CARBOXYMETHYLATION AT THE ACTIVE SITE OF DISULFIDE EXCHANGED MYOSIN A*

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Previous studies on myosin A in which ATPase activity had been destroyed by reagents reacting specifically with sulfhydryl residues, had led to the conclusion that these residues were somehow involved at the active site of this enzyme. Indeed, two types of sulfhydryl groups have been postulated because of their varying reactivity to p-chloromercuribenzoate and N-ethyl maleimide (Blum, 1960; Sekine, et al., 1962). It has recently been shown that the reaction of one sulfhydryl residue per polypeptide chain with N-ethyl maleimide was sufficient to cause complete inhibition of the ethylenediaminetetraacetate activated ATPase activity of myosin A (Sekine, et al., 1962).

During the course of our studies on the disulfide exchange reaction of myosin A with bis-β-carboxyethyl disulfide $(-S-CH_2-CH_2-COOH)_2$, it was consistently observed that of the 15 sulfhydryl residues present in myosin A per 200,000 molecular weight (Sekine, et al., 1962), only 14 of these residues would exchange with the disulfide reagent. The

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exchange reaction led to complete inhibition of the Ca^{++} activated ATPase of myosin A, however, treatment of the disulfide exchanged myosin (DSEM) with an excess of β-mercaptoethanol completely restored the original activity. It was subsequently shown that if DSEM was treated with iodoacetamide, excess β-mercaptoethanol did not lead to a restoration of ATPase activity.

These observations led to a more detailed investigation of the reaction of iodoacetamide with DSEM and resulted in the localization of a sulfhydryl residue at the active site of myosin A.

Myosin A was prepared by standard procedures (Szent-Gyorgyi, 1947) and the several preparations used in this study all gave similar results. Disulfide exchanged myosin was prepared by treating the enzyme with a 200 fold molar excess of bis-β-carboxyethyl disulfide at pH 9.3 for 96 hours at 4°C. No ATPase activity could be observed in this derivative. Treatment of DSEM with a 200 fold molar excess of β-mercaptoethanol at pH 9.3 for 16 hours at 4°C led to a complete restoration of ATPase activity. The excess β-mercaptoethanol could be removed by three precipitations of the reactivated protein.

The number of non-exchanged sulfhydryl residues was determined by reaction of DSEM with iodoacetamide-1-C^{14} for thirty minutes at pH 8.3 and 25°C. From the specific activity of the labelled protein it was possible to determine the number of carboxymethyl residues introduced into the protein. Isolation of S-carboxymethyl cysteine from the hydrolysed protein, as described below, enabled a determination of the number of unexchanged sulfhydryl residues in DSEM. Only 1.14 residues per $2X10^5$ g could be detected.

In studying the reaction of DSEM with iodoacetamide, the protein and the reagent (0.1M) were incubated at pH 8.3 and 25°C. At suitable time intervals aliquots from the reaction mixture were removed and the enzyme was precipitated by dilution to remove excess iodoacetamide. The precipitate was redissolved and treated with β-mercaptoethanol as described above. Measurement of the ATPase activity on each aliquot gave the results shown in Figure 1. It can be seen that the loss of ATPase activity for three different samples of DSEM is first order. At pH values below 7.5 and molar concentrations of iodoacetamide below 0.05 only a 20 per cent loss in activity occurred in 15 minutes whereas at pH 8.3 complete inactivation of ATPase took place in the same time period.

Figure 1 also shows that in the presence of 2×10^{-3} M ATP and 1×10^{-3} M $MgCl_2$, DSEM is protected from inactivation by iodoacetamide suggesting that reaction with a residue at the ATPase active site was occurring.

In order to determine the amino acid residue(s) responsible for the loss in ATPase activity, DSEM was treated with iodoacetamide-1-C^{14} (1.5 mc/mmole, Tracerlab, Inc.) as described in the text. The protein was repeatedly precipitated until no radioactivity could be detected in the supernatant. The labelled protein was hydrolysed in 6N HCl at 110°C for 24 hours in a sealed, evacuated tube. The amino acids were separated by automatic amino acid analyses (Piez and Morris, 1960). The effluent was collected in individual tubes and an aliquot from each dried on a planchet and counted. Only two radioactive peaks could be detected, one corresponding to S-carboxymethyl cysteine and the other carboxymethyl histidine (Gundlach, et al., 1959). The

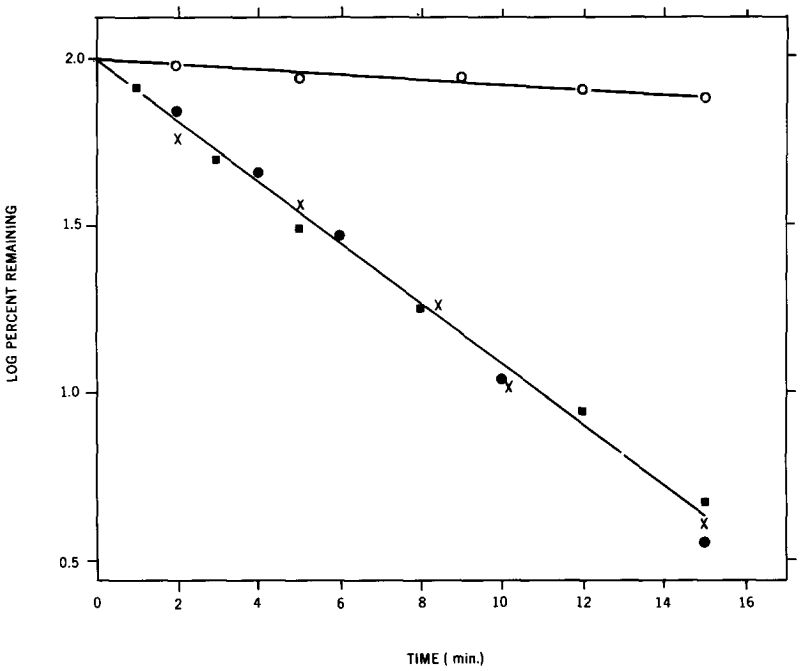

Fig. 1. Reaction of iodoacetamide with DSEM. See text for experimental details. ATPase activities were measured in 0.5 M KCl, 0.05 M Tris, pH 7.5, 1 x 10^{-3} M $CaCl_2$.

O - O - reaction in the presence of ATP
X - X - sample 1 of DSEM
● - ● - sample 2 of DSEM
■ - ■ - sample 3 of DSEM

results are shown in Table 1. It can be seen that in DSEM 2 moles of carboxymethyl residues had been introduced into the protein and one mole each of S-carboxymethyl cysteine and carboxymethyl histidine could be detected.

The reactive histidyl residue could be ruled out as being part of the active site since it can be seen in Table 1, line 2, that ATP protects the sulfhydryl residue from reacting with iodoacetamide and not the histidyl residue. Furthermore, inactivation of DSEM with N-ethylmaleimide showed that only the sulfhydryl residue reacted with NEM

TABLE 1

REACTION OF IODOACETAMIDE-1-C^{14} WITH DISULFIDE EXCHANGED MYOSIN

SAMPLE	MOLES OF CARBOXYMETHYL DERIVATIVE PER 2 x 10^5 G		
	PROTEIN	CYSTEINE	HISTIDINE
DSEM	2.08	1.14	0.84
DSEM + ATP	1.33	0.25	0.94
DSEM + NEM	0.82	0.00	0.78
DSEM (ATP)	2.94	1.75	1.08
DSEM (ACTIN)	4.16	3.02	0.80

See text for experimental details.

(Table 1, line 3) since no S-carboxymethyl cysteine could be detected.

In Table 1, line 4, it can be seen that when the disulfide exchange reaction was carried out in the presence of 2 x 10^{-3} M ATP and 1 x 10^{-3} M $MgCl_2$, two sulfhydryl groups were prevented from exchanging. Apparently ATP is binding to two sulfhydryl groups at the active site. When this derivative was treated with iodoacetamide and ATPase measured, an initial increase in ATPase was noted and then a decrease (Figure 2). It would appear that the second -SH residue is involved in the well known activation phenomenon of myosin ATPase (Kielley and Bradley, 1956). It can also be seen in Table 1, line 5, that when the exchange reaction is carried out in the presence of F-actin, three sulfhydryl residues do not exchange. When this derivative is treated

with iodoacetamide (Figure 2), only a loss in ATPase is noted. It would appear that the sulfhydryl residues involved in ATP and actin binding are dissimilar. Further studies on these derivatives are now in progress.

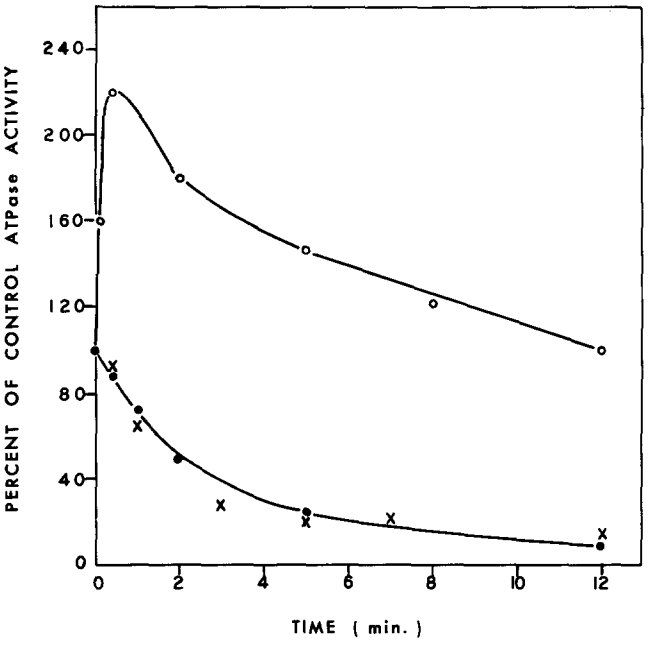

Fig. 2. Reaction of iodoacetamide with DSEM derivatives.

O - O - DSEM (ATP)
X - X - DSEM (Actin)
● - ● - DSEM

References

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