Loewe, R., & Linzen, B. (1975) *J. Comp. Physiol. 98*, 147–156.
Mangum, C., & Towle, D. (1977) *Am. Sci. 65*, 67–75.
Miller, K., & Van Holde, K. E. (1974) *Biochemistry 13*, 1668–1674.
Miller, K. I., Eldred, N. W., Arisaka, F., & Van Holde, K. E. (1977) *J. Comp. Physiol. 115*, 171–184.
Murray, A. C., & Jeffrey, P. D. (1974) *Biochemistry 13*, 3667–3671.
Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.
Pickett, S. M., Riggs, A. F., & Larimer, J. L. (1966) *Science 151*, 1005–1007.
Riggs, A. F., & Wolbach, R. A. (1956) *J. Gen. Physiol. 39*, 585–605.
Schepman, M. (1975) Ph.D. Thesis, University of Groningen.
Seamonds, B., Forster, R. E., & Gottlieb, A. J. (1971) *J. Biol. Chem. 246*, 1700–1705.
Seizen, R., & Van Driel, R. (1973) *Biochem. Biophys. Res. Commun. 51*, 741–745.
Sevilla, C. (1977) *Arch. Int. Physiol. Biochim. 85*, 125–131.
Studier, W. J. (1973) *J. Mol. Biol. 79*, 237–248.
Sullivan, B., Bonaventura, J., & Bonaventura, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2558–2562.
Sullivan, B., Bonaventura, J., Bonaventura, C., & Godette, G. (1976) *J. Biol. Chem. 251*, 7644–7648.
Svedberg, J., and Pedersen, K. O. (1940) *The Ultracentrifuge* (Fowler, R. H., & Kapitza, P., Eds.) Oxford University Press, London.
Terwilliger, N. B., Terwilliger, R. C., & Schabtach, E. (1976) *Biochim. Biophys. Acta 453*, 101–110.
Terwilliger, R. C., Garlick, R. L., Terwilliger, N. B., & Blair, D. P. (1975) *Biochim. Biophys. Acta 400*, 302–309.
Van Holde, K. E., & Van Bruggen, E. F. J. (1971) in *Biological Macromolecules Series* (Timasheff, S. N., & Fasman, G. D., Eds.) Vol. 5, pp 1–53, Marcel Dekker, N.Y.
Weber, K., & Osborn, M. (1975) *Proteins, 3rd Ed.*, 192.
Wiechelman, K. J., & Parkhurst, L. J. (1972) *Biochemistry 11*, 4515–4520.

Inhibition of the (Ca^{2+})ATPase from Sarcoplasmic Reticulum by Dicyclohexylcarbodiimide: Evidence for Location of the Ca^{2+} Binding Site in a Hydrophobic Region†

Uri Pick‡ and Efraim Racker*

ABSTRACT: Dicyclohexylcarbodiimide (DCCD) inhibits the (Ca^{2+})ATPase, Ca^{2+} uptake by sarcoplasmic reticulum vesicles and Ca^{2+} binding to the (Ca^{2+})ATPase from sarcoplasmic reticulum. Ca^{2+} (at μM concentrations) specifically protects against DCCD inhibition. The inhibition can, therefore, be readily demonstrated only in the presence of Ca^{2+} chelating agents such as EGTA. In the presence of EGTA, the ionophore A-23187 increased the sensitivity to DCCD. The ionophore also increased the phosphorylation of the enzyme by inorganic phosphate in the presence of Mg^{2+}. These results indicate that tightly bound Ca^{2+} is located in a hydrophobic region of the enzyme which is not accessible to EGTA. Complete inhibition of the (Ca^{2+})ATPase is accompanied by binding of 4–5 nmol of [^{14}C]DCCD per mg of ATPase protein in the absence of Ca^{2+} compared with 2 nmol bound per mg in the presence of Ca^{2+} with no ATPase inhibition. Assuming a molecular weight of 100 000 for the ATPase monomer, about 1 nmol of DCCD inhibits 4 nmol of ATPase. This result suggests that the minimal functional unit of the enzyme is a tetramer. Following trypsin digestion of the [^{14}C]DCCD-labeled ATPase most of the radioactivity appears in the 20 000-dalton fragment. We propose that DCCD reacts with the Ca^{2+}-binding site of the ATPase.

The (Ca^{2+})ATPase from sarcoplasmic reticulum is a membrane-bound enzyme which catalyzes Ca^{2+} transport into the vesicles coupled to the hydrolysis of ATP (Hasselbach & Makinose, 1961). Ca^{2+} uptake is accompanied by a reversible sequence of phosphorylation and dephosphorylation of the ATPase protein. The involvement of Ca^{2+} in the different partial reactions can be summarized as follows: Ca^{2+} binding to sarcoplasmic reticulum vesicles (Meissner, 1973) and to the purified (Ca^{2+})ATPase protein (Ikemoto, 1975) indicates the existence of two high-affinity binding sites and one to three low-affinity binding sites. The high-affinity Ca^{2+} binding sites are probably located at the outer surface of the sarcoplasmic reticulum membrane since the apparent K_m for Ca^{2+} uptake (Hasselbach & Makinose, 1961; Mermier & Hasselbach, 1976), for Ca^{2+}-dependent ATPase (Hasselbach & Makinose, 1961; Shigekawa et al., 1976), and for phosphorylation of the ATPase protein by ATP (Martonosi, 1969) is similar to the dissociation constant of the high-affinity Ca^{2+} binding site (1–3 μM).

de Meis & Carvalho (1974) suggested that the low-affinity Ca^{2+} binding site is at the inner surface of the sarcoplasmic reticulum membrane. This conclusion was based mainly on the observation that in leaky vesicles the P_i–ATP exchange reaction requires high Ca^{2+} concentrations (ca. 2 mM), whereas in intact vesicles, which accumulate Ca^{2+}, low external concentrations sufficed. In line with this conclusion is the fact that high internal Ca^{2+} concentrations are required for the reversal of the pump (Barlogie et al., 1971; Panet & Selinger, 1972).

Knowles & Racker (1975) demonstrated that the purified (Ca^{2+})ATPase catalyzes ATP formation from P_i and ADP without involving an ion gradient in a two-step reaction:

† From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853. *Received August 14, 1978.* Supported by Grant No. BMS-75-17887 from the National Science Foundation and Grant CA-08964 from the National Cancer Institute, DHEW.
‡ Supported by a Chaim Weizmann Fellowship obtained from the Weizmann Institute of Science, Rehovot, Israel.

0006-2960/79/0418-0108$01.00/0 © 1979 American Chemical Society

phosphorylation of the protein by P_i[1] in the presence of Mg^{2+} (this step is inhibited by low Ca^{2+} concentrations) followed by transfer to ADP in the presence of high Ca^{2+} concentrations, indicating the involvement of the low-affinity Ca^{2+} binding site. It was suggested that the formation of ATP from ADP and P_i is driven by the binding energy of the interaction of the enzyme with the divalent cations (Knowles & Racker, 1975).

In this article we report that DCCD, a hydrophobic carboxyl reagent, inhibits the (Ca^{2+})ATPase, Ca^{2+} transport and Ca^{2+} binding by the (Ca^{2+})ATPase from sarcoplasmic reticulum. The inhibition is specifically prevented by Ca^{2+}. We propose that DCCD reacts with the carboxyl group which is involved in Ca^{2+} binding at the high-affinity Ca^{2+} binding site of the enzyme.

Experimental Procedures

Materials. Chelex-100, Na^+ form (100–200 mesh), was obtained from Bio-Rad and washed with 0.1 M Tris-Cl (pH 8.0) until pH 8.0 was reached. Sephadex (G-50 fine), ATP (from equine muscle), and Mes were obtained from Sigma. Trypsin inhibitor was obtained from Worthington Biochemical Corp. Arsenazo III, obtained from Aldrich, was passed as a 30 mM solution through a Chelex 100 column to remove Ca^{2+}. A-23187 was donated by Dr. R. Hosley of Eli Lilly, and [^{14}C]DCCD was a gift from Dr. R. Kaback of the Roche Institute of Molecular Biology. This preparation contained four different ^{14}C-labeled components when separated on a thin-layer chromatographic plate (chloroform:methanol:water, 75:25:4). The component which was identical in R_f with the unlabeled DCCD contained 51% of the total counts and this was taken into account in calculating the stoichiometry of DCCD binding to the (Ca^{2+})ATPase. This estimate was consistent with the finding that labeled DCCD was half as active as inhibitor of the ATPase as unlabeled DCCD.

Preparations. Sarcoplasmic reticulum (washed R_1 of preparation A) and purified (Ca^{2+})ATPase (R_3c) were prepared according to MacLennan (1970) with slight modifications (Zimniak & Racker, 1978).

Assays. The ATPase activity was assayed for 5 min at 37 °C (MacLennan, 1970) and the amount of $^{32}P_i$ released from [γ-^{32}P]ATP was measured according to Carmeli & Racker (1973). Ca^{2+} uptake was assayed by a modification of the Sephadex method (Penefsky, 1977). A 1-mL tuberculin syringe containing 1 mL of swollen Sephadex G-50 (fine) was equilibrated with ice-cold 0.2 M sucrose and centrifuged in a 13 × 100 mm test tube for 3 min at 300g a few minutes before use. Ca^{2+} uptake was initiated by addition of 10 μg of sarcoplasmic reticulum protein into 250 μL of medium containing: 50 mM imidazole chloride (pH 7.0), 5 mM Na-ATP, 5 mM $MgCl_2$, 5 mM potassium oxalate, and 0.1 mM $^{45}CaCl_2$ (3×10^6 cpm/μmol). Seventy-five microliters of this mixture was applied to a Finnpipette tip placed on top of the syringe and the reaction was stopped after 3 min by starting the centrifuge. After 3 min at 300g the vesicles in the test tube were dissolved by addition of 0.2 mL of 2% $NaDodSO_4$ and the radioactivity was counted with 8 mL of ACS scintillation fluid.

Ca^{2+} Binding. Ca^{2+} binding to the purified (Ca^{2+})ATPase (in the absence of ATP) was assayed by measuring the absorption changes of arsenazo III at 695–660 nm in an Aminco-Chance DW-2 spectrophotometer. About 1 mg of enzyme was added to a 3-mL cuvette containing 0.1 M KCl, 20 mM Tris–Mes (pH 7.2), and 50 μM arsenazo III. The reference cuvette contained the same components without arsenazo III. Increasing concentrations of $CaCl_2$ (2–30 μM) were added, and the absorption changes were recorded. The absorption changes of arsenazo III solution in the presence of various concentrations of Ca^{2+} and in the absence of protein were used as standards.

Table I: Protection against Thermal Inactivation of (Ca^{2+})ATPase[a]

addition	(Ca^{2+})ATPase	
	μmol mg^{-1} min^{-1}	% of original act.
	0.2	2.3
sucrose (0.66 M)	4.65	54
ammonium acetate (0.6 M)	7.2	84
KCl (0.1 M)	7.1	82.5
Tris-Cl (0.1 M)	2.1	24.5
imidazole-Cl (0.1 M)	1.8	21
$CaCl_2$ (3 mM)	6.0	70
$MgCl_2$ (5 mM)	1.1	12.8
no incubation	8.6	100

[a] (Ca^{2+})ATPase (0.42 mg) was incubated for 1 h at room temperature in 5 mL of medium containing 0.1 M Tris-Mes (pH 6.0), 1 mM EGTA, and the compounds listed in the table. The enzyme was washed and assayed for ATPase activity as described under Experimental Procedures.

Incubation with DCCD. Unless otherwise specified, 0.3–0.4 mg of enzyme (sarcoplasmic reticulum or purified (Ca^{2+})-ATPase) was incubated at room temperature in 5 mL of medium containing 0.1 M KCl, 30 mM Tris–Mes (pH 6.0), 1 mM EGTA or 1 mM $CaCl_2$, and usually 20 μM DCCD. After 1 h, the tubes were cooled to 4 °C, and the enzyme was collected at 105 000g for 45 min. The pellet was resuspended in 0.2 mL of 0.2 M sucrose, 50 mM Tris-Cl (pH 8.0) and samples were taken for activity measuremnts.

Labeling with [^{14}C]DCCD and Treatment with Trypsin. The (Ca^{2+})ATPase (0.4 mg) was incubated as described above but with 20 μM [^{14}C]DCCD (specific activity, 11.2 μCi/μmol). The enzyme was collected by centrifugation at 145 000g for 30 min and resuspended in 1 mL of a medium containing 1 M sucrose, 0.1 M KCl, and 20 mM Tris–maleate (pH 7.0). Trypsin (20 μg) was added followed by 50 μg of trypsin inhibitor after 30 s or 30 min. After incubation of the mixture at 30 °C, 4 mL of buffer B (MacLennan, 1970) containing 0.66 M sucrose, 0.05 M Tris-Cl (pH 8.0), 1 mM histidine and 0.4 mL of 50% saturated ammonium acetate were added. After centrifugation at 145 000g for 30 min, the pellet was suspended and washed sequentially with 5 mL of the following solutions: (1) 10% trichloroacetic acid plus 2% pyridine; (2) 40% ethanol; and (3) diethyl ether. Finally, the pellet was dried, dissolved in $NaDodSO_4$, and applied to 10% polyacrylamide gels. Absorptions of Coomassie Blue stained gels were measured at 750 nm in a Gilford (Model 2400) spectrophotometer.

Results

Lability of (Ca^{2+})ATPase. In the presence of EGTA, the purified (Ca^{2+})ATPase was rapidly inactivated when incubated at room temperature at pH 6.0. Similar acid inactivation of the ATPase activity of sarcoplasmic reticulum has been previously observed (Berman et al., 1977). As shown in Table I, the enzyme can be protected by addition of a variety of salts or sucrose. Of particular interest is the effectiveness of Ca^{2+} (Berman et al., 1977) which, unlike other ions (including

[1] Abbreviations used: SR, sarcoplasmic reticulum; DCCD, dicyclohexylcarbodiimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P_i, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

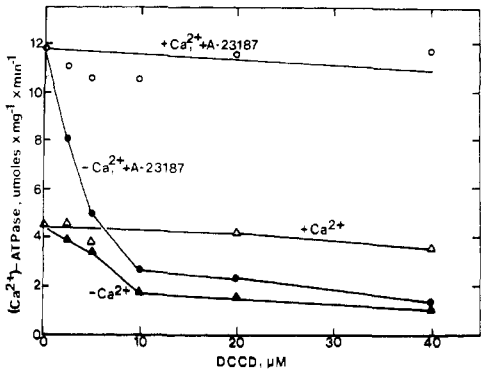

FIGURE 1: Protection of sarcoplasmic reticulum vesicles by Ca^{2+} against DCCD inhibition of (Ca^{2+})ATPase. Sarcoplasmic reticulum vesicles (0.33 mg of protein) were incubated with DCCD for 1 h at 4 °C in 5 mL of medium containing 20 mM Tris-Mes (pH 6.0), 0.1 M KCl, and either 1 mM EGTA (designated as $-Ca^{2+}$) or 1 mM $CaCl_2$. DCCD at the indicated concentration was also added. The protein was collected by centrifugation and samples were taken to assay ATPase and Ca^{2+} uptake. A-23187 (5 μg) was included in the assay reaction mixtures where indicated. (Ca^{2+})ATPase was assayed as described under Experimental Procedures.

Table II: Effect of A-23187 on DCCD Inhibition of Purified (Ca^{2+})ATPase[a]

additions	(Ca^{2+})-ATPase (μmol mg^{-1} min^{-1})
	10.8
DCCD (5 μM)	9.3
EGTA (3 mM)	10.3
EGTA + DCCD	4.2
EGTA + A-23187 (1 μg/mL)	10.0
EGTA + A-23187 + DCCD	2.2
A-23187 + DCCD	8.9
EGTA + DCCD + $CaCl_2$ (3 mM)	10.1
EGTA + A-23187 + DCCD + $CaCl_2$	10.1
EGTA + 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (0.5 mM)	10.5

[a] (Ca^{2+})ATPase (330 μg) was incubated for 1 h as described in Table I in the presence of 0.1 M KCl and the compounds indicated above. ATPase was assayed as described under Experimental Procedures.

Mg^{2+}), protected at low concentrations. In all subsequent experiments with either intact sarcoplasmic reticulum or purified enzyme, 0.1 M KCl was added for the protection of the enzyme.

Inhibition of ATPase Activity and Ca^{2+} Uptake by DCCD. We have shown elsewhere (Banerjee et al., 1979) that A-23187 markedly stimulated the ATPase activity of sarcoplasmic reticulum vesicles. This observation is compatible with either of the following explanations: (1) removal of inhibitory Ca^{2+} from a region that was not readily accessible to EGTA or (2) uncoupling of the ATPase from Ca^{2+} uptake by dissipation of the Ca^{2+} gradient. As shown in Figure 1, in the presence of EGTA and A-23187, the ATPase activity of sarcoplasmic reticulum vesicles is highly sensitive to DCCD with as little as 10 μM inhibiting over 80%. In the presence of EGTA alone the ATPase activity is much lower and the DCCD effect is somewhat less pronounced. A similar inhibition by DCCD was observed with purified (Ca^{2+})ATPase. As shown in Table II neither DCCD nor EGTA alone affected enzyme activity significantly after exposure for 1 h at room temperature. When both were present a 60% inhibition was observed. In the presence of A-23187 and EGTA there was about 80% inhibition by DCCD. The control without DCCD was not

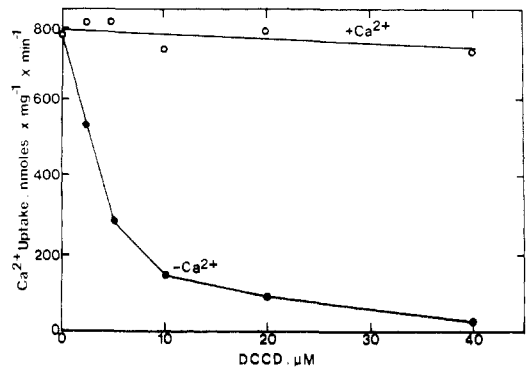

FIGURE 2: Inhibition of Ca^{2+} uptake by DCCD and protection by Ca^{2+}. The experimental procedure was as described in the legend of Figure 1 except that Ca^{2+} uptake was assayed as described under Experimental Procedures.

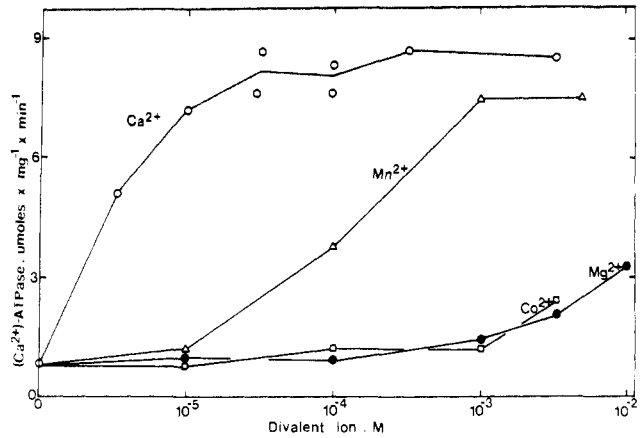

FIGURE 3: Effect of Ca^{2+} concentration on the inhibition of (Ca^{2+})ATPase by DCCD. (Ca^{2+})ATPase (4 mg) was washed to remove Ca^{2+} by incubation for 10 min at 4 °C in 7 mL of buffer B (see Experimental Procedures) containing 5 mM EGTA and 150 μg of A-23187. After centrifugation for 30 min at $4.5 \times 10^3 g$ the pellet was resuspended in 0.25 mL of Ca^{2+}-free buffer B. Other incubation and assay conditions are described under Experimental Procedures. $CaCl_2$ or other ions at the indicated concentrations were added to the DCCD incubation medium which contained 20 μM DCCD.

affected by EGTA and A-23187. $CaCl_2$ at 3 mM completely protected the enzyme against DCCD. A water-soluble carbodiimide was not effective even at a concentration 100 times that of DCCD. Ca^{2+} uptake by sarcoplasmic reticulum was also inhibited after exposure to DCCD in the presence of EGTA (Figure 2).

Effect of pH and Divalent Cations on Inhibition by DCCD. The inhibition by DCCD was pH dependent with maximal effects at pH 6.0. After 1-h incubation with 20 μM DCCD, about 90% inhibition was observed at pH 6.0, 70% at pH 7.0, and 44% at pH 8.0. At pH 5.0 significant inactivation took place even in the presence of 0.1 M KCl.

At pH 6.0 10 μM $CaCl_2$ provided almost complete protection, 1 mM $MnCl_2$ was somewhat less effective, and $MgCl_2$ or $CoCl_2$ was rather ineffective (Figure 3). To demonstrate the effectiveness of Ca^{2+} at low concentration, residual Ca^{2+} had to be removed from the purified ATPase by exposure to EGTA and A-23187 as described in the legend of Figure 3.

Effect of A-23187 and EGTA on Phosphorylation of (Ca^{2+})ATPase by P_i. Another reaction catalyzed by the (Ca^{2+})ATPase which is influenced by the presence of Ca^{2+} is the formation of a phosphorylated intermediate in the presence of P_i and Mg^{2+} (Knowles & Racker, 1975). As shown in Table III, in the presence of EGTA and A-23187 the yield of phosphoprotein formation is markedly increased,

Table III: Effect of the Ionophore A-23187 on Phosphoprotein Formation from Inorganic Phosphate[a]

addition	nmol of $^{32}P_i$/mg of protein	% of control
none	1.8	100
EGTA (1 mM)	1.9	106
A-23187	2.25	125
10 μg of A-23187 + EGTA	3.2	178
$CaCl_2$ (2 mM) + EGTA	0.1	5.5

[a] (Ca^{2+})ATPase (0.3 μg) was incubated 1 h at room temperature in 4 mL of medium containing 10 mM Tris-Mes (pH 6.0), 4 mM $^{32}P_i$ containing 2.7×10^3 cpm/nmol, 10 mM $MgCl_2$, and the compounds indicated above. Phosphorylation was measured as previously described (Knowles & Racker, 1975).

Table IV: Effect of Internal and External Ca^{2+} on the Inhibition of Sarcoplasmic Reticulum ATPase by DCCD[a]

SR	ATPase (μmol mg^{-1} min^{-1}) without DCCD	ATPase (μmol mg^{-1} min^{-1}) with DCCD	% inhibition
control	5.1	1.0	81
control + external Ca^{2+}	7.0	6.0	15
loaded with Ca^{2+}	2.9	0.7	76
loaded with Ca^{2+} + external Ca^{2+}	3.3	3.1	6

[a] Sarcoplasmic reticulum vesicles were loaded with Ca^{2+} in a medium containing 0.1 M KP_i (pH 7.5), 5 mM Na-ATP, 5 mM $MgCl_2$, and either 0.2 mM EGTA or 0.2 mM $CaCl_2$. After 5-min incubation at room temperature, the tubes were cooled and the vesicles collected at 105 000g for 30 min. The vesicles were resuspended in 0.2 mL of buffer B. Exposure to DCCD was started by addition of 5 mL of ice-cold medium containing 30 mM Tris-Mes (pH 6.0), 0.1 M KCl, and either 1 mM Ca^{2+} or EGTA with or without 10 μM DCCD. After 1 h at 0 °C the vesicles were collected, washed, and assayed for ATPase.

approaching levels obtained with ATP.

Location of the Site at Which Ca^{2+} Protects against DCCD. There are at least two binding sites for Ca^{2+} (de Meis & Carvalho, 1974), one accessible from the outside of sarcoplasmic reticulum, the other from the inside. In order to determine which of these two sites protects against inhibition by DCCD, sarcoplasmic reticulum vesicles were loaded with Ca^{2+} in the presence of P_i before exposure to DCCD. As shown in Table IV, the Ca^{2+}-loaded vesicles were as sensitive to DCCD as the control vesicles, although they were considerably less active. In both loaded and control vesicles, external Ca^{2+} protected effectively. We observed in control experiments (data not shown) that vesicles loaded with $^{45}Ca^{2+}$ retained virtually all radioactivity after incubation at 0 °C in either the absence or presence of DCCD.

Effect of DCCD on the Binding of Ca^{2+} to Purified (Ca^{2+})ATPase. Figure 4 shows that DCCD also inhibits the binding of Ca^{2+} to the purified (Ca^{2+})ATPase. It lowers the binding constant from 0.9 to 0.55 μM^{-1} and the binding sites from 40 to 32 nmol/mg of protein. Compared with previous reports (Meissner, 1973; Ikemoto, 1975) these binding constants are lower and the extent of binding higher than previously described. Possible reasons for these differences are as follows. (a) The (Ca^{2+})ATPase preparations used were quite different: in one case sarcoplasmic reticulum membranes were used (Meissner, 1973); in the other case a preparation purified with Triton was used (Ikemoto, 1975). (b) The arsenazo dye method used here is sensitive to variations in ionic strength, pH, Mg^{2+} ion, etc. Since the calibration was made in the absence of enzyme, the absolute values may be inaccurate. In any case, the marked differences between the

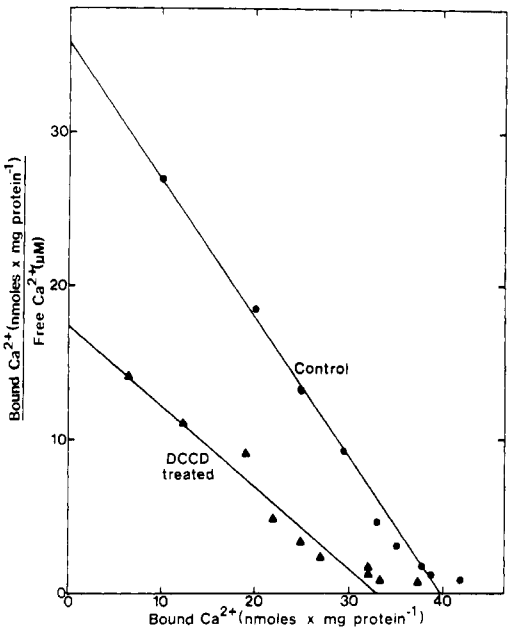

FIGURE 4: Inhibition of Ca^{2+} binding to (Ca^{2+})ATPase by DCCD. (Ca^{2+})ATPase (2.4 mg) was incubated with 1 mM EGTA in the presence or absence of 20 μM DCCD as described in the legend of Figure 1. The protein was collected by centrifugation and resuspended in 0.2 mL of medium containing 0.1 M KCl and 20 mM Tris-Mes (pH 6.0). Ca^{2+} binding was measured by following the absorption changes of arsenazo III as described under Experimental Procedures. Each cuvette contained 1.05 mg of protein in 3 mL.

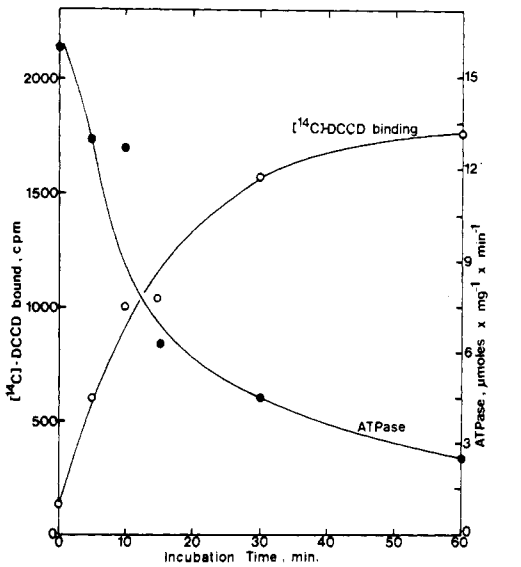

FIGURE 5: Correlation between [^{14}C]DCCD binding and inhibition of (Ca^{2+})ATPase. (Ca^{2+})ATPase (0.66 mg) was incubated in 5 mL of medium containing 0.1 M Tris-Mes (pH 6.0), 0.1 M KCl, 0.2 mM $CaCl_2$, and 10 μM DCCD for 30 min at room temperature. EGTA (1 mM) plus [^{14}C]DCCD (7 μCi/μmol, 2 μM final concentration) were added and the incubation was continued for another 60 min. At the indicated times samples were withdrawn to cooled tubes containing either 5% trichloroacetic acid or 3 mM $CaCl_2$ (final concentration). The former was used to determine DCCD binding (as described under Experimental Procedures). The latter were washed by centrifugation and assayed for ATPase.

DCCD-treated and nontreated enzyme shown in Figure 4 suggest a significant decrease in Ca^{2+} affinity after exposure of the enzyme to 20 μM DCCD.

Effect of Time on Binding of [^{14}C]DCCD and Inhibition of ATPase Activity. At 10 μM DCCD the inhibition of ATPase activity was dependent on the time of exposure (Figure 5). About 10-min incubation was required to achieve 50%

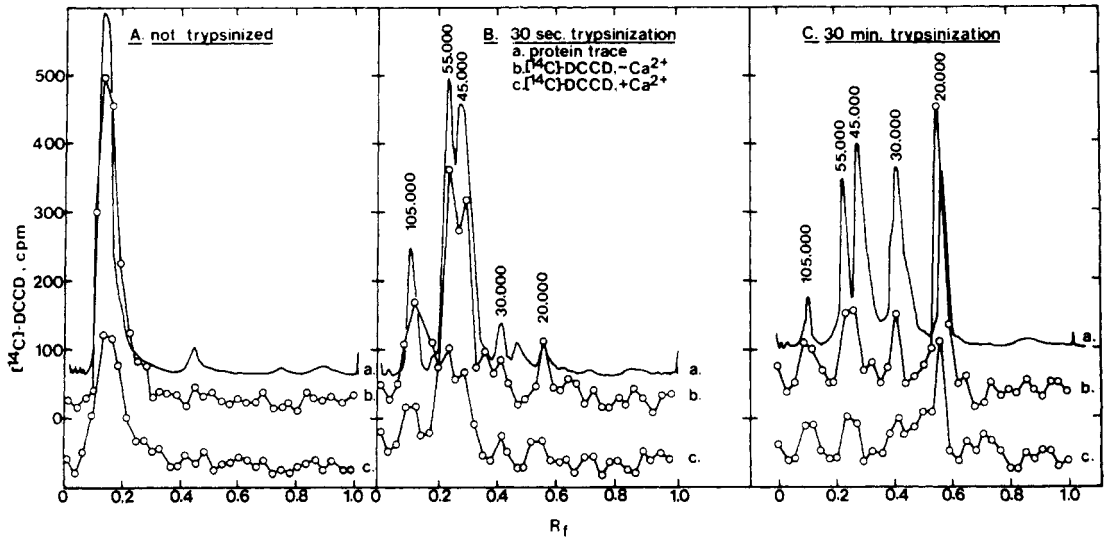

FIGURE 6: Trypsin digestion of [^{14}C]DCCD-labeled (Ca^{2+})ATPase. (Ca^{2+})ATPase (0.4 mg) was incubated for 1 h at room temperature with 20 μM [^{14}C]DCCD (22.5 μCi/μmol) in the presence of either 1 mM EGTA or 3 mM $CaCl_2$. The enzyme was washed and incubated with trypsin for 30 s or 30 min as described under Experimental Procedures. The protein fragments (110–130 μg of protein) were applied to $NaDodSO_4$–acrylamide gels. One set of gels was stained for protein and a second set sliced (2-mm slices), solubilized in 1 mL of NCS, and counted in 10 mL of ACS scintillation fluid. (—) Protein; (o—o) radioactive counts. The upper curve represents enzyme labeled in the absence of Ca^{2+}. (A–C) Samples were taken 0, 30 s, and 30 min after exposure to trypsin.

Table V: [^{14}C]DCCD Binding to (Ca^{2+})ATPase[a]

addition during incubation	[^{14}C]-DCCD bound (cpm)	nmol of DCCD bound/mg of protein	mol of DCCD/ mol of ATPase
	3348	4.2 ± 0.8[b]	0.44 ± 0.08
$CaCl_2$ (3 mM)	1536	1.88 ± 0.12[b]	0.20 ± 0.013

[a] Labeling of the ATPase protein with [^{14}C]DCCD was done as described under Experimental Procedures except that the trichloroacetic acid precipitation step was done in the presence of 2% sodium deoxycholate instead of pyridine. [b] Average of three experiments.

inhibition. In the same experiment binding of [^{14}C]DCCD was measured. As shown in Figure 5, again about 10 min was required for one-half-maximal binding. After 1 h of incubation, almost maximal binding and maximal inhibition were observed.

Binding of [^{14}C]DCCD to (Ca^{2+})ATPase. The binding of [^{14}C]DCCD to the purified enzyme was markedly inhibited by Ca^{2+}. As shown in Table V in the absence of Ca^{2+} 4.2 nmol of DCCD was bound per mg of protein. Under these conditions the ATPase activity was about 85% inhibited. In the presence of Ca^{2+} about 1.9 nmol of DCCD was bound per mg of protein and the activity was preserved. Assuming a molecular weight of 10^5, this result shows that only about 2.3 mol of DCCD binding per mg of enzyme is required for 85% inhibition or about 1 mol of DCCD per 4 mol of enzyme for complete inhibition of ATPase activity.

Figure 6A shows that on $NaDodSO_4$–acrylamide gels the [^{14}C]DCCD counts are associated with the 100 000-dalton band which represents the (Ca^{2+})ATPase.

Trypsin digestion cleaves the ATPase protein first into two polypeptides of 55 000 and 45 000 daltons. On prolonged incubation the 55 000 fragment is further cleaved to a 30 000- and 20 000-dalton fragment (Shamoo et al., 1976).

Figures 6B and 6C show that, following trypsin digestion of the [^{14}C]DCCD labeled ATPase, the counts shift from the 50 000-dalton region to the 20 000-dalton region. The DCCD reactive site appears therefore to be localized in the 20 000-dalton fragment obtained after prolonged incubation with trypsin.

Discussion

What Is the Mechanism of DCCD Inhibition? DCCD is a relatively specific carboxyl reagent, although side reactions have been described. Since we could not detect inhibition by hydrophilic carbodiimide analogues, we conclude that DCCD reacts with a carboxyl group located in a hydrophobic region.

We presented evidence for the hydrophobic nature of the Ca^{2+}-binding site based on the effect of the ionophore A-23187 which increases the accessibility of the site to DCCD (Table II) and enhances phosphorylation of the enzyme by P_i (Table III). These results suggest that the ionophore removes Ca^{2+} from a hydrophobic binding site which is inaccessible to EGTA. The fact that protection by Ca^{2+} is provided from the outside of the sarcoplasmic reticulum vesicles (Table IV) combined with the low concentration of Ca^{2+} required for protection (Figure 3) indicates that it is the high-affinity external Ca^{2+} binding site which protects against DCCD inhibition. A similar protection by Ca^{2+} against thermal inactivation of sarcoplasmic reticulum at an acid pH was observed by Berman et al. (1977). These findings as well as calorimetric measurements (Epstein & Racker, 1977) suggest that conformational changes are induced in the protein in the presence of Ca^{2+}.

The high specificity for Ca^{2+} (Figure 3) and the inhibition of Ca^{2+} binding (Figure 4) suggest that the interaction of DCCD with the ATPase is very specific and occurs at the Ca^{2+} binding site. An alternative explanation, namely, that the removal of Ca^{2+} from the protein induces a conformational change which discloses the DCCD-reactive site at a different locus of the enzyme and, conversely, that interaction with DCCD creates an irreversible conformational change which inhibits Ca^{2+} binding, seems less likely in view of the fact that DCCD reacts with the same fragment of the protein (Figure 6C) which confers Ca^{2+} conductivity to artificial membranes (Shamoo et al., 1976).

Stoichiometry of [^{14}C]DCCD Binding. Based on the stoichiometry of [^{14}C]DCCD binding (Table V), the increment in DCCD binding in the absence of Ca^{2+} which leads to an essentially complete inhibition of the catalytic activity is only ~0.25 mol/mol of enzyme based on the known molecular weight of 10^5. This result implies that the functional unit of

the enzyme is a tetramer and that it is sufficient to block one of the monomers to inactivate the complex. These results are in agreement with previously published data which demonstrated evidence for fluorescence energy transfer between purified reconstituted (Ca^{2+})ATPase monomers (Vanderkooi et al., 1977) and with equilibrium centrifugation of catalytically active (Ca^{2+})ATPase soluble in detergent (Le Maire et al., 1976). An alternative explanation for the observed results is that the interaction of DCCD with the enzyme follows multiple routes: part of the ^{14}C label is released as a diacylurea derivative following a nucleophilic attack of the intermediary complex (by an adjacent amino group of an amino acid side chain?) and part of the DCCD is covalently bound as a result of a rearrangement conversion of the intermediary complex (Carraway & Koshland, 1972).

References

Banerjee, R., Epstein, M., Kandrach, M., Zimniak, P., & Racker, E. (1979) *Membr. Biochem.* (in press).

Barlogie, B., Hasselbach, W., & Makinose, M. (1971) *FEBS Lett. 12*, 267.

Berman, N. C., McIntosh, D. B., & Kench, J. E. (1977) *J. Biol. Chem. 252*, 994.

Carmeli, C., & Racker, E. (1973) *J. Biol. Chem. 248*, 8281.

Carraway, K. L., & Koshland, D. E. (1972) *Methods Enzymol. 25*, 616.

de Meis, L., & Carvalho, M. G. C. (1974) *Biochemistry 13*, 5032.

Epstein, M., & Racker, E. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) p 192, Elsevier North-Holland, Amsterdam.

Hasselbach, W., & Makinose, M. (1961) *Biochem. Z. 333*, 518.

Ikemoto, N. (1975) *J. Biol. Chem. 250*, 7219.

Knowles, A. F., & Racker, E. (1975) *J. Biol. Chem. 250*, 1949.

Le Maire, M., Møller, J. V., & Tanford, C. (1976) *Biochemistry 15*, 2336.

MacLennan, D. H. (1970) *J. Biol. Chem. 245*, 4508.

Martonosi, A. (1969) *J. Biol. Chem. 244*, 613.

Meissner, G. (1973) *Biochim. Biophys. Acta 298*, 906.

Mermier, P., & Hasselbach, W. (1976) *Eur. J. Biochem. 69*, 79.

Panet, R., & Selinger, Z. (1972) *Biochim. Biophys. Acta 255*, 34.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891.

Shamoo, A. E., Ryan, T. E., Stewart, P. S., & MacLennan, D. H. (1976) *J. Biol. Chem. 251*, 1447.

Shigekawa, M., Finegan, J. M., & Katz, A. M. (1976) *J. Biol. Chem. 251*, 6894.

Vanderkooi, J. M., Ierokomas, A., Nakamura, H., & Martonosi, A. (1977) *Biochemistry 16*, 1262.

Zimniak, P., & Racker, E. (1978) *J. Biol. Chem. 253*, 4631.

Antithrombin Reactions with α- and γ-Thrombins†

Tien-ling Chang, Richard D. Feinman,* Bryan H. Landis, and John W. Fenton II

ABSTRACT: Human α-thrombin with high clotting activity and its proteolyzed derivative γ-thrombin with virtually no clotting activity reacted in an essentially identical manner with antithrombin. The two enzyme forms bound proflavin with similar constants and showed identical behavior with small substrates. No significant differences were found for the antithrombin reactions (measured by proflavin displacement or active site titration) with respect to kinetics, extent of reaction, or effect of added heparin. The enzyme–antithrombin complexes could not be dissociated with sodium dodecyl sulfate ($NaDodSO_4$) but the $NaDodSO_4$-denatured complexes were dissociated by hydroxylamine treatment. The γ-thrombin–antithrombin complex has an approximate molecular weight of 75000 by disc gel electrophoresis as compared with 100000 for the α-complex, consistent with the polypeptide structures of the two proteins. The γ-thrombin–antithrombin complex did not inhibit clotting catalyzed by α-thrombin. In addition, fibrinogen did not affect the reaction of γ-thrombin with antithrombin or antithrombin–heparin. Thus, the antithrombin and antithrombin–heparin reactions do not involve the fibrinogen recognition sites which are destroyed by proteolytic conversion of α-thrombin to the noncoagulant γ form.

The enzyme responsible for the clotting of fibrinogen in blood, α-thrombin (EC 3.4.21.5), can be converted by proteolysis to a form, γ-thrombin, which retains esterase activity but has minimal clotting ability. The existence of multiple active forms is also known for other proteases such as trypsin and chymotrypsin, but the thrombin preparations have two unusual properties. First, α-thrombin's ability to clot fibrinogen represents high specificity for particular arginylglycine bonds. An enzymically active form such as γ-thrombin, which has lost this specificity, is clearly useful. Second, the amino acid residues responsible for enzymatic activity appear on three separate chains in γ-thrombin as a result of the α- to γ-proteolytic conversion, and is, thus, of interest in the study of enzyme mechanism. The structure and properties of the various human thrombin forms are summarized in Figure 1. The derivative γ-thrombin has been found to retain some of the biological activities of α-thrombin. These include activation of factor XIII (Credo et al., 1976; Lorand & Credo, 1977), factor D like activity in the alternate complement pathway (Davis et al., 1978), and reduced although qualitatively similar activity in initiating platelet reactions (Charo et al., 1977). Also, reaction of native and γ-thrombins was shown to be the same toward an affinity label for sites adjacent to the active site (exo-sites; Bing et al., 1977).

† From the Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, New York 11203 (T.C. and R.D.F.), and the Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201 (B.H.L. and J.W.F.). *Received July 14, 1978.* These studies were supported in part by National Institutes of Health Research Grants HL 13160 (J.W.F.) and HL 14992 (R.D.F.) and funds from the American Heart Association, Northeastern New York Chapter (J.W.F.). Richard D. Feinman is the recipient of Career Development Award HL 00406 from the National Institutes of Health.

0006-2960/79/0418-0113$01.00/0 © 1979 American Chemical Society