easier for cobalt than for iron, as the odd Co(II) electron is more unstable.] The only model mononuclear oxygen complexes for which structures are available have two vacant co-ordination sites for O₂ and are not strictly comparable.

Both in the cases of the haemoproteins and of model porphyrin and vitamin B₁₂ complexes oxidation by molecular oxygen is relatively slow. It can be 'catalysed' by the addition of one-electron reducing agents, implying that oxidation by oxygen needs two electrons, one from the metal, to be fast. Once a peroxide stage of a mononuclear complex is reached the breaking of the O-O bond is easy. Thus we suggest that in oxygen carriers and oxygen-storage systems it is the absence of a source of a second electron that prevents oxidation reactions. In such enzymes as terminal oxidases, cytochrome P-450 etc. we consider that there will be found a distal redox group of the protein, thiol or tyrosine for example, that can act as the source of the second electron, though it is obviously also possible for a substrate to act in this capacity.

The binding of oxygen to the metal in haemoproteins alters the binding to its other ligands, especially as a spin-state change is involved, so that a change in protein conformation is easily achieved, causing allosteric effects.

Two-metal centres. Crystal-structure studies of several model cobalt complexes have been reported. There are two main types of binuclear cobalt complexes: one with a twisted sequence Co-O-O-Co and one with a sequence Co-O(OH)-Co. In the first case two complexes with different charges have been studied. In one of them the cobalt is close to Co(III) and the oxygen is close to O₂^{•-}, whereas in the other the electronic structure is Co(III) and the oxygen is O₂²⁻. In all cases the oxygen is oxygen-bonded and is relatively inactive as an oxidizing agent. In the first case absorption spectroscopy shows a charge-transfer band at 700nm whereas in the second it is at 430nm. With reference to iron-containing proteins, Okamura, Klotz, Johnson, Winter & Williams (1969) have studied in detail the absorption and Mössbauer spectroscopy of Fe-O₂-Fe in haemerythrin. First, they have proved that there is an oxo-bridged Fe(III)-O-Fe(III) complex in the oxidized form of the proteins, so that there are two iron atoms, closely spaced, in the protein. On oxygenation of the Fe(II) protein there is no electron-paramagnetic-resonance spectrum; there is, however, a change in the Mössbauer and absorption spectra, which they interpret as being due to:

The uptake of oxygen by haemocyanin probably leads to a similar unit, Cu-O₂-Cu, and the difficulties of giving an electronic distribution to this complex are very similar to those with the iron-containing protein. In many respects the absorption spectrum is most like that expected for Cu(II)-O₂²⁻-Cu(II), but a Cu(I) or a Cu(III) complex could not be excluded. New physical methods, such as X-ray-induced electron-emission spectroscopy, are required to decide which structure is correct.

The overall picture of the first steps in oxygen reaction is that the interaction of oxygen with metal ions can lead to one of several electronic states, O₂, O₂^{•-}, O₂²⁻ (or even 2O²⁻ in some mononuclear models with two vacant co-ordination sites), all of which lie close in energy when the metal atom, or pair of metal atoms, has two or more valence states that are not grossly different in energy. There is no evidence that these complexes are exceedingly reactive in themselves, for internal attack on protein side chains does not occur. Oxygenation or oxidation, rather than the carrying of oxygen, would seem to depend on nearby reactive side chains of the protein (terminal oxidase) or possibly of a second substrate (oxidase) or of both (mixed-function oxidase).

Bayston, J. H., King, N. K., Looney, F. D. & Winfield, M. E. (1969). *J. Am. chem. Soc.* **91**, 2775.

Okamura, M. Y., Klotz, I. M., Johnson, C. E., Winter, M. R. C. & Williams, R. J. P. (1969). *Biochemistry*, *Easton*, **8**, 1951.

Co-operation of Electron-Accepting Sites in Oxygen Reduction by Oxidases

By Bo G. MALMSTRÖM. (*Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Göteborg, Sweden*)

Oxidases generally require metals for their catalytic function. A natural hypothesis for the role of the metal would be a mechanism in which a high-valence form oxidizes the substrate by accepting an electron, and the metal ion in the reduced enzyme is then reoxidized by molecular oxygen. Such a scheme can be expressed by the following equations in which E and M represent enzyme protein and the metal respectively and AH₂ the oxidizable substrate:

Substrate oxidation (eqn. 1) is here depicted as a one-electron transfer, which is certainly true for cytochrome c oxidase, and also for the only other oxidases reducing oxygen to water, namely the blue copper-containing oxidases (Malkin & Malmström, 1970), e.g. ascorbate oxidase and laccase (*p*-diphenol oxidase) (see Broman, Malmström, Aasa & Vänngård, 1963). Such a reaction is easy to visualize, and the major mechanistic problem is concerned with the

question how it is coupled to the four-electron transfer required for the reduction of molecular oxygen to water (eqn. 2).

Oxygen is a good oxidizing agent only from a thermodynamic point of view; kinetically it is rather inert. These properties form part of the concept of 'the fitness of oxygen' (George, 1965), but also pose severe mechanistic problems. Reduction in four consecutive one-electron steps would be expected to be extremely slow in view of the high free-energy barrier for the first step. This could be overcome by a stabilization of the O_2^- intermediate. However, in cases having water as the final product of reduction, there is another difficulty with the step-wise mechanism: the last free-radical intermediate, $OH\cdot$, is very reactive, and this could easily lead to undesirable side reactions.

The two difficulties just mentioned would be overcome in mechanisms involving multi-electron steps, e.g. two double-electron transfers, which could be made possible by the co-operation of several electron acceptors in the enzymes. Such a concept receives support from some simple considerations (Malmström, 1969). For example, the cytochrome oxidase unit contains four potential electron acceptors (haems α and α_3 plus two copper ions), and the 'blue' oxidases all contain at least four copper ions/molecule. Further, proteins that contain only one prosthetic group of a similar nature in each molecule, e.g. cytochrome *c* (haem) or azurin (copper), can carry out reactions of the type in eqn. (1) rapidly, but the reduced proteins are not autooxidizable (eqn. 2). Recent studies have now given a direct demonstration of the correctness of the multi-electron-step mechanism for one enzyme, namely laccase from the fungus *Polyporus versicolor*.

Laccase contains four copper ions/molecule. These represent three distinct forms of the metal: (1) type 1 Cu^{2+} ; (2) type 2 Cu^{2+} ; (3) two diamagnetic copper ions (Malmström, Reinhammar & Vänngård, 1968). Type 1 Cu^{2+} is responsible for the strong blue colour of the enzyme, and it also has unusual electron-paramagnetic-resonance parameters. Type 2 Cu^{2+} represents a spectrally more normal form of the metal. However, it has an exceptionally high affinity for F^- , which also acts as an inhibitor of the enzyme by interfering with the reaction in eqn. (2), whereas type 1 Cu^{2+} and its reduction (eqn. 1) are not affected. The two diamagnetic copper ions have been suggested to be a binuclear pair of spin-coupled Cu^{2+} (Fee, Malkin, Malmström & Vänngård, 1969). This pair is responsible for the strong absorption at about 330 nm found in all 'blue' oxidases (Malkin, Malmström & Vänngård, 1969).

In anaerobic redox titrations it has been found that the type 1 Cu^{2+} and the diamagnetic pair are reduced together in a linear fashion on the addition of about 3.5 electron equivalents. (Type 2 Cu^{2+} is only

partially reduced under these conditions, accounting for the non-integral end point.) All the electron-accepting sites have a potential greater than 500 mV. The linearity of the titration curve can be explained on the basis that the diamagnetic copper ions act as a co-operative two-electron-accepting unit, and that the type 1 Cu^{2+} has the same standard potential as this pair.

Kinetic studies (Malmström, Finazzi Agrò & Antonini, 1969) show that the type 1 Cu^{2+} and the diamagnetic pair are reduced by substrates rapidly enough to involve such reductive steps in the catalytic mechanism, although this does not seem to be true for the type 2 Cu^{2+} . Rapid reoxidation with molecular oxygen, on the other hand, occurs only when the two-electron-accepting unit is reduced. A mechanism involving two double-electron transfers to oxygen is consistent with all experimental results. The function of the type 2 Cu^{2+} in this mechanism may be to stabilize the HO_2^- intermediate.

- Broman, L., Malmström, B. G., Aasa, R. & Vänngård, T. (1963). *Biochim. biophys. Acta*, **75**, 365.
 Fee, J. A., Malkin, R., Malmström, B. G. & Vänngård, T. (1969). *J. biol. Chem.* **244**, 4200.
 George, P. (1965). In *Oxidases and Related Redox Systems*, p. 3. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
 Malkin, R. & Malmström, B. G. (1970). *Adv. Enzymol.* (in the Press).
 Malkin, R., Malmström, B. G. & Vänngård, T. (1969). *Eur. J. Biochem.* **10**, 324.
 Malmström, B. G. (1969). In *Symmetry and Function of Biological Systems at the Macromolecular Level*, p. 153. Ed. by Engström, A. & Strandberg, B. Stockholm: Almqvist and Wiksell.
 Malmström, B. G., Finazzi Agrò, A. & Antonini, E. (1969). *Eur. J. Biochem.* **9**, 383.
 Malmström, B. G., Reinhammar, B. & Vänngård, T. (1968). *Biochim. biophys. Acta*, **156**, 67.

Mixed-Function Oxidation

By G. S. BOYD. (Department of Biochemistry, University of Edinburgh Medical School, Edinburgh EH8 9AG, U.K.)

Enzymes that catalyse the introduction of molecular oxygen into a substrate are termed oxygenases. There are various types of oxygenase, and this account will be concerned with mono-oxygenases in which one atom of molecular oxygen is incorporated into the substrate as follows:

In these reactions there is no evidence as yet to indicate that a peroxide ($S\cdot O\cdot OH$) is an intermediate. Similarly there is as yet no evidence to suggest that the oxygen molecule is cleaved before incorporation into the substrate.