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Modification of Myosin Subfragment 1 by Carbodiimide in the Presence of a Nucleophile. Effect on Adenosinetriphosphatase Activities†

Gabrielle Lacombe,* Nguyen Van Thiem, and Bernard Swynghedauw

ABSTRACT: The modification of myosin subfragment 1 by *N*-cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methyl *p*-toluenesulfonate in the presence of the nucleophile nitrotyrosine ethyl ester was investigated. For elimination of interference of the thiol groups, the two most reactive thiols were protected by cyanylation with 2-nitro-5-(thiocyanato)benzoic acid. The ATPase activity of the cyanylated myosin subfragment 1 was not lost, but had changed. At pH 5.9, carbodiimide in the presence of the nucleophile rapidly inactivated the cyanylated enzyme. The inactivation followed first-order kinetics. The K^+(EDTA)–, Ca^{2+}–, and Mg^{2+}–ATPase activities decreased at the same rate. Inactivation and incorporation of nucleophile occurred simultaneously. A full loss of activity resulted from the incorporation of 1 mol of nitrotyrosine per mol of myosin subfragment 1. Pyrophosphate, ITP, ADP, and ATP protected against inactivation, and the efficiency of the protection was parallel to the ligand binding strength. These results suggested that one carboxyl group was essential for the active conformation of myosin.

The cleavage of ATP[1] catalyzed by myosin in the presence of actin is the direct source of energy for muscle contraction. Kinetic features of this biological reaction have already been studied (Lymn & Taylor, 1970; Bagshaw & Trentham, 1974). In contrast, structural information on the active site of myosin is scarce. Data regarding the amino acid residues involved in the hydrolytic role of myosin are rare and sometimes controversial. For example, the cysteinyl side-chain residues SH1 and SH2 (Sekine & Kielley, 1964) have been implicated in the mechanism of the ATP hydrolysis (Reisler et al., 1974). This now appears very unlikely, since the blockage of the thiols only affected the conformation of the active site (Wiedner et al., 1978; Botts et al., 1979). Other amino acids have been proposed, including lysine, histidine, and tryptophan.

The modification of lysine (Fabian & Mühlrad, 1968), histidine (Hegyi & Mühlrad, 1968), and tryptophan (Yoshino, 1976) did not promote a parallel inactivation of the ATPase activities. Chantler & Szent-Gyorgyi (1978) reported that the well-known tryptophan fluorescence enhancement following the nucleotide binding to skeletal myosin was absent in invertebrate myosins. Their research suggests that tryptophan does not seem to be involved in the active site (Chantler, 1980). A concomitant loss of Mg^{2+}– and Ca^{2+}–ATPase activities was observed by blocking tyrosine with diazonium-1*H*-tetrazole, but the reagent was not specific. In addition to tyrosine, some modified histidine residue was detected (Shimada, 1970). It is therefore difficult to attribute a definite role to these residues in the mechanism of catalysis. We suggest that the most likely residue involved in the active site seems to be arginine. The labeling of arginine by phenylglyoxal affected the K^+(EDTA)–, Ca^{2+}–, and actin-activated ATPase activities, and the nucleotide substrates afforded efficient protection against

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[1] Abbreviations used: S-1, α-chymotryptic myosin subfragment 1; CMC, *N*-cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methyl *p*-toluenesulfonate; NTEE, 3-nitro-L-tyrosine ethyl ester; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; ITP, inosine triphosphate; Tris, tris(hydroxymethyl)-aminomethane.

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the inactivation (Mornet et al., 1979; Morkin et al., 1979).

The carboxyl groups have already been investigated by Kinoshita et al. (1969) who succeeded in activating the carboxyl groups by ATP with a subsequent nucleophilic substitution by *p*-nitrothiophenol. From their results it was concluded that a glutamic residue was implicated in the formation of the intermediate complex myosin–ADP–P_i. This is, in fact, rather controversial since the linkage of the reagent *p*-nitrothiophenol to myosin was not a thiol ester but a disulfide (Wolcott & Boyer, 1973). This fact does not rule out the hypothesis of a dicarboxylic acid involved in the myosin ATPase. Indeed, several ATPases possess a carboxyl group in the active site. Degani & Boyer (1973) have identified an aspartyl residue at the active site of the phosphorylated (Ca^{2+},Mg^{2+})–ATPase of sarcoplasmic reticulum by reduction of the acyl phosphate linkage with borohydride. Satre et al. (1979) have obtained a full loss of activity of the *Escherichia coli* coupling factor ATPase by the dicyclohexylcarbodiimide with a stoichiometry of 1 mol of reagent per mol of enzyme. They suggest that a carboxyl group is essential for the enzymatic activity.

To test the possibility of the involvement of a carboxyl group in the myosin ATPase, we followed the procedures of Hoare & Koshland (1967). In a first step, the carboxyl group was activated by a carbodiimide (Khorana, 1953), and then a suitable nucleophilic reagent was added to the activated group in order to yield a stable and well-defined product. We reported herein the modification of the myosin head by the carbodiimide in the presence of the nucleophile nitrotyrosine ethyl ester. The incorporation of the reagent and the decrease in the ATPase activity proceeded at the same rate. The binding of 1 mol of nitrotyrosine per mol of myosin subfragment 1 induced a complete loss of the Ca^{2+}–, Mg^{2+}– and K^+(EDTA)–ATPase activities.

Materials and Methods

Chemicals. Nitrotyrosine ethyl ester hydrochloride (NTEE) was synthesized according to Dall'Asta & Ferrario (1962). *N*-Cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methyl *p*-toluenesulfonate (CMC) and *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide hydrochloride were purchased from Fluka; 2-nitro-5-(thiocyanato)benzoic acid was from Eastman; and 3-nitro-L-tyrosine was from ICN Pharmaceuticals. Taurine, tyramine, adenosine, ITP, ADP, and ATP were obtained from Sigma, and Worthington supplied α-chymotrypsin. All other chemicals were reagent grade.

Preparation of the Chymotryptic Myosin Subfragment 1 (S-1). The rabbit skeletal myosin was extracted according to Offer et al. (1973) by using an ammonium sulfate salting out as a final step of the preparation. Myosin subfragment 1 (S-1) was prepared from fresh myosin by digesting myosin filaments with α-chymotrypsin according to Weeds & Taylor (1975) and Wagner & Weeds (1977) and purifying by ammonium sulfate fractionation. The precipitate obtained between 48% and 59% saturation was thoroughly dialyzed against 40 mM NaCl and 1 mM EDTA at pH 6.5. After centrifugation for 30 min at 40000*g*, the solution of S-1 was analyzed for protein and ATPase activities and then cyanylated. The molecular weight used for S-1 was 115000 (Weeds & Pope, 1977).

Cyanylation of S-1. Cyanylation of sulfhydryl groups of S-1 was carried out according to Degani et al. (1970) and Degani & Patchornik (1974). S-1 (100 μM) was incubated with 2-nitro-5-(thiocyanato)benzoic acid (400 μM) at pH 8.0 in 100 mM Tris-HCl, 2 mM EDTA, and 100 mM KCN at 22 °C. The appearance of the thionitrobenzoic ion was monitored at 412 nm, using ϵ_M = 13600 (Ellman, 1959). The reaction was stopped when 2 equiv of thionitrobenzoic acid was produced by adjusting the pH to 5.8 with 0.2 volume of 200 mM phthalate buffer, pH 4.0. The protein was freed from the excess of the reagent and from the thionitrobenzoic ion by precipitating several times at 0 °C with 4 volumes of a saturated ammonium sulfate solution, pH 7.0, containing 1 mM EDTA, and then dialyzed against 40 mM NaCl and 5 mM EDTA, pH 6.5. The solution was then centrifuged for 1 h at 100000*g* and analyzed for protein and ATPase activities. The cyanylated S-1, stored in 40 mM NaCl and 5 mM EDTA, pH 6.5, at 0 °C, remained stable for 3 weeks.

Reaction of S-1 with CMC Plus Nucleophile. By use of the Radiometer pH-stat system, the extemporary reagent was prepared by rapidly adjusting the aqueous solution of CMC and nucleophile to pH 5.8 with 20 mM NaOH at 25 °C. Inactivation was performed at 25 °C by injecting 2 volumes of reagent to 1 volume of S-1. The controlled pH of the reaction mixture was 5.9–6.0. Samples were periodically withdrawn, assayed for ATPase activities, and compared to a control subjected to the same conditions but excluding the reagent. The 20–100-fold dilution of the aliquots in the assay medium completely quenched the modification reaction. In the protection experiments, the enzyme was preincubated with the ligands for 5 min before the addition of reagent. Control experiments have shown that the ligands preincubated with the enzyme have no disturbing effect on the ATPase activities under these conditions.

Determination of the Stoichiometry of the Labeling. Another sample was withdrawn to determine the incorporation of NTEE into the enzyme. The sample was rapidly stirred in the presence of 5 volumes of 5% (w/v) trichloroacetic acid at 0 °C. The precipitate was collected by centrifugation, washed 5 times with 5% trichloroacetic acid (Pick & Racker, 1979), redissolved in 0.1 M NaOH, and thoroughly dialyzed against the same solvent (Ba Pho et al., 1977) at 0 °C. The protein solution was then centrifuged for 1 h at 100000*g* and its absorbance at 428 nm (Sokolovsky et al., 1966) determined, using ϵ_M = 4200 for NTEE. A slight absorbance of S-1 treated by NTEE alone and subjected to the same isolation procedure was subtracted for calculation.

ATPase Assays. The ATPase activities were estimated at 25 °C in the presence of 2.5 mM ATP, 50 mM Tris-HCl, pH 7.5, either 5 mM $CaCl_2$, 5 mM $MgCl_2$, or 1 M KCl, and 5 mM EDTA (Van Thiem et al., 1978). Inorganic phosphate was determined with malachite green (Itaya & Ui, 1966).

Protein Concentration. S-1 concentration was measured by absorbance at 280 nm ($E^{1\%}_{280nm}$ = 7.5 cm^{-1}) (Wagner & Weeds, 1977). The concentration of the modified S-1 was estimated according to Bradford (1976) by using the native enzyme as a standard.

Results

ATPase Activities of the Cyanylated S-1. For elimination of possible interference with the most sensitive sulfhydryl groups, S-1 was cyanylated. With a 4 M excess of 2-nitro-5-(thiocyanato)benzoic acid, only two sulfhydryl groups were rapidly blocked after 8 min, and the reaction did not continue (Figure 1). A 10 M excess of the reagent accelerated the process but did not cyanylate any more thiol groups. After this treatment, the Mg^{2+}–ATPase activity had increased by 16%, and the Ca^{2+}– and K^+(EDTA)–ATPase activities had decreased by 16% and 38%, respectively (Table I). The modification can be easily reversed by reduction, and a 10 mM dithiothreitol treatment at pH 8.0 for 50 min at 22 °C restored all initial activities.

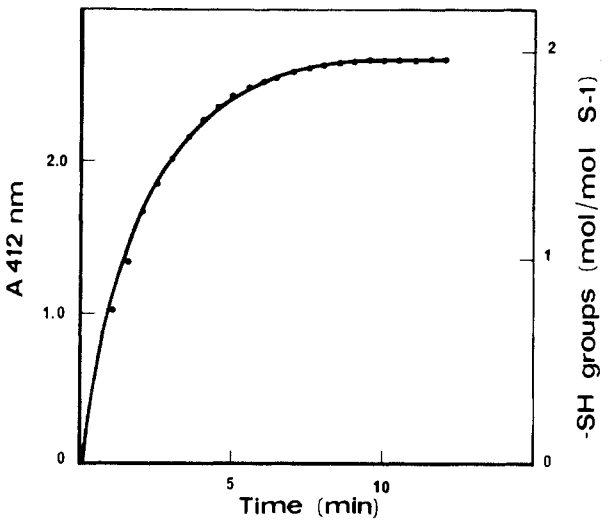

FIGURE 1: Cyanylation of S-1. Extent of sulfhydryl group modification by 2-nitro-5-(thiocyanato)benzoic acid. 100 μM S-1, 400 μM 2-nitro-5-(thiocyanato)benzoic acid, 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 100 mM KCN, 22 °C. The absorbance at 412 nm was monitored (see Materials and Methods).

Table I: ATPase Activities of Native S-1 and Cyanylated S-1[a]

	ATPase [(nmol of P_i) mg^{-1} min^{-1}]					
	K^+(EDTA)		Ca^{2+}		Mg^{2+}	
enzyme preparation	native	cyanylated	native	cyanylated	native	cyanylated
1	5890	3240	4040	2970	59	66
2	4860	3020	3970	3460	54	97
3	4970	3070	4050	3400	59	73
4	4350	2700	3980	3660	63	71

[a] After 8-min incubation of S-1 with 2-nitro-5-(thiocyanato)benzoic acid (see Figure 1), the cyanylated S-1 was freed from excess of reagent, and the ATPase activities were assayed and compared to those of native S-1 (see Materials and Methods).

Table II: Effect of Various Carbodiimides and Nucleophiles on the Inactivation of Cyanylated S-1[a]

reagents	half-time of inactivation (min)
ethyl(diaminopropyl)carbodiimide (100 mM) + NTEE (15 mM)	8
CMC (100 mM) + NTEE (15 mM)	0.5
CMC (100 mM) + imidazole (100 mM)	6
CMC (50 mM) + taurine (100 mM)	15
CMC (50 mM) + tyramine (100 mM)	11
CMC (50 mM) + NTEE (15 mM)	6
CMC (50 mM) + NTEE (30 mM)	4

[a] Inactivation was carried out as described under Materials and Methods; 30 μM cyanylated S-1 and 5 mM EDTA, pH 5.9–6.0, at 25 °C. The concentrations of reagents were as indicated. The half-time of inactivation was calculated from the semilogarithmic plots of Ca^{2+}-ATPase activity.

Choice of Carbodiimide and Nucleophile. The kinetics of inactivation of the cyanylated S-1 by carbodiimides plus nucleophiles were pseudo first order. The rate of inhibition varied with the various reagents tested. The half-time of inactivation is shown (Table II). CMC was more potent than ethyl(diaminopropyl)carbodiimide, and NTEE was more efficient than tyramine, taurine, and imidazole.

Inactivation of Cyanylated S-1 by CMC with NTEE. At pH 5.9–6.0, NTEE alone had no effect on the cyanylated S-1 ATPase; CMC alone inactivated the enzyme, but the addition of NTEE strongly enhanced the rate of inactivation which followed pseudo-first-order kinetics (Figure 2).

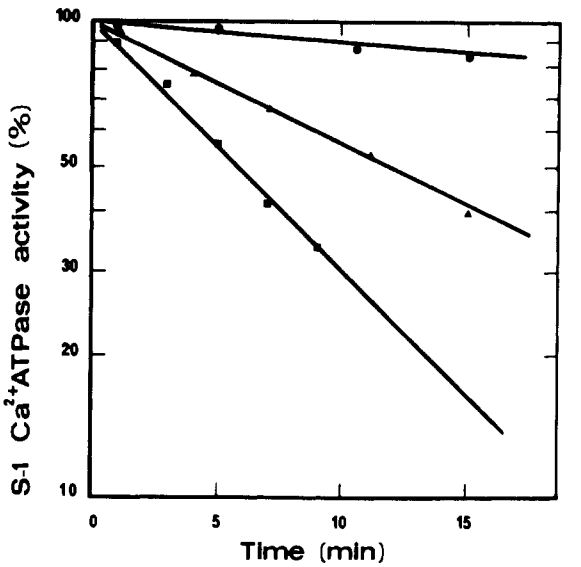

FIGURE 2: Inactivation of cyanylated S-1 by CMC with NTEE. 33 μM cyanylated S-1 and 5 mM EDTA, pH 5.9–6.0, at 25 °C. (●) 15 mM NTEE alone, (▲) 50 mM CMC alone, (■) 50 mM CMC + 15 mM NTEE. For Ca^{2+}-ATPase assay, see Materials and Methods.

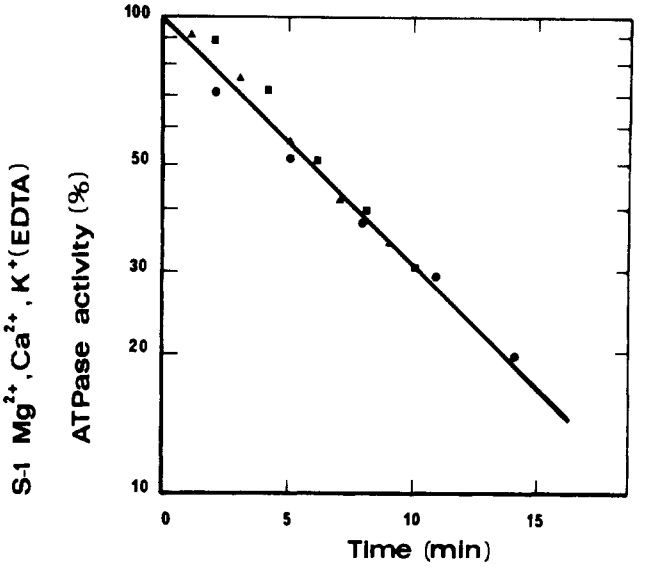

FIGURE 3: Effects of CMC plus NTEE on the Mg^{2+}-, CA^{2+}-, and K^+(EDTA)-ATPase activities of cyanylated S-1. 33 μM cyanylated S-1, 5 mM EDTA, 50 mM CMC, and 15 mM NTEE, pH 5.9–6.0, at 25 °C. (●) Mg^{2+}-ATPase, (▲) Ca^{2+}-ATPase, and (■) K^+-(EDTA)-ATPase. For Mg^{2+}-, Ca^{2+}-, and K^+(EDTA)-ATPase assays, see Materials and Methods.

The modification by CMC plus NTEE was performed in the presence of 5 mM EDTA since, in its absence, at 25 °C, the preincubated enzyme has a strong tendency to aggregate. At a higher ionic strength (100–500 mM KCl) the inactivation was no longer first order, possibly due to a change in conformation. The temperature of the reaction had a pronounced effect on the rate of inactivation, which showed a 4-fold depression from 20 to 10 °C when 50 mM CMC plus 15 mM NTEE were used.

Throughout all the modified conditions explored, all the steady-state K^+(EDTA)-, Ca^{2+}-, and Mg^{2+}-ATPase activities of the cyanylated S-1 declined at the same rate (Figure 3).

Stoichiometry of the Reaction. The incorporation of the nitrotyrosyl group and inactivation were of the same rate, at least up until 80% of inactivation. The total loss of activity was associated with the incorporation of 1 mol of nitrotyrosine

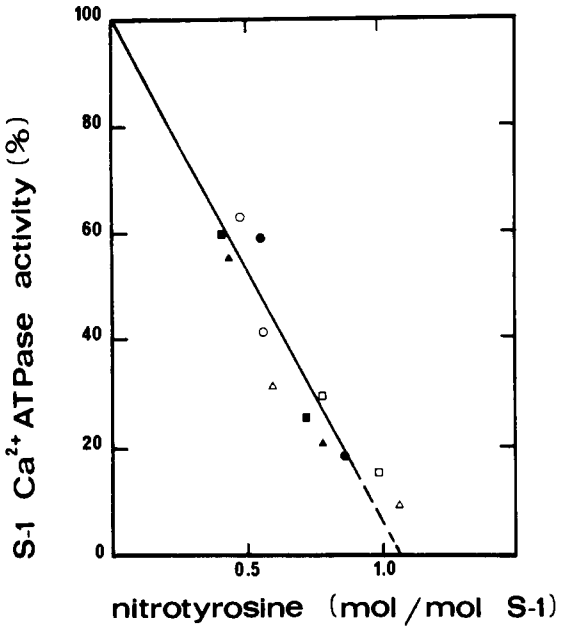

FIGURE 4: Correlation of inactivation of ATPase activity and incorporation of nitrotyrosine. The figure shows the results of six experiments with two determinations per experiment (one symbol per experiment). 33 μM cyanylated S-1, 5 mM EDTA, 100 mM CMC, and 15 mM NTEE, pH 5.9–6.0, at 25 °C. For Ca^{2+}–ATPase activity and nitrotyrosine incorporation assays, see Materials and Methods.

per mol of cyanylated S-1 (Figure 4).

These results were obtained with a high concentration of CMC, i.e., 100 mM, in conjunction with 15 mM NTEE and 5 mM EDTA, pH 5.9–6.0, 25 °C. The inactivation was very rapid, with a half-time of inactivation of 0.5 min. When a lower concentration of CMC (50 mM) was used under the same conditions and although the inactivation was of first-order kinetics with a half-time of inactivation of 6 min, the incorporation of the nucleophile was no longer first order, and several groups have been labeled. This indicates that the first blocked groups are implicated in the enzymatic activity.

Protection by Substrate Analogues. Table III illustrates that substrate analogues as well as pyrophosphate have prevented inactivation. The order of protection efficiency was MgADP > ADP = ATP > ITP = pyrophosphate.

Discussion

Kinoshita et al. (1969), in the only paper published concerning the carboxyl groups of myosin, labeled myosin with *p*-nitrothiophenol by prolonged incubation in the presence of MgATP. The modified enzyme lost its phosphate burst activity, but the steady-state ATPase activities remained normal; 1 mol of *p*-nitrothiophenol was incorporated per mol of myosin. By enzymatic digestion of the modificed myosin, a nitrothiophenyl amino acid was isolated. It contained 1 mol of glutamic acid per mol of *p*-nitrothiophenol. These results suggested to the authors that a glutamic acid residue of myosin had formed a thioester linkage with *p*-nitrothiophenol. However, Wolcott & Boyer (1973) repeated these experiments and have shown that the linkage is not a thioester but a disulfide. The modified myosin was much more stable than the thioester model compounds and did not give rise to aminohydroxyvaleric acid, which is the expected reduction product of glutamyl thioester, by treatment with borohydride.

Cyanylation of myosin has been made by Wiedner et al. (1978) with 6-(thiocyanato)-9-β-D-ribofuranosylpurine. When 2 mol of cyanide per mol of myosin head was incorporated, K^+(EDTA)–ATPase activity dropped to 5% of its original value, Ca^{2+} activity rose 40%, and Mg^{2+} activity increased

Table III: Effect of Substrate Analogues on Inactivation of Cyanylated S-1 by CMC + NTEE[a]

additions	concn (mM)	half-time of inactivation (min)
none		6
adenosine	2.5	6
pyrophosphate	2.5	10
ITP	2.5	10
ATP	2.5	14
ADP	2.5	14
ADP	1.0	9
MgADP	1.0	14

[a] Cyanylated S-1 was preincubated with the ligand for 5 min at 25 °C before addition of the reagent (CMC + NTEE) (see Materials and Methods). 30 μM cyanylated S-1, 5 mM EDTA, 50 mM CMC and 15 mM NTEE, pH 5.9–6.0, at 25 °C. The concentrations of ligand were as indicated.

8-fold. With a methyl methanethiosulfonate reagent, the same degree of labeling of papain myosin subfragment 1 brought about an increase of 3-, 1.5-, and 3.5-fold of the Ca^{2+}–, actin-activated, and Mg^{2+}–ATPase activities, respectively (Botts et al., 1979). Cyanylation with a simpler and commercially available reagent, 2-nitro(thiocyanato)benzoic acid, on the α-chymotryptic myosin subfragment 1 promoted a smaller change in the ATPase activities (see Results). The quantitative and qualitative variability observed from these results can easily be explained by the type of preparation, the reagents used, and the experimental conditions. For the latter consideration, it is well-known that the ionic strength has great importance for the configuration of myosin and derivatives. Our results confirm previous reports that blocking the thiols of enzymes with small volume reagents does not seriously alter their activities, as in the case of myosin ATPase (Wiedner et al., 1978; Botts et al., 1979) and creatine kinase (Der Terrossian & Kassab, 1976).

Since the main purpose of our work was to study modifications caused by a carbodiimide, cyanylation of S-1 was not studied in detail. There exists the possibility of formation of a disulfide linkage, but it would seem unlikely given that, in this cyanylation, an amount in excess of cyanide was used. In fact, cyanide is able to break both the cystine disulfide linkage (Castsimpoolus & Wood, 1966) and the mixed disulfide protein–thionitrobenzoic acid linkage (Vanaman & Stark, 1970). In the same way, it displaces any additional thionitrobenzoic group (Chung et al., 1971; Der Terrassian & Kassab, 1976). According to Wells & Yount (1980), the 5,5′-dithiobis(2-nitrobenzoic acid) reagent, whose reactivity resembles that of nitro(thiocyanato)benzoic acid, only causes formation of a cystine disulfide linkage between the SH1 and SH2 thiol groups of myosin in the presence of MgADP; in the absence of nucleotide, the SH2 thiol group is unchanged. We are led to believe that, following the analogy of our reagent with that of Wells & Yount, the two thiol groups affected in our work are that of the SH1 thiol group and that of the thiol group which is not essential for the activity described by those authors.

Carbodiimides have been used by several authors since 1966 for the modification of carboxyl groups of proteins [see Carraway & Koshland (1972)]. According to Hoare & Koshland (1967), the initial attack of carbodiimide upon the carboxylic acid results in the formation of an *O*-acylurea. This latter can undergo either a rearrangement to *N*-acylurea or a hydrolysis with regeneration of the acid. Inactivation of the enzymes by carbodiimide alone could be explained by the formation of *N*-acylurea. This explains why S-1 is inactivated by carbodiimide alone (Figure 2). This is also observed for

triphosphopyridine nucleotide dependent isocitrate dehydrogenase (Colman, 1973) and for *E. coli* coupling factor ATPase (Satre et al., 1979). When the carbodiimide concentration goes from 50 to 100 mM, the rate of inhibition of S-1 is nearly doubled. This result (not shown) is identical with that found by Ba Pho et al. (1977) and Satre et al. (1979). The presence of nucleophile accelerates the rate of inactivation observed with carbodiimide alone, with the nucleophile acting in competition with water to block the carboxyl group in order to form an amide. This acceleration depends on the nucleophilic capacity of the reagent (Table II). In the presence of NTEE (15 mM), when the concentration of CMC passes from 50 to 100 mM, the rate of inhibition increases by a factor of 12 (see Results). This fact agrees with the results of Ba Pho et al. (1977), where the authors obtained a 6-fold increase in rate, passing from 50 to 100 mM of CMC in the presence of 30 mM of NTEE. This phenomenon cannot presently be explained.

Carboxyl groups are specifically modified by carbodiimide, although sulfhydryl groups could react at the same rate and tyrosyl groups at a slower rate (Carraway & Koshland, 1972). To avoid interference of the thiol groups of myosin, which are known to be very reactive, the thiol groups of S-1 have been protected in our experiments by cyanylation. The strong accelerating effect of the nucleophile NTEE on the rate of reaction (Table II and Figure 2) eliminates the participation of sulfhydryl and tyrosyl residues (Carraway & Koshland, 1972). Cyanylation can be reversed by dithiothreitol. Nonetheless, we did not attempt to regenerate the thiol groups after modification of S-1 by carbodiimide since the rapid quenching of the reaction as well as the elimination of reagent excess and regeneration necessitate a number of manipulations which are difficult to carry out in kinetic studies. Nevertheless, it is reasonable to suppose that the use of the cyanylated form of S-1 would not lead to erroneous interpretation.

Several arguments support labeling of the S-1 active site by carbodiimide. First, the Mg^{2+}-, Ca^{2+}-, and K^+-(EDTA)-ATPase activities all decreased with the same velocity (Figure 3). This is rather rare and had not been frequently encountered in the previous modifications of myosin. *N*-Ethylmaleimide treatment both activates the Ca^{2+}-ATPase and inactivates the K^+(EDTA)-ATPase (Sekine & Kielley, 1964; Reisler et al., 1974; Pfister et al., 1975; Klotz et al., 1976). The labeling of the lysyl groups by trinitrobenzenesulfonate (Fabian & Mühlrad, 1968) or the blocking of the histidyl residues with diethyl pyrocarbonate (Hegyi & Mühlrad, 1968) promoted an increase of Mg^{2+}-ATPase activity while the K^+(EDTA)-ATPase activity decreased or remained unchanged. The initial phosphate burst size decreased, but the steady-state Mg^{2+}-ATPase activity remained unchanged when the tryptophanyl residue was modified with 2-hydroxy-5-nitrobenzyl bromide (Yoshino, 1976). Nevertheless, there are three cases where a parallel inactivation of ATPase activities had been observed, the modification of arginine (Mornet et al., 1979; Morkin et al., 1979), the modification of tyrosine (Shimada, 1970), and the cross-linking of the thiol groups SH1 and SH2 by cobalt (Wells et al., 1979; Wells & Yount, 1979).

Moreover, the great efficiency of CMC as compared to ethyl(diaminopropyl)carbodiimide (Table II) means that the former reagent might preferentially bind the hydrophobic active site. The active site might be in a special configuration which conferred to the presumed essential carboxyl group a great reactivity among the 217 dicarboxylic residues per 10^5 g of S-1 (Jones & Perry, 1966).

Finally, the protection afforded by various substrate analogues against the inactivation reinforces the idea that the active site has been affected by the modification. Pyrophosphate, ITP, ADP, and ATP, which are known to be firmly bound to myosin (Banerjee & Morkin, 1978; Schliselfeld & Barany, 1968; Nauss et al., 1969), protected against inactivation. ITP which has a lower affinity for myosin than ATP (Azuma & Tonomura, 1963) showed lower protection than ATP (Table III).

The first-order kinetics of inactivation and incorporation of nucleophile (Figures 2–4) interpreted according to the kinetic method of Ray & Koshland (1961) suggests that a residue essential for the enzymatic activity was blocked. The very rapid and complete loss of activities, in addition to the occurrence, at the same rate, of enzymatic inactivation and incorporation of 1 mol of nitrotyrosine, strongly suggests that one carboxyl group located in the active site or in the immediate vicinity is critical for the ATPase activity. This is in agreement with the model of Ramirez et al. (1979) where an aspartyl group was shown to form a hydrogen bond with the purine 6-amino group of ATP.

In summary, the incorporation of 1 mol of nitrotyrosine per mol of myosin subfragment 1 with CMC + NTEE concomitant with a complete inactivation of all ATPase activities suggests that the reagent blocked an amino acid residue essential to the enzymatic activity. It is presumed that this residue is a dicarboxylic acid. Further investigations on the nature of the labeled residue and on the localization in the primary structure of the S-1 heavy chain are in progress.

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A (Carbonmonoxy)heme Complex with a Weak Proximal Bond. Molecular Stereochemistry of Carbonyl(deuteroporphinato)(tetrahydrofuran)iron(II)†

W. Robert Scheidt,* Kenneth J. Haller, M. Fons, Toshio Mashiko, and Christopher A. Reed*

ABSTRACT: The synthesis and structural characterization of a six-coordinate (carbonmonoxy)(porphyrin)iron(II), carbonyl(deuteroporphinato)(tetrahydrofuran)iron(II), is described. The choice of tetrahydrofuran as the sixth ligand represents one of the weakest field and weakest binding ligands possible and leads to stronger than usual bonding of CO to the heme. The Fe–C(CO) bond distance is 1.706 Å, unusually short. The Fe–O(THF) bond distance in the low-spin complex is 2.127 Å. The Fe–C–O and O–Fe–C groups are essentially linear with angles of 178.3° and 177.4°, respectively. The iron atom is displaced out of plane 0.10 Å toward the tightly bound CO. The results suggest that the different CO stretching frequencies observed in myoglobin might result from modulating the bonding of the histidine trans to CO. Crystal data are as follows: $a = 11.524$ (3) Å, $b = 14.915$ (5) Å, $c = 10.583$ (2) Å; $\alpha = 91.51$ (2)°, $\beta = 108.99$ (2)°, $\gamma = 102.86$ (3)°; triclinic, space group $P\bar{1}$, $Z = 2$; $\rho_{calcd} = 1.363$ g/cm^3, $\rho_{obsd} = 1.370$ g/cm^3.

Binding of the strong-field ligand carbon monoxide has been frequently exploited in studies of hemoproteins and porphyrin species. These studies have focused particularly on exploring the nature of the heme binding site and the evaluation of cis and trans effects (Caughey et al., 1972; Buchler et al., 1978). Such studies have shown, for example, that there are spectroscopically distinguishable (and probably structurally distinct) components of (carbonmonoxy)myoglobin, both in solution (McCoy & Caughey, 1971; Alben, 1978) and in the crystalline state (Makinen et al., 1979). The affinity of CO for a deuteroheme has been found to be significantly influenced by the nature of the ligand trans to the CO (Rougee & Brault, 1975).

Crystallographic study of carbon monoxide liganded hemoglobins and myoglobins (Huber et al., 1970; Heidner et al., 1976; Norvell et al., 1975; Tucker et al., 1978; Steigemann & Weber, 1979; Baldwin, 1980) has shown that the CO ligand

† From the Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556 (W.R.S., K.J.H., and M.F.), and the Department of Chemistry, University of Southern California, Los Angeles, California 90007 (T.M. and C.A.R.). *Received September 25, 1980.* The work done at Notre Dame was supported by National Institutes of Health Grant HL-15627 and that done at Southern California by National Science Foundation Grant CHE-78-09813.

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