Nature of the Active Sites in Haemocyanin and Iron Nickel Hydrogenases: the Bond Valence Sum Approach†

Sanchita Hati and Dipankar Datta*
Department of Inorganic Chemistry, Indian Association for the Cultivation of Science, Calcutta 700 032, India

The available crystal structure data on deoxyhaemocyanin and the extended X-ray absorption fine structure data on oxygenated haemocyanin and various forms of iron nickel hydrogenases have been analysed by the bond valence sum method to examine the nature of the co-ordination spheres around the metals in these enzymes.

Haemocyanin is the oxygen carrier protein in arthropods and molluscs.[1,2] Iron nickel hydrogenases, found in certain bacteria, are enzymes which catalyse the reversible oxidation of H_2.[3,4] The active site of haemocyanin consists of two copper centres and that of iron nickel hydrogenases is believed to contain a mononuclear nickel centre near an iron–sulfur cluster. While haemocyanin can exist in two forms, oxygenated (oxy) and deoxygenated (deoxy), the hydrogenases can exist in several forms (see below). No crystal structures of oxyhaemocyanin and any form of the iron nickel hydrogenases are at present available. However, extended X-ray absorption fine structure (EXAFS)[5] studies have been reported for oxyhaemocyanin from several species and the various forms of iron nickel hydrogenases in a few species (see below). The metal–ligand bond distances obtained from EXAFS are, in general, known to match very well with X-ray crystallographic data. Thus in the absence of the latter the EXAFS data can be very useful. However, in EXAFS it is not possible to determine the exact geometry of the co-ordination sphere and the oxidation state of a metal. Very recently Thorp has shown[6,7] that EXAFS data when used in conjunction with the bond valence sum (BVS) method can yield very valuable information regarding the oxidation state of a metal and its co-ordination sphere in metalloproteins and enzymes. The BVS method essentially correlates the metal–ligand bond distances with the oxidation state of the metal. In the present article we have used this method to analyse the available EXAFS data on oxyhaemocyanin and iron nickel hydrogenase in an attempt to understand the nature of their active sites. Before we present our results, we discuss the BVS method in some detail.

The Bond Valence Sum (BVS) Method

In the BVS method[8,9] the valence s of a bond between two atoms i and j is given empirically by equation (1) where r_{ij} is the

$$s_{ij} = \exp(r_0 - r_{ij})/0.37 \quad (1)$$

length and r_0 a parameter characteristic of the bond. The oxidation number N_i of an atom i is a simple algebraic sum of the s values of all the bonds formed by the atom [equation (2)];

$$N_i = \sum_j s_{ij} \quad (2)$$

it is also known as the BVS. To determine the parameter r_0, equation (3), similar to (2), is solved for r'_0. A value of r'_0 is

$$N_i = \sum_j s_{ij} = \sum_j \exp(r'_0 - r_{ij})/0.37 \quad (3)$$

obtained for an atom environment where the oxidation state of the atom is known, the ligands are chemically identical, the r_{ij} values are also known from X-ray crystallography and there is no disorder. The r'_0 values resulting from a number of known structures are then averaged to obtain the best value of r_0. Evidently the value of r_0 becomes more reliable as the number of structures used for averaging r'_0 increases. Very recently, by using a large data base, O'Keeffe and Brese[10a] have shown that such bond valence parameters can be related to the atomic sizes. The r_0 value for an atom pair usually depends on the oxidation states of the atoms.‡[,10b] In ref. 9 the r_0 values for a number of metal–ligand bonds in some oxidation states of the metals are listed. The remaining values can be calculated by an empirical equation described in ref. 9. However, it should be pointed out that if some steric strain is present in the co-ordination sphere of the metal [*e.g.* in complexes of first-row transition metals with co-ordination number ≥ 8, or where the usual steric effects are exerted by bulky groups around the donor atom(s)] the BVS value can be affected appreciably.[11] Such cases are to be avoided carefully while determining r_0.

Results and Discussion

Using the r_0 values of Brown and Altermatt,[9] Thorp[6] originally showed that the BVS values in certain complexes of Fe, Cu, Mn and Zn in some oxidation states and geometries reproduce the oxidation state of the metal within ±0.25. Later, Liu and Thorp[7] found that these r_0 values should be refined. For this purpose, they modified the original procedure[9] slightly to apply the method to heteroleptic complexes as well. However, they determined the r_0 values only for complexes of V, Mn, Fe, Cu, Ni and Mo of N-, O- and S-donors in some selected oxidation states.

In our attempt to probe the nature of the active sites of haemocyanin and iron nickel hydrogenase by using the BVS method, we re-examined the viability of the available r_0 values

† *Supplementary data available* (No. SUP 570 73, 24 pp.): bond distances and bond valence sums for copper and nickel complexes. See Instructions for Authors, *J. Chem. Soc., Dalton Trans.*, 1995, Issue 1, pp. xxv–xxx.

‡ However, the bond valence parameters derived by Brese and O'Keeffe[10b] do not change with oxidation states for atom pairs involving donor atoms other than O, F and Cl. Since here we shall deal with complexes of Cu and Ni involving mainly N- and S-donors, we have chosen not to use them. The reasons will be clear in the following sections.

for copper(I) complexes of N-donors, copper(II) complexes of N- and O-donors, and complexes of Ni^{I}, Ni^{II} and Ni^{III} of N- and S-donors. It was found that even the recently derived r_0 values of Liu and Thorp[7] can lead to considerable errors in some cases. As an example of the extent of error encountered, we note that the r_0 value of 1.751 Å for Cu^{2+}–N gives rise to a BVS value of 2.495 for the copper(II) complex of Jäger's macrocycle,[12] and for the same complex the value (1.747 Å) calculated from ref. 9 yields a BVS value of 2.469. More examples of such cases can be found in Table S1 of SUP 570 73. In the case of the nickel(II) complexes examined by us (Table S2, SUP 570 73) the r_0 values of Brown and Altermatt[9] for Ni^{2+}–L (L = N or S) actually apply only in NiL_6^{2+} chromophores [for many square-planar nickel(II) complexes with L_4 co-ordination spheres these give errors > 0.5 unit in the calculated oxidation state of the metal; see Table S2] and those of Liu and Thorp[7] for Ni^{2+}–L are actually suitable for NiL_4^{2+} moieties (for many NiL_6^{2+} chromophores errors in the calculated oxidation state are greater than 0.45 unit, see Table S2). Thorp and Liu[7] did not determine the r_0 values for Ni^{+}–L. Large errors are obtained for nickel(I) complexes of N- and S-donors using the r_0 values of Brown and Altermatt[9] (see Table S2). Consequently we have tried to refine the bond valence parameters pertaining to the present work by following Brown's approach. For this purpose, the crystal structures of various relevant homoleptic complexes were collected at random from the recent literature. All the references are given in Table S1 (copper complexes) and Table S2 (nickel complexes). For the complexes of Ni^{I} and Ni^{III} only, all the structurally characterised examples, which are few, were used.

Our studies show that for Cu^{+}–N and Cu^{2+}–O the r_0 values of Brown and Altermatt,[9] and for Ni^{3+}–L those of Liu and Thorp,[7] are good enough. The various r_0 values used in the present work are given in Table 1. At present, to our knowledge, only one structurally characterised complex of Ni^{I} with a Ni^{I}–S bond is known;[13] the ligand is a diphenyldi-*p*-tolyl 21-thiaporphyrin. Structural data for only this complex were used to evaluate the r_0 value for Ni^{+}–S with the r_0 value for Ni^{+}–N being 1.457 Å, which was obtained separately (Table 1). Using the r_0 values given in Table 1, we have found that for the various copper and nickel complexes considered in Tables S1 and S2 the BVS values lie within ± 0.40 unit of the actual oxidation state of the metal. In only two examples of copper(I) complexes having a CuN_2^{+} chromophore,[14] which we came across in our random sampling, the BVS values are lower than the metal oxidation state by 0.43 to 0.47; such low values are obtained possibly because of the large steric effects present[14] in these two cases. Here we have determined the r_0 values for Ni^{0}–L from the extrapolation of the r_0 values for Ni^{3+}–L, Ni^{2+}–L and Ni^{+}–L bonds. It should be noted that the BVS model discussed here cannot be used for oxidation state 0 (or a negative oxidation state) as such. That is why we have used extrapolations. The idea is to restrict the BVS value for complexes of zerovalent metals within 0.40, *i.e.* within the error limit stipulated here. To that extent our bond valence parameters for Ni^{0}–N and Ni^{0}–S work quite well. Concrete examples are: (*a*) $[Ni^{0}(SO_2)(PPh_3)_3]$,[15a] Ni–S 2.038 Å (BV = 0.079) and $[Ni^{0}(SO_2)_2(PPh_3)_2]$,[15a] Ni–S 2.058 (0.075), 2.076 Å (BV = 0.071), (*b*) $Ni^{0}SP_3$ chromophore in ref. 15*b*, Ni–S 2.013 Å (BV = 0.084), and (*c*) $Ni^{0}NP_3$ moiety in ref. 15*c*, Ni–N 2.178 Å (BV = 0.052); such calculations show that it is possible to derive a r_0 parameter for Ni^{0}–P in these cases to restrict the sum of the BVs within 0.40.

Table 1 Various bond valence parameters (r_0) used in the present study[a]

Bond	r_0/Å	Bond	r_0/Å
Cu^{+}–N	1.587[b]	Ni^{3+}–N	1.731[c]
Cu^{2+}–N	1.719	Ni^{0}–S	1.098[d]
Cu^{2+}–O	1.679[b]	Ni^{+}–S	1.649
Ni^{0}–N	1.083[d]	Ni^{2+}–S	1.963
Ni^{+}–N	1.457	Ni^{3+}–S	2.040[c]
Ni^{2+}–N	1.673		

[a] The r_0 values were determined by us unless otherwise specified; see text. [b] From ref. 9. [c] From ref. 7. [d] Extrapolated value (see text).

Haemocyanin.—As mentioned earlier, the active site of haemocyanin contains a pair of copper atoms. In the oxygenated form there is one molecule of oxygen per two copper atoms. In the deoxy form the oxidation state of copper is I and the separation between the two Cu^{+} ions is ≈3.5 Å.[16] On binding O_2, Cu^{I} is oxidised to Cu^{II} and O_2 is reduced to O_2^{2-} giving rise to a $Cu^{II}_2(O_2^{2-})$ moiety.[17–19] Oxyhaemocyanin is not detectably paramagnetic suggesting a strong antiferromagnetic coupling between the two Cu^{2+} ions ($-2J \geqslant 550\ cm^{-1}$).[20]

The crystal structure of the deoxyhaemocyanin of an arthropod, *Panulirus interruptus*, was reported in 1989 by Volbeda and Hol.[16] The system studied by them is a hexamer (*M* 460 000). The structural models for the active sites in the six subunits which emerged from their study are given in Table 2. These show one common feature. Each Cu^{+} ion is surrounded by three histidine N atoms, two of them forming short bonds and the other a rather long bond with the metal [Fig. 1(*a*)]. Our calculations by the BVS method show that the active sites are essentially like CuN_2^{+} systems. The contribution from the long bond towards the oxidation state of copper lies in the range 0.094 (Cu^{+}–N 2.46)–0.032 (Cu^{+}–N 2.86 Å). The average

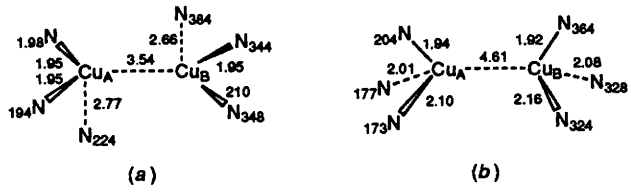

Fig. 1 Schematic idealised structures of the copper(I) centres in (*a*) *P. interruptus* deoxyhaemocyanin isolated at pH 3.8 and (*b*) *L. polyphemus* deoxyhaemocyanin isolated at pH 6.5–7.0. The various Cu–N bond distances (Å) in (*a*) are from ref. 16 and in (*b*) from ref. 21. The subscripts to the nitrogen atoms identify their parent histidine residues. In (*a*) Cu_A, Cu_B, N_{194}, N_{198}, N_{344} and N_{348} are roughly in a plane, the BVS of Cu_A is 0.791 and that of Cu_B is 0.680

Table 2 Bond distances Cu^{+}–N in the deoxyhaemocyanins of *Panulirus interruptus* and of *Limulus polyphemus*, and the bond valence sums

Unit	Copper	Cu^{+}–N/Å	BVS
Panulirus interruptus[a]			
SU1	A	2.02, 2.46, 2.02	0.628
	B	2.02, 2.53, 2.26	0.550
SU2	A	2.22, 2.02, 2.84	0.525
	B	2.03, 2.72, 2.15	0.567
SU3	A	1.95, 1.94, 2.86	0.792
	B	1.95, 2.68, 2.11	0.670
SU4	A	1.96, 1.94, 2.73	0.795
	B	1.93, 2.61, 2.07	0.730
SU5	A	1.96, 1.96, 2.86	0.762
	B	1.96, 2.64, 2.12	0.660
SU6	A	1.95, 1.97, 2.62	0.791
	B	1.95, 2.72, 2.11	0.665
Limulus polyphemus[b]			
SU2	A	2.10, 2.01, 1.94	0.954
	B	2.16, 2.08, 1.92	0.883

[a] The bond length data were taken from ref. 16. The average value of BVS is 0.678 ± 0.097. [b] The bond length data were taken from ref. 21. The average value of BVS is 0.918.

Table 3 Bond valence sums for copper in the oxyhaemocyanin in some arthropods and molluscs

Species	Cu^{2+}–N/Å	No. of N	Cu^{2+}–O/Å	No. of O	BVS for a CuN_2O_2 moiety[a]
Busycon canaliculatum[b]	2.00	2.0	1.90	2.4	2.036
Limulus polyphemus[b]	1.98	2.0	1.86	2.5	2.214
Cancer irroratus[b]	2.02	2.0	1.89	2.3	2.016
Megathura crenulata[c]	2.01	2.0	1.92	2.0	1.952
β Component of *Helix pomatia*[c]	2.02	2.2	1.92	2.0	1.928
α Component of *Helix pomatia*[c]	2.02	1.8	1.94	2.4	1.874

[a] Average is 2.003 ± 0.119. [b] The data were taken from ref. 24. [c] The data were taken from ref. 23.

oxidation state of the copper in the six subunits as calculated by the BVS method is 0.678. We have examined some 22 copper(I) complexes containing CuN_2^+ chromophores selected at random (Table S1). In all these cases the BVS values are < 1; specifically, the values lie in the range 0.526–0.977 with an average of 0.809 (standard deviation, $\sigma = 0.124$). The average oxidation state of copper in the deoxyhaemocyanin of *P. interruptus* corresponds to 0.809 − σ. According to the BVS approach, in the subunits SU1 and SU2 the Cu^+–N bonds are rather long. Such long Cu^+–N bonds are observed in copper(I) complexes with CuN_2^+ chromophores where large steric effects are operative (for examples see ref. 14). Incidentally in some 19 copper(I) complexes with CuN_3^+ chromophores (again selected at random; Table S1) the average value of the oxidation state of the metal is found to be 1.016 ± 0.062. Ideally, as allowed by the BVS, for a symmetrical trigonal CuN_3^+ chromophore the Cu–N length should be 1.993 Å. Distances close to this ideal value are found in the crystal structure of *Limulus polyphemus* (another arthropod) deoxyhaemocyanin determined very recently by Hazes *et al.*[21] This structure shows a near-symmetrical trigonal-planar three-co-ordination for each of the two Cu^+ ions comprising the active site; the average Cu–N bond lengths for the two Cu^+ ions are 2.017 and 2.053 Å [Fig. 1(*b*), Table 2]. The corresponding BVS values are 0.954 and 0.883. Thus our BVS calculations show that the newer structure is much more compatible with the oxidation state I for Cu than is the unsymmetrical one found earlier in *P. interruptus*. It is indeed felt that the latter structure, though real, may not have been a representative one since crystals were obtained at low pH (3.8),[16] and that the newer structure found in *L. polyphemus* probably represents the physiological situation.[21] The crystals of *L. polyphemus* deoxyhaemocyanin were grown at pH 6.5–7.0 which is slightly below the physiological pH (7.1–7.55).[21]

From the EXAFS studies on the oxyhaemocyanin from several species,[22–24] the co-ordination sphere around Cu^{2+} in oxyhaemocyanin is of the planar N_2O_2 type (Table 3). However, it has been felt by several workers[17,22,25] that a long axial Cu^{2+}–N bond may exist leading to a square-pyramidal CuN_3O_2 moiety. Such a possibility is also supported by the recent extensive model studies carried out by Kitajima *et al.*[26] The models provided by them contain CuN_3O_2 chromophores. These mimic the various unusual spectroscopic properties of oxyhaemocyanin in an excellent manner.[27] Table 3 shows that when an N_2O_2 co-ordination sphere is assumed the oxidation state of copper in the various oxyhaemocyanin systems studied is close to 2.0, except in the arthropod *Limulus polyphemus* where it is 2.21. It is really interesting to find that for a CuN_3O_2 moiety with an average bond distance of 1.96 Å[22] the BVS method yields a value of 2.499 as the oxidation state of copper. Such a large error in the metal oxidation state has not been observed for any of the 24 copper(II) complexes having CuN_3O_2 chromophores examined by us (Table S1). The list of such complexes includes all synthetic models of oxyhaemocyanin put forward by Kitajima *et al.*,[26] Karlin and co-workers,[28] and Sorrell *et al.*[29] For these complexes the BVS method gives an average copper oxidation state of 2.112 ± 0.077, specifically the values lie in the range 1.979–2.221. If we assume that the average BVS value for copper in various oxyhaemocyanin systems (see Table 3) is 0.1 unit too low (*i.e.* allowing an average error of 0.1 in the calculation of oxidation state), then an additional Cu^{2+}–N bond of average length ≈2.6 Å is suggested. It appears that in *L. polyphemus* this additional bond should be the longest, if there is any, among the oxyhaemocyanin systems in Table 3. Thus in keeping with the EXAFS results, the BVS method shows that only a rather long axial Cu^{2+}–N bond can exist in oxyhaemocyanin. This conclusion of ours is in line with the very recent proposition made in a preliminary report on the crystal structure of oxyhaemocyanin from *L. polyphemus*.[30] Here we would like to mention that in the case of some 14 copper(II) complexes having CuN_2O_2 chromophores (Table S1) an average value of 2.093 ± 0.153 is obtained for the oxidation state of the metal.

Arthropodal and molluscan haemocyanins are known to have completely different molecular architectures. The former class consists of hexamers or multihexamers with subunits of molecular weight ≈75 000, while the latter class is cylindrical oligomer with subunits of molecular weight ≈400 000.[31] Nevertheless there are indications that the oxygen binding sites in these classes are quite similar.[24,32–34] Accordingly our discussion in this section applies to the active sites in both types of haemocyanin.

Iron Nickel Hydrogenase.—As mentioned earlier, the iron nickel hydrogenases catalyse the reversible oxidation of H_2 in certain bacteria. It is believed that the active site contains an Ni atom near an FeS moiety. In the catalytic process several forms of Ni are encountered.[3,4] The oxidised forms (as isolated in air) display two types of rhombic EPR signals: Ni_A ($g_1 = 2.31$, $g_2 = 2.23$ and $g_3 = 2.02$) and Ni_B ($g_1 = 2.33$, $g_2 = 2.16$ and $g_3 = 2.02$). Form A is termed 'unready' and B 'ready'. Upon exposure to H_2, first a state is obtained where both types of signals disappear and then a new type of rhombic signal termed Ni_C ($g_1 = 2.19$, $g_2 = 2.14$ and $g_3 = 2.02$) appears. Further reduction by H_2 results in another EPR-silent form (fully reduced). While the active site composition is known to differ from species to species,[3,35–39] the above observations are common in, at least, certain varieties.[3,40]

If the nature of the co-ordination sphere around nickel is disregarded, the EPR signals can originate only from Ni^{III} or Ni^{I}. So far, owing to the lack of any crystal structure data on any form of iron nickel hydrogenase from any species, the assignments of the EPR-active oxidation state(s) of Ni in the various forms of the hydrogenases have been difficult and rather controversial. Currently there are two hypotheses for explaining the catalytic cycle. According to one (hypothesis H1), Ni shuttles between two oxidation states III and II, the two EPR-silent forms being nickel(II) species.[4,41] The other one (hypothesis H2) associates Ni^{III} only with the forms A and B; in subsequent stages Ni^{III} is assumed to undergo stepwise one-electron reductions, 0 being the oxidation state of Ni in the fully reduced form.[38,42,43] The oxidation states corresponding to the two hypothesis H1 and H2 are in Table 4. However, very recently Maroney and co-workers[37] expressed doubts as to whether the Ni atom is involved at all in the redox cycle.

We now analyse the available EXAFS data on iron nickel hydrogenase from three species, *Thiocapsa roseopersicina, Methanobacterium thermoautotrophicum* and *Desulfovibrio gigas*, using the BVS method. The EPR and redox behaviours of the enzymes isolated from these species are essentially similar.

T. roseopersicina hydrogenase has been studied most extensively by Maroney and co-workers[37-39,44,45] in recent times by using various X-ray absorption techniques. They examined the EXAFS of form C on several occasions and their curve fitting was best with an $NiNS_4$ chromophore having bond distances Ni–N 1.924 Å, Ni–S 2.222 (three) and Ni–S 2.748 Å.[39] Earlier they found from the EXAFS analysis that the co-ordination sphere around Ni remains virtually unchanged in all forms of the enzyme.[37] Assuming that the $NiNS_4$ moiety found in form C persists in all other forms, our BVS calculations show that the co-ordination sphere is compatible with all the four oxidation states of the metal from III to 0 (Table 5). [We would point out that, like EXAFS, the BVS also cannot properly distinguish between N- and O-donors, as the r_0 values for M^{n+}–N and M^{n+}–O are usually very close. For example, Cu^{2+}–N 1.719 and Cu^{2+}–O 1.679 Å (Table 1). For more examples, see ref. 7.] However, our BVS analysis of the EXAFS data on the oxidised form (as isolated) of F_{420} deazaflavin reducing hydrogenase of *M. thermoautotrophicum*[36] clearly shows (Table 5) that none of the co-ordination spheres emerging from the EXAFS studies is compatible with a nickel(III) description of the oxidised form (A and/or B), as postulated by hypotheses H1 and H2 (Table 4). Since the two enzymes are similar in nature, it is apparent from the comparison of our BVS results obtained for *T. roseopersicina* hydrogenase with those obtained for *M. thermoautotrophicum* hydrogenase that any hypothesis which requires the involvement of Ni^{III} is not tenable. Thus it is questionable whether the redox chemistry involves the nickel centre. This conclusion draws support from the observation that the Ni K-edge XAS spectra remain more or less unchanged for all forms of *T. roseopersicina* hydrogenase,[37] which means that Ni remains in the same oxidation state in all forms.

Recently, Wang *et al.*[46] have studied the magnetic properties of *Desulfovibrio baculatus* hydrogenase poised in the EPR-silent active form and concluded that Ni is present as low-spin Ni^{II}. This enzyme is analogous to the *T. roseopersicina* one except for a substitution of Se for S. It should be noted that both hypotheses H1 and H2 (Table 4) agree that the oxidation state of Ni in the EPR-silent active form is II. In view of the results of our BVS analysis of the EXAFS data on *T. roseopersicina* and *M. thermoautotrophicum* hydrogenases, and since the Ni K-edge spectra do not change significantly in any of the forms of the *T. roseopersicina* enzyme, it seems logical to conclude that the oxidation state of Ni in all the forms of iron nickel hydrogenase is II. This hydrogenase has been briefly examined by Liu and Thorp[7] also by using the BVS approach; working with older EXAFS data of Maroney and co-workers,[37] they proposed an N_3S_2 co-ordination sphere with Ni–N 2.00 and Ni–S 2.23 Å for the nickel site in *T. roseopersicina* hydrogenase. We would point out that such a co-ordination sphere is compatible with all the oxidation states of Ni from III to 0 (Table 5), *i.e.* like the NS_4 co-ordination sphere considered by us, with this co-ordination sphere also little changes in the bond lengths are expected with change in the oxidation level of Ni in the range III to 0.

The oxidised form (A and/or B) of iron nickel hydrogenase isolated from *Desulfovibrio gigas* has been examined by Scott and co-workers[3,35] using EXAFS on two occasions. Their curve fitting suggests two to three sulfur atoms at an average distance of 2.12 Å and two to four sulfur atoms at an average distance of 2.28 Å from the nickel centre.[3] Table 5 shows that according to the BVS method only the $S'_2S''_2$ (S' indicates the sulfur atom at 2.12 Å and S'' that at 2.28 Å) co-ordination sphere is compatible with the oxidation state II for Ni. However, a five-co-ordinate structure with a long Ni–S bond ($\geq$2.6 Å) can be accommodated in the BVS. Existence of a such a long bond will not affect the conclusions drawn from the Ni K-edge spectra of and the EXAFS data for *D. gigas* hydrogenase.[35]

The question now remains as to the redox-active centre in iron nickel hydrogenase. Redox studies on nickel alkanethiolate complexes[47-49] suggest a possibility that cysteine sulfurs are involved. Maroney and co-workers considered this possibility but thought it unreasonable since they presumed that in such a case the Ni–S bond distance would be affected significantly by redox changes. However, from our BVS studies on the hyperoxo and peroxo complexes of Co^{III} we have found that in

Table 4 Possible oxidation states of nickel in iron nickel hydrogenases encountered in the process of catalysing the oxidation of H_2

	Hypothesis	
Form	H1	H2
Ni_A, Ni_B	III	III
Active state (EPR silent)	II	II
Ni_C	III	I
Fully reduced (EPR silent)	II	0

Table 5 Bond valence sums for Ni in various possible co-ordination spheres emerging from the EXAFS studies on some forms of iron nickel hydrogenases

			BVS assuming			
Species	Form	Co-ordination sphere	Ni^{III}	Ni^{II}	Ni^{I}	Ni^{0}
T. roseopersicina	All forms	N_3S_2[a]	2.645	2.212	1.107	0.346
	All forms	NS_4[b]	2.576	2.117	0.972	0.259
M. thermoautotrophicum	Oxidised[c] (A/B)	S_3[c]	1.701	1.381	0.591	0.132
		S_4[c]	2.268	1.842	0.788	0.176
		NS_3[c,d]	1.709	1.388	0.595	0.133
		NS_4[c,d]	2.276	1.849	0.792	0.177
D. gigas	Oxidised[e] (A/B)	$S'_2S''_2$[e,f]	2.658	2.157	0.924	0.208
		$S'_2S''_3$[e,f]	3.181	2.582	1.106	0.249
		$S'_2S''_4$[e,f]	3.704	3.006	1.288	0.290
		$S'_3S''_2$[e,f]	3.464	2.812	1.204	0.271
		$S'_3S''_3$[e,f]	3.987	3.237	1.386	0.312
	Fully reduced[g]	S_4[g]	2.596	2.108	0.900	0.204
		S_5[g]	3.245	2.635	1.125	0.255

[a] Ni–N 2.00, Ni–S 2.23 Å. See refs. 7 and 37. [b] Ni–N 1.924, Ni–S 2.222 (three) and Ni–S 2.748 Å. See text. [c] From ref. 36. Average Ni–S 2.25 Å. [d] Ni–N is taken as 3.5 Å. [e] From ref. 3. [f] S' = Sulfur atom at 2.12 Å, S'' = sulfur atom at 2.28 Å. [g] From ref. 35. Average Ni–S 2.20 Å.

all cases the same Co^{3+}–O r_0 parameter can be used successfully.[50] [For some examples of structurally characterised peroxo and hyperoxo complexes of Co^{III}, see refs. 51 and 52 respectively.] The point is that even if a sulfur radical is bound to Ni^{II} at some stage(s), the same Ni^{2+}–S r_0 parameter given in Table 1 will hold good and consequently within the BVS approach the Ni–S bond distances are not expected to change appreciably with the changes in the redox level.

Conclusion

Earlier Thorp[6,7] proposed that the BVS method can reproduce the oxidation state of certain metals (including Ni and Cu) in certain complexes within ±0.25. Such accuracy, as found by us, cannot be afforded by the BVS method at least in copper and nickel complexes of N-, O- and S-donors. As such the available r_0 values[7,9] in many cases need refinement. Even after refinement, the oxidation states of Cu/Ni are reproduced at best within ±0.40 unit. Nevertheless, like Thorp,[6,7] we have also found the BVS method very useful in analysing the available EXAFS data on oxyhaemocyanin and some forms of iron nickel hydrogenases. In the case of deoxyhaemocyanin, we analysed the X-ray structural data. The various conclusions drawn are as follows.

(1) Since the *L. polyphemus* deoxyhaemocyanin was isolated at pH 6.5–7.0 which is very close to the physiological pH (see above), we feel that its active-site structure is representative of *in vivo* deoxyhaemocyanin. Accordingly, it seems that the active site can best be modelled by a symmetrical CuN_3^+ chromophore with the bond distances found in similar inorganic complexes. For oxyhaemocyanin the BVS analysis is consistent with the existence of a CuN_2O_2 chromophore. However, in keeping with the EXAFS results, an N_3O_2 co-ordination sphere with one of the N atoms being at a distance much greater than the other four atoms seems to be more appropriate.

(2) In iron nickel hydrogenase the oxidation state of Ni in all the forms seems to be the same, *i.e.* II. In *T. roseopersicina* hydrogenase the nickel site is of $NiNS_4$ type with a long Ni–S bond; in *M. thermoautotrophicum* hydrogenase the chromophore is probably the same with a long Ni–N bond and in *D. gigas* hydrogenase also it is possible that a five-co-ordinate Ni of NiS_5 type with a long Ni–S bond is present. The BVS does not rule out the possibility of the cysteine sulfurs, bound to Ni, being involved in the redox processes.

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