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The effect of trace metal contaminants and EDTA on the velocity of enzyme-catalysed reactions. Studies on ATP:creatine phosphotransferase

It has been shown[1] that the activity of ATP:creatine phosphotransferase (creatine kinase, EC 2.7.3.2), in the absence of added metal ions, can be attributed to the presence of trace amounts of activating metal ions (Mg^{2+} and Ca^{2+}) in the reaction components. Treatment of the latter compounds with Dowex-50 (Na^+ form) resulted in a reduction of the concentration of these metal ions and at the same time, the residual activity was reduced. Furthermore, it was reported[1] that the addition of EDTA at a concentration slightly higher than that of Mg^{2+} and Ca^{2+} eliminated the residual activity while the presence of lower concentrations increased the enzymic activity under standard conditions with substrates which had not been treated with Dowex-50 resin.

These results suggest that the activity of the enzyme was limited by virtue of the presence of heavy metal ions in the reaction mixtures. Further investigations have now been made of the effect of various chelating agents and metal ions on the activity of creatine kinase. Of particular interest is the finding that this enzyme undergoes inactivation during the course of the reaction when a chelator is omitted and that this inactivation may be prevented by the use of low concentrations of EDTA.

The enzyme preparation and substrates were as described previously[1]. Enzymic activity was determined by following the release of creatine from phosphoryl-creatine in the presence of ADP and *N*-ethylmorpholine buffer (pH 8.0).

Although the activity of creatine kinase is considerably increased by the addi-

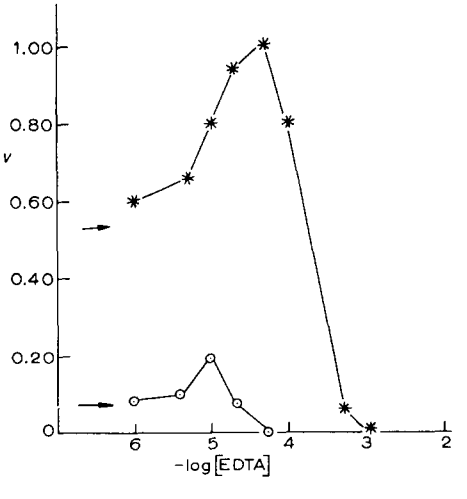

Fig. 1. The effect of EDTA on the velocity of the reaction catalysed by creatine kinase. The reaction mixture contained 0.1 M *N*-ethylmorpholine (pH 8.0), $2.8 \cdot 10^{-4}$ M ADP, 10^{-2} M phosphorylcreatine, and 0.9 μg creatine kinase. Total volume, 1.0 ml; temperature, 30°. The velocity is expressed in arbitrary units. *—*, $5 \cdot 10^{-4}$ M $MgCl_2$; ○—○, no added metal ion. The position of the arrows indicates the observed velocity in the absence of EDTA.

tion of Mg^{2+} ions (Fig. 1), the pattern of activation and inhibition obtained with increasing concentrations of EDTA is similar both in the absence and presence of Mg^{2+}. However, it may be noted that the concentration of EDTA required for maximum activity is less in the absence of added $MgCl_2$ than in its presence. These results are in accord with the idea that the enzymic activity is limited by virtue of the presence of heavy metal ions and suggest that $MgCl_2$ may be an additional source of such ions.

Apart from increasing the enzymic activity, low concentrations of EDTA also stabilized the enzyme during the course of the reaction (Fig. 2). The activity–time curve for the reaction in the absence of EDTA has been drawn as a biphasic curve since linearity was obtained consistently when the activity was determined between 0.5 and 2 min and also at shorter times between 10 and 40 sec. However, since no detailed investigations were carried out, it is not possible to decide if the results give a true biphasic rather than a smooth curve which would be expected if the enzyme undergoes a time-dependent denaturation. It should be noted that while the blank value, representing the free creatine in the phosphorylcreatine sample, obtained by extrapolation of Curve 1 is identical with the value determined directly, that obtained from Curve 2 is considerably higher. The results obtained on adding EDTA, at a concentration of 10^{-5} M, gave a simple linear plot (Curve 3) which cut the ordinate at a point corresponding to the correct blank value. The addition of EDTA also resulted in an increase of the reaction velocity. Such results are consistent with the reduction of the concentration of inactivating heavy metal ions.

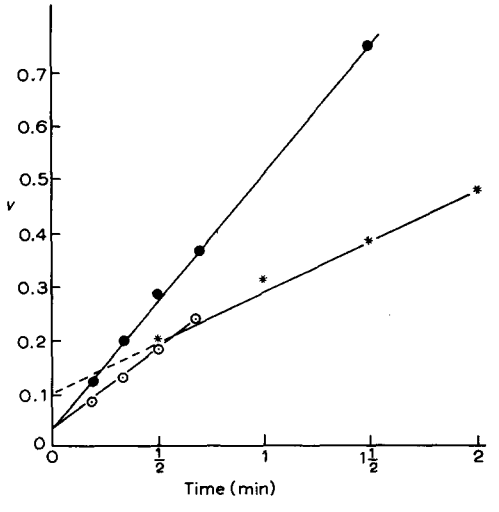

Fig. 2. The kinetics of the reaction catalysed by creatine kinase in the presence and absence of EDTA. The reaction mixture contained 0.1 M *N*-ethylmorpholine (pH 8.0), 10^{-2} M phosphorylcreatine, $2.4 \cdot 10^{-4}$ M ADP, $3.1 \cdot 10^{-4}$ M $MgCl_2$, and 0.9 μg creatine kinase. Total volume, 1.0 ml; temperature, 30°. Activity is expressed in arbitrary units. ○—○ (Curve 1) and *—* (Curve 2), no added EDTA; ●—● (Curve 3), 10^{-5} M EDTA.

Other metal chelators also showed the ability to activate creatine kinase. Concentrations of histidine up to 5 mM increased the enzymic activity as did 8-hydroxyquinoline within the concentration range from $7.5 \cdot 10^{-5}$ M to $30 \cdot 10^{-5}$ M. The same

maximum activity was obtained with each of the above compounds at concentrations of approx. 10 mM and 2 mM, respectively. On the other hand, cysteine and imidazole from $4 \cdot 10^{-5}$ M to 10^{-3} M had no effect either in the presence or absence of Mg^{2+}. From these results, it may be concluded that while the former compounds, within the concentration range tested, chelate the heavy metal ions without affecting the Mg^{2+} concentration, the latter compounds do not react to any appreciable extent with either Mg^{2+} or the heavy metal ions.

The enzyme was completely and irreversibly denatured by Cd^{2+}, VO^{2+}, Zn^{2+}, Cu^{2+} and UO_2^{2+} at a concentration of $5 \cdot 10^{-4}$ M, and reversibly inactivated by Ni^{2+} and Cr^{3+} at the same concentration. These findings offer support for the conclusion that the effect of EDTA in protecting the enzyme from inactivation and increasing its activity is due to its ability to chelate heavy metals. Moreover, they are in accord with the fact that free SH groups are essential for creatine kinase activity[2,3]. In these respects, the results are similar to those reported by MILSTEIN[4], who showed that the activation of D-glucose 1,6-diphosphate:D-glucose 1-phosphate phosphotransferase (phosphoglucomutase, EC 2.7.5.1) by chelating agents is due to their reaction with heavy-metal contaminants. It is of interest to note that the alkaline earth metals, Be^{2+}, Sr^{2+} and Ba^{2+}, were also capable of causing enzyme inhibition and that there was activation by Co^{2+} as well as by Mg^{2+}, Ca^{2+} or Mn^{2+}.

It is clear (Fig. 2) that the failure to take precautions to ensure that inactivating metal ions are not present in the test system can lead to erroneous reaction velocities, and hence incorrect conclusions. Such an omission can also cause difficulties, for example, with product inhibition studies when the product added is a better chelator than any of the substrates. While these difficulties can be overcome by treatment of each of the reaction components with Chelex resin or with dithizone[5], the simpler procedure of adding low concentrations of EDTA to the reaction mixture has been adopted.

In subsequent kinetic studies of reactions catalysed by other metal-activated enzymes, it has been found that the addition of $(5\text{–}10) \cdot 10^{-6}$ M EDTA also gives rise to a marked improvement in the reproducibility of reaction velocities. Under most circumstances, this concentration is sufficiently low as to have only a negligible effect on the concentration of any added metal ion.

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