Biochemistry

 Volume 20, Number 24 November 24, 1981

Interaction of Myosin Subfragment 1 with Cibacron Blue F3GA†

Emil Reisler* and Jane Liu

ABSTRACT: Cibacron Blue F3GA and its immobilized derivatives have been shown before to bind and inhibit nucleotide-dependent enzymes and, among them, myosin subfragment 1. Experiments have been carried out to examine the mechanism of the subfragment 1–dye interaction. Binding of subfragment 1 to immobilized dye (Affi-Gel Blue) does not involve the ATP binding site on myosin. Subfragment 1 hydrolyzes MgATP and CaATP while bound to the Affi-Gel Blue column. Inactivated subfragment 1, which contains [^{3}H]ADP noncovalently trapped at the active site, binds and elutes from the Affi-Gel Blue column in the same manner as unmodified, active protein. Free Cibacron Blue inhibits the ATPase activity of subfragment 1. The inhibition is pH, salt, and time dependent. Complete inhibition correlates with the noncovalent binding of four to five dye molecules per mole of subfragment 1. Three to four of these dye molecules can be preferentially removed from subfragment 1 in the presence of 1 M KCl without relieving the inhibition. This inhibition, which can be traced to one dye molecule per subfragment 1, is reversible and is facilitated in the presence of MgADP and MgATP, suggesting that the dye does not bind at the active site of subfragment 1. Our observations are explained in terms of hydrophobic and electrostatic protein–dye interactions.

Immobilized Cibacron Blue F3GA and other triazine dyes have become popular and widely used tools for purification of a variety of proteins. The Cibacron Blue dye was found to interact with many dehydrogenases (Thompson & Stellwagen, 1976), nucleotide- and polynucleotide-dependent enzymes (Ashton & Polya, 1978; Kumar et al., 1979; Drocourt et al., 1978), and such unrelated proteins as troponin (Reisler et al., 1980), interferon (Jankowski et al., 1976), serum albumin (Travis & Pannell, 1973; Leatherbarrow & Dean, 1980; Dean & Watson, 1979), and others. Consequently, the original suggestion that Cibacron Blue is dinucleotide-fold specific (Thompson et al., 1975; Stellwagen, 1977) has been revised in favor of a less specific protein–dye interaction (Beissner et al., 1979; Ashton & Polya, 1978). These interactions appeared to be hydrophobic (Beissner & Rudolph, 1978; Seelig & Colman, 1979) and sometimes of mixed hydrophobic and electrostatic nature (Reisler et al., 1980; Chambers & Dunlap, 1979). In a few cases, the inhibition of enzyme activities by Cibacron Blue was traced to irreversible protein modification by contaminating materials (Weber et al., 1979) or by the dye itself (Witt & Roskoski, 1980).

An intriguing aspect of the above findings is the apparent diversity of protein–Cibacron Blue interactions on the one hand and the common pattern and features of binding and inhibition of nucleotide-dependent proteins on the other hand. An interesting example in this class of proteins is myosin subfragment 1 (S-1).[1] Subfragment 1 binds strongly to a Blue Sepharose column at low ionic strength and is quickly and quantitatively released on elution with high salt (Toste & Cooke, 1979). Moderate substrate concentrations, between 1 and 5 mM MgATP, do not elute S-1 from the gel, although the free dye inhibits the ATPase activity of myosin and the tension generation in glycerinated muscle fibers. On the basis of these results, it appeared that the blue chromophore might be useful in studies of the myosin–ATP interaction.

In this work, we show that the ATP binding site on S-1 is not directly involved in the adsorption of the protein to the immobilized gel. Furthermore, we demonstrate that the inhibition of myosin ATPase by the free dye results from reversible, time-dependent inactivation of the protein. The inactivation is accompanied by the binding of between four and five dye molecules per myosin head, of which only one inhibits the ATPase reaction.

Materials and Methods

α-Chymotrypsin, ADP, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]ADP was purchased from New England Nuclear. Cibacron Blue F3GA and Procion Blue MX-R (dichlorotriazine dye) were obtained from Polysciences (Warrington, PA). 2-Anthraquinonesulfonic acid was the product of Eastman Kodak Co. (Rochester, NY). Cibacron Blue F3GA covalently attached to cross-linked agarose was purchased from Bio-Rad Laboratories. Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann Co. (Orangeburg, NY), and *p*-phenylenedimaleimide was the

† From the Department of Chemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received May 14, 1981.* This work was supported by U.S. Public Health Service Grant AM 22031.

[1] Abbreviations used: MalNET, *N*-ethylmaleimide; pPDM, *p*-phenylenedimaleimide; S-1, myosin subfragment 1; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-6745$01.25/0

product of Aldrich Chemical Co. All other reagents were of analytical grade.

In view of the reported heterogeneity of commercial preparations of Cibacron Blue F3GA, we have purified this dye following the procedure of Weber et al. (1979). Although spectacularly colored impurities could be separated from the blue dye on a silica gel column, their total amount was negligible (less than 0.1%). The reactivity of the purified blue dye toward S-1 was the same as that of unfractionated, commercial Cibacron Blue. Thus, in subsequent work, we have used the commercial dye preparation.

Preparation of Proteins. Myosin was prepared from rabbit skeletal muscle according to the procedure of Godfrey & Harrington (1970). Subfragment 1 was obtained by chymotryptic digestion of myosin filaments. Digestion and purification followed previously described procedures (Weeds & Taylor, 1975). The purified S-1 was dialyzed into 5 mM KCl and 10 mM Bis-Tris (pH 7.0) buffer. The same buffer was used in all experiments.

Column Experiments. Between 2 and 10 mg of S-1 was applied to a 0.7 × 8 cm Affi-Gel Blue column equilibrated with 5 mM KCl and 10 mM Bis-Tris (pH 7.0) buffer. The gel column was operated at room temperature and at flow rates between 0.5 and 2.0 mL/min. The collected fractions (1.5 mL) were monitored spectrophotometrically. About 90–95% of the applied S-1 was retained on the column. About 90% of the bound S-1 could then be eluted with a pulse of 0.5 M KCl and 10 mM Bis-Tris (pH 7.0) solvent. The bound protein could not be released from the gel by MgATP or CaATP (no more than 5–10% of the adsorbed protein could be detected in column washings with 1–5 mM MgATP or CaATP). The amount of free phosphate in the ATP-containing eluant was determined by the method of Fiske & Subbarow (1925).

Modification of S-1 with p-Phenylenedimaleimide (pPDM) and Trapping of [^{3}H]ADP. The modification of S-1 with the bifunctional thiol reagent pPDM was carried out at 5 °C in 0.03 M KCl and 0.025 M Tris (pH 7.9) and in the presence of 1 mM MgADP ([^{3}H]ADP) (Burke & Reisler, 1977). The protein concentration was about 5 mg/mL, and the molar ratio of pPDM per head was 1.3:1. The reaction was terminated by addition of dithiothreitol. The free [^{3}H]ADP was removed by $(NH_4)_2SO_4$ precipitation followed by protein purification on Sephadex G-50 centrifuge columns (Panefsky, 1977) equilibrated with 5 mM KCl and 10 mM Bis-Tris (pH 7.0). Our ADP trapping procedure follows closely the one described by Wells et al. (1980). On the average, between 0.60 and 0.70 mol of nucleotide was trapped per mol of S-1.

Activity Assays and Dye Binding Measurements. The Ca^{2+} and EDTA (K^+) stimulated ATPases of S-1 were measured at 37 °C by employing the procedures of Kielley et al. (1956). Unless specified otherwise, the Ca^{2+}-dependent activity was measured in the presence of 5 mM KCl and 10 mM Bis-Tris (pH 7.0), and the EDTA-dependent activity was determined in the presence of 0.5 M KCl.

The rate of S-1 inactivation by dyes was followed by periodically removing samples (750 μL) of the reaction mixture and assaying them for ATPase activity. Prior to activity tests, these samples were centrifuged through Sephadex G-50 (fine) columns (Panefsky, 1977) in order to remove the free dye.

The Sephadex G-50 columns used in our experiments were 5-mL syringes fitted with a porous filter near the tip. The columns had a bed volume of 5 mL before they were centrifuged at about 100*g* for 2 min. After the first centrifugation, 750 μL of protein solution (1.1 mg/mL) was placed on top of the column which was then recentrifuged as above. The protein emerged from the syringe practically undiluted and free of unbound dye. In preliminary control experiments conducted with Cibacron Blue dye (0.5 mM) and ADP (1 mM), we established that under the conditions described above the free ligands were quantitively retained on the columns. More detailed description of the Sephadex centrifuge column procedure was presented before by Panefsky (1977).

The purified S-1 samples were used for determinations of ATPase activities and dye binding. The amount of Cibacron Blue bound to S-1 was determined spectrophotometrically by using an extinction coefficient of 13 600 M^{-1} cm^{-1} at 610 nm (Thompson & Stellwagen, 1976). Protein concentrations in these samples were derived from their absorbance at 280 and 290 nm after correcting for the spectral contribution of the bound dye. Whenever necessary, protein concentrations were obtained by microbiuret determinations.

Results

Binding of S-1 to Affi-Gel Blue. A previous study (Toste & Cooke, 1979) described the specific and strong binding of S-1 to the Blue Sepharose column and the inhibition of its CaATPase activity by the free dye. Somewhat surprisingly, 20–30-fold larger amounts of dye were required in this study for the inhibition of the MgATPase activity of S-1. Also, contrary to what might be expected in such a case, neither MgATP nor CaATP could elute S-1 from the Blue Sepharose column (Toste & Cooke, 1979).

In order to clarify the nature of the S-1 interaction with immobilized Cibacron Blue and its relationship to the protein's active site, we have examined the binding of inactivated protein to the immobilized blue dye. S-1 was inactivated by cross-linking the SH_1 and SH_2 groups with the bifunctional thiol reagent pPDM (Burke & Reisler, 1977). When carried out in the presence of MgADP, this modification offers the advantage of nucleotide trapping at the active site (Wells & Yount, 1979). Employing [^{3}H]ADP, we have determined that our pPDM-modified S-1 (90% inactivated) contained 0.65 mol of trapped ADP per mol of S-1. Such S-1 preparations, with [^{3}H]ADP trapped at the active site, could bind and elute from a Blue Sepharose column in the same manner as control, unreacted S-1. The eluted fractions of pPDM S-1 contained between 0.60 and 0.70 mol of [^{3}H]ADP per mol of S-1. The obvious implication of this result is that if, indeed, the trapped nucleotide is locked at the ATPase site (Wells & Yount, 1979) then this site is not directly involved in the binding of S-1 to Cibacron Blue gel.

This conclusion was confirmed in measurements of ATP hydrolysis by column-bound S-1. In these experiments, the protein was applied to an Affi-Gel Blue column in 5 mM KCl and 10 mM Bis-Tris (pH 7.0) and was washed with the same solvent, first in the absence and then in the presence of MgATP (1 mM). Phosphate determinations showed that the fractions eluted in the presence of MgATP contained about 0.4 μmol of P_i/mL. Since these fractions contained, according to our protein determinations, only marginal amounts of S-1, we concluded that MgATP was hydrolyzed by the gel-bound S-1, while passing through the column. On the basis of the amount of S-1 bound to the column, the flow rate, and the amount of free P_i in the eluant, the calculated turnover rates for MgATPase and CaATPase activities were 0.06 and 0.80 s^{-1}, respectively. Given the inaccuracy of such determinations for column-bound protein, the turnover rates are reasonably close to standard ATPase activities of S-1. Thus, although the enzymatic properties of S-1 are probably modified by its binding to the blue gel, these changes do not appear to be large. It should be noted that similar ATP hydrolysis was observed

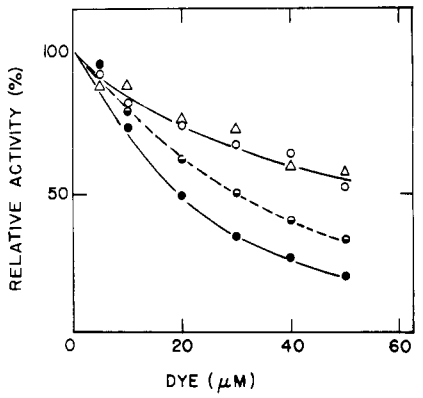

FIGURE 1: Effect of Cibacron Blue F3GA on the ATPase activity of S-1. CaATPase activities were measured in the presence of 5 mM KCl and 10 mM Bis-Tris at pH 7.0 (●), 5 mM KCl and 10 mM Tris at pH 8.0 (⊖), and 0.5 M KCl and 10 mM Bis-Tris at pH 7.0 (○). EDTA ATPase activity (△) was assayed in the presence of 0.5 M KCl and 10 mM Bis-Tris at pH 7.0. The assay mixtures contained 0.01 mg/mL S-1, 1 mM ATP, and either 5 mM Ca^{2+} or 1 mM EDTA. Small aliquots of concentrated dye solution were added to the assay system prior to ATP addition. The incubations were carried out for 5 min at 37 °C. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925).

with S-1 bound to Blue Sepharose columns distributed by other manufacturers.

Inhibition Studies. In view of the unexpected finding that the binding of S-1 to the immobilized Cibacron Blue dye does not involve the ATP site, we have reinvestigated in some detail the interaction of the free dye with S-1. Figure 1 shows, in agreement with Toste & Cooke (1979), that the free dye inhibits the CaATPase activity of S-1. In low ionic strength (pH 7.0), as reported by these authors, the 50% inhibition occurs already at 20 μM dye concentration. It should be noted, however, that in such activity assays the concentration of S-1 is very low (about 0.1 μM) and the effective dye to protein ratio ranges from 50:1 to 500:1. At higher pH (pH 8.0) or in the presence of 0.5 M KCl, the inhibition of ATPase activity is less efficient (middle and upper curves in Figure 1). This is consistent with the effects of pH and salt concentration on the binding of S-1 to the immobilized dye (Toste & Cooke, 1979). Under high salt conditions, both the Ca^{2+} and EDTA (K^+) activated ATPases of S-1 could be measured and were found to decrease at the same rate with increasing dye concentration. Similar inhibition experiments carried out with the agarose-bound dye showed that the blue gel is a rather poor inhibitor of the myosin ATPase.

In order to explore the possibility of irreversible protein modification by Cibracon Blue (Witt & Roskoski, 1980), we have examined the ATPase activity of S-1 preincubated with different amounts of the dye. Subsequent to such preincubations, the unbound dye was removed from the samples by centrifugation through Sephadex columns. Consequently, only the protein-bound dye was transferred into the activity assay mixtures, and its concentration (Figure 2b) was much below that employed in the experiments shown in Figure 1. A characteristic time course of S-1 inactivation due to its preincubation with Cibacron Blue is shown in Figure 2a. The rate of the seemingly "irreversible" S-1 inactivation was significantly increased in preincubations carried out in the presence of 1 mM MgADP (lower curve in Figure 2a) or 1 mM MgATP. A similar effect of ADP on the rate of myosin inactivation is normally detected during modification of the "critical thiols", SH_1 and SH_2. However, it seems unlikely that the SH_1–SH_2 peptide is involved in the dye-induced inhibition of S-1. Neither the premodification of the SH_1 groups

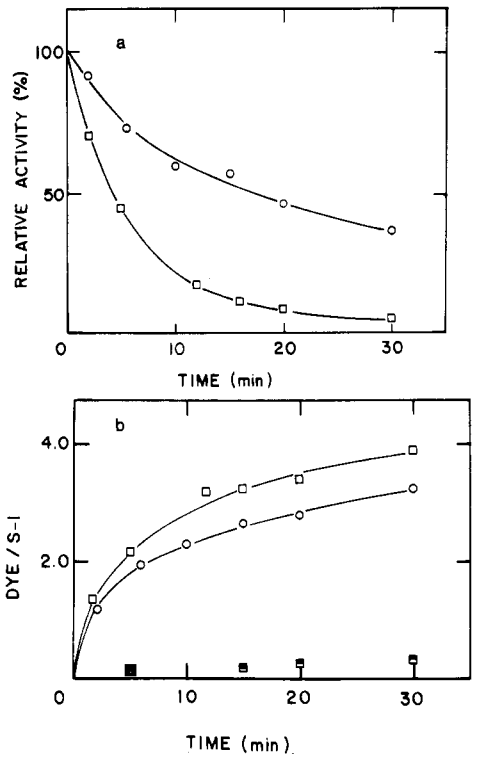

FIGURE 2: (a) Time course of Cibacron Blue F3GA inhibition of S-1 CaATPase. S-1 (10^{-5} M) (1.1 mg/mL) was incubated with 1.5 × 10^{-4} M dye, at room temperature in 5 mM KCl and 10 mM Bis-Tris (pH 7.0), either in the presence of 1 mM MgADP (□) or in its absence (○). At given time intervals, samples were withdrawn from the incubation mixture. They were purified on Sephadex centrifugation columns and assayed for protein content, dye content, and ATPase activity. (b) Binding of Cibacron Blue F3GA to S-1 in the presence (□) and the absence (○) of MgADP as a function of incubation time. These binding data are for the same reaction and protein samples as those shown in Figure 2a. The amount of dye covalently bound to S-1 (■) was estimated from the protein and dye content of reaction samples initially purified on Sephadex centrifugation columns equilibrated with the low-salt solvent followed by purification on columns equilibrated with 6 M guanidine hydrochloride.

with MalNET nor the presence of dithioerythritol had any effect on the time course of S-1 inhibition by Cibacron Blue. Also, no trapping of ADP was detected in S-1 samples inactivated with the blue dye in the presence of MgADP.

Binding Measurements. The amount of Cibacron Blue associated with partially inactivated S-1 was measured by using the same protein samples as shown in Figure 2a. Overall, the binding of Cibacron Blue to S-1 is somehwat faster in the presence of ADP (Figure 2b). Due to a strong adsorption of Cibacron Blue to Sephadex, the actual binding values shown in Figures 2b and 3 set only a lower limit for the true equilibrium binding parameters. (Unfortunately, the free dye does not equilibrate well across a dialysis membrane.) Nevertheless, due to their accuracy and excellent reproducibility under constant separation conditions on Sephadex columns, the reported binding data are of comparative value.

A plot of the residual ATPase activity of S-1 as a function of the amount of bound dye is shown in Figure 3. These data are taken from Figure 2 and from similar experiments in which S-1 has been incubated with a larger excess of the blue dye (0.25 and 0.5 mM). In the incubations carried out in the absence of MgADP, following an initial lag phase the inactivation appears to increase linearly with the amount of the bound S-1 (except for the last 15–20% of residual activity). The total loss of activity can be correlated with the binding of 4.7 ± 0.3 mol of dye/S-1. The lag phase is not detected in S-1 samples preincubated with Cibacron Blue in the

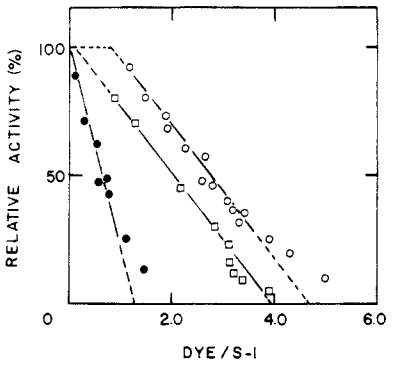

FIGURE 3: Effect of Cibacron Blue binding on the ATPase activity of S-1. S-1 (10^{-5} M) (1.1 mg/mL) was incubated with 1.5, 3.0, and 5.0×10^{-4} M Cibacron Blue in the presence of 1 mM MgADP (□) and in its absence (○), ●). The incubations were carried out at room temperature in 5 mM KCl and 10 mM Bis-Tris (pH 7.0). At various time intervals, samples were withdrawn from the incubation mixtures and were purified on Sephadex centrifugation columns equilibrated either with 5 mM KCl and 10 mM Bis-Tris at pH 7.0 (○, □) or with 1.0 M KCl and 10 mM Bis-Tris at pH 7.0 (●). Protein content, dye content, and ATPase activities were measured on the purified protein samples.

presence of MgADP. In this case, the total inhibition of ATPase activity occurs with the binding of 4.0 ± 0.3 mol of dye/S-1.

The results presented up to now were consistent with an irreversible modification of S-1 by Cibacron Blue. However, when solutions of S-1–dye complexes were rerun on Sephadex columns equilibrated with 6 M guanidine hydrochloride, almost all of the bound dye was separated from the protein (Figure 2b). Also, in sodium dodecyl sulfate gel electrophoresis of the inactivated protein, we could not detect any comigration of the dye with S-1. Obviously, even if marginal covalent modification of S-1 occurs, such labeling is not responsible for ATPase inhibition.

The reversibility of the S-1 inactivation by Cibacron Blue was examined by using Sephadex columns equilibrated with high salt concentrations. Centrifugation of the protein–dye incubation mixture on columns equilibrated with 0.5 and 1.0 M KCl leads to a substantially lower estimate of dye binding than separations on columns equilibrated with the incubation solvent (5 mM KCl). Strikingly, however, S-1 samples incubated in low salt and centrifuged on high-salt columns do not recover activity in spite of the decreased dye binding in such samples. When the results of such experiments are plotted in the form of S-1 activity vs. the amount of the bound dye, they extrapolate to zero ATPase activity at a binding ratio of 1.3 mol of dye/S-1 (Figure 3). These results indicate that dye molecules bound to S-1 at nonessential sites can be preferentially dissociated from the protein and that only one dye molecule is responsible for the inhibition of ATP hydrolysis.

Although one dye molecule appears to be more tightly bound to S-1 than the others, it too can be removed through successive centrifugations (three to four) of the protein–dye complex on 1 M KCl–Sephadex columns followed by chromatography on an Affi-Gel Blue column. The eluted protein is fully active and contains no bound dye.

Inhibition by Other Dyes. The dichlorotriazine dye Procion Blue MX-R, which does not contain the terminal phenyl ring of Cibacron Blue, inhibits only marginally the ATPase activity of S-1 (about 5%, compared with 95% inhibition of Cibacron Blue under similar reaction conditions). The anthraquinone moiety alone has even smaller impact on the hydrolysis of ATP. Preliminary binding tests reveal that both dyes bind to S-1, albeit significantly less efficiently than the Cibacron Blue dye.

Discussion

The interaction of Cibacron Blue with myosin subfragment 1, as shown in this work, is quite complex and encompasses many of the previously discussed facets of protein–dye binding. The elution of S-1 from an Affi-Gel Blue column by high salt concentrations is a nonspecific phenomenon which implicates electrostatic interactions in the protein's binding to the immobilized dye. This binding does not involve either the protein's active site or its SH_1–SH_2 peptide. Unmodified and SH_1–SH_2 cross-linked S-1 samples bind equally well to the Affi-Gel Blue column. Moreover, the unmodified protein can hydrolyze ATP while bound to the immobilized dye. Under similar solvent conditions, the free dye reversibly inhibits the ATPase activity of S-1. The different effects of the immobilized and free dye may be due to steric constraints in the vicinity of the myosin's active site (or some other site essential to the ATPase reaction) and its inability to accommodate the agarose-linked dye.

The free Cibacron Blue dye inhibits the ATPase activity of S-1 in a time-, pH-, and salt-dependent fashion. The Ca^{2+}-stimulated and K^+-stimulated (EDTA) ATPase activities decay at about the same rate as a function of dye concentration or time or incubation. The time-dependent inhibition of S-1 caused by its preincubation with Cibacron Blue, at room temperature and at moderate dye to protein ratios, is reversible and does not involve covalent labeling of the protein. The bound dye can be removed from S-1 in the presence of either guanidine hydrochloride or sodium dodecyl sulfate. In a more time-consuming procedure, purified and fully active S-1 can be obtained by combined chromatography on Sephadex (1.0 M KCl) and Affi-Gel Blue columns.

The inactivation of S-1 by Cibacron Blue does not resemble an active site directed process. The fact that neither MgATP nor MgADP protects the protein from inhibition argues against such a possibility. On the contrary, these nucleotides facilitate dye binding to S-1 and even more so the inhibition of the ATPase activity (Figures 2 and 3). The simplest interpretation of these results is that MgADP facilitates the binding of Cibacron Blue at a critical site on S-1. That a single site is involved in ATPase inhibition is indicated by the results shown in Figure 3, according to which all but one dye molecule can be removed from S-1 without relieving the inhibition. Interestingly enough, actin strongly inhibits the inactivation of S-1 by Cibacron Blue (unpublished results).

We may speculate that the critical dye binding site on S-1 recognizes primarily the hydrophobic part of Cibacron Blue. The dye appears to be more tightly bound to this particular site than to other sites from which it can be preferentially dissociated. The binding of ADP to S-1 probably results in the "opening" of some hydrophobic pockets on the protein, thus accelerating the dye binding and the inhibition of the ATPase activity. The location of the critical dye binding site in relationship to the active site cannot be assessed at present. In the absence of nucleotides, one or more dye molecules have to bind to S-1 in order to "expose" the critical site. It should be noted again that these binding estimates probably underrate the true dye binding to S-1 because of the strong affinity of Cibacron Blue to Sephadex. This, however, has no bearing on the activity–binding correlations obtained on purified protein samples. The binding of dye molecules to S-1 also has a definite electrostatic component. It is easily affected by high salt concentration, and it depends critically on the presence of the sulfonated terminal phenyl ring of Cibacron Blue as

indicated by the experiments with the truncated aromatic analogues of Cibacon Blue, Procion Blue MX-R, and anthraquinonesulfonic acid.

In conclusion, myosin subfragment 1 contains multiple Cibacron Blue binding sites. The binding of S-1 to the immobilized dye does not involve the protein's active site. The reversible inhibition of the ATPase activity of S-1 is related to the binding of Cibacron Blue to a single site on S-1 which appears to be distinct from the nucleotide binding site.

In a more general sense, our results underscore the complexity of protein–dye interactions and point out the risks inherent in extrapolating from the properties and behavior of one protein to other proteins. Protein binding to immobilized dyes and inhibition of catalytic activity need not necessarily imply the direct involvement of the nucleotide site in these events, let alone serve as a basis for structural conjectures.

References

Ashton, A. R., & Polya, G. M. (1978) *Biochem. J. 175*, 601–607.

Beissner, R. S., & Rudolph, F. B. (1978) *Arch. Biochem. Biophys. 189*, 76–80.

Beissner, R. S., Quiocho, F.-A., & Rudolph, F. B. (1979) *J. Mol. Biol. 134*, 847–850.

Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559–5563.

Chambers, B. B., & Dunlap, R. B. (1979) *J. Biol. Chem. 254*, 6515–6521.

Dean, P. D. G., & Watson, D. S. (1979) *J. Chromatogr. 165*, 301–319.

Drocourt, J. L., Thank, D.-C., & Thang, M.-N. (1978) *Eur. J. Biochem. 82*, 355–362.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Godfrey, J., & Harrington, W. F. (1970) *Biochemistry 9*, 886–892.

Jankowski, W. J., Muenchhausen, E., Sulkowski, E., & Carter, W. A. (1976) *Biochemistry 15*, 5182–5188.

Kielley, W. W., Kalckar, H. M., & Bradley, L. B. (1956) *J. Biol. Chem. 219*, 95–112.

Kumar, S. A., Beach, T. A., & Dickerman, H. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2199–2203.

Leatherbarrow, R. J., & Dean, P. D. G. (1980) *Biochem. J. 189*, 27–34.

Panefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Reisler, E., Liu, J., Mercola, M., & Horwitz, J. (1980) *Biochim. Biophys. Acta 623*, 243–256.

Seelig, G. F., & Colman, R. F. (1979) *J. Biol. Chem. 252*, 3671–3678.

Stellwagen, E. (1977) *Acc. Chem. Res. 10*, 92–98.

Thompson, S. T., & Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 361–365.

Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669–672.

Toste, A. P., & Cooke, R. (1979) *Anal. Biochem. 95*, 317–328.

Travis, J., & Pannell, R. (1973) *Clin. Chim. Acta 49*, 49–52.

Weber, B. H., Willeford, K., Moc, J. G., & Piszkiewicz, D. (1979) *Biochem. Biophys. Res. Commun. 86*, 252–258.

Weeds, A.-G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.

Wells, J. A., Sheldon, M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 1598–1602.

Witt, J. J., & Roskoski, R., Jr. (1980) *Biochemistry 19*, 143–148.

Ionization of Reactive Lysyl Residue of Myosin Subfragment 1†

Andras Muhlrad*,‡ and Reiji Takashi

ABSTRACT: The ϵ-NH_2 groups of lysyl residues of myosin subfragment 1 belong to two classes on the basis of their reaction with 2,4,6-trinitrobenzenesulfonate: one reactive lysyl residue and 82 slow-reacting lysyl residues. The trinitrophenylation of the reactive lysyl residue is accompanied by a sharp decrease in the K^+(EDTA)-activated ATPase of myosin subfragment 1. The rate of trinitrophenylation of this group was followed as an increase in A_{345} or as a decrease in K^+(EDTA)-activated ATPase at various pHs between 6.5 and 10. The second-order rate constant obtained by these methods sharply increased with pH, plateauing at about pH 9.7. A typical dissociation curve with pK = 9.0 was obtained by plotting the pH dependence of the rate constant. For this reactive lysyl, the pK value was low and the maximal rate of trinitrophenylation was high in comparison to the corresponding quantities of the slow-reacting lysyls of myosin subfragment 1 and of a model compound, N^{α}-carbobenzoxy-L-lysine. The pH dependence of the trinitrophenylation of lysyl residues of myosin subfragment 1 was anomalous. The pK value and maximal rate of trinitrophenylation of poly-L-lysine resembled those of the reactive lysyl residue. The presence of an aromatic moiety in the model compound was found to promote trinitrophenylation. It is suggested that the anomalous behavior of the reactive lysyl residue is caused by a vicinal positive charge and by other neighboring groups.

Myosin subfragment 1 (S-1)[1] is the segment of the myosin molecule responsible for ATPase activity and interaction with actin. Well-defined functional groups, such as reactive thiols, reactive lysine, certain tryptophanes, are located in this segment. The modification of these residues characteristically affects the activities of myosin. One of these functional groups is a reactive lysyl residue (RLR) described first on the basis

† From the University of California, San Francisco, Cardiovascular Research Institute, San Francisco, California 94143. *Received November 14, 1980; revised manuscript received June 23, 1981.* This research was supported by Grants HL-16683, PCM 75-22698 (National Science Foundation), and AHA CI-8.

‡ Senior investigator of the Muscular Dystrophy Association. Present address: Department of Oral Biology, Hebrew University, Hadassah School of Dental Medicine, Jerusalem, Israel.

[1] Abbreviations used: S-1, myosin subfragment 1; TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, trinitrophenyl; RLR, reactive lysyl residue; N^{α}-Cbz-lysine, N^{α}-carbobenzoxy-L-lysine.

0006-2960/81/0420-6749$01.25/0