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LIGAND BINDING PROPERTIES OF THE SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase LABELLED WITH *N*-CYCLOHEXYL-*N'*-(4-DIMETHYLAMINO-α-NAPHTHYL)CARBODIIMIDE

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The (Ca^{2+} + Mg^{2+})-ATPase of rabbit sarcoplasmic reticulum, when labelled at two Ca^{2+}-protected sites with *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide (NCD-4) retains Ca^{2+} binding capacity at the sites with K_d values of approx. 3 μM and 0.12 mM as assessed by fluorescence titration. The sites correspond to the two high-affinity Ca^{2+} binding sites present in the native ATPase. The NCD-4 labelled ATPase exhibits slow conformational changes at each site on addition of Ca^{2+}. It retains the ability to form phosphoenzyme, and can most likely translocate Ca^{2+}.

Introduction

The (Ca^{2+} + Mg^{2+})-ATPase of rabbit sarcoplasmic reticulum is irreversibly inhibited by *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide (NCD-4), a fluorescent analogue of dicyclohexylcarbodiimide (DCCD) [1]. Inhibition of ATPase activity only occurs during incubation with NCD-4 in the absence of Ca^{2+}: inclusion of Ca^{2+} (250 μM) completely protects against inhibition. Inhibition is correlated with the covalent incorporation of NCD-4 into calcium-protected ('specific') sites located in the 24 kD tryptic fragment [2] of the ATPase. There is an approximately equivalent extent of calcium-independent incorporation of NCD-4 into 'non-specific' sites of the ATPase, but the other proteins of the sarcoplasmic reticulum are not significantly labelled. Probe responses from the specifically labelled sites to the addition of Ca^{2+} and ATP are now described and interpreted.

Materials and Methods

Labelling with NCD-4

Rabbit sarcoplasmic reticulum vesicles (vesicles) were prepared and labelled with NCD-4 as described [1]. Vesicles (1 mg protein/ml) were incubated in 100 mM KCl/50 mM NaMes (pH 6.2) in the absence (1 mM NaEGTA) or presence (1 mM NaEGTA/1.25 mM $CaCl_2$) of Ca^{2+} with 150 μM NCD-4 for 4 h at 23°C. After addition of 10 vol% 500 mM NaMops (pH 7.9) unreacted NCD-4 was removed by elution of vesicles through a Sephadex LH20 column with 100 mM KCl/1.0 mM NaEGTA/25 mM NaMops (pH 7.0).

Labelling with DCCD

Vesicles (1 mg protein/ml) were incubated with DCCD (150 μM) at 0°C for 3 h as described [3], with the exception that the ionophore A23187 was not included in the incubation mixture.

Abbreviations: Mops, 3-(*N*-morpholino)propanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*,-tetraacetic acid; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl)carbodiimide; DCCD, *N*,*N'*-dicyclohexylcarbodiimide; Ado*PP*[NH]*P*, adenosine 5'[β,γ-imido]triphosphate; Ado*PP*[CH_2]*P*, adenosine 5'[β,γ-methylene]triphosphate.

Calcium binding

(a) Sample preparation. Vesicles (50 mg protein) were incubated with or without NCD-4 in the absence of Ca^{2+} as described above. Unreacted NCD-4 was removed by elution through a Sephadex LH20 column (60 cm × 3 cm) with 100 mM KCl/10 mM NaMops (pH 7.0)/1 mM $CaCl_2$ at a flow rate of 1.0 ml/min. After centrifugation (100000 × *g*, 105 min, 4°C) onto a 1 ml pad of 75% sucrose, vesicles were washed once with 100 mM KCl/10 mM NaMops (pH 7.0) before final resuspension in 2.5 ml 0.3 M sucrose/100 mM KCl/10 mM NaMops (pH 7.0). The preparation was then dialysed overnight at 4°C against 200 vol. of 0.3 M sucrose/100 mM KCl/50 μM NaEGTA/10 mM NaMops (pH 7.0).

(b) Calcium binding. Equilibration buffer: 100 mM KCl/50 μM NaEGTA/10 mM NaMops (pH 7.0), was doped with $^{45}CaCl_2$ to give approx. 10^5 cpm/ml. Aliquots of this buffer, after addition of vesicles to a protein concentration of 1 mg/ml and $CaCl_2$ to the desired concentration were incubated for 30 min at 23°C. Aliquots were then removed and vesicles pelleted by centrifugation in a Beckman Airfuge (10 min, 15 lb/inch^2, 18° rotor). Supernatant aliquots were removed and counted for radioactivity using a Triton X-100 cocktail [4], to give an estimate of the fraction of unbound calcium. Total calcium was estimated by liquid scintillation counting of aliquots prior to centrifugation and the fraction of bound calcium was estimated from the difference. Atomic absorption spectroscopy [5] indicated contaminating Ca^{2+} of approximately 8 μM which was taken into account when calculating bound Ca^{2+} levels.

Phosphoenzyme formation

Phosphoenzyme levels were determined at 0°C or 23°C in 4.0 ml of solution prepared by quenching 2.0 ml of incubation medium (± carbodiimide/$-Ca^{2+}$) with 1.8 ml of 111 mM KCl/55.6 mM NaMops (pH 7.9)11.1 mM $MgCl_2$/± 4.4 mM $CaCl_2$. Reaction was initiated by the addition of 0.2 ml 2 mM [γ-^{32}P]ATP (final concentration 0.1 mM) and the reaction quenched after 5–10 s by rapid addition of 25 ml ice-cold 4.5% trichloroacetic acid/1 mM phosphate/0.5 mM ATP. Precipitated protein was collected by centrifugation (5000 × *g*, 20 min, 2°C), and washed twice with 25 ml 2% trichloroacetic acid, once with 10 ml 5% trichloroacetic acid, and once with 10 ml distilled water. The final precipitate was dissolved in 3.0 ml 1% SDS/50 mM NaMops (pH 7.) and protein estimated assuming A_{280} per mg per ml = 1.0 [6]. Aliquots were then counted for radioactivity as described above.

Fluorescence methods

Fluorescence was measured using either Perkin-Elmer 1000 or Schoeffel RRS 1000 spectrofluorimeters linked appropriately to a home built stopped flow apparatus. Conditions for particular experiments are indicated in the figures. All fluorescence measurements were made at 23°C using excitation wavelengths of 338 or 330 nm, and an emission wavelength of 430 nm.

Materials

$^{45}CaCl_2$ and [γ-^{32}P]ATP were obtained from Amersham International. All reagents were of analytical grade and double distilled water was used throughout Ca^{2+} binding studies. NaEGTA solutions were standardised against a Radiometer standard solution of 100 mM $CaCl_2$ using a F2110Ca Ca^{2+} Selectrode (Radiometer). An apparent dissociation constant of $2 \cdot 10^{-7}$ M was assumed for CaEGTA at pH 7.0.

Computer analyses

Analysis of fluorescence responses and Ca^{2+} binding was performed using a least mean squares fitting program [7] modified by D.A. Duddell and R. Catterall of Salford University. All kinetic analyses represent an average of three determinations.

Results

Covalent incorporation of 12.6 nmol NCD-4 per mg vesicular protein occurs when incubation is performed with NCD-4 in the absence of Ca^{2+}. Incubation in the presence of Ca^{2+} results in the covalent incorporation of approx. 5.6 nmol NCD-4 per mg vesicular protein (non-specific labelling). In both cases NCD-4 is incorporated exclusively into the (Ca^{2+} + Mg^{2+})-ATPase. The Ca^{2+}-dependent difference (approx. 7 nmol/mg) refered to as specific labelling, is associated with inhibition of

Ca^{2+}-dependent ATPase activity [1]. Determinations of steady-state phosphoenzyme levels formed by our preparations (Table I) indicate that an average of 3.6 nmol of (Ca^{2+} + Mg^{2+})-ATPase is present per mg of vesicular protein. Approx. 2 moles of NCD-4 per mole of ATPase are thus incorporated into specific sites, while approx. 1.6 moles are incorporated into non-specific sites.

Fig. 1 shows the fluorescence response of NCD-4 labelled vesicles to the addition of excess (1 mM) Ca^{2+}. Vesicles labelled at non-specific sites show only a slight fluorescence quenching, whereas vesicles labelled at both specific and non-specific sites show a marked time-dependent fluorescence quenching of 40–50%.

A fluorescence titration of specifically labelled vesicles with Ca^{2+} is shown in Fig. 2. Curve fitting of these data indicates that Ca^{2+} binding to two types of sites on the ATPase is responsible for fluorescence quenching. Binding at the first site(s) with an apparent Ca^{2+} dissociation constant of 2.7 μM induces 29% of the fluorescence quenching, while binding at the second site(s) with an apparent dissociation constant of 0.12 mM induces 71% of the fluorescence quenching.

Measurements of Ca^{2+} binding using an Airfuge technique gave direct confirmation of the fluorescence results. Fig. 3 shows a comparison of Ca^{2+} binding to native and specifically labelled vesicles. Native vesicles show one class of sites (presumably the two high-affinity sites involved in Ca^{2+} translocation) with $K_d = 0.1$ mM, and approx. 7.4 nmol

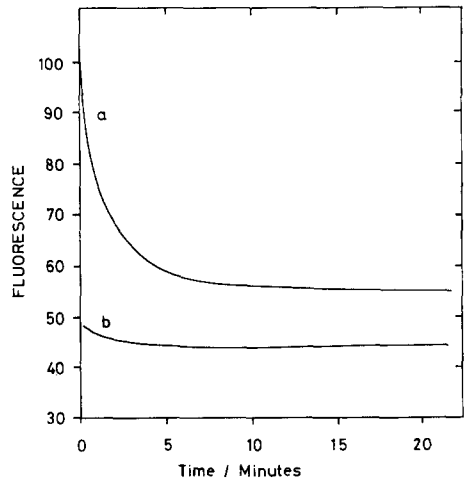

Fig. 1. Stopped-flow fluorescence quenching of NCD-4 labelled vesicles by Ca^{2+}. Syringe A contained vesicles (240 $\mu g \cdot ml^{-1}$)/100 mM KCl/1 mM EGTA/10 mM NaMops (pH 7.0). Syring B contained 100 mM KCl/1 mM EGTA/4 mM $CaCl_2$/10 mM NaMops (pH 7.0). (a) Response of specifically labelled vesicles, (b) response of non-specifically labelled vesicles.

Ca^{2+} bound per mg protein. Low-affinity binding, characterised by an arbitrary $K_d = 1$ mM, binding approx. 80 nmol Ca^{2+} per mg protein, is also present. NCD-4 labelled vesicles, in contrast, show one site with $K_d \sim 4$ μM representing 3.9 nmol Ca^{2+} bound per mg protein, accompanied by low-affinity Ca^{2+} binding ($K_d = 1$ mM, approx. 70

TABLE I

STEADY-STATE PHOSPHOENZYME LEVELS FORMED BY SARCOPLASMIC RETICULUM VESICLES

The levels indicated are those formed after 5–10 s incubation with [^{32}P]ATP at 0°C, as described in Materials and Methods. No significant differences were observed for determinations performed at 23°C.

Ca^{2+} concn.	Phosphoenzyme level (nmol/mg protein)		
	NCD-4 treated	DCCD treated	Control
1 mM	1.3±0.3	2.1±).1	3.8±0.2
~ 20 μM	0.5±0.1	0.9±0.1	3.5±0.2
–	0.02	–	0.02

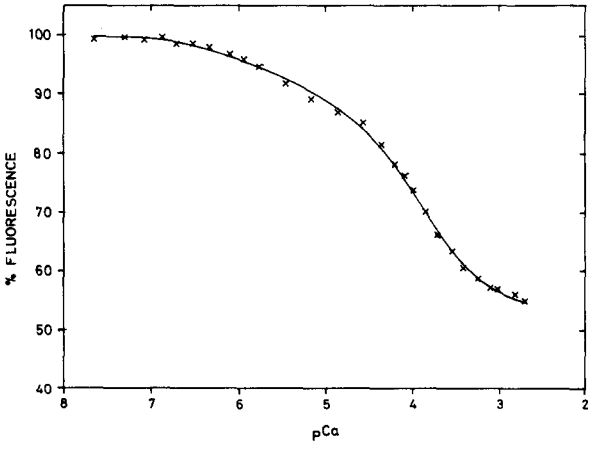

Fig. 2. Fluorescence titration of specifically labelled vesicles (40 $\mu g \cdot ml^{-1}$) in 100 mM KCl/1.0 mM EGTA/10 mM NaMops (pH 7.0), with $CaCl_2$. ×,experimental data; ———, fitted curve.

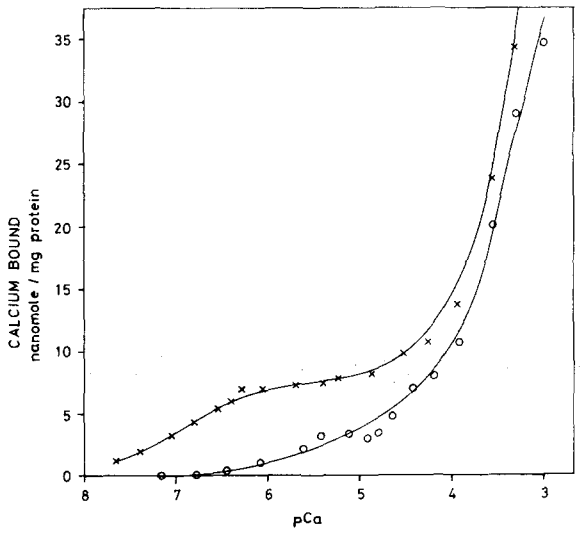

Fig. 3. Ca^{2+} binding by native and specifically labelled vesicles at various Ca^{2+} concentrations. ×, native vesicles; ○, specifically labelled vesicles. Continuous line represent fitted curves.

nmol Ca^{2+} bound per mg protein).

The $K_d \sim 4$ μM binding is ascribed to a single site, corresponding to the higher affinity binding ($K_d \sim 2.7$ μM) observed in the fluorescence titration. The $K_d = 0.12$ mM site observed by fluorescence titration cannot be distinguished by direct Ca^{2+} binding experiments, due to the masking effect of the large extent of low affinity binding (presumably by contaminating low-affinity Ca^{2+} binding proteins). The above results show that NCD-4 has labelled two Ca^{2+} binding sites of the (Ca^{2+} + Mg^{2+})-ATPase, the modified sites being sharply differentiated into high ($K_d \sim 2.7$ μM) and low ($K_d \sim 0.12$ mM) affinity binding sites.

Further evidence for the existence of two distinct Ca^{2+} binding sites derives from analysis of the kinetics of the fluorescence quench induced by Ca^{2+} addition to specifically labelled vesicles. An excellent fit to a double exponential process is obtained (chi squared double exponential = 0.20, chi squared single exponential = 14.2). One component, associated with 23% of the fluorescence change, has a rate constant of 0.067 s^{-1}. The slower component associated with 77% of the fluorescence change, has a rate constant of 0.0089 s^{-1}. These rate processes probably involve the responses of the $K_d \sim 2.7$ μM and $K_d \sim 0.12$ mM sites to Ca^{2+} binding.

The fluorescence response of specifically labelled vesicles to the addition of ATP is shown in Fig. 4. Vesicles preequilibrated with Ca^{2+} (1 mM) show a rapid rise in fluorescence on adding sub-micromolar concentrations of ATP, this is followed by a relatively slower decrease in fluorescence. These responses are strictly Ca^{2+} dependent and are not induced by the ATP analogues Ado*P*[NH]*P* or Ado*PP*[CH_2]*P*. Vesicles labelled at non-specific sites only show no response. These results indicate that the specifically labelled enzyme retains Ca^{2+}-dependent ATPase activity. In TABLE I, steady-state phosphoenzyme levels formed by specifically labelled vesicles in the presence of Ca^{2+} (1 mM) are shown: both NCD-4 and DCCD inhibited preparations form significant amounts of phosphoenzyme under our conditions. Decreasing [Ca^{2+}] to approx. 20 μM halves the amount of phosphoenzyme formed by the carbodiimide-inhibited preparations, control levels remain unaffected. Residual (Ca^{2+} + Mg^{2+})-ATPase activity in NCD-4 inhibited preparations (always 5–10% of initial activity [1]) may thus be ascribed to labelled enzyme of attenuated activity. No basic (Ca^{2+}-independent) ATPase activity could be detected in our control preparations. The fluorescence response to ATP may thus be ascribed to Ca^{2+} removal from NCD-4 labelled sites (presumably by translocation), giving rise to the fluorescence increase. Reoccupancy of the sites by Ca^{2+} would occur after complete hydrolysis of ATP, resulting in a quenching of fluorescence. Addition of equimolar ATP (relative to ATPase) produces the response shown in Fig. 4A (inset), and analysis of the fluorescence decrease (Fig. 4A) indicates a first order process with a rate constant of 0.053 s^{-1}. Addition of a 5-fold excess of ATP (Fig. 4B inset) results in an initial increase in fluorescence to its original unquenched level. After a lag period, fluorescence decays to its Ca^{2+} quenched level, kinetic analysis in this case (Fig. 4B) indicates a double exponential process with rate constants 0.058 s^{-1} and 0.0088 s^{-1}. These rates are in good agreement with those obtained on rapid mixing of Ca^{2+} (1 mM) with the specifically labelled enzyme, and this indicates that both Ca^{2+} binding sites are coupled to the phosphorylation site.

Preliminary measurements on the fluorescence

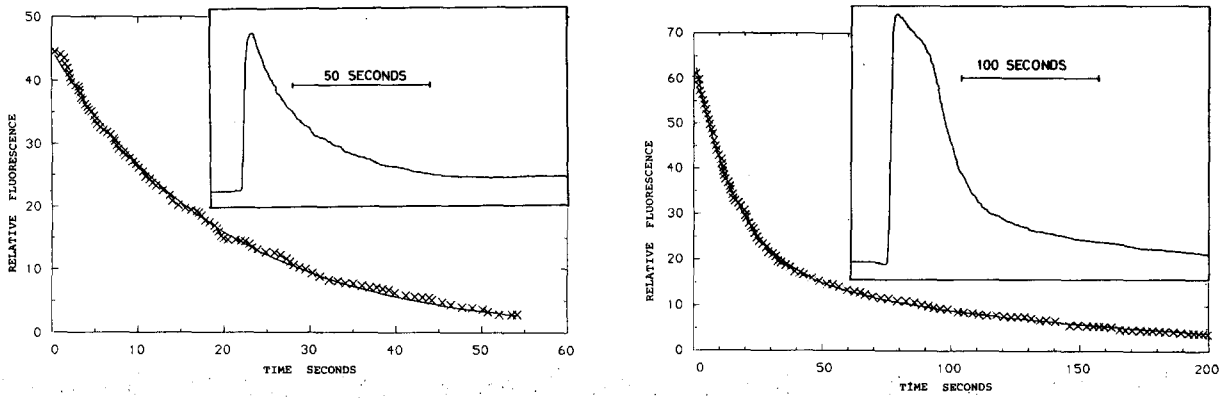

Fig. 4. Stopped-flow fluorescence response of Ca^{2+}-equillibrated specifically labelled vesicles to ATP. Syringe 1 contained vesicles (240 $\mu g \cdot ml^{-1}$)/100 mM KCl/1 mM EGTA/2 mM $CaCl_2$/25 mM NaMops (pH 7.0). Syringe 2 contained 100 mM KCl/1 mM EGTA/2 mM $CaCl_2$/25 mM NaMops (pH 7.0)/1 μM or 5 μM ATP. (A) Kinetic analysis of response to a stoichiometric concentration of ATP (0.5 μM) relative to ATPase (approx. 0.5 μM). (B) Response to the addition of excess ATP (2.5 μM). Inset figures are the original traces from which data were obtained for the respective kinetic analyses.

rise induced by ATP (a 5-fold excess) show an increase in fluorescence with a rate constant of 2.1 s^{-1}. Removal of Ca^{2+} by rapid mixing with excess EGTA (final concentration EGTA = 2.5 mM, final concentration of $CaCl_2$ = 0.5 mM) results in a return of fluorescence to its original unquenched level with an apparent rate constant of 1.3 s^{-1}. This may reflect Ca^{2+} removal from a different conformational species of the ATPase molecule in the two experiments, but further studies at improved time resolution are required to resolve this question.

Discussion

The above results are best interpreted on the basis that the NCD-4 inhibited ATPase, incorporating approx. 2 moles NCD-4 per mole enzyme at the Ca^{2+}-protected sites, and retaining significant Ca^{2+} binding capacity, also retains the ability to hydrolyse ATP via phosphoenzyme formation and to presumably translocate Ca^{2+}. NCD-4 is therefore regarded as a covalent modulator of (Ca^{2+} + Mg^{2+})-ATPase activity, with the covalently incorporated NCD-4 acting as an extrinsic fluorescent probe reporting both Ca^{2+} binding, ATP hydrolysis, and Ca^{2+} translocation.

Specifically labelled enzyme is formed by chemical modification of two Ca^{2+} binding sites found in the native enzyme: these two sites are most likely those involved in Ca^{2+} translocation. Thermodynamic and kinetic analysis of the Ca^{2+}-binding data (obtained by fluorescence titration and direct binding measurements) indicate the presence of two independent Ca^{2+} binding sites with K_d = 2.7 μM and 0.12 mM. The binding capacity of the weaker of the two sites could not be assessed, due to swamping by the large number of low affinity sites in the vesicles. The higher-affinity site had a binding capacity of 3.5 nmole Ca^{2+} per mg vesicular protein, equivalent to approx. 50% of the high affinity binding capacity of native vesicles. The labelled sites also proved kinetically distinct, with slow fluorescence quenching processes induced by excess (1 mM) Ca^{2+} of 0.067 s^{-1} and 0.0089 s^{-1}. These slow processes are unlikely to directly result from Ca^{2+} binding to the sites, but presumably reflect slow conformational changes reported independently from each site. These are presumably protein conformational changes triggered by Ca^{2+} binding, resulting in quenching of fluorescence. Studies in progress are designed to reveal whether Ca^{2+}-induced quenching is a consequence of increased solvent exposure of the fluorophores, or whether an increased degree of rotational motion of the bound fluorophores is responsible. If such changes are considered essential for the activity of the labelled enzyme, an immediate explanation for its attenuated activity is available in that the rate limiting steps (even in the

presence of saturating (1 mM) calcium concentrations), are the conformational changes subsequent to Ca^{2+} binding. Although several reports of conformational changes triggered by Ca^{2+} binding have appeared [8,9], few kinetic data are available, and may not be immediately comparable with the present study. Monitoring tryptophan fluorescence of native ATPase enable kinetic characterisation of a Ca^{2+}-triggered process which is relatively faster than described here (approx. 5 s^{-1} at 22°C) and which may be related to the cooperative transition involved in binding the two calcium ions [9]. As the two sites in the NCD-4 labelled ATPase behave independently, local conformational changes at each site are presumably being monitored.

Coupling of both NCD-4 modified sites to ATP hydrolysis is also demonstrable, indicating that the two NCD-4 modified Ca^{2+}-binding sites are most likely those involved in Ca^{2+} transport in the native enzyme. Kinetic analysis of the slow phase of the ATP response indicates that the higher affinity site ($K_d \sim 2.7$ μM) only is operative during the first turnover of the specifically labelled enzyme (addition of one equivalent of ATP). During subsequent catalytic cycles (induced by the presence of excess ATP) Ca^{2+} removal from the lower-affinity site ($K_d = 0.12$ mM) also takes place. This finding implies that translocation via the low-affinity site only takes place when the high-affinity site is unoccupied (has already translocated Ca^{2+}), and also indicates that transfer of Ca^{2+} between the sites does not occur. A further implication would be that the two sites in the NCD-4 labelled enzyme translocate Ca^{2+} independently and in parallel. Rate constants for the slow phase of the ATP response correspond closely to those obtained by mixing Ca^{2+} with specifically labelled vesicles, identifying this phase as a quenching triggered by reoccupancy of Ca^{2+} binding sites. The relatively fast rise in fluorescence observed on ATP addition is comparable in magnitude and rate to that observed on mixing Ca^{2+}-equilibrated vesicles with EGTA, and is thus identified as Ca^{2+} removal from both sites, presumably by translocation. Further studies on the ATP response are in progress.

Finally, the ability of the carbodiimide-modified ATPase to form phosphoenzyme needs comment. In view of the attenuated Ca^{2+} binding levels, adequate [Ca^{2+}] must be present in order to trigger the requisite conformational changes activating ATPase activity. One report [3] claims that ($Ca^{2+} + Mg^{2+}$)-ATPase inhibited by DCCD forms no phosphoenzyme. Ca^{2+} levels were not reported, however, and insufficient Ca^{2+} levels may explain these results.

NCD-4 emerges as a useful structural and dynamic probe of ATPase activity.

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