THE NUMBER OF SH GROUPS IN RABBIT MUSCLE PHOSPHORYLASE

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The number and function of the SH groups in the muscle phosphorylases (E.C. 2.4.1.1) do not appear to be known with certainty. Velick and Wicks (1951) investigated the amino acid composition of phosphorylase by microbiological and colorimetric techniques and they found 18 half-cystine residues in phosphorylase a. Madsen and Cori (1956) using pCMB (parachloromercuribenzoate) titration concluded that phosphorylase a contained a total of 18 SH groups and phosphorylase b 9 SH groups per molecule, and further that these enzymes did not possess any disulfide linkages. More recently Appleman et al. (1963) have reinvestigated the amino acid composition of phosphorylase b and found that the enzyme contained 22 half-cystine residues. By alkylation with iodoacetic acid they obtained evidence that at least 18 of these existed as free SH groups. No explanation was given for the rather marked discrepancy with earlier results. In spite of this report most workers in the field still seem to use the value obtained by

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Madsen and Cori (1956).

In the present work the number of SH groups in phosphorylase b was determined with pCMB and DTNB (5,5'-dithio-bis-(2-nitro-benzoic acid)), in the presence and absence of protein denaturing agents. The results support the conclusion of Appleman et al. (1963) and they provide an explanation for the discrepancy between these data and those of Madsen and Cori (1956).

Materials and Methods

Four times recrystallized rabbit muscle phosphorylase b was prepared according to Fischer and Krebs (1958). Cysteine and AMP (adenosine-5-monophosphate) were removed by passing the preparation, immediately before use, through a column of Sephadex G-100 (2.5 x 40 cm), equilibrated with 0.033 M glycerophosphate and 0.001 M EDTA, pH 6.8. The homogeneity of the purified enzyme was examined in an analytical ultra-centrifuge. A single sedimenting peak was obtained in the presence of mercaptoethanol. The specific activity (Illingworth and Cori, 1953) of the enzyme was approximately 1600 units/mg of protein and $A_{260\ mu}/A_{280\ mu}$ was 0.53. For phosphorylase b a molecular weight of 242.000 was used (Krebs and Fischer, 1962).

Protein was determined by the method of Lowry et al. (1951) and by the method of Appleman et al. (1963). Protein sulfhydryl groups were determined with pCMB according to Boyer (1954) and with DTNB according to Ellman (1959).

Results and Discussion

Fig. 1 shows the titration of SH groups of phosphorylase b with pCMB. It is apparent that there is a significant diffe-

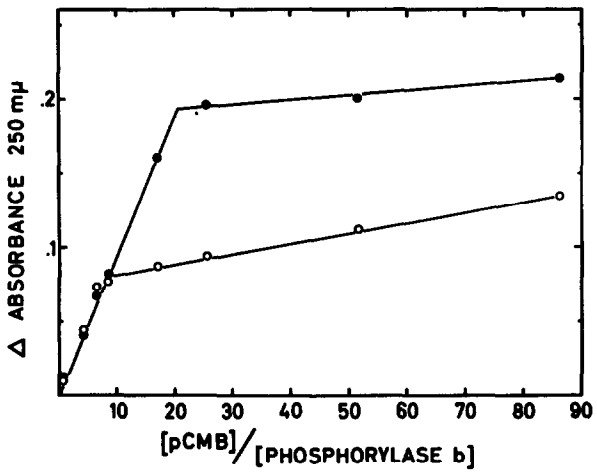

Fig. 1. The reaction of phosphorylase b with pCMB in the presence, ●——●, and absence, o——o, of 0.9% lauryl-sulphate. Protein concentration was 1.165×10^{-6}M. Temperature, 25°; pH 6.8.

rence in the number of SH groups titratable in the presence and absence of lauryl-sulphate. Thus, in the presence of this agent approximately 20 SH groups per molecule were found, whereas in its absence titration yielded only 9 SH groups. This lower value is in good agreement with that reported by Cori and Madsen who did not employ protein denaturing agents in their studies. The high value obtained here in the presence of lauryl-sulphate agrees well with that reported by Appleman et al. (1963).

The number of SH groups in phosphorylase b was also determined with DTNB which is assumed to be a more specific SH blocking agent. With lauryl-sulphate present approximately 20 SH groups reacted in the course of 1 hour (Fig. 2) whereas only 6 SH groups reacted in the absence of lauryl-sulphate. In the latter case longer reaction periods gave a significant increase in the number of SH groups. In a number of parallel experiments the total number of SH groups in the presence of lauryl-sulphate was found to vary between 18 and 22 SH groups per molecule.

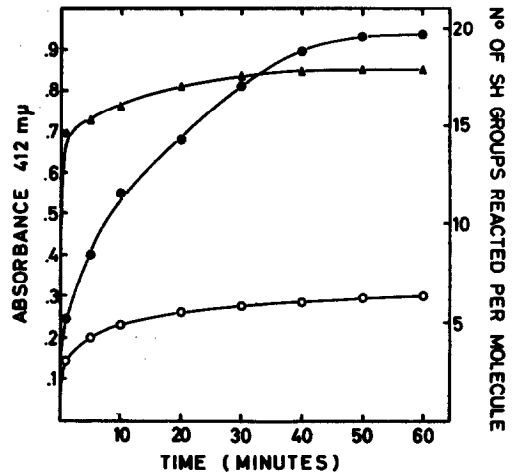

Fig. 2. The reaction of phosphorylase b with DTNB in the presence of 0.9% lauryl-sulphate, ●——●, and 8 M urea, Δ——Δ. o——o is a control containing no denaturing agent. The incubation mixture contained: 5×10^{-6} M phosphorylase b and 6.6×10^{-4} M DTNB. Temperature, 25°; pH 6.8.

When urea was used as an unfolding agent, the total number of titratable SH groups was somewhat lower. However, the time course shows that in this case the reactivity was higher than when lauryl-sulphate was used. In view of the higher reactivity of the SH groups in the presence of urea the possibility exists that the lower total number found might be due to some oxidation of the SH groups. The difference in reactivity under the different conditions may be a reflection of different mechanisms whereby these denaturing agents act. Thus lauryl-sulphate is thought to affect the hydrophobic bonds of the tertiary and quaternary structure, whereas urea is known to cause disruption of hydrogen bonds.

The present results are in agreement with the earlier data of Appleman et al. (1963). The reason for the inconsistency

with the results obtained by Madsen and Cori now seems clear. Some of the SH groups of phosphorylase b are unavailable due to steric hindrance. Unfolding of the peptide chains by agents such as lauryl-sulphate and urea exposes the masked SH groups to the sulphydryl reagents. Since earlier investigators did not employ unfolding agents they failed to detect these SH groups.

The experiments reported here do not provide an answer to the question whether or not muscle phosphorylases possess any disulphide linkages. However, preliminary data indicate that, in the presence of mercaptoethanol or cysteine, the enzyme contains no such bonds. Furthermore, dissociation into subunits also occurs after DTNB treatment as well as after pCMB treatment. These results will be reported in a forth-coming paper.

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