Involvement of the 50-kDa Peptide of Myosin Heads in the ATPase Activity Revealed by Fluorescent Modification with 4-Fluoro-7-nitrobenz-2-oxa-1,3-diazole*

Toshiaki Hiratsuka
From the Department of Chemistry, Asahikawa Medical College, Asahikawa, 078-11, Japan

The fluorescent reagent 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) reacted specifically with 1.9 lysyl residues/mol of the myosin subfragment-1 (S-1) ATPase. When 1.9 lysyl residues were modified, the K* and Ca**-ATPase activities were almost completely inhibited, whereas the Mg**-ATPase activity was increased to 180% of original activity. The actin-activated Mg**-ATPase activity was decreased to 30% of original activity by this modification. However, affinity of S-1 for actin in the presence of ATP was unchanged. The NBD fluorescence of the modified S-1 was quenched on addition of ATP, suggesting that ATP induced conformational changes around the NBD of S-1. Tryptic digestion of the modified S-1 revealed that the NBD groups are attached mainly to the 50-kDa peptide of S-1, more precisely the 45-kDa peptide. These results confirm the recent reports that the 50-kDa peptide of S-1 is involved in the myosin ATPase reaction (Körner, M., Thiem, N. V., Cardinaud, R., and Lacombe, G. (1983) Biochemistry 22, 5843-5847; Hiratsuka, T. (1986) Biochemistry 25, in press).

Myosin subfragment-1 (S-1) is the segment of the myosin molecule containing the sites responsible for ATPase activity and binding of actin (1). In recent years, significant gains in characterizing the topology of S-1 have been made possible by the application of limited trypptic proteolysis of S-1. As established by Balint et al. (2), limited trypptic proteolysis of the heavy chain of S-1 produces mainly three peptide fragments of 26, 50, and 26 kDa which are aligned in this order within the heavy chain (3). A number of amino acid residues on the S-1 heavy chain have long been suggested as being essential for the myosin ATPase. Well-defined functional groups have been located mainly in the 20- and 26-kDa tryptic peptides of S-1. This is the case for the reactive lysyl residue located in the 26-kDa peptide (4-6) and the reactive cysteinyl residues, SH1 and SH2 (7), located in the 20-kDa peptide (8, 9). Modification of these reactive lysyl and cysteinyl residues markedly affects the ATPase properties of S-1. On the other hand, arginyl residues have been shown to be essential in the ligand-binding sites of several enzymes that transfer phosphorus-containing groups (10). Indeed, the phenylglyoxal modification of arginyl residues of S-1 affected the K*, Ca**, and actin-activated ATPase activities, and the nucleotide substrates afforded sufficient protection against the inactivation (11, 12). Unfortunately, attempts made to determine the location of the residues on the S-1 heavy chain have been unsuccessful because of the chemical instability of the phenylglyoxal-arginine bond.

Recently, we have suggested that the ATPase site of S-1 resides between the 50- and 26-kDa peptides, and the 50-kDa peptide acts as a communicating apparatus between the ATPase and actin-binding sites (13, 14). This important role of the 50-kDa peptide has also been suggested by a number of recent observations that, when ATP binds to S-1, conformational changes occur at the 50-kDa peptide (15-20). Although the 50-kDa peptide comprises almost half of the total mass of S-1, little is known about functional groups in this peptide responsible for the ATPase activity. The only known functional group is a single carboxyl group, which is modified with carbodiimides in the presence of the nucleophile nitrotyrosine ethyl ester (21, 22). The modification results in a parallel inactivation of the K*, Ca**, and Mg**-ATPase activities of S-1. Recently, Morlet and Ue (23) have reported that lysyl residues in the 50-kDa peptide of S-1 are selectively modified with the fluorescent reagent 6-carboxyfluorescein in the presence of carbodiimide. However, these lysyl residues were found to be nonessential for the S-1 ATPase activity.

The fluorescent reagent 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) has been used to modify lysyl, cysteinyl, and tyrosinyl residues on a number of proteins (25-31). The reaction products with lysyl and cysteinyl residues, especially the former product, have intense absorption and fluorescence emission properties which are sensitive to polarity of the environment of protein (24-26). Because of these spectroscopic characteristics, the reagent has been chosen for a site-directed fluorescent probe for the active site of several enzymes (26, 30, 31). In the present paper, we describe the fluorescent modification of the S-1 ATPase with 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F), the 4-fluoro analog of NBD-Cl (32). NBD-F is superior to NBD-Cl in regard to the reactivity toward lysyl residues on proteins (27, 32). Indeed, our results indicate that NBD-F reacts selectively with 2 lysyl residues in the 50-kDa peptide of S-1, markedly affecting the ATPase properties.

**EXPERIMENTAL PROCEDURES**

Materials—NBD-F and NBD-Cl were purchased from Dojira Co. α-Chymotrypsin, diphenylcarbamyl chloride-treated trypsin, and soybean trypsin inhibitor were from Sigma. ATP, ADP, and AMP were from Koijin Co. All other reagents were of reagent or biochemical research grade.

Preparations of Proteins—Rabbit skeletal myosin was prepared by the method of Perry (33) with slight modification. S-1 was prepared...
by chymotryptic digestion of myosin (34) with modification as described by Weeds and Taylor (35). Trypsin-split S-1 was prepared as described previously (14). F-actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (36).

Fluorescent Modification of S-1—Fluorescent modification of S-1 with NBD-F was routinely performed in 40 mM borate (pH 8.5), 0.5 mM MgCl$_2$, and 50 mM NaCl. S-1 was incubated at 2 mg/ml with an 8-fold molar excess of NBD-F (in acetone) over S-1 at 25°C in the dark. The final acetone concentration was 1%. The reaction was stopped by the addition of 2-mercaptoethanol at a final concentration of 100 mM. The reaction mixture was further incubated for 10 min in ice to remove the NBD groups attached to cysteinyl and tyrosinyl residues. The modified S-1 was then separated from the unreacted NBD-F and the NBD-mercaptoethanol by passage through a 1.5 x 9-cm column of Sephadex G-50 equilibrated with 20 mM Tris/Cl (pH 8.5) and 50 mM NaCl. For the measurements of ATPase activities, the samples were compared to a control subjected to the same conditions but excluding NBD-F. The stoichiometry of the NBD-lysyl residue was determined from the absorption spectrum using an extinction coefficient of 2.6 x 10$^4$ M$^{-1}$cm$^{-1}$ at 480 nm (25, 29). The modified S-1, in which 1.6-1.9 lysyl residues had been modified, was used for all experiments unless otherwise noted.

Trypsin Digestion of S-1—Limited cleavage by trypsin was performed at 25°C in 50 mM NaCl and 20 mM Tris/Cl (pH 8.5) using a molar ratio of 1:100 for trypsin/S-1. In the presence of 5 mM Mg$^{2+}$-ATP, the molar ratio of trypsin to S-1 was increased to 1:5 (16). SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel (7.5%) electrophoresis was carried out according to Weber and Osborn (37). To follow the time course of the process of trypdic digestion of S-1, 50-100-μl aliquots of sample were withdrawn periodically and pipetted into equal volumes of a solution containing 2% SDS, 10% 2-mercaptoethanol, 34% sucrose, and 0.1 M sodium phosphate (pH 7.0). Each mixture was heated in boiling water for 3 min and then subjected to SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Brilliant Blue.

For observing the fluorescent bands, gels were washed with 5% ethanol and 7.5% acetic acid at room temperature for 4 h with two exchanges of the solution (30 ml each/gel). Fluorescent bands were visualized by illumination of the gel with a 366-nm light source (Camag Deluxe UV lamp, 16 watts) in a dark room. Gels were photographed using Kodak Tri-X pan film and a green filter (Kenko ND4). In order to determine the distribution of the NBD fluorescence among the 20-, 26-, and 50-kDa peptides, the modified S-1 was subjected to limited trypdic digestion for 1 h as described above. The digests (400 μg) were divided into four parts and subjected to SDS-polyacrylamide gel electrophoresis (100 μg/gel). After electrophoresis, gels were stained with Coomassie brilliant blue. There was no evidence from the spectrum of the product for significant reaction of the tyrosinyl residue in S-1 with NBD-F. Treatment of the modified S-1 with 2-mercaptoethanol caused the immediate bleaching of the yellow solutions, eliminating the absorption maximum at 425 nm of the NBD-cysteine adduct. There was evidence from the spectrum of the modified S-1 that the NBD-cysteine and NBD-tyrosine bonds can be split by sulfhydryl reagents, but the stable NBD-lysyl bond is resistant to the reagents (26, 30). Thus, the reaction products with amino acid residues can be easily distinguished from others on the basis of the spectral and chemical characteristics of the product.

As shown in Fig. 1, the absorption spectrum of S-1 modified with NBD-F, which was recorded after removal of excess reagent by gel filtration, shows four absorption maxima at 280, 340, 425, and 480 nm (curve a). Taking into account the spectral characteristics of NBD derivatives described above, the maxima at 340 and 480 nm are associated with the NBD-lysine adduct and the maximum at 425 nm with the NBD-cysteine adduct. There was no evidence from the spectrum of the modified S-1 that the NBD-cysteine adduct with NBD-F. Treatment of the modified S-1 with 2-mercaptoethanol caused the immediate bleaching of the yellow solutions, eliminating the absorption maximum at 425 nm of the NBD-cysteine adduct (curve b). However, two absorption peaks of the NBD-lysine adduct at 340 and 480 nm were not affected by the treatment. Consequently, even after 2-mercaptoethanol treatment, the sample fluoresced strongly with an emission maximum at 530 nm (curve b), characteristic of the NBD-lysine adduct (25, 26, 30). Thus, fluorescent emission spectra of unmodified S-1 and actin were assumed to have molecular weights of 120,000 (35) and 42,000 (42), respectively.

**RESULTS**

Reaction of S-1 with NBD-F—NBD-CI can react with lysyl, cysteinyl, and tyrosinyl residues of proteins (25-31). The spectral characteristics of NBD derivatives depend on the nature of reacting group; NBD-cysteine and NBD-tyrosine adducts have a major visible absorption maximum at 425 nm (28, 30, 31) and 380 nm (26, 29), respectively, whereas an NBD-lysine adduct has those at 340 and 480 nm (25, 26, 29, 30). Additionally, it is well-known that the labile NBD-cysteine and NBD-tyrosine bonds can be split by sulfhydryl reagents, but the stable NBD-lysine bond is resistant to the reagents (26, 30). Thus, the reaction products with amino acid residues can be easily distinguished from others on the basis of the spectral and chemical characteristics of the product.
Fluorescent Modification of Myosin ATPase

the modified S-1, in which only lysyl residues are modified, can be prepared by removing any residual NBD group attached to cysteinyl and tyrosinyl residues by incubation with 2-mercaptoethanol, followed by gel filtration, to remove excess 2-mercaptoethanol and any released NBD-mercaptoethanol.

Since the molar absorption coefficient for the NBD-lysine adduct is known (25, 29), it is possible to evaluate the number of modified lysyl residues of S-1. Fig. 2 shows the time course of the modification of the lysyl residue, which was followed with a %fold molar excess of NBD-F over S-1. The data indicate that the modification of the lysyl residue is relatively rapid for the first 5 min and becomes much slower thereafter. After 30 min of reaction, 1.9 lysyl residues/mol of S-1 were modified. Electrophoretograms of modified S-1 show a single fluorescent band, corresponding to the S-1 heavy chain, even after 30 min of the reaction (Fig. 2, inset). The absence of other fluorescent bands indicates that the modification is specific for the S-1 heavy chain.

Localization of the NBD-modified Lysyl Residues in S-1—To ascertain to which tryptic peptide of the S-1 heavy chain the NBD group binds covalently, we studied the limited cleavage of modified S-1 by trypsin. Fig. 3 shows the time course of tryptic digestion of the modified S-1. Modified S-1 was found to be digested by trypsin in a manner similar to unmodified S-1, as indicated by generation of three main tryptic peptides of 20, 26, and 50-kDa (Fig. 3A). Upon tryptic digestion, the yellow fluorescence of the NBD group, originally seen only in the S-1 heavy chain, progressively appears in the 75-kDa peptide, which is the precursor to the 50- and 26-kDa peptides, and finally mainly in the 50-kDa peptide (Fig. 3B). Fluorescence at the regular position of the 26-kDa peptide, however, suggests that the NBD group is also associated slightly with the 26-kDa peptide. The fluorescence in the 20-kDa peptide was negligible. In a separate experiment, the fluorescence of the NBD group which had been extracted from each peptide band was measured. The distribution of fluorescence was 89, 9, and 2% in the 50-, 26-, and 20-kDa peptides, respectively, confirming the results of the fluorescence photography shown in Fig. 3B. A similar result was obtained when trypsin-split S-1 was modified with NBD-F; the fluorescence of the NBD group was observed again mainly in the 50-kDa peptide and slightly in the 26-kDa peptide (Fig. 3C). These results suggest that the lysyl residues in trypsin-split S-1 are modified with NBD-F in a manner similar to those in intact S-1.

In a second experiment, we submitted the modified S-1 to trypsin cleavage, using a trypsin/S-1 molar ratio of 1:5 in the presence of 5 mM Mg²⁺-ATP. In this case, the 50-kDa peptide is further degraded into a 45-kDa peptide (16). This 45-kDa peptide results from cleavage of a 5-kDa peptide in the COOH terminus of the 50-kDa peptide (43). Comparison of panels A and B of Fig. 4 shows that the modified S-1 undergoes a Mg²⁺-ATP-induced conformational change in a manner similar to unmodified S-1, generating the 45-kDa peptide. Illumination of the gel reveals that the NBD fluorescence is associated not...
Fluorescent Modification of Myosin ATPase

ATPase Properties of Modified S-1—The effect of modification of NBD-F on the K⁺-, Ca²⁺-, and Mg²⁺-ATPase activities of S-1 was also followed. Fig. 5 shows the relationship between the percentage of remaining ATPase activity of modified S-1 and the number of NBD-lysyl residues/mol of S-1. For the K⁺- and Ca²⁺-ATPase activities, the activity was decreased linearly with an increasing amount of NBD-lysyl residues (Fig. 5, A and B). In both cases, the modification of 1 lysyl residue/mol of S-1 led to a 50% loss of activity. Extrapolation to complete inactivation indicates that loss of activity correlates with the modification of 2.1–2.2 lysyl residues/mol of S-1. This was taken as an indication that there are 2 lysyl residues/mol of S-1 responsible for the K⁺- and Ca²⁺-ATPase activities. We attributed the somewhat higher experimental values of 2.1–2.2 to the contribution of lysyl residues which react with the reagent without affecting the ATPase activities. In contrast to these results, the Mg²⁺-ATPase activity of S-1 was enhanced by the modification (Fig. 5C). When 1.9 lysyl residues were modified, the activity was increased to 180% of the original activity.

The actin-activated Mg²⁺-ATPase activity of modified S-1, in which 1.6 lysyl residues had been modified, was also studied using a double-reciprocal plot of the ATPase rate against actin concentration. As shown in Fig. 6, the linear regression analysis of the data for the modified and unmodified S-1 were obtained. The Vₘₐₓ for modified S-1 was found to be decreased to 30% of the original value: 7.1 and 2.1 μmol of Pᵢ/min/mg for unmodified and modified S-1, respectively. However, the Kₘ for actin (19 μM) was unchanged by the modification of S-1. These findings indicate that the lysyl residues are essential for the acto-S-1 ATPase activity but nonessential for the binding of actin to S-1 in the presence of ATP.

Interaction of Modified S-1 with ATP—The emission spectrum of modified S-1 excited at 480 nm is a single, well-defined peak with the maximum (uncorrected) at 530 nm (Fig. 7, curve a). The sensitivity of the NBD fluorescence to the binding of ligands to S-1 was investigated. When ATP was added to the modified S-1, the fluorescence of the NBD group was quenched (curve b). However, binding of ATP did not cause any shift in the emission maximum. The fluorescence quenching induced by ATP was also studied at varying nucleotide concentrations (Fig. 7, inset). The fluorescence intensity, plotted against the concentration of ATP added, decreased gradually up to 100 μM ATP, and little or no further decrease occurred. The limiting fluorescence quenching was approximately 11%. A similar result was obtained with ADP (data not shown). AMP and Pᵢ, which are neither substrates nor inhibitors of S-1, had little effect on the fluorescence emission (curve c).

DISCUSSION

NBD-F, the 4-fluoro analog of NBD-Cl, was shown to react with amino groups of amino acids 10 times faster than NBD-
Fluorescent Modification of Myosin ATPase

FIG. 6. Double-reciprocal plots of the actin-activated ATPase activity of the modified (●) and unmodified (□) S-1. The ATPase activity was measured under the same conditions used for the Mg\textsuperscript{2+}-ATPase measurement (Fig. 5) except for the presence of F-actin (0.08-0.4 mg/ml).

FIG. 7. Fluorescence emission spectra of modified S-1 in the presence and absence of various ligands. Fluorescence spectra of modified S-1 were measured in 20 mM Tris/Cl (pH 8.5), 1 mM MgCl\textsubscript{2}, and 50 mM NaCl in the presence and absence of 1 mM ligand with 450-nm exciting light. Curve a, no addition; curve b, +ATP; curve c, +AMP or Pi. Inset; effect of ATP on the fluorescence emission intensity at 530 nm of modified S-1. Aliquots of ATP were added to modified S-1 (0.42 mg/ml) under the conditions described above.

Cl (32). This is the case for the modification of mitochondrial ATPase (27) and the S-1 ATPase (data not shown). The reaction is completed by loss of halide ion to give the modified enzyme (27). A faster rate with an aryl fluoride compared with the chloride is typical of such a reaction (44). All the NBD derivatives of amines exhibit similar excitation and emission maxima at wavelengths in the ranges 470-480 and 520-540 nm, respectively (24, 25, 29). The NBD fluorophore has a favorably high quantum yield in environments of low polarity (0.36 in ethanol) and a very low quantum yield in water (0.005) (24). Furthermore, the acid-stable nature of the derivative enables the amino acid analysis of modified proteins (25). Therefore, the NBD group has long been used as a valuable fluorescent probe to follow the conformational changes of various proteins.

Because of the high reactivity of NBD-F toward lysyl residues, there was the possibility of modification of the well-defined lysyl residue (Lys-83) on the 26-kDa peptide of S-1, which is known to be rapidly modified with 2,4,6-trinitrobenzenesulfonate (4-6). However, the present study clearly indicates that NBD-F reacts preferentially with 2 lysyl residues on the 50-kDa peptide instead of Lys-83 on the 26-kDa peptide. This is the case for the modification of the myosin ATPase with other amine-directed reagents, 6-(N-methylanilino)naphthalene-2-sulfonyl chloride (17, 45), formaldehyde (46), and 6-carboxyfluorescein in the presence of carbodiimide (23); none of these reagents was capable of the specific modification of Lys-83. It is logical to assume that the modification of Lys-83 is highly dependent on the structure of the reagent employed.

The NBD fluorescence of the modified S-1 was quenched on addition of ATP. Conformational changes in S-1 may be invoked to explain the effects of the nucleotide on the fluorescence of modified S-1 since the fluorescence intensity of the NBD-lysine adduct is known to be sensitive to the environment (24, 26, 30). Since fluorescence intensity of the NBD group decreases with increased solvent polarity as described above, the fluorescence quenching induced by ATP corresponds to a transfer of the NBD groups to a more polar or exposed environment.

As shown in Fig. 8, the NBD moiety of NBD-F resembles the adenine moiety of ATP. Thus, we had hoped that NBD-F would be useful for the S-1 ATPase as an active site-directed reagent. As was expected, the modification of S-1 with NBD-F markedly affected the ATPase properties of S-1. The complete loss of the K\textsuperscript{+}- and Ca\textsuperscript{2+}-ATPase activities, in addition to the occurrence, at the same rate, of enzymatic inactivation and modification of 2 lysyl residues, strongly suggests that 2 lysyl residues located in the active site or in the immediate vicinity are critical for these ATPase activities. However, the residues were found to be nonessential for the binding of actin to S-1 in the presence of ATP.

6-Carboxyfluorescein in the presence of carbodiimide (23) and carbodiimide in the presence of nitrotyrosine ethyl ester (21, 22) react with lysyl residues and one carboxyl group, respectively, which are located in the 50-kDa peptide of S-1. More precisely, these residues are located in the 45-kDa peptide, which results from cleavage of a 5-kDa peptide in the COOH terminus of the 50-kDa peptide (43). This is also the case for 2 lysyl residues modified with NBD-F. Thus, it is of interest to compare the effects of the NBD-F modification on the S-1 ATPase with those of the modifications with 6-carboxyfluorescein and carbodiimide.

First, the modification of lysyl residues with 6-carboxyfluorescein did not affect the K\textsuperscript{+}-, Ca\textsuperscript{2+}-, and Mg\textsuperscript{2+}-ATPase activities of S-1 (23). In this regard, these lysyl residues are...
Fluorescent Modification of Myosin ATPase

clearly different from those modified with NBD-F. On the other hand, the modification of the carboxyl group led to a parallel inactivation of the K⁺-, Ca²⁺-, and Mg²⁺-ATPase activities. However, ADP did not protect S-1 against the modification. Thus, this residue has been considered to be involved only in the ATP splitting but not in the ATP binding (22). In contrast to this, taking into account the result that S-1 modified with NBD-F exhibits enhanced Mg²⁺-ATPase activity, the possibility of 2 lysyl residues on the 50-kDa peptide being directly involved in the splitting of ATP by S-1 ATPase would be unlikely. It is more likely that they are essential for the ATPase site to assume an active conformation. Whatever the mechanism for the inhibition of S-1 ATPase by the NBD-F modification is, the present results confirmed the recent reports that the 50-kDa peptide is involved in the myosin ATPase reaction (14, 22).

We can now introduce a specific marker in the 50-kDa peptide of S-1 which is stable and highly fluorescent. The modified ligands other than ATP. Especially, studies with the NBD-F modified as a communicating gate the role of the 50-kDa peptide in the ATPase reaction (22).

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REFERENCES