Alkylation of Bovine Brain Creatine Kinase

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(Received 26 May 1970)

Creatine kinases (EC 2.7.3.2) from muscle and brain tissues have two highly reactive thiol groups, blocking of which leads to inactivation (Thomson, Eveleigh, Laws & Miles, 1968). The reaction of these groups with alkylating agents has been studied by several workers, including (i) Mahowald, Noltmann & Kuby (1962), who found that the rate of inhibition of the rabbit muscle enzyme with iodoacetic acid was very dependent on protein concentration, (ii) O'Sullivan, Diefenbach & Cohn (1966), who found that creatine plus Mg^{2+} +ADP gave almost complete protection to the rabbit muscle enzyme against inhibition by iodoacetamide, and (iii) Hooton (1968), who found that chicken brain-type enzyme ($88\mu M$), as distinct from muscle enzyme, could not be inhibited more than 75% by a 100-fold excess of iodoacetamide or iodoacetate at 25°C. This author suggested that this behaviour was the result of a conformational rearrangement occurring on one alkylated thiol group leading to burial of the other active-site thiol group.

Kumudavalli, Moreland & Watts (1970) studied the reaction between human muscle creatine kinase and iodoacetamide. The most striking difference between this and the rabbit muscle enzyme was that creatine plus Mg^{2+} +ADP enhanced the inhibition by iodoacetamide instead of giving protection. Concentrations of this substrate combination that less than half-saturated the enzyme appeared to first activate the reactive thiol groups and then protect them as the reaction proceeded. The authors suggested that creatine plus Mg^{2+} +ADP enhanced the reactivity of the thiol group in the subunit to which they bound but caused protection of the reactive thiol group in the subunit without substrate: 'Some form of co-operatively induced conformational change between the subunits was indicated, and if this were stabilized by the alkylation of the more reactive thiol group this would explain why the reaction does not go to completion.' The same group (Milner-White & Watts, 1970) have also described the significance of anions in the protection of creatine kinase by substrates against inhibition by iodoacetamide.

We have obtained results with bovine brain creatine kinase that also suggest a co-operatively induced conformation change between the two subunits. In this case, however, these changes

were caused by alkylation alone in the complete absence of substrates. We have also found, as was observed with the chicken brain-type enzyme (Hooton, 1968), that under certain conditions of enzyme concentration and temperature complete inhibition by iodoacetamide cannot be achieved.

Bovine brain creatine kinase (specific activity 71 Kubu units/mg) was prepared by a method developed by R. S. Atherton, J. F. Laws, B. J. Miles & A. R. Thomson (unpublished work). Stoichiometric inhibition studies (enzyme concentration $140\mu M$, incubated for 30 min at 30°C, other conditions as in Table 1) showed that 1.4 mol of SH groups/mol of enzyme reacted with iodoacetamide to give complete inhibition. The extent of inhibition under these conditions was at a maximum and was near 100% with a 2:1 ratio of iodoacetamide to enzyme.

Table 1 illustrates the reaction of this creatine kinase with iodoacetamide. The non-alkylated control was significantly more unstable than the enzyme alkylated for 70 s, which had lost only 1.7% of its activity. As the extent of alkylation increased the remaining activity became increasingly more stable until, at 50% inhibition (equivalent to alkylation of one reactive thiol group?), the remaining activity was completely stable for at least 24 h.

A second-order plot of the extent of alkylation as a function of time was linear up to at least 2 h (when only 13.6% of the control activity remained); this assumes that loss of activity exactly parallels alkylation as with the rabbit muscle enzyme (Watts, Rabin & Crook, 1961) and takes into account that only 1.4 mol of SH groups/mol of enzyme was available for alkylation and enzyme activity (see above). Thus the reaction appears to follow second-order kinetics, suggesting that, although alkylation of one thiol group protects the other from oxidation, it does not alter its rate of reaction with further alkylating agent.

In the above experiment the enzyme was stored between assays at a concentration of $0.4\mu M$. The stability of the same enzyme preparation in the absence of alkylating agent was also studied at $3\mu M$. At this higher concentration the enzyme was stable for at least 24 h after dilution, regardless of whether the buffer contained $1\mu g$ or $0.1 mg$ of

Table 1. *Stabilization of creatine kinase by iodoacetamide*

Incubation conditions were as follows: enzyme concentration, 160 μ M; iodoacetamide/enzyme ratio, 2:1; temperature, 3°C; buffer, 10 mM-tris-HCl, pH 7.7, containing dithiothreitol (1 μ g/ml). Samples were removed at various times after the addition of iodoacetamide and diluted 400-fold with 10 mM-tris-HCl buffer, pH 7.7, containing dithiothreitol (0.1 mg/ml). These solutions were stored at 3°C and assayed as described by Kuby, Noda & Lardy (1954) (except that acetate replaced sulphate) 3 h, 6 h and 24 h after dilution. Zero-dilution-time activities were obtained by extrapolation of the 6 h and 3 h values; they were directly proportional to the logarithm of the alkylation time up to 45 min.

Incubation time (min)	Specific activity (Kuby units/mg) at zero dilution time	Decrease in specific activity/h after dilution	
		0-6 h	6-24 h
Control	71.0	4.3	1.0
1.17	69.8	2.8	0.7
5	62.5	1.8	—
10	56.1	1.7	0.3
20	44.1	1.1	—
30	34.3	0	0

dithiothreitol/ml or 0.1 mg of dithioerythritol/ml. However, activities in dithioerythritol solution were 30% higher than in dithiothreitol.

The results of other alkylation experiments have also suggested that concentration has an effect on the conformation of bovine brain creatine kinase. These may be summarized as follows. (1) At 3 μ M-enzyme complete inhibition with iodoacetamide cannot be achieved at either 3°C or 30°C, even with a 100:1 iodoacetamide/enzyme ratio. A plateau effect similar to that reported by Hooton (1968) was found. (2) At concentrations above 100 μ M-enzyme complete inhibition is achieved at 30°C with only a 2:1 iodoacetamide/enzyme ratio and virtually complete inhibition is achieved at only 3°C.

Our results, and those of others referred to in this paper, suggest that alkylation of one of the essential thiol groups of creatine kinase can lead to protection of the other even in the absence of substrate.

Both binding of substrates and partial alkylation lead to conformational changes in creatine kinase (O'Sullivan *et al.* 1966; Kumudavalli *et al.* 1970), and the stability of partially alkylated species of the enzyme varies with degree of alkylation (this paper). In addition, the degree and detailed mechanism of

inhibition varies with the anion (Milner-White & Watts, 1970). Further, the binding constants for substrate and inhibitor may vary by as much as an order of magnitude [e.g. K_m (creatine) for rabbit muscle creatine kinase is 16.0 mM, for the human muscle enzyme is 1.47 mM and for the ox brain enzyme is 3.3 mM]. In view of this extremely complex situation great caution must be exercised in interpreting protection experiments that have not been carried out under identical conditions.

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