Effects of Temperature and Reagent Size on the Reaction of the Thiol Groups of Rabbit Muscle Creatine Kinase

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The dimeric enzyme creatine kinase (EC 2.7.3.2) from rabbit muscle has a reactive thiol group on each subunit, modification of which by iodoacetate or iodoacetamide leads to inactivation of the enzyme (Watts, 1973). It had been previously shown (Price & Hunter, 1976) that when the enzyme is treated with iodoacetate or some other reagents in the presence of the 'transition-state analogue' complex (i.e. Mg$^{2+}$+ADP+creatine+nitrate), the thiol groups exhibit differential reactivity. Analysis of these reactions has suggested that differences in reactivity of up to about 7-fold between the groups can be exhibited under these conditions.

A study of the reaction of the enzyme with various derivatives of iodoacetamide and at different temperatures has revealed further conditions under which differential reactivity of the groups can be exhibited. The reagents used in the study were iodoacetamide, 4-iodoacetamidosalicylic acid, N-(iodoacetylaminooethyl)-5-aminonaphthalene-1-sulphonic acid and 2-[4'-(2''-iodoacetamido)phenyl]aminonaphthalene-6-sulphonic acid. Modification of the enzyme was studied by monitoring the enzyme activity of portions of the reaction mixtures withdrawn at known times and diluted into 0.1 M-glycine/NaOH buffer, pH 9.0, containing 2mM-dithiothreitol. Control experiments showed in each case that this dilution procedure effectively stopped the reaction.

It was also shown, for the reaction of the enzyme with the iodoacetamide derivatives, that the decrease in enzyme activity was proportional to the amount of reagent incorporated, and to the loss of thiol groups which reacted rapidly with 5,5'-dithiobis-(2-nitrobenzoic acid), and hence with iodoacetamide (Watts, 1973; Price & Hunter, 1976). All reactions were performed in 50mM-Tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine)/NaOH buffer, pH 8.0, at either 25 or 0°C, as indicated. The reactions were analysed as described previously (Price & Hunter, 1976) with the results summarized in Table 1.

The results show that the reactions of iodoacetamide with the enzyme at 25°C and at 0°C follow second-order kinetics for at least 85% of the total reaction, and thus that the two thiol groups react at the same rate as each other. In the case of 4-iodoacetamidosalicylic acid and N-(iodoacetylaminooethyl)-5-aminonaphthalene-1-sulphonic acid,

Table 1. Reaction of creatine kinase with iodoacetamide derivatives

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Second-order rate constant (m$^{-1}$·min$^{-1}$)</th>
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<tbody>
<tr>
<td>Iodoacetamide</td>
<td>450</td>
</tr>
<tr>
<td>4-Iodoacetamidosalicylic acid</td>
<td>100000</td>
</tr>
<tr>
<td>N-(iodoacetylaminooethyl)-5-aminonaphthalene-1-sulphonic acid</td>
<td>16000</td>
</tr>
<tr>
<td>2-[4'-(2''-iodoacetamido)phenyl]aminonaphthalene-6-sulphonic acid</td>
<td>Biphasic reaction</td>
</tr>
</tbody>
</table>

The reactions were performed in 50mM-Tricine/NaOH buffer, pH 8.0.
the characteristics of the reactions differ at the two temperatures; at 25°C the reactions follow second-order kinetics for at least 85% of the total reaction. However, at the lower temperature, the second-order kinetic plots show distinct curvature, reflecting a differential reactivity of the thiol groups on the two subunits of the enzyme.

With the largest iodoacetamide derivative studied, 2-[4'-(2'-iodoacetamido)phenyl]-aminonaphthalene-6-sulphonic acid, very marked differential reactivity was observed at both temperatures studied. For instance, at 25°C, with concentrations of enzyme subunits and reagent of 87 and 190 μM respectively, reaction of one thiol group per dimer was complete within 30s, whereas reaction of the second thiol group was only 80% complete after about 100min. Haugland (1975), using fluorescence measurements to monitor the reaction, has also indicated that the reaction is markedly biphasic. This marked differential reactivity should permit the preparation of derivatives modified in a single subunit by reaction of the enzyme with one molecule of reagent per dimer.

The effect of reagent size on the characteristics of the reaction probably arises from a conformational change induced in the second subunit after incorporation of a bulky aromatic moiety at the thiol group in the first subunit (Levitzki, 1974). The effect of temperature suggests that there may well be some difference in the strength and/or type of subunit interactions in the enzyme at 25 and at 0°C. It would be of interest to study various properties of the enzyme such as thiol-group reactivity and ligand binding over a range of temperatures, to see whether there exists a temperature transition of the type noted for glycogen phosphorylase b (Birkett et al., 1971).

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Nucleotide and Sugar Substrate-Binding Sites on Yeast Hexokinase in Solution as the Native Dimeric or Monomeric Form

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Yeast hexokinase isoenzymes A and B differ in isoionic point and in that form B has a 3-fold greater maximum reaction velocity with glucose (Ramel et al., 1971). Each contains two identical subunits.

The inert co-ordination complex of ATP with chromium (CrATP) was reported by Danenberg & Cleland (1975) to bind tightly, along with glucose, to hexokinase, to an extent suggested to be about one each per molecule, but there was great variation in the apparent combining molecular weight of the commercial hexokinase used [known to be partly degraded proteolytically, existing as a partly active monomer (Rustum et al., 1971; Colowick, 1973)]. We have applied CrATP to the native A and B isoenzymes in the presence of glucose, with either ligand isotopically labelled. The results (Table 1) show that CrATP binds in a ternary complex to an extent approaching 2 nucleotide molecules per molecule of dimeric protein. This amount decreased as the period was increased between the incubation and the separation of the complex by gel filtration, showing that the uncorrected values in Table 1 are lowered by partial dissociation of each ligand from the complex during the minimum period required for its separation, glucose dissociating faster than CrATP. The gel column profiles or radioactivity in the two cases, relative to the protein, were slightly displaced in accord with this deduction. Extrapolation back to