Ca^{2+} to the enzyme [15,16], the inhibition of ATPase activity (Table I) and the loss of the ATP-induced components of the difference spectrum (Fig. 2, line 3) is presumably due to inhibition of the Ca^{2+} binding to the DCCD-treated enzyme. Protection by Ca^{2+} of the Ca^{2+} binding site preserves both ATP hydrolysis (Table I) and the ATP-induced difference spectrum (Fig. 2, line 2).

The characteristic bands at 1650–1651 and at 1626 cm^{-1} are reduced in intensity after incubation for 6 min at 6°C following the photolysis of caged-ATP (Fig. 3, line 2). This is due to the hydrolysis of ATP by the Ca^{2+}-ATPase. Traces of myokinase and adenylate deaminase that are present in the sarcoplasmic reticulum preparations convert the ADP during continued incubation into inosine monophosphate, with the appearance of a positive band at 1673 cm^{-1} and a negative band at 1624 cm^{-1} (Fig. 3, lines 3, 4). The 1673 cm^{-1} band is attributed to the C=O group of inosine and the negative band at 1624 cm^{-1} to the ring stretching of adenosine. The myokinase inhibitor P^1P^5-di(adenosine-5') pentaphosphate (10–60 μM) slowed but did not prevent the spectral changes due to conversion of AMP into IMP (not shown).

The effect of cyclopiazonic acid on the difference spectra of Ca^{2+}-ATPase induced by photolysis of caged-Ca^{2+} and caged-ATP

Sarcoplasmic reticulum vesicles were incubated with cyclopiazonic acid in a medium containing 0.1 M KCl, 10 mM imidazole (pH 7.4), 0.5 mM $MgCl_2$, 2 mM caged Ca^{2+}, 0.1 mM $CaCl_2$ and 1% dimethylsulfoxide

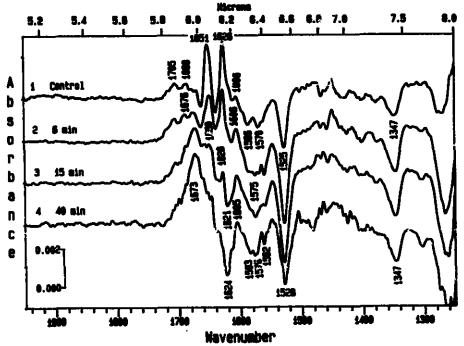

Fig. 3. Time dependent changes in the difference FTIR spectra of sarcoplasmic reticulum after the release of ATP from caged-ATP. (Trace 1) Sarcoplasmic reticulum vesicles (30 mg protein/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.4), 5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 1.5–5 mM caged-ATP, 3–10 mM DTT, 50 μM A23187. FTIR spectra were taken at 6°C, before and during 1 min illumination with 360 nm light. The trace represents the average of 16 difference spectra. Difference spectra were also taken on the same samples 6 (trace 2), 15 (trace 3) and 40 (trace 4) min later.

for 0.5–4 h at 2–4°C. Difference FTIR spectra were taken before and after photolysis of caged Ca^{2+}, as described under Methods (Fig. 4). All these measurements were performed in the absence of dithiothreitol, that may influence the reaction of CPA with the Ca^{2+}-ATPase. The bands at 1676 and 1653 cm^{-1} associated with Ca^{2+} binding to the Ca^{2+}-ATPase (Fig. 4, trace 1, control sample) were significantly reduced or absent in samples treated with 50 or 150 nmol cyclopiazonic acid/mg protein (Fig. 4, traces 2 and 3). This is consistent with earlier observations that CPA interferes with Ca^{2+} binding and with the Ca^{2+}-induced changes in the conformation of Ca^{2+}-ATPase [8,9]. The small change at 1662 cm^{-1}, may also be related to the inhibition of Ca^{2+} binding. Cyclopiazonic acid also affected the difference absorbance bands at 1705 and 1645 cm^{-1} (Fig. 4, traces 2 and 3). As discussed in our earlier report [3] the bands at 1645 and 1705 cm^{-1} are due to the reaction of the nitrosoketone photoproducts of Nitr-5 with the sarcoplasmic reticulum in the absence of DTT. The mechanism of CPA effect on these bands was not investigated.

The effects of cyclopiazonic acid on the difference spectra of sarcoplasmic reticulum induced by photolysis of 2 mM caged ATP were tested at 6°C in the presence of 1 mM $CaCl_2$ under conditions similar to those described in Fig. 2. Presumably due to competition between caged ATP and CPA there was no significant inhibition of the ATP-induced difference spectrum, even at 150 nmol CPA/mg protein (not shown), although CPA at this concentration produced essentially complete inhibition of the Ca^{2+}-induced difference FTIR spectrum in the absence of ATP (Fig. 4).

The photolysis of caged-ATP in the presence of Na^+,K^+-ATPase

The reaction mechanisms of Na^+,K^+-ATPase and Ca^{2+}-ATPase include the formation of a similar phosphoenzyme intermediate, but the two enzymes differ in their specificity for the transported cations [17,18]. We hoped that by comparing the effects of ATP on the FTIR spectra of the two enzymes the contributions of cation binding and phosphoenzyme formation to the ATP-induced difference spectra could be identified.

The FTIR spectrum of purified Na^+,K^+-ATPase is shown in Fig. 5. Like the spectrum of Ca^{2+}-ATPase, it contains a band at 1726 cm^{-1} due to the C=O stretching vibration of phospholipids, the Amide I band at 1645 cm^{-1} that arises mainly from the C=O stretching vibration of the peptide backbone, the residual Amide II band at ≅ 1550 cm^{-1}, and a large band at 1465

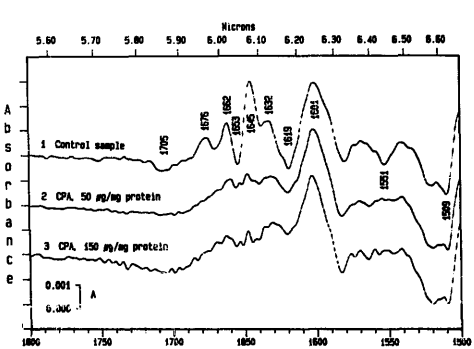

Fig. 4. Effect of cyclopiazonic acid on the difference FTIR spectra of sarcoplasmic reticulum induced by Ca^{2+} release from caged-Ca^{2+}. (Trace 1) Control sarcoplasmic reticulum. Native sarcoplasmic reticulum vesicles (30 mg protein/ml were suspended in 0.1 M KCl; 10 mM imidazole (p^2H 7.4), 0.5 mM $MgCl_2$, 2 mM caged-Ca^{2+}, 0.1 mM $CaCl_2$ and 1% v/v DMSO. FTIR spectra were taken before and after illumination with 360 nm light for 1 min. The spectra represent the average of six different spectra (after minus before illumination). (Trace 2) Sarcoplasmic reticulum treated with 50 nmol CPA/mg protein. The conditions were similar to those in trace 1, except that the sarcoplasmic reticulum vesicles were incubated with 50 nmol CPA/mg SR for 30 min to 4 h at 2–4°C under conditions described for control sarcoplasmic reticulum (trace 1). Eight different spectra were averaged. (Trace 3) Sarcoplasmic reticulum treated with 150 nmol CPA/mg protein. The conditions were similar to those in trace 1 except that the sarcoplasmic reticulum vesicles were incubated with 150 nmol CPA/mg for 30 min to 4 h at 2–4°C. Four different spectra were averaged.

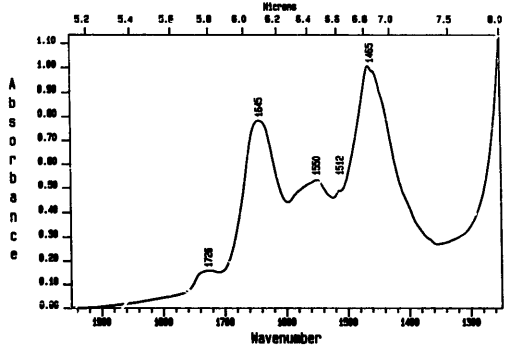

Fig. 5. FTIR spectrum of Na^+,K^+-ATPase containing caged-ATP. Purified Na^+,K^+-ATPase was suspended in a ^2H_2O medium containing 90 mM Na^+, 10 mM K^+, 20 mM Hepes (p^2H 7.6), 0.1 mM EGTA, 1 mM $MgCl_2$, 1.5 mM caged-ATP and 1.5 mM DTT. The spectrum was taken at 6°C without illumination.

cm^{-1} that is a composite of HO^2H and CH_2 deformations in proteins and in phospholipids.

Photolysis of caged-ATP in the absence of DTT produced only one broad band located at ≅ 1688 cm^{-1} (Fig. 6, line 1) that shifted to 1640 cm^{-1} when 10 mM DTT was present during photolysis (Fig. 6, line 2). However, these bands were also observed in the absence of Na^+,K^+-ATPase, and the 1640 cm^{-1} band was unaffected by the omission of Na^+ from the medium (Fig. 6, line 3) or by the addition of 1 mM

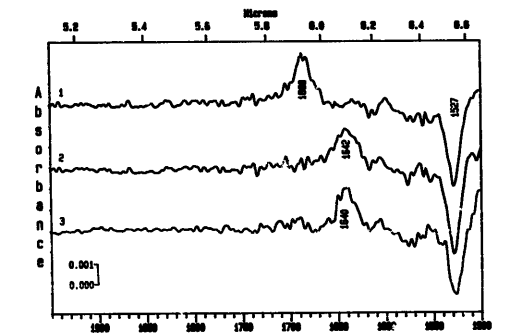

Fig. 6. Difference FTIR spectra of Na^+,K^+-ATPase after photolysis of caged-ATP in the presence or absence of DTT and Na^+. (Trace 1) Purified Na^+,K^+-ATPase was suspended in a ^2H_2O medium containing 90 mM Na^+, 10 mM K^+, 20 mM Hepes (p^2H 7.6), 0.1 mM EGTA, 1 mM $MgCl_2$, 5 mM caged-ATP. The FTIR spectra were taken at 6°C before and during illumination with 360 nm light for 1 min; 16 difference spectra were averaged. (Trace 2) The conditions were identical to those in trace 1 except that 10 mM DTT was added to the buffer medium; 28 difference spectra were averaged. (Trace 3) Na^+/K^+-ATPase was suspended in ^2H_2O buffer containing 70 mM KCl, 14 mM Hepes (p^2H 7.8), 1 mM $MgCl_2$, 0.1 mM EGTA, 10 mM DTT and 2 mM caged-ATP but no Na^+.

ouabain (not shown). Therefore we suggest that the shift of the 1688 band to 1640 cm^{-1} is due to the reaction of the nitrosophenone photoproduct of caged-ATP with DTT. At the present level of sensitivity there is no spectral component that could be confidently associated with the phosphorylation of the Na^+,K^+-ATPase by ATP.

In the Ca^{2+}-ATPase experiments (Fig. 2), although DTT was present, the 1640 cm^{-1} band could not be resolved because it was overshadowed by the much larger 1650 cm^{-1} band and the negative band at 1639 cm^{-1} that are caused by the reaction of ATP with the Ca^{2+}-ATPase. The small residual band at 1688 cm^{-1} in the Ca^{2+}-ATPase system (Fig. 2) may be due to residual nitrosophenones.

We commented earlier on the fact that the FTIR difference spectrum of sarcoplasmic reticulum Ca^{2+}-ATPase produced by photolysis of caged-Ca^{2+} is in some respects a mirror image of the difference spectrum produced by photolysis of caged-ATP [3]. A possible explanation is that the reaction steps following the formation of the phosphointermediate $E(Ca)_2 \sim P$ decrease the Ca^{2+} affinity of the Ca^{2+}-ATPase and cause the release of bound Ca^{2+} [15,16]. Therefore, the ATP-induced spectra would represent a reversal of the Ca^{2+}-induced spectral changes. If the FTIR difference spectra of sarcoplasmic reticulum produced by photolysis of caged-ATP are indeed dominated by the effects of Ca^{2+} binding to the Ca^{2+}-ATPase, the absence of an ATP-induced difference spectrum in the Na^+,K^+-ATPase may reflect the differences between the ion binding sites of the two enzymes.

Based on the observations available so far, we cannot assign any of the observed components of the difference spectra of sarcoplasmic reticulum to the formation of the acylphosphate intermediate or to changes in the secondary structure of Ca^{2+}-ATPase.

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