reacted with other reactive groups in the protein. However, it would appear that, whatever other reactions may have occurred, the increase in $A_{242}$ specifically monitors the extent of modification of histidine residues only. Thus diethyl pyrocarbonate appears to have reacted with pig pepsinogen to modify, at least, all three histidine residues in the protein. Once again, therefore, the potential activity observed of 30% must be inherent in the completely modified protein and cannot be derived from molecules which have undergone only fractional modification.

Since both of these types of reaction produce derivatives which are virtually completely modified but which possess only a fraction of the potential activity of native pepsinogen, derivatives could be used to study further the activation process. They may activate themselves more slowly than unmodified pepsinogen, and this would facilitate following the course of activation.

This work was supported by the Medical Research Council (grant no. G974/126/B). We are very grateful to Dr. John R. Coggins of the University of Glasgow for generously providing us with methyl acetamidate.


Superoxide Dismutase from Bacillus stearothermophilus: Reversible Removal of Manganese and its Replacement by other Metals

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Superoxide dismutase is widely distributed among oxygen-metabolizing organisms, where it catalyses the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (for a review see Fridovich, 1975). The enzyme occurs in two evolutionarily distinct forms. Cu/Zn-containing enzymes, found principally in the cytoplasm of eukaryotic organisms, are dimers of chemically identical subunits each containing one atom of Cu and one atom of Zn. The metal ligands and three-dimensional structure of the Cu/Zn enzyme from bovine erythrocytes have now been elucidated (Richardson et al., 1975). Superoxide dismutases from eukaryotic mitochondria and prokaryotes contain Mn or Fe and are dimers or tetramers of chemically identical subunits.

Superoxide dismutase from Bacillus stearothermophilus is a dimeric enzyme with a subunit mol.wt. of 20000. It gives a purple-red colour in solution, and metal analysis by neutron-activation and atomic-absorption methods show that it contains one atom of Mn per dimer (Brock et al., 1976). The metal is very firmly bound and it has proved difficult to prepare metal-free (apo-) protein from any mesophilic source, owing to its instability under the conditions necessary for complete removal of the bound metal (Ose & Fridovich, 1976). Thermophilic enzymes in general are considerably more stable than their mesophilic counterparts (Suzuki & Harris, 1971; Hocking & Harris, 1976), and it has proved possible to prepare a stable dimeric apoprotein from B. stearothermophilus Mn-containing superoxide dismutase (Brock et al., 1976) by exposing the native enzyme to EDTA in the presence of 8M-urea at acidic pH. Moreover, reconstitution of the fully active Mn enzyme was also accomplished by adding an
Table 1. Polyacrylamide-gel electrophoresis at pH8.9 of reconstituted superoxide dismutase

All samples (1–8) migrated to the same position on the gel. Gels were stained for protein with Coomassie Brilliant Blue R, and for superoxide dismutase activity as described by Beauchamp & Fridovich (1971). Key to samples: (1) native superoxide dismutase; (2) apo-(superoxide dismutase); (3)–(7), superoxide dismutase reconstituted with Mn (3), Fe (4), Co (5), Ni (6) and Cu (7); (8), superoxide dismutase reconstituted with Mn from Co-reconstituted enzyme. +, Stained; −, not stained.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
<th>(8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Superoxide dismutase activity</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

excess of MnCl₂ to apoprotein under the same dissociating conditions. Although enzymically inactive, the apoprotein was virtually indistinguishable from native enzyme when examined by gel filtration and gel electrophoresis (Table 1). Further evidence for gross conformational identity has come from a comparison of the proton-magnetic-resonance spectra of the native and apoenzymes (C. J. Brock, A. E. G. Cass, J. I. Harris & H. A. O. Hill, unpublished work).

Mn superoxide dismutase reconstituted from apoprotein with MnCl₂ was fully active and indistinguishable from the native enzyme by its absorption spectrum, Mn content and electrophoretic mobility on polyacrylamide gels (Table 1). Apo-(superoxide dismutase) has been reconstituted with a number of transition metals by the same method, but replacing MnCl₂ with the appropriate transition-metal salt. Co, Fe, Ni and Cu were all found to bind to the apoprotein with approximately the same stoichiometry as Mn to give electrophoretically native but inactive derivatives (Table 1) (cf. Ose & Fridovich, 1976).

The complete reversibility of reconstitution was demonstrated by taking superoxide dismutase reconstituted with Co, making apoenzyme from it, and reconstituting again with Mn. The end product was fully active and indistinguishable from the native Mn enzyme by all the criteria mentioned above.

In the resting state the metal in Mn- and Fe-containing superoxide dismutases has been shown to occur as the tervalent cation (Mn³⁺ or Fe³⁺) (Fee et al., 1976; Lumsden et al., 1976), and it is noteworthy that the absorption spectra of the Co and Fe proteins reconstituted from the B. stearothermophilus Mn enzyme with the appropriate bivalent-metal salts are also typical of the respective tervalent-metal complexes. The main requirement for metal binding would thus appear to be the ability to form complexes of this type, suggesting that this class of superoxide dismutase could have evolved from a single ancestral tervalent-metal-binding protein. This hypothesis gains support from the considerable sequence homology found among Mn- and Fe-containing enzymes from a wide variety of different organisms (Harris & Steinman, 1977). In the case of the B. stearothermophilus Mn enzyme the ability to bind different metals nevertheless contrasts sharply with the absolute specificity for Mn that is a requirement for superoxide dismutase activity.

Interaction of Mitochondrial Adenosine Triphosphatase with Nucleotides and with Glycerol

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A nitrobenzofurazan group can be specifically introduced to one tyrosine residue of mitochondrial ATPase, which is then inactive (Ferguson et al., 1975a). At pH 9.0 the nitrobenzofurazan group undergoes an intramolecular shift to a nitrogen atom in a β-subunit of the enzyme, which remains inactive (Ferguson et al., 1975b). The fluorescence of the N-nitrobenzofurazan group was shown previously to be quenched upon binding of ADP to the modified ATPase, and an approximate dissociation constant can be estimated for this ADP binding (Table 1; Ferguson et al., 1975b). This effect is specific for ADP, as we have now found that no fluorescence quenching is observed when GDP or IDP is added to the modified enzyme (Table 1). There is evidence for an ADP-specific site on the ATPase, which is characterized by a dissociation constant of approximately 1 μM (Senior, 1973; Pedersen, 1975). At present we have not distinguished whether the N-nitrobenzofurazan fluorescence senses a previously unrecognized weak ADP-specific binding site, or whether the effect of modification of the enzyme is to weaken the binding of ADP to the specific site described by others.

ATP, ITP and GTP all enhanced the fluorescence of the N-nitrobenzofurazan group and the dissociation constants were measured (Table 1). It is not yet known if these ligands are binding to the same site at which ADP produces a fluorescence quenching, but the presence of 4 mM-ATP increased the apparent dissociation constant for ADP to 2.0 mM, which suggests that either ADP or ATP is competing for the same site or that there is an interaction between two putative distinct sites. The high dissociation constants obtained for the nucleoside triphosphates can be explained by similar reasons to those suggested to account for the high dissociation constant (Table 1) for ADP binding to the modified enzyme. Further work should resolve these alternatives and may help explain why modification of ATPase by the nitrobenzofurazan group inhibits activity.

Garrett & Penefsky (1975) have reported that nucleotides are completely removed from the ATPase by passing the enzyme through Sephadex G-50 in 50% (v/v) glycerol/50 mM-Tris/H₂SO₄, pH 8.0. In our hands, with our preparations of the enzyme, this procedure

* Abbreviation: ATPase, adenosine triphosphatase.

Table 1. Apparent dissociation constants for the binding of nucleotides to N-nitrobenzofurazan-modified ATPase estimated from changes in N-nitrobenzofurazan fluorescence

Nucleotides were titrated into a 0.1 mg/ml solution of N-nitrobenzofurazan-modified ATPase in 200 mM-sucrose/50 mM-triethanolamine hydrochloride/4 mM-EDTA, pH 7.5. Fluorescence was measured as described previously (Ferguson et al., 1975b).

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Limiting change in fluorescence</th>
<th>Apparent dissociation constant (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>30% quenching</td>
<td>0.8</td>
</tr>
<tr>
<td>GDP</td>
<td>Nil</td>
<td>—</td>
</tr>
<tr>
<td>IDP</td>
<td>Nil</td>
<td>—</td>
</tr>
<tr>
<td>ATP</td>
<td>12% enhancement</td>
<td>0.85</td>
</tr>
<tr>
<td>ITP</td>
<td>12% enhancement</td>
<td>1.35</td>
</tr>
<tr>
<td>GTP</td>
<td>12% enhancement</td>
<td>1.84</td>
</tr>
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</table>

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