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Fluorescence properties of the Ca^{2+},Mg^{2+}-ATPase protein of sarcoplasmic reticulum labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole

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The fluorescence intensity of the Ca^{2+},Mg^{2+}-ATPase protein of rabbit skeletal sarcoplasmic reticulum that incorporated about 2 mol of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was enhanced at high MgATP concentrations with or without 50 μM calcium. The observed enhancement indicates that the fluorophore, NBD-Cl, can detect conformational changes in the ATPase protein.

Sarcoplasmic reticulum; Skeletal muscle; ATPase; Fluorescence labeling

1. INTRODUCTION

The primary structure of the Ca^{2+},Mg^{2+}-ATPase (ATP phosphohydrolase (Ca^{2+}-transporting), EC 3.6.1.38) protein of skeletal muscle sarcoplasmic reticulum, SR [1–4] has been essentially elucidated [5–8] and its N- and C-terminal segments appear to be located on the cytoplasmic side [9]. Recent mutagenesis [10] and affinity labeling studies [11] have reported on the nucleotide sites of the ATPase protein [10,11]. The calcium binding sites of the ATPase protein are probably located within the center of the transmembrane domain [12]. A limited number of thiols of the Ca^{2+}, Mg^{2+}-ATPase have been described as being essential based on their reactivity toward sulfhydryl reagents [13–19]. In general, these modifications induced changes in the conformation of the ATPase protein some of which occurred in a Ca^{2+}-dependent manner [13–17]. Conformational changes associated with the ATPase protein have been monitored by fluorometric methods as detected by different fluorophores some of which were quite bulky or their binding was non-covalent (e.g. see [20–30]. In this regard, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), a fluorescent analog of the adenine moiety of ATP, has been used for covalent modification of a variety of ATPase pump proteins [18,31–34] and structural features of the various NBD-proteins were revealed based on their fluorescence properties [31,35–38]. This paper will show that Mg-ATP induced an enhancement of the fluorescence of the NBD-ATPase protein from SR.

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2. MATERIALS AND METHODS

Rabbit skeletal sarcoplasmic reticulum was prepared and its ATPase protein was isolated by a procedure described previously [18,39]. The ATPase protein was suspended in 0.4 M sucrose, 20 mM Tris-HCl (pH 7.4) at a concentration of 15 mg/ml for storage at 4°C or in 1 M sucrose, 20 mM Tris-HCl for storage at −80°C. The biuret method was used with bovine serum albumin as a standard [40]. The Ca^{2+}-dependent ATPase activity of the ATPase protein of SR was determined as described recently [18]. Typically, the independent and total activities for the isolated SR-ATPase protein were 0.11 and 3.0 μmol of P_i per min per mg protein, respectively. Unless otherwise stated, the Ca^{2+}-ATPase activity refers to the Ca^{2+}-dependent ATPase activity.

The ATPase protein from SR (2 mg/ml) in 0.4 M sucrose (pH 7.5) was treated with 25 mM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, NBD-Cl, or radiolabeled [^{14}C]NBD-Cl, to a final concentration of 0.8 mM at 25°C for 2.5 min as described recently [18]. Fluorescence spectral measurements were obtained at 25°C with a Perkin-Elmer model LS-5B Spectrofluorometer with a 3600 LS data station and stirrer. The slit widths for the excitation and emission scans were 15 and 5 nm, respectively. The spectra were smoothed by computer analysis [29] and these experiments were performed in triplicate.

A 25 mM stock solution of 7-chloro-4-nitro[^{14}C]benzo-2-oxa-1,3-diazole (Research Products International Corp.) had a specific radioactivity of 5.25×10^6 cpm/μmol [18]. Stock Ca-EGTA buffer solutions were prepared and free Ca^{2+} concentrations were calculated from a Ca-EGTA binding constant [18,39].

3. RESULTS AND DISCUSSION

Modification of the ATPase protein of SR with 0.8 mM NBD-Cl for 2.5 min resulted in the incorporation of 2.5 mol of the reagent and concomitantly, about 68% of the Ca^{2+}-dependent ATPase activity remained. This is consistent with our recent findings [18]. The

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spectral data and absorption measurements obtained recently for the NBD-ATPase protein [18] suggested that the NBD probe could be a useful monitor of changes in protein conformation [36–38]. To test this, fluorescence properties of the NBD-ATPase protein were studied. The fluorescence excitation and emission scans of the NBD-ATPase protein showed an excitation maximum at 430 nm and an emission maximum at about 512 nm (fig.1). These values are close to those obtained for NBD-proteins whose labeling was specific for sulfhydryl groups of cysteine residues [35,36]. This establishes further that NBD-Cl modified sulfhydryl groups of the ATPase protein [18].

When MgATP was added to the NBD-ATPase protein of SR in the absence of calcium the fluorescence intensity was enhanced (fig.2). For example, the emission difference spectrum of the NBD-ATPase protein in the presence of increasing concentrations of the ATP showed concurrent increases in the maximum intensity at 515 nm (compare spectrum 1, no additions with spectra 2–4 with Mg-ATP in fig.2). Interestingly, there was little shift to either the red or blue region of the spectrum. It is noteworthy that essentially the same pattern of enhancement was observed when MgADP was added (not shown). The fluorescence increase was dependent, in part, upon the concentration of MgATP (fig.3). In

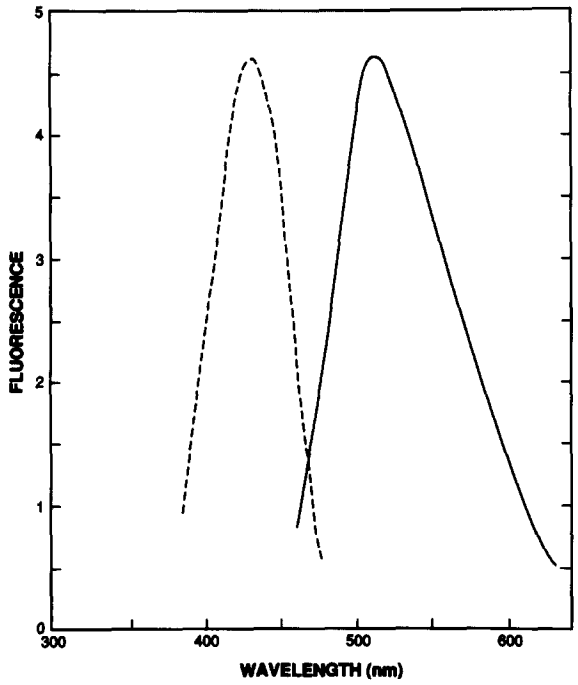

Fig.1. Fluorescence excitation and emission difference spectra of the NBD-ATPase protein of SR. The ATPase protein of SR was modified with NBD-Cl at 0.8 mM final concentration for 2.5 min at pH 7.5 as described in section 2. Excitation and emission data were obtained using NBD-ATPase protein at 25 μg/ml in 0.4 M sucrose, 20 mM Tris-HCl (pH 7.0). The scans represent the difference spectra between NBD-ATPase and ATPase protein alone. Fluorescence values correspond to arbitrary units. Solid line = emission difference spectrum; broken line = excitation difference spectrum. See the text for further details.

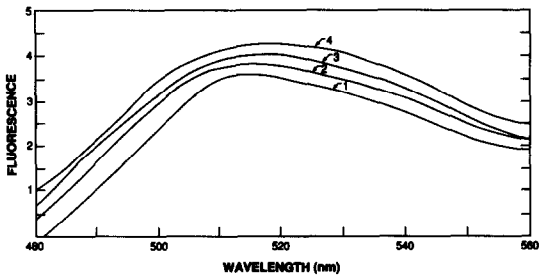

Fig.2. Effect of MgATP on the fluorescence emission difference spectra of the NBD-ATPase protein of SR. The NBD-ATPase protein of SR was prepared as described in the legend to fig.1. The scans represent the difference spectra between NBD-ATPase protein alone incubated at pH 7.0, 25°C, in the presence and absence of metalnucleotides. Spectrum 1 = no additions; spectra 2–4 = 0.50, 5 and 50 μM MgATP which correspond to mol ratios of 2.0, 20 and 200:1 (MgATP: ATPase protein, mol/mol), respectively.

the absence of Ca^{2+}, and at low ATP concentrations that ranged between 10^{-8} M and 5×10^{-6} M, the fluorescence intensity was about 6%. This is probably a consequence of conformational changes in the ATPase protein induced by NBD-Cl binding. However, at

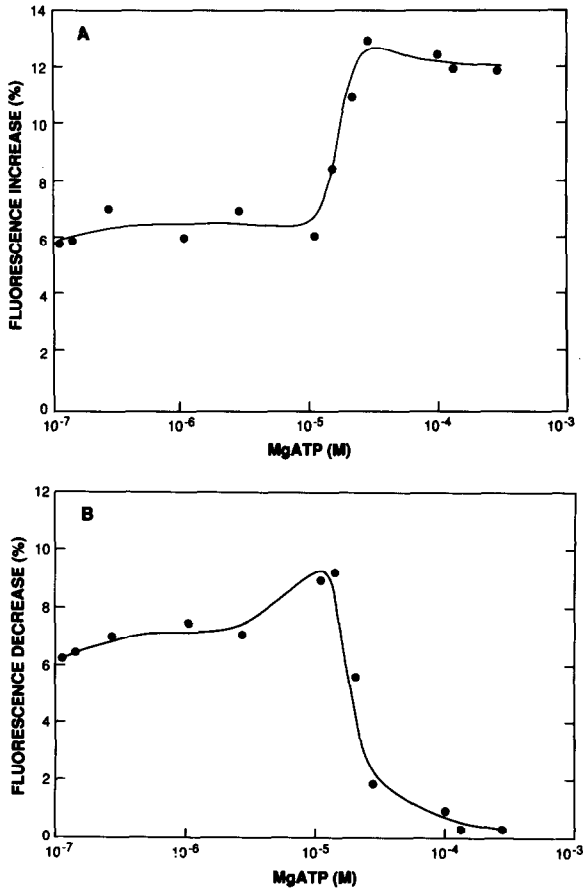

Fig.3. Effect of MgATP on fluorescence intensity of NBD-ATPase protein of SR. The NBD-ATPase protein was incubated in the presence and absence of various concentrations of MgATP as described in the legend to fig.2 and in the text. *Panel A = no calcium: panel B = 50*μM $CaCl_2$.

higher concentrations of MgATP there was an enhancement of the fluorescence intensity (fig.3A). It would appear that an enzyme transition (from lower to higher fluorescence enhancement was induced by MgATP binding to the NBD-ATPase. The half-maximal change in fluorescence occurred at 35 μM MgATP suggesting that the NBD probe was able to detect conformational changes at the low affinity nucleotide binding sites [20,22,23,41]. This is consistent with recent findings which showed that NBD-Cl modified thiols of the ATPase protein at sites other than the hydrolytic site [18].

When 50 μM Ca^{2+} was added under similar conditions quenching of the fluorescence intensity was observed but it was reduced at high ATP concentrations (fig.3B). The pattern of half-maximal changes at 35 μM ATP establishes further the idea that low affinity site interaction between nucleotide and modified ATPase protein can be monitored by the label. ATP induced a change in the conformation of the ATPase protein in the presence or absence of Ca^{2+} (fig.3). It is noteworthy that incubation of the NBD-ATPase protein with various concentrations of ionized Ca^{2+} did not show any Ca^{2+}-dependent change in fluorescence intensity compared to controls. There was a non-specific quenching of fluorescence intensity of about 6% at Ca^{2+} concentrations that ranged between 0.1 μM and 50 μM (not shown). This is in agreement with recent data which showed that NBD-Cl modification of the ATPase protein or intact SR was not Ca^{2+}-dependent [18]. The labeling of thiols of the ATPase protein was not associated with regions involving Ca^{2+} binding. In conclusion, NBD-Cl is a useful reagent in detecting changes in the structure of the ATPase protein of SR as measured by its fluorescence emission.

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