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INHIBITION OF CREATINE KINASE BY IODOALKANES

FURTHER APPRAISAL OF THE ESSENTIAL NATURE OF THE REACTIVE THIOL GROUP

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Summary

Creatine kinase (ATP: creatine *N*-phosphotransferase) is completely inhibited by low molecular weight iodoalkanes in a pseudo first order reaction. Analysis of this and other data suggests that covalent modification per se is not a sufficient criterion to estabilish whether or not an enzyme group is essential for catalysis.

Creatine kinase (ATP: creatine *N*-phosphotransferase, (EC 2.7.3.2) has long been recognised as a thiol enzyme [1,2] containing one highly reactive thiol group per subunit of approx. 40 000 molecular weight [3]. Comparative studies reveal that this cysteine residue occurs in the same almost constant sequence in both creatine and other phosphagen kinases from a diversity of species [4], indicating that a significant functional role has been highly conserved in evolution. The available evidence favours a role mediating an enzyme conformational change rather than an involvement as a contact amino acid in the catalytic process per se [5,6].

Alkylation of creatine kinase with iodoacetate or iodoacetamide causes essentially complete enzyme inhibition [5,7]. However, in 1968 Hooton [8] reported that blocking the chicken enzyme with iodomethane left a significant residual enzyme activity, 8% and 32% for the muscle and brain types, respectively. More recently it was found for the rabbit muscle enzyme that generation of the mixed disulphide by reaction with a series of alkanethiolsulphonates resulted in up to 19% residual enzyme activity [9,10] while with the S-cyano derivative the residual activity was approx. 80% [11]. This led naturally to the rather ill-defined proposal that the thiol group was 'non-es-

sential'. It was suggested [9,10] that because covalent modification with small, hydrophobic residues could be accomodated without complete loss of enzyme activity the thiol group had no important role in the overall catalytic process. The present work was undertaken to further investigate this problem.

Creatine kinase was isolated from rabbit skeletal muscle as described previously [12] and finally dialysed overnight against *N*, *N'*-dihydroxyethylglycine (bicine) buffer, pH 8.6, $I = 0.01$, containing 1 mM EDTA and 5 mM dithiothreitol. The specific activity was 50—70 μmol product$\cdot$min$^{-1}\cdot$mg^{-1} protein in the direction of phosphocreatine synthesis measured by the phosphate assay procedure at 30°C [13] and 300—600 units$\cdot$min$^{-1}\cdot$mg^{-1} protein in the reverse direction by a coupled enzyme assay [14]. Agarose gel electrophoresis at pH 8.6 (Corning AC1 System, 490 San Antonio Rd., Palo Alto, CA, U.S.A.) showed a major band correlating with enzyme activity and a trace of a more cathodal component. The phosphate assay was used to monitor the effect of inhibitors on enzyme activity at 30°C in an assay mixture containing 25 mM creatine, 4 mM ATP (disodium salt), 4 mM magnesium acetate, and sodium bicinate buffer, pH 8.6 and $I = 0.1$. The enzyme concentration was chosen so that the rate of product formation was linear with time over the 10-min incubation period used.

Inhibition was carried out by equilibrating four vols. of enzyme solution (58.75 μg protein/ml) or inhibitor, both solutions being made up in the assay buffer, for 5 min at 30°C and then adding one vol. of inhibitor or enzyme to start the reaction. The final concentrations of enzyme and the inhibitors used are given below. At appropriate time intervals the reaction was stopped by transferring 0.1 ml samples to 0.5 ml of ice cold cysteine solution in the same buffer. The cysteine concentration was varied according to the inhibitor being used; 5 mM for iodoacetate and iodoacetamide, 20 mM for iodomethane and iodoethane, and 100 mM for iodopropane. Residual enzyme activity was measured in samples of the enzyme/inhibitor/cysteine mixture. Control experiments showed that all the stopping solutions acted instantaneously and that the enzyme assay was unaffected by the variation in cysteine concentration.

Iodocompounds were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Iodoacetate and iodoacetamide were purified as described previously [2]. Iodoalkanes were first treated with sodium thiosulphate to remove free iodine, dried over sodium sulfate and distilled shortly before use. They were solubilized in bicine buffer by vigorous shaking in a capped tube using a Whirlimix vortex mixer until the initial cloudy suspension turned into a clear solution.

Table I lists the second order rate constants (k) for the inhibitors investigated. An enzyme molecular weight of 83 000 was assumed and the listed values of k should be halved to obtain constant on a reactive thiol basis.

Halasz and Polgar [15] found that the ratio of k for thiolsubtilisin/glutathione was twenty fold greater with iodomethane than with iodoacetamide. This 'rate enhancement' was shown to reflect the greater reactivity of iodomethane in the local hydrophobic environment of the enzyme as compared with the more polar iodoacetamide. The rate constants for both inhibitors with glutathione could be increased three to four fold by making the environ-

TABLE I

SECOND ORDER RATE CONSTANTS FOR THE INHIBITION OF RABBIT MUSCLE CREATINE KINASE BY DIFFERENT ALKYLATING AGENTS AT 30°C IN *N,N*-BIS-(2-HYDROXYETHYL) GLYCINE/NaOH BUFFER, pH 8.6, $I = 0.1$

Alkylating agent	No. of determinations	K ($M^{-1} \cdot min^{-1}$) (mean ± S.D.)
Iodoacetamide	1	1010
Iodoacetate	1	234
Iodomethane	5	21.9 ± 6.6
Iodoethane	6	1.68 ± 0.35
1-Iodopropane	2	0.05
2-Iodopropane	1	0

ment more hydrophobic by the addition of dioxan.

Comparison of the present data with that of Halasz and Polgar reveals that at 30° C iodoacetamide yields similar rate constants/thiol with both thiolsubtilisin (9.9 $mol^{-1} \cdot s^{-1}$) and creatine kinase (8.4 $mol^{-1} \cdot s^{-1}$) but with iodomethane k is thirty fold greater with thiolsubtilisin (5.9 $mol^{-1} \cdot s^{-1}$) than with creatine kinase (0.18 $mol^{-1} \cdot s^{-1}$). The ratios of k for creatine kinase/glutathione are 0.227 with iodoacetamide and 0.121 with iodomethane. Thus the rate enhancement for creatine kinase (0.121/0.227) is 0.53 compared with approx. 20 for thiolsubtilisin. It may reasonably be concluded that both the small value of k with iodomethane and the lack of rate enhancement indicate that the thiol of creatine kinase, unlike that of thiolsubtilisin, reacts as though it is located in an essentially aqueous environment. This is in accord with an earlier finding that blocking creatine kinase with iodoacetate is without effect on electrophoretic mobility but blocking with iodoacetamide reduces the mobility by one unit of charge for each thiol/subunit in the enzyme molecule [16,17].

The above analysis indicates that the reactive thiol is located at or near the enzyme surface and interacts with the environment. The second order rate constant for the reaction of glutathione with 2-bromopropionamide is about 100-fold slower than with bromoacetamide [15]. A similar decrease is found for creatine kinase in going from iodoethane to iodopropane (Table I). Thus there is no evidence here to suggest any specific enzyme-iodoalkane interaction. Fig. 1A provides the additional information that iodomethane and iodoethane, like iodoacetate and iodoacetamide, give linear semilog plots for up to 90% inhibition of enzyme activity. Both iodoalkanes cause complete

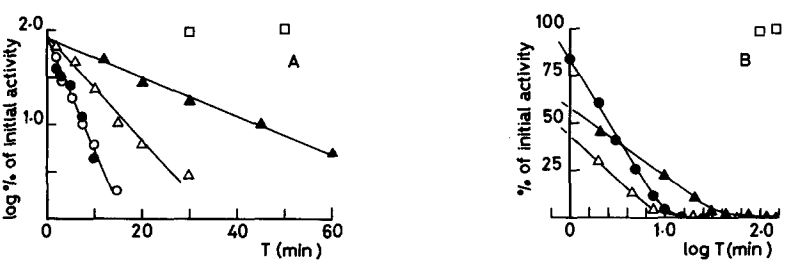

Fig. 1. Inhibition of rabbit muscle creatine kinase by iodocompounds at pH 8.6 and 30° C. The concentrations of inhibitors used were for A: ●, 0.2 mM iodoacetamide; ○, 1.0 mM iodoacetate; ▲, 19.6 mM iodoethane; △, 6.5 mM iodomethane; and for B: ●, 0.2 mM iodoacetamide; ▲, 80 mM iodoethane and △, 19.6 mM iodomethane. Controls without inhibitor are indicated by □. Other experimental details are given in the text.

inhibition (accurate to within 1%—2%) of the fully reduced enzyme (Fig. 1B) just as do iodoacetate and iodoacetamide [7]. However, it was observed that prolonged storage of the enzyme in the refrigerator after the initial dialysis against dithiothreitol resulted in a progressive appearance of a variable amount of residual enzyme activity—up to about 5% of the value before modification. Finally, the possibility was checked whether 1-iodopropane was reacting with creatine kinase without causing a commensurate degree of inhibition. Fig. 2 shows that the addition of iodoacetamide after incubation with 200 mM iodopropane results in the rapid inhibition expected if no unexpressed modification by 1-iodopropane had occurred.

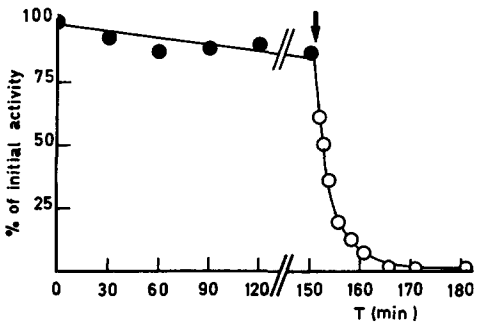

Fig. 2. Inhibition of rabbit muscle creatine kinase by 1-iodopropane at pH 8.6 and 30°C. The inhibitor concentration was 200 mM. At the time shown by the arrow iodoacetamide was added to give a final concentration of 0.2 mM.

It must be concluded that small, hydrophobic reagents, as exemplified by the iodoalkanes, do not possess special properties resulting in the alkylated enzyme possessing a significant residual catalytic activity. The question remains, then, why this is so upon introduction of $-SCH_3$ or $-SC_2H_5$ [10] both of which are larger than the methyl- or ethyl-radicles? Possible explanations emerge from the consideration of additional evidence. With iodoacetamide creatine kinase is only about one quarter as reactive as glutathione under similar conditions [15]. Further, creatine kinase reacts in a pH-independant manner with iodoacetamide over the normal stable range of the enzyme (pH 6.0—pH 9.5) [5]. These results indicate that the thiol group of the enzyme must be in the S^- form over the whole pH range in which the enzyme is stable. Histidine and lysine side chains have been implicated in the catalysis and might act by stabilizing the negative charge [5]. Unlike simple alkylation modification of the enzyme thiol by disulfide bond formation could, by an appropriate charge polarization to preserve a partial negative charge at or near the enzyme thiol group, allow the postulated interaction with a neighbouring amino acid residue to continue. The amount of residual activity would relate to the extent to which the original interaction was observed and depend primarily upon the chemistry of the modification rather than the size of the substituent although no residual activity has been found with large substituents such as the thionitrobenzoate ion [10]. This would explain why introduction of the small but hydrophilic -CN group results in more than 70% residual activity. It offers a less satisfactory explanation of the finding that covalent attachment of the relatively small sulphite ion causes complete inactivation [10, 18], even though

a negative charge is preserved near the enzyme thiol. An alternative explanation of these results is that both a thiol and cyanide may readily transfer from the enzyme thiol to another group on the enzyme, but only after the binding of substrates to induce the working enzyme conformation. This is in line with current views on the mechanism of action of other kinases derived from structural studies in which substrates induce a large conformation change to bring together catalytically active side chains situated in separate domains of the enzyme [19].

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