

# METALLOPROTEINS, METALLO-ENZYMES AND HETEROGENEOUS CATALYSTS

R. J. P. WILLIAMS

*Inorganic Chemistry Laboratory, Oxford, UK*

## ABSTRACT

An inspection of the active site region of metallo-enzymes leads to two conclusions. (i) Individual attacking groups usually have uncommon physical properties. (ii) The active site region is cooperatively linked with a large part of the enzyme. Such an interaction is common to heterogeneous catalysts but is absent in homogeneous catalysts. It is concluded that the energetics of catalysis requires an analysis of the nature of cooperativity in microdomains of well organized material and some general remarks as to the problems which arise are described. Methods of examining large well-organized molecules (microdomains) are described and some suggestions are made as to how man-made 'enzymes' could be produced.

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There is a very large number of metalloproteins and metallo-enzymes which are now well characterized. In this article it will be assumed that the reader is familiar with much of the work before 1970<sup>1</sup>. The article will be restricted to those metallo-systems which contain iron, cobalt, copper, zinc, or molybdenum. No reference will be made to either magnesium or calcium enzymes. The assumption will be made that metalloproteins, which have no enzyme activity, differ from metallo-enzymes in that, while the metal sites of metallo-proteins have relatively conventional physical properties, the sites of metallo-enzymes have more unusual properties. Elsewhere we have developed the idea that the metallo-enzymes are unusual because the metal ions in them do not have conventional ground state geometries<sup>2,3</sup>. The geometry may be unusual by virtue of bond length, bond angle, or both properties. These energized ground states have evolved to meet the functional catalytic requirements of biological systems, i.e. so as to carry out a certain number of reactions at high speed. As will be shown below it is most illuminating to compare enzymic catalysts with heterogeneous catalysts evolved by man.

As well as examining one or two new examples of enzymes which illustrate the above themes I shall also attempt to develop a better understanding of the energetics of the catalysis due to enzymes. This requires a very detailed inspection of an enzyme in solution, both in the presence and absence of the substrate. In a final section of speculative review I shall consider if it is possible to produce artificial polymers with a catalytic power equal to that of enzymes.

## NEW EXAMPLES OF METALLO-ENZYME SITES

Table 1 gives a summary of some of the older data on metal ions at enzyme active sites<sup>1, 2</sup>. There are many features of this table which remain unexplained. For example the curious character of the cytochrome P-450 iron as seen in absorption spectra has not been related to any feature of the haem molecule with any certainty. In what follows I draw attention to more successful new work, taking proteins one at a time.

Table 1. Some properties of metallo-enzymes

Enzyme	Metal	Physical property
Cytochrome P-450	Fe(II)	Unusual absorption spectrum
Myoglobin	Fe(II)	Five-coordinate
Plastocyanin	Cu(II)	Unusual absorption spectrum
Caeruloplasmin	Cu(?)	'Diamagnetic copper'
Laccase	Cu	High redox potential
Carboxypeptidase	Zn	Very distorted geometry
Carbonic anhydrase	Co(II)	Anomalous absorption spectrum
	Zn	Distorted geometry
	Co(II)	Anomalous absorption spectrum

## Iron-containing enzymes

*Rubredoxin*

The most complete structure determination on a metalloprotein involved in catalytic action is the work of Jensen and co-workers on rubredoxin<sup>4</sup>. The curious pattern of bond lengths and bond angles is shown in Figure 1. There is no clear explanation for this geometry. It must be remembered that the crystal structure data are for the oxidized state, Fe(III), of the protein. It may well be that the function of the curious structure can only be discovered once it is known how the protein structure changes with oxidation state of the metal. In fact although it is known that this protein is involved in electron transfer little is known of the system in which it is functional *in vivo*.

*Nitrogenase*

The iron-sulphur proteins of nitrogenase are now known to be different from all other such proteins<sup>5</sup>. Particularly their e.p.r. spectra is of a new kind which may be due to the Fe(III), spin =  $\frac{3}{2}$ , state. In any event the iron must be in a site of unusual geometry for such e.p.r. signals have not been observed before. Mössbauer data indicate that the iron sites are under the influence of the binding of adenosine triphosphate (ATP)<sup>6</sup>. Now the function of ATP-binding is to permit its hydrolysis and the release of energy. Thus in this case the iron in the protein is in an unusual ground state energized in a 'hot-spot'. It is as if the iron had been lifted to a steady-state configuration by input of appropriate *local* thermal energy.

*Ribonucleotide reductase*

The evidence for iron dimers in the ribonucleotide reductase of animals is

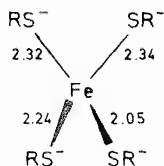
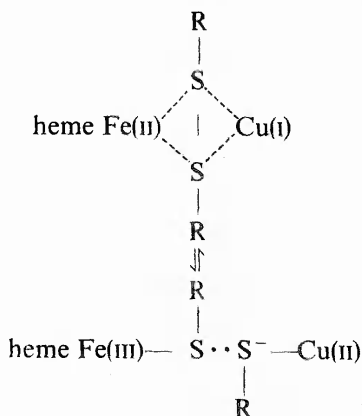


Figure 1. The coordination sphere of iron(III) in rubredoxin

now impressive. However, a more startling finding is that this enzyme contains a stable radical—probably of organic origin<sup>7</sup>. There is another ribonucleotide reductase from *E. coli* which requires coenzyme B<sub>12</sub> (Co)<sup>8</sup>. Curiously the first intermediate in the reaction path involves the production of an organic radical in this protein too. Paths involving radicals in proteins are not of obvious significance but radicals have been observed trapped in several heterogeneous solid-state catalysts (see below).

### Heme-enzymes

Apart from the well-documented cases of the adjustment of spin-state energies by protein geometries as in cytochrome P-450, peroxidase, and hemoglobin there have been several interesting advances in the study of cytochrome oxidase<sup>9</sup>. Thus certain of the e.p.r. signals during the electron transfer process have very high rhombicity; it is clear that high-spin Fe(III) cytochrome states are intermediates; the ground state of some of the heme and some of the copper (blue copper?) does not contribute to the e.p.r. signals and perhaps has a 'zero' magnetic moment. A possible explanation of the low magnetic moment would be a complex of the type



Here oxidation states are difficult to find for certain. A possible one-electron intermediate would be the state heme Fe(III) . . . . RS—SR Cu(I).

The juxtaposition of organic (—S—S—) and inorganic (Fe) (Cu) reactive centres is one way of generating extremely effective catalysts. Another way of making catalysts is to put together metals of rather different types. (It is, however, very difficult for biological systems to make such catalysts, e.g. in Li—Cu, Al—Ti compounds.)

*Problems of heme spectra*

The recent work of Hager<sup>10</sup> on the halo-peroxidases throws light on the problem of the nature of the P-450 reactions. Hager found that he could reproduce the Soret absorption band shift from 420 nm to 450 nm as seen in the microsomal P-450 oxidases. Now P-450 and the halo-peroxidases have different ligands coordinated to the iron, probably cysteine and histidine respectively. The fact that they can give almost identical spectra on binding to a strong sixth ligand, carbon monoxide, and that this spectrum differs from that of all other heme proteins in which the coordination of the iron is often fully known, suggests that the iron has lost the fifth ligand. It could well be a feature of these two proteins, unlike oxygen carriers, that the fifth ligand can be lost while the heme is retained in the protein provided that the sixth ligand is very strong<sup>11</sup>. Oxygen is a ligand not too different from carbon monoxide. As pointed out by Dunford and Williams<sup>12</sup> the dependence of the reactivity of peroxidases on a pK of around 0.0 in some peroxidatic activities could arise from a required assistance from protonation of the fifth group histidine in the reaction intermediate. Overall reactivity in oxygenation and peroxidation reactions rests partly, if our conjectures are correct, on the strength of the iron-fifth-ligand bond. The control is a property of the protein as a whole. Elsewhere we have stressed how conformational fluctuations of a fairly large part of molecules such as cytochrome-*c* could be an essential feature of electron transfer<sup>13</sup>. In purified cytochrome *aa*<sub>3</sub> the addition of *one* mole of CO, O<sub>2</sub>, or CN<sup>-</sup> alters the properties of *both* heme groups to a much more striking degree than the cooperativity seen in hemoglobin oxygen uptake<sup>14</sup>. This discussion allows us to stress the role of cooperativity between large parts of a protein both in activating a single reactive centre and in making a communication net between active centres. I shall return again to this theme of cooperative energetics.

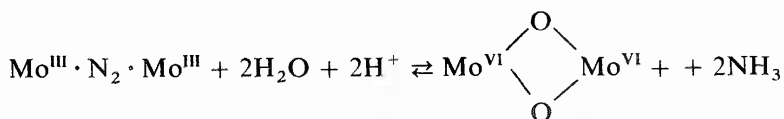
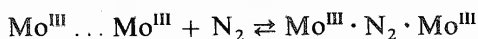
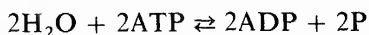
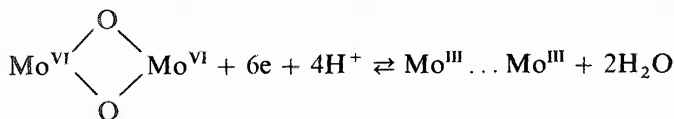
**Molybdenum-containing enzymes**

Molybdenum has special catalytic functions in biology, e.g. in nitrogen fixation. Molybdenum is a common element in heterogeneous catalysts and some features of its chemistry are worth noting. (a) It forms high coordination number complexes even with ligands such as sulphur. This is not the case for the lighter transition metals of biology. (b) It forms oxo-complexes Mo=O. (c) It has a wide range of redox states, III-VI. Molybdenum is known (from e.p.r. data) to enter rhombic sites in enzymes and is probably bound to sulphur ligands. It takes part in redox reactions and a possible one, which would be rather restricted to molybdenum at low oxidation-reduction potentials, is



Iron (oxidation state greater than three) brings about such reactions at a potential around + 0.80 V but molybdenum can do so at around 0.0 V. Thus molybdenum could be a special hydrogen atom transfer catalyst.

Molybdenum, like iron in nitrogenase, is activated by ATP. Could the reactions be as follows?

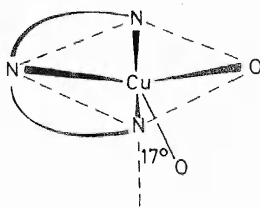


The lower oxidation state of molybdenum is then stabilized by removal of water (by ATP) and this change is also seen in the iron proteins.

### Copper-containing enzymes

#### *Superoxide dismutase*

When Vallee and Williams<sup>2</sup> discussed this copper protein of the erythrocyte membrane they thought they were dealing with a metalloprotein which had no enzyme activity. The protein was classified with conventional metal complexes for the literature data at that time indicated that the e.p.r. and absorption spectra were those of a conventional tetragonal complex. Since that time it has been found that the protein is a very common *enzyme* for dismutation of superoxide and it therefore seemingly represented a case of a non-entatic metal site. However, more detailed study of physical properties have since shown that the copper(II) has a rhombic symmetry, that its absorption spectrum is abnormal for the three nitrogen ligands known to be bound, and that its redox potential is high. Rotilio and his co-workers<sup>16</sup> have subsequently discovered a model copper compound of known crystal structure which has very similar e.p.r. and visible absorption spectra to those of the Cu(II) dismutase. The structure is shown in *Figure 2*. This

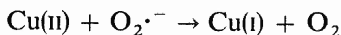


*Figure 2.* A schematic version of the coordination sphere of copper(II) in superoxide dismutase

structure may well be like that in the dismutase and it is obviously unusual. The structure does not look very probable for the Cu(I) site of the reduced enzyme, and perhaps there is a change of geometry on reduction. It should be possible to follow the local changes in geometry of this enzyme during redox cycles using the methods described later in this paper<sup>17</sup>.

An additional problem presented by the dismutase is the function of the

water in the fourth (and fifth?) coordination positions. It is known that water exchanges rapidly from around  $\text{Cu(II)}(\text{H}_2\text{O})_6$ , rate of exchange  $\sim 10^{10} \text{ s}^{-1}$ . In distorted five coordinate complexes the rate of water exchange may be much slower<sup>18</sup>,  $\sim 10 \text{ s}^{-1}$ . The rate of reaction of the dismutase is much faster than this so in this case it could well be that the electron-transfer step



is an outer-sphere reaction, not requiring replacement of the water of coordination. Can water be curiously activated in enzyme sites? The question arises again in the discussion of zinc enzymes.

### *Caeruloplasmin*

There are three types of copper atom in caeruloplasmin. The first, type II copper, could be said to resemble the copper of superoxide dismutase. The second, the deep blue copper, (type I) is like the blue copper of other enzymes and may well be copper bound to a thiolate. The third type of copper is of an unknown kind associated with absorption at around 330 nm. A possible structure for this copper has been suggested and is shown in *Figure 3*<sup>19</sup>. Whether this is correct or not, an additional peculiarity of the

Reduced enzyme	Oxidized enzyme	Anion bound oxidized enzyme
$\text{Cu(I)} \dots \text{Cu(I)}$ $\text{RS}^- \quad \text{RS}^-$	$\text{Cu(II)} \dots \text{Cu(II)}$ $\text{RS} - \text{SR}$	$\text{Cu(II)X}^- \quad \text{Cu(II)X}^-$ $\text{RS} \quad \text{RS}^-$
no absorbance bands above 300 nm	330 nm band	420 nm band

*Figure 3.* A proposed relationship between the states of the 'diamagnetic' copper in caeruloplasmin and laccase

copper sites arises from the appearance of an absorption band at around 350–400 nm in both inhibited forms of the enzyme and in some of the reaction intermediates.

It will be noticed that a common feature of iron, molybdenum and copper centres is the association with sulphur. All the inorganic sulphides of these elements are good semiconductors.

### *Different Cu(II) sites*

Our knowledge of copper sites in enzymes leads us to postulate a possible relationship between them, *Figure 4*. This scheme could be extended to include heme-containing enzymes for it is known that half the copper(II) in cytochrome oxidase cannot be detected by e.p.r., see above. Perhaps there is a similar series of non-heme non-ferredoxin iron sites, in which case oxidases of three different origins could have much in common.

### **Selectivity of metal-binding**

It can be asked, why do sites such as that in the superoxide dismutase contain copper and not zinc? As we shall see later some of the copper sites suggested here, three histidines, are exactly the same as the sites for zinc in carbonic anhydrase and probably class II aldolase, while the inter-

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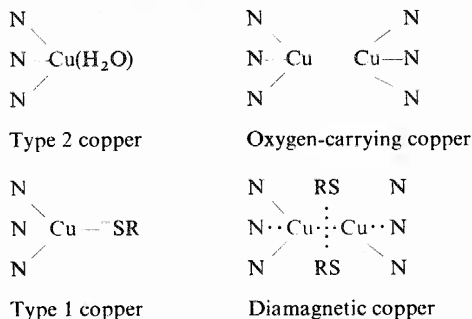


Figure 4. A possible relationship between the coordination spheres of different types of copper found in various enzymes

action of copper with nitrogen and sulphur ligands in, say, caeruloplasmin differs little in chemical composition from that which we believe to exist in alcohol dehydrogenase, for zinc. As was pointed out in 1953 the difference must lie in the geometry which the protein subtends<sup>20</sup>. If we compare the small molecule  $\text{N}(\text{CH}_2\text{CH}_2\text{NH}_2)_3$  which fits a tetrahedron better than a square, with  $\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$  which fits a square better than a tetrahedron, we find that zinc prefers the former and copper the latter. A relative selectivity factor of four orders of magnitude can be achieved. Whereas sites can be designed to give strain in the metal coordination sphere they can also be designed to give selectivity of uptake. While noting such factors it should be remembered that biological systems can exert kinetic control of insertion of metals into sites as they do in the insertion of magnesium into chlorophyll.

### Cobalt-containing enzymes

#### *Vitamin B<sub>12</sub> enzymes*

It is now generally believed that cobalt(II) is an intermediate in coenzyme B<sub>12</sub> catalysis in enzymic reactions<sup>8, 21</sup>. Hence we must accept homolytic fission and the generation of diradicals in the catalytic sites of these enzymes. Such radicals have been seen as major species, up to 90 per cent of the total enzyme, in diol dehydratase, ethanolamine deaminase, ribonucleotide reductase and even in methyl-transfer enzymes. Apart from the nature of the radical centres, one of which is cobalt(II) and the other in some way associated with the ribose fragment which had been bound to the cobalt(III), the considerable separation of the two radicals as indicated by an examination of their e.p.r. spectra, is quite striking. It would appear that homolytic fission is followed by a separation of the radicals through some conformational change of the enzyme. As has been stressed, change of redox state will generate a change in conformation.

In an earlier section the reaction of P-450 has been described as involving heterolytic fission of a metal-nitrogen bond on binding of carbon monoxide or more particularly on the binding of molecular oxygen. This is another type of reaction which can be associated with large conformation changes in ligand and protein. In yet other cases a ligand may be completely rejected.

e.g. on reduction of aquo-methemoglobin. a water molecule is rejected and there is a very large conformation change in the protein.

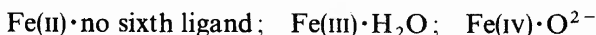
### Redox changes in enzymes

Table 2 lists some of the most important changes of oxidation state in enzymes. Each change is expected to force some change of geometry at the

Table 2. Changes of redox state in enzymes

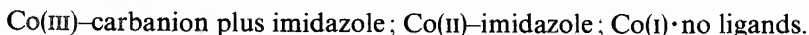
Metal	Redox states	Enzymes
Copper	Cu(I), Cu(II)	Most copper-containing enzymes
Iron	Fe(II), Fe(III)	Cytochromes
	Fe(III), Fe(IV), Fe(V)	Peroxidase, catalase
Molybdenum	Mo(III), to Mo(VI)	Nitrogenase
Cobalt	Co(I)?, Co(II), Co(III)	B <sub>12</sub> -requiring enzymes
Manganese	Mn(II) to Mn(IV)?	Photosynthetic enzymes

active site, e.g. Cu(I) prefers tetrahedral to tetragonal, Cu(II), geometry. Again on change of redox state ligand partners may change. For example in peroxidase



a similar sequence may apply in manganese and molybdenum enzymes.

In the case of cobalt in vitamin B<sub>12</sub> coenzyme we expect the sequence to be



These changes in partner must have profound effects on the geometry of the protein associated with the metal as we shall see. Even if there is no change in bond angle or of ligand partner bond lengths must alter. Such changes in stereochemistry introduce much of the opposing activation energy to homogeneous redox reactions. A good catalyst must reduce this energy and an enzyme may well do so by utilizing a large part of the protein to partition energy effectively at the active site. Similar observations apply to acid-base (substitution) reactions in the next section.

### Zinc sites

#### Carbonic anhydrase

This enzyme has zinc at the active site. Some data on metal substitution are given in Table 3. Only cobalt(II) and zinc(II) are of high activity but the manganese(II) enzyme shows some activity. The physical properties indicate that 4(5) coordination is required for activity. Interest then centres on the function of the water molecule held in the coordination sphere for water is a *substrate* of this enzyme. It is still not known if the metal-bound water is activated or if the reactive water is in the second coordination sphere. Work on proton relaxation suggests that it is unlikely that coordinated water takes part in the reaction<sup>22</sup>. The mechanism discussed by Dennard and Williams<sup>23</sup> for model catalysts and invoked by Khalifa<sup>24</sup> for carbonic anhydrase seems plausible.



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Table 3. The properties of different metallo-carbonic anhydrases

	Mn(II)	Co(II)	Ni(II)	Cu(II)	Zn(II)
Method of study	n.m.r.	e.p.r., or absorption spectra	—	e.p.r., or absorption spectra	crystal structure
Geometry observed	Four-coordinate	Four- (or five) coordinate (tetrahedral)	—	Four- (or five) coordinate (planar)	Four-coordinate (tetrahedral)
Activity	5%	75%	Very low	zero	100%

Note: None of the geometries are supposed to be regular but the observed differences between the metals are well beyond experimental error. The metals are open-sided with one or possibly two water molecules.

### Carboxypeptidase

The zinc site of carboxypeptidase is still a difficult problem. Table 4 gives a list of observed physical properties of different metal ion forms of the enzyme<sup>1</sup>. It is clear that the coordination number can vary from four to

Table 4. The properties of different metallo-carboxypeptidases

	Mn(II)	Co(II)	Ni(II)	Cu(II)	Zn(II)
Method of study	n.m.r. on H <sub>2</sub> O · F <sup>-</sup>	e.p.r. Absorp. spectra	Absorp. spectra	e.p.r. Absorp. spectra	Crystal structure
Geometry observed	Five coordinate	Four- or five coordinate (tetrahedral)	Six coordinate	Four- or five coordinate (planar)	Four coordinate (tetrahedral)
Activity	4%	75%	50%	zero	100%

Note: None of the geometries are supposed to be regular but the observed differences are well beyond experimental error. It is noteworthy too that different metals discriminate in favour of particular types of substrates<sup>1</sup>. The metals are open-sided with one, two or three water molecules.

six and that here the activity of the enzyme cannot be simply related to the observed ground state geometry of the free enzyme or the enzyme bound to inhibitors. Thus the nickel enzyme described by Gray *et al.* apparently has an octahedral structure<sup>2,5</sup>. Curiously, its spectrum is almost identical with that of [Ni(H<sub>2</sub>O)<sub>6</sub>]<sup>2+</sup> while one expects it to be shifted to much higher energy if two nitrogen ligands are bound, Table 5. Thus although the symmetry is not unusual the bond lengths are strange if the same coordination groups are used as in the zinc enzyme.

The fact that there is so little change in activity for such a large geometry change and indeed a large change in the number of water molecules in the active site, means that the role of the metal is obscure. Much more experimental work will be necessary before this enzyme can be understood.

### The entatic state

The general hypothesis presented by Vallee and Williams<sup>2</sup> was that metal ion sites in enzymes would be found to be of irregular coordination geometry. It is clear from the above and earlier data that this is the case. The general

Table 5. The absorption spectra of some nickel complexes

Nickel complex	Absorption maxima (nm)		
Ni(H <sub>2</sub> O) <sub>6</sub>	850	1350	2530
Ni in MgO	860	1380	2450
Ni(H <sub>2</sub> O) <sub>4</sub> ethylenediamine	980	1580	2680
Ni(H <sub>2</sub> O) <sub>2</sub> (glycinate) <sub>2</sub>	1000	1600	2700
Ni(H <sub>2</sub> O) <sub>2</sub> (ethylenediamine) <sub>2</sub>	1050	1790	2810
Ni-carboxypeptidase	900	1460	2427

Note: The closest parallel exists between nickel in carboxypeptidase and in water and nickel oxide. In complexes with nitrogen ligands the nickel bands move to considerably higher energy

advantage of such a situation in catalysis had been described earlier and was elaborated in diagrammatic form in later publications, see *Figure 5*. It is supposed that the catalyst is in an energized ground state prior to contact with the substrate. If this is the case, it should be possible to generate similar catalyst centres in other *organized* phases for the raising of the ground state is brought about by the overall packing of a large assembly. Away from the active site region the packing of the protein has stabilized the system as a whole and it is the conflict between the stereochemical demands of many centres which has elevated the catalytic site. In what follows we shall look at other *organized* states of matter before asking how we can find out more about them.

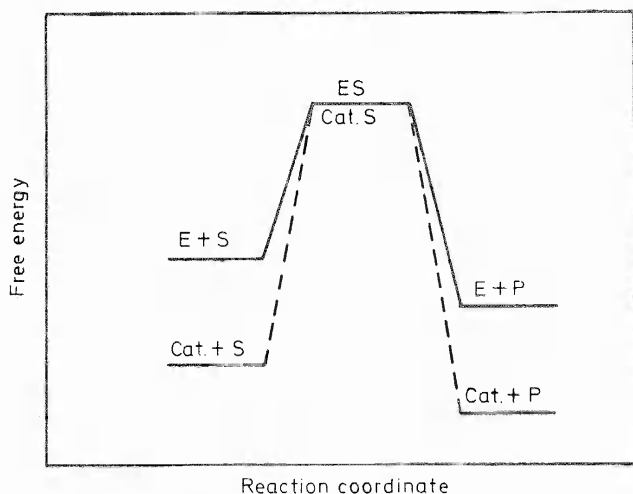


Figure 5. A diagrammatic representation of the way in which the ground state differs in an enzyme catalyst, E, and a homogeneous catalyst, Cat. A heterogeneous catalyst is to be likened to an enzyme in that it too has a promoted energy due to the nature of its highly structured matrix

## SOLID STATE CATALYSTS

In an article by Boudart<sup>26</sup> it is pointed out there are many examples of *structure insensitive* bulk *metal* catalysts. In these cases it is of no consequence

if the sizes of the metal particles are large or small. It has often been suggested that there is another class of metals for which the metal particle sizes are very important and that there are special catalytic sites in these metal catalysts. Boudart points out that the existence of such sites has never been proved. Turning to catalysts of the semi- or non-conducting metal-compound type a very different picture of catalysis has emerged. Very instructive examples are provided by various metal oxides. *Table 6*. Burwell and co-workers have

*Table 6*. Dependence of catalysis on surface states

Catalyst	Form used	Catalysis	Ref.
Cr <sub>2</sub> O <sub>3</sub>	Amorphous	High	1
	Microcrystalline	Low	
α-TiCl <sub>3</sub>	0001 plane	Low	2
	1010 plane	High	
	Screw dislocation	High	
Zeolites	No long-range order	High	3
Al-doped SiO <sub>2</sub>	γ-ray treated	High	4
	Untreated	Low	
MgO	Neutron or u.v. treated	High	5
	Untreated	Low	

<sup>1</sup> R. L. Burwell, G. L. Haller, K. C. Taylor and J. F. Read, *Adv. Catalysis* **20**, 1 (1969).

<sup>2</sup> E. J. Arlman, *J. Catalysis*, **3**, 89 (1964); M. Boudart, *Amer. Scientist*, (57), 1, 97 (1969).

<sup>3</sup> J. A. Rabo, C. L. Angell, P. H. Kasai and V. Schomaker, *Disc. Faraday Soc.* **41**, 328 (1966).

<sup>4</sup> H. W. Kohn, *J. Catalysis*, **2**, 208 (1963).

<sup>5</sup> J. H. Lunsford and T. W. Leland, *J. Phys. Chem.* **73**, 130 (1969).

shown that a Cr(III) oxide gel becomes active only on heating the gel to 400°C. The heating creates a number of *coordinatively unsaturated* acid-base pairs, Cr<sup>3+</sup> · O<sup>2-</sup>. The availability of these sites is highly dependent upon the size and shape of the particles. In the case of MgO, *Table 6*, the catalytic sites are the imperfect corners of octahedra of MgO<sub>6</sub> (NaCl structure). These unneutralized corners are in fact OH<sup>-</sup> groups which undergo exchange. In yet other catalysts of this type it has been shown that trapped radicals (V-centres) are the source of the catalysis, compare B<sub>12</sub> enzymes.

Generalizing from this limited range of results we see that catalysis owes itself to an especially energized centre. This energy can result from defects during crystallization, crystal growth along a screw axis, or the unavoidable appearance of corners. The first two cases are kinetically stable perhaps but the third is thermodynamically unavoidable. Further ways of generating the energy are by exposure of crystals to radiation at such a temperature that they cannot recover e.g. γ-rays, neutrons, or light. We have already postulated that biological systems can have similar states, arising from energy partition between the large protein and the smaller active site.

A further lesson can be learnt from the solid state. Thomas and his co-workers<sup>27</sup> have surveyed the literature and summarized their own experience on enhanced *intrinsic reactivity* at structural faults in solids. They state, 'It has long been recognized that emergent dislocations and related defects,

function as centres of enhanced reactivity at the surfaces of a wide range of solids'. The implication here is that the excess *ground-state* energy of the defects causes them to decompose more readily. In the case where adsorption occurs at such a site (but now the reaction is that of an *absorbed* molecule) it is the *ground-state* energy of the defect which causes heightened catalysis. Elsewhere Vallee and Williams<sup>2</sup> have termed such a condition of a catalyst centre 'an entatic state'. Even in *Table 6* we note the elevated energy of the  $\text{MgO}_5\text{OH}$  unit of  $\text{MgO}$  catalysts and the V-centre radicals. Should they not be compared with the reactivity of zinc in carbonic anhydrase and of the enzyme radical in the iron-containing ribose reductase?

### Homogeneous catalysis

If we consider homogeneous catalysis then it must suffer from one disadvantage—the inability to build-in relatively large 'strain' energy at one site through cooperative stability which gives only a small energy improvement per atom but a large one per assembly. Thus the strain must be put in by building ligands for the central atom which are rigidly fixed so that strain cannot be relieved. To some extent we see this principle applied in co-enzymes which contain metal ions. The hole at the centre of chlorophyll and porphyrin has so developed that it cannot be adjusted to fit high-spin iron(II) or magnesium(II). The general idea is to build the strain into covalent bonds which have little (kinetic) chance of reassembling in an alternative geometry. Unfortunately this has the disadvantage that there is no flexible matching of the four different periods in the reaction ground state, bound substrate, bound intermediate (or bound transition) states and bound product. Homogeneous systems are inherently at a disadvantage.

### Enzymic catalysis

If our view of the nature of enzymic catalysis is correct then the energetics of catalysis could involve a relatively large fraction of a protein. The situation arises through the *cooperative* energies of a large number of interacting centres. Consider the case of the catalytic oxygen atom of  $\text{MgO}$ . This oxygen is reduced to  $-\text{OH}$  during the course of the reaction. Now replacing  $\text{O}^{2-}$  by  $\text{OH}^-$  means that all the oxygen and magnesium atoms near the site will readjust their bond distances (slightly). In the case of a metal (or nonmetal) atom which alters its state in a protein the adjustment is likely to be much greater and to be open to inspection at much greater distances from the metal for the cooperative interactions in the protein are much less 'structured' than those in  $\text{MgO}$ . How shall we discover if such changes do occur? What further advantages does a protein assembly gain from the additional flexibility it has relative to a heterogeneous catalyst? Moreover if we are correct in likening a protein to a solid state structure then we should make a much more general enquiry into the nature of cooperativity in phases.

## COOPERATIVITY

From this discussion we see that the great distinction between biological systems and true homogeneous solutions lies in cooperativity. Cooperativity

in chemistry is often thought to belong to the highly organized solid phase, e.g. the cooperative energy of a sodium chloride crystal. While this is not the case such simple systems give us insight into the nature of cooperative interactions. What striking properties are generated by cooperativity in a lattice? Clearly consideration of energy belongs to the whole lattice and not to individual atoms so that reactivities at certain points arise from interactions in the whole system. As a consequence, and although in a very simple phase we can usefully describe the energy change per mole, in a very complex solid phase the partitioning of energy to the individual atoms is impossibly difficult—so it is in a protein. Thus although a protein is not a cooperative unit of simple repeating units of almost indefinite extent, like NaCl, but is a complex assembly ABCDEFG . . . etc (no repeats) of definite micro-volume, yet it is still a highly cooperative entity—it gives only one stable structure. Such cooperative microphases are of extreme interest for their physico-chemical properties are only somewhat like those of macrophases such as NaCl. They are in fact more similar to the surface layers of crystals which have not yet been satisfactorily treated by thermodynamics. The parallel is not only in the limited extent of the two phase systems but also in their irregular structures and in the mobilities of centres in the structures. It may be for these reasons that both give similar catalytic sites in enzymes and in heterogeneous surface catalysts.

Unlike a repeating lattice such as MgO where the effects of cooperativity can only be seen at the surface, because the interior cannot be reached, a protein is a more open structure and any part of it can have enhanced reactivity or much reduced reactivity. A simple consideration illustrates again how this can be understood at the structural level as seen in macrophases. When NaCl molecules enter a lattice there is a complete change in the structure. The sodium ions are surrounded by six chloride ions, the  $\text{Na}^+ \text{---} \text{Cl}^-$  bond distance is totally different from that in the NaCl molecule. Its reactivity must obviously be different from NaCl molecules. Yet our development of considerations of reactivity of amino acid groups (and metal ions) in proteins has been from simple molecular models—solution chemistry. In so far as a protein is a *large* molecule the cooperative energy can be such as to alter bond angles and bond lengths in familiar chemical groups so as to make them hyper-reactive. Can we detect the structural features by physical measurements? The early part of this paper describes how we can see such factors through the use of many types of spectroscopy in certain metallo-enzymes. Can we detect them in proteins which have no metals? We return to this point later.

We have now seen how cooperativity adjusts the ground state energy and structure of a large group of atoms. A third feature of cooperativity is seen in the effect of temperature changes, which produce phase changes. A phase change of a large amount of a solid crystalline material occurs at a fixed temperature, all the material melts at once, e.g. NaCl. Now, gross conformation changes of proteins or of DNA (denaturations), unlike an equilibrium between two conformations of a small molecule, also occur over a very short temperature range, indicative of the fact that the heat and entropy change for a change of state of a protein molecule and a DNA molecule are large and intermediate between those of macroscopic crystals and isolated molecules.

The denaturation is a type of phase change. All these large molecular weight systems have cooperative structures. But as this is the case we must be able to construct other systems of any degree of cooperativity (molecular weight and structures), so that the melting of a solid is only one extreme and protein denaturation another. Amongst such systems lies a whole world of biological phases, such as membranes, protein-protein connections, protein-membrane interactions, and the whole realm of surface chemistry. A thorough re-analysis of the phase relationships of such bodies is therefore required. I believe that this will show that we must not restrict ourselves by discussing solids, liquids and vapours but must accept a vast range of partially organized phases between crystalline three-dimensional regular lattices and liquids. Phases can be of one or two dimensions, of small volume, and of a great variety of restricted mobilities. The properties of these phases will be different from our present expectation. They need not show *very* sharp phase changes, and the condition for a phase change will depend on the total amount of material in the system. They will show highly selective mutual solubility for all mutual solution must now be highly cooperative: there can be no relevance for ideal laws of solution. This may well be the explanation of the association of particular molecular species in well defined membranes in biology. Likewise the sensitivity of such structured cooperative systems to impurities and foreign chemicals could be quite unexpected, for the impurities could interact through the generation of phase changes, each impurity centre acting cooperatively with the next. Just as it is not to be expected that we shall be able to give a detailed atomic level description of protein energetics so these phases will not be open to discussion in the language of small gas molecules. A first need then is for an appropriate thermodynamic framework, see reference 28 for example.

I note in passing that all kinds of devices may be built into biological reactions through cooperativity, e.g. energy conservation can be action at a distance, memory can arise through the association of 'phase' changes with ion movements. After all memory in a computer is nothing more than a 'phase change' in magnetization. Memory may well be a property of the steady states achieved by action involving any large component of a biological cell system because of the nature of biological microassemblies—they are cooperative phases.

It follows too that there is no single critical point which differentiates solids from liquids. The idea of a melting point independent of the amount of material comes from lattices of infinite extension. We must expect phases in which the fluctuation of cooperative ordering makes us treat them as 'liquids' to some degree and 'solids' to another. In this light our recent findings<sup>29</sup> that molecules such as adenosine-5'-phosphate, (AMP), adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate have '*structures*' in aqueous solution means that we must expect '*structures*' for growing nucleotide (and protein) chains, throwing light on a further problem in biology—the unfolding and folding of macromolecules. In solid state chemistry the idea of 'microdomains' has often been canvassed. Liquids can obviously have microdomains though their fluctuations may be quite rapid.

Returning to the problem of protein reactivity and supposing that we accept the idea that a protein phase is somewhat intermediate between a solid

and a liquid with a high degree of cooperative energy and considerable fluctuational mobility, it is very easy to see how the problem may have been misconstrued. Consider electron transfer in cytochrome-*c* or in any other electron-transfer protein<sup>3, 13, 30, 31</sup>. If the protein is anything like a crystal it is hard to conceive how electron transfer of low activation energy is possible. Electron transfer between the iron centres would be easy if these centres existed in free solution but the protein is obviously much more sterically-hindered than is true of simple small complex ions. If, however, the whole pocket in which the heme-group sits has considerable fluctuational mobility then the expected barriers of the solid state to electron transfer disappear, for normally in a solid the cooperativity prevents easy electron transfer while in such an irregular cooperative phase as cytochrome-*c* it could well be that its structure aids electron transfer. Moreover there is evidence that cytochrome-*c*, like many proteins, has a high fluctuational mobility for in both the oxidized and reduced states proton exchange with nearly all the peptide bonds is fast. Water can penetrate deeply into the protein matrix. Cytochrome-*c* is but one example of a highly cooperative phase with no long-range order which repeats. Our problem now becomes that of devising methods of sufficient sensitivity to study this type of phase, whether it be a protein, a membrane, or DNA.

### NEW N.M.R. METHODS

We have published recently new procedures for the study of protein n.m.r. spectra<sup>32</sup>. They are two types of difference spectra and exemplify a great variety of difference spectra which are now possible in n.m.r. In the first case a difference is taken between the Fourier transform of the n.m.r. spectrum and the same Fourier transform multiplied by a line broadening function. Such subtraction makes for an increase in the effective resolution at the expense of signal strength. The enhanced spectrum of hen egg white lysozyme is shown in *Figure 6*. Utilizing a variety of relatively conventional procedures the peaks have been assigned and we can in fact assign nearly all aromatic and methyl residues. At this stage it is possible to follow the response of the methyl and aromatic protons to changes in pH, to metal ion binding, or to inhibitor and (in principle) substrate binding. All of these experiments have been carried out. The titration of the carboxylates at residues 35 and 52, or their binding by  $\text{Ca}^{2+}$  or  $\text{La}^{3+}$  causes a movement in tryptophan 108 which we can follow readily. The binding of tri-*N*-acetylglucosamine, an inhibitor, has a different effect. However, it will now be possible to follow such effects deep into the lysozyme molecule, testing if this protein is somewhat flexible. The exact functional significance of the limited flexibility already proved cannot be assessed yet but it is tempting to suppose that the enzyme utilizes energy partition in the way described here.

In still larger assemblies this energy partition becomes the method for communication between different proteins so that cooperativity extends over a considerable volume. Here we stray from catalysis to the nature of life—another subject.

Before closing I wish to speculate about the possibility of utilizing the ideas discussed here in synthetic systems, made by man.

Figure 6(a)

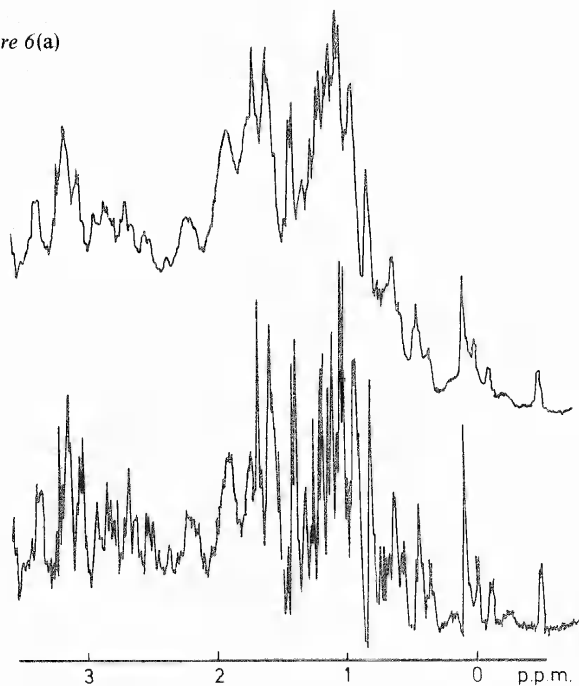


Figure 6(b)

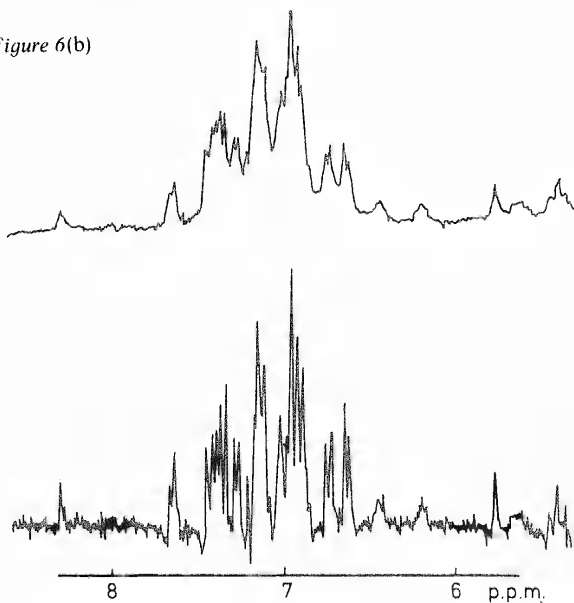


Figure 6. Methyl (a) and aromatic (b) regions of the n.m.r. spectrum of hen egg-white lysozyme. The upper curves are conventional 270 MHz FT spectra while the lower curves show the effect of resolution enhancement. Such spectra are sufficiently detailed to permit a full structure determination of a small protein using special probe procedures<sup>32</sup>



## SYNTHETIC POLYMERS AS CATALYSTS

The above discussion leads to a suggestion for the structure frame which must be generated in synthetic polymers if we are to make them ourselves. Firstly they must be built so that (in our chosen solvent) they will have a maintained conformation overall. The ground rules for doing this are known from protein chemistry. There must be relatively strong interactions between at least some of the units in the polymer. In part this maintains solubility too. Secondly we do not need to have specially designed reactive centres as in homogeneous catalysis for the energy of the polymer conformation will generate these centres. It follows that it is no use using a polymer from a single monomer; we must copolymerize or even mix several monomers with acidic and basic sidechains. If we choose the units correctly we do not need to work at such low temperatures as biological systems so we do not need to have as highly activated groups as in those systems. A starting point would be the combination of leucine, glutamic acid, and lysine. Various polymers of molecular weight around 10000 could be made and their structures studied. Their activity as esterases at very different pH values could then be evaluated. This would be a hit-and-miss research effort but this is the way in which I believe biological catalysts have evolved.

I wish to record my indebtedness to the members of my own research group, and to my fellow-members in the Oxford Enzyme Group.

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