Bond Valence Sum Analysis of Metal–Ligand Bond Lengths in Metalloenzymes and Model Complexes. 2. Refined Distances and Other Enzymes

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The bond valence sum (BVS) method is further applied to metalloenzyme active sites. When a particular coordination model is assumed, the BVS method allows for oxidation states of metal ions in metalloproteins to be determined from metal–ligand bond distances measured using extended X-ray absorption fine structure (EXAFS) analysis. Thus, the BVS can be used to determine the compatibility between a given coordination model and a particular oxidation state. A new procedure for calculating r_0 values on which BVS's are based is presented. This procedure allows for calculation of r_0 values on heteroleptic complexes and was used to determine a new set of r_0 distances using complexes that more closely model the active sites of interest. In particular, the new distances allow for calculations involving vanadium, molybdenum, and nickel. New calculations using EXAFS data on CO dehydrogenase, NiFe hydrogenase, manganese catalase, sulfite oxidase, MoFe nitrogenase, and VFe nitrogenase are presented. The interplay between oxidation state and coordination geometry can be quantitatively assessed using the BVS method.

Determining the structures of metal ion binding sites is critical in understanding the mechanism of action of metalloenzymes. Extended X-ray absorption fine structure (EXAFS) provides a means for determining the metal–ligand bond lengths for a given metal ion in a protein.[1-3] X-ray absorption near-edge spectroscopy (XANES) can also provide some information on the oxidation state of the metal ion.[1,4] Recently, we reported on the application of the bond valence sum (BVS) method to the analysis of metal–ligand bond lengths determined by EXAFS in metalloenzymes.[5] In particular, when a structural model is deduced from EXAFS and other spectroscopic methods, the BVS can be used to confirm the compatibility of that model with a given oxidation state of the metal ion. Conversely, if the oxidation state is known, BVS can be used to test structural models. We have demonstrated that this method is effective in a variety of mononuclear and polynuclear metal ions in a wide range of oxidation states for a series of metalloenzymes.

Bond valences (s) are calculated according to eq 1, where B

$$s = \exp[(r_0 - r)/B] \quad (1)$$

= 0.37 and r_0 is obtained either from published tables or standard equations.[6] The BVS for a given metal ion is then the sum of bond valences for each bond made to that metal ion. The r_0 values are determined empirically such that the BVS is generally quite close to the oxidation state of the metal ion. Least-squares fitting procedures were used to determine the initial set of r_0 values by minimizing the difference between the BVS and the known oxidation state for crystallographically characterized materials.

The original BVS distances of Brown,[6] which were used in our initial studies, were determined using crystal structures of homoleptic, extended solids. Despite the apparent lack of similarity between such solid materials and model complexes for metalloprotein active sites, the original BVS distances give good results for the coordination complexes of the ions originally chosen for analysis.[5] Nonetheless, we have undertaken a program to calculate r_0 values on the basis of actual model complexes that are relevant to the metalloprotein of interest. The objective was to determine if the analysis can be further refined with better distances or if the original distances represent the upper limit of refinement. We report here on our method for determining r_0 values from crystallographically characterized model complexes. We find that calculations on the metal ions discussed in our present study are somewhat improved by this effort, and r_0 values for other metal ions that were not addressed originally, namely those of molybdenum, vanadium, and nickel, are significantly improved for this application using our method. We also present here calculations for vanadium, molybdenum, and nickel enzymes and for the manganese catalase enzyme,[7] the EXAFS of which were reported only recently.

Computational Procedure and Revised Distances

The new r_0 values were calculated on the basis of crystal structure data obtained from the literature for each metal ion/oxidation state combination. The program can determine r_0 values using structures that contain several different types of donor atom. The program is given a starting r_0 value for each metal ion–ligand combination present in the total group of structures. Ordinarily, M–O, M–N, and M–S bonds are analyzed, so a three-parameter fit is performed. The BVS is calculated for each metal center and compared to the known oxidation state. A standard Marquardt algorithm[8] is then used to adjust the r_0 values to minimize the deviation of all of the BVS values from the known oxidation state.

For Fe^{3+} complexes, for example, the input file consists of one entry for each metal center (polynuclear complexes therefore have more than one entry) and each entry consists of a bond length for each bond and the type of donor atom. Thus, an entry in the Fe^{3+} file contains five or six bond lengths and a parameter indicating whether each bond length is associated with an Fe^{3+}–S, Fe^{3+}–O, or Fe^{3+}–N bond. Usually, the values given by Brown are used for the starting r_0 values. The program than calculates the BVS for each entry and compares these BVS's with the known oxidation state (3.0 in the case of Fe^{3+}). The difference between the BVS and the known oxidation state is then minimized by the Marquardt procedure.

The new distances are given in Table I along with the number of metal centers on which each value is based. A total of 829 different

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Table I. New Values for r_0 Based on Crystallographically Characterized Model Complexes

bond	r_0 (Å)	n^a	bond	r_0 (Å)	n^a
Fe^{3+}–O	1.765	57	Mn^{2+}–O	1.765	78
Fe^{3+}–N	1.815		Mn^{2+}–N	1.849	
Fe^{3+}–S	2.134		Mn^{3+}–O	1.732	103
Fe^{2+}–O	1.700	116	Mn^{3+}–N	1.837	
Fe^{2+}–N	1.769		Mn^{4+}–O	1.750	36
Fe^{2+}–S	2.125		Mn^{4+}–N	1.822	
Cu^{2+}–N	1.751	73	Ni^{2+}–O	1.670	104
Cu^{2+}–O	1.649		Ni^{2+}–N	1.647	
Cu^{2+}–S	2.060		Ni^{2+}–S	1.937	
Mo^{6+}–O	1.907	46	Ni^{3+}–S	2.040	13
Mo^{6+}–N	2.009		Ni^{3+}–N	1.731	
Mo^{6+}–S	2.331		V^{3+}–O	1.749	27
Mo^{5+}–O	1.917	58	V^{3+}–N	1.813	
Mo^{5+}–N	2.006		V^{3+}–S	2.185	
Mo^{5+}–S	2.288		V^{4+}=O	1.735	82
Mo^{4+}–O	1.866	36	V^{4+}–O	1.780	
Mo^{4+}–N	2.043		V^{4+}–N	1.875	
Mo^{4+}–S	2.235		V^{4+}=S	2.181	
			V^{4+}–S	2.226	

[a] Number of crystal structures used to determine r_0 values for a given oxidation state; complete references are given in the supplementary material.

crystallographically characterized metal centers were used to determine the r_0 values, and the complete set of references is given in the supplementary material. We stated previously that BVS values calculated from Brown's distances were reliable to approximately ±0.25 units for complexes of iron, manganese, zinc, and copper.[5] For these metal centers, the BVS's calculated with our new distances are improved somewhat but not enough to change the conclusions drawn. For vanadium, molybdenum, and nickel, however, we obtain useful and reliable BVS values only with our distances. Statistical analysis gives errors corresponding to an agreement of approximately ±0.25 units for a given determination.

In Table II is shown a comparison between BVS's calculated using the original r_0 values and those given in Table I. The values shown are the calculated BVS's for the entire set of complexes used to calculate the new distances. In order to perform reliable calculations, these BVS's should be close to the actual oxidation state of the metal ions in the complexes. The original r_0 values for iron, manganese, and copper give generally good agreement with the actual oxidation states. However, for molybdenum, vanadium, and nickel, the original r_0 values do not give BVS's in good agreement with the actual oxidation states of the metal ions. The new distances were calculated by minimizing the difference between the calculated BVS and the actual oxidation state, and as shown in Table II, sets of distances could be determined that provided excellent agreement. Thus, the new r_0 values given in Table I offer improvement over the original distances when analyzing discrete coordination complexes, such as models for metalloenzymes. This demonstrates that the reliability of the BVS method depends on the relevance of the crystal structure data used to determine the values of r_0 to the system of interest. In particular, calculations on molybdenum, vanadium, and nickel coordination complexes would be impossible except with the r_0 values reported here. Of course, for solid-state materials, the original distances of Brown[6] are preferable to those given here.

Metalloproteins

General Considerations. The BVS analysis is based on the simple principle that when ligands bind to a metal center, the positive charge on the metal center is compensated by an amount that is related to the type of donor atom and the bond distance. For a given metal–ligand bond, the valence s is larger when the bond length is shorter. Thus, shorter bonds give larger BVS's, which is consistent with the fact that metal–ligand bonds generally contract when the metal center is oxidized. Particularly short bonds therefore contribute a large amount to the BVS. In earlier calculations, we showed that clusters containing aqua, hydroxo, alkoxo, and oxo ligands give satisfactory BVS's using a single r_0 value for oxygen.[5] Thus, the contribution of a particular bond valence to the BVS is simply controlled by the length of that bond compared to the average value for a particular donor atom, as is clear from eq 1. In addition, particularly long bonds contribute very little to the BVS, although earlier calculations showed that better agreement with the actual oxidation state of blue copper proteins was obtained when the long copper–sulfur bond was included in the calculation.[5]

Table II. Comparison of Oxidation State Determinations for Original and Newly Determined r_0 Values for the Set of Model Complexes[a]

ion	BVS[b]	BVS[c]	ion	BVS[b]	BVS[c]
Fe^{2+}	2.15	1.99	V^{4+}	4.44	4.00
Fe^{3+}	3.28	3.00	V^{5+}	5.47	5.00
Mn^{2+}	2.07	1.99	Ni^{2+}	2.88	1.99
Mn^{3+}	3.29	3.06	Ni^{3+}	3.44	3.00
Mn^{4+}	4.03	4.00	Mo^{4+}	4.65	3.99
Cu^{2+}	1.84	1.98	Mo^{5+}	5.49	4.98
V^{3+}	3.35	3.00	Mo^{6+}	6.68	5.98

[a] Average BVS for the set model complexes used to calculated the new distances given in Table I. [b] Calculated using r_0 values taken from ref 6. [c] Calculated using the r_0 values given in Table I.

With regard to types of ligands, the sensitivity of the BVS to changes in the donor atoms is proportional to the difference in r_0 values of the two atoms. It is therefore difficult to distinguish between oxygen and nitrogen ligands, since the r_0 values for these two donors are not that different (Table I). The r_0 values for sulfur donors are much larger, and it is therefore straightforward to distinguish between an oxygen or nitrogen donor and a sulfur donor. Thus, the limitations on determinations between different types of donor atoms are the same as for EXAFS, because the changes in r_0 will generally reflect the changes in the size of the donor atom. Any sensitivity of the BVS analysis to hard versus soft donors is then only as much as the differences in the r_0 values.

The usefulness of the BVS analysis comes in determining the compatibility between a given oxidation state for a metal ion and a particular coordination model. Since the BVS is just the sum of valences of each bond, it is particularly sensitive to coordination number. For example, if a given ion is determined by EXAFS to be coordinated to either five or six of a single type of ligand at a single distance, the BVS for the five-coordinate model will simply be five-sixths that of the six-coordinate model. Thus, higher coordination numbers will imply higher oxidation states. The interplay of oxidation state and coordination number is therefore what is most directly addressed using the analysis. This suggests two possible applications of the analysis. First, if the oxidation state is known from some spectroscopic or magnetic measurement, the BVS analysis can be used to confirm a particular coordination model (i.e. numbers and types of donors) for that particular ion. Examples of this type will be discussed below. Conversely, if the coordination model is known from an X-ray crystal structure, the BVS is then a very powerful method for determining the oxidation state. An example of this type is also discussed below.

Manganese Catalase. Recently, Waldo and Penner-Hahn have reported the EXAFS for the manganese catalase enzyme in the reduced and super-oxidized states.[7] The active site is known to contain a dinuclear manganese complex, which gives a multiline EPR signal in the super-oxidized state. In the reduced state, the EXAFS results are consistent with 6 nitrogen or oxygen ligands to each manganese at a distance of 2.19 Å (Table III). These workers have suggested that these results are consistent with an oxidation state of Mn^{2+}. Using their distances, we calculate a BVS of 2.05. We use a model for the cluster where both metal centers are coordinated to 4 oxygen and 2 nitrogen ligands; varying this ratio does not alter the BVS significantly. In the super-oxidized state, the EXAFS results are consistent with 2 short Mn–O bond lengths of 1.82 Å and 4 nitrogen or oxygen ligands at 2.14 Å. We calculate the BVS for the dinuclear cluster to be 3.16, which is consistent with a $Mn^{3.5+}$ oxidation state, as suggested earlier. Here we use the same coordination model, with two short Mn–O bonds, two long Mn–O bonds, and two Mn–N bonds.

Table III. BVS Values for Metalloenzymes

enzyme	coord	BVS[a]	ox state[b]	ref[c]
Mn catalase, reduced	4O, 2N	2.05	2.0	7
Mn catalase, super oxidized	4O, 2N	3.16	3.5	7
CO dehydrogenase, *C. thermoaceticum*	4S	2.19	2.0	10
CO dehydrogenase, *R. rubrum*	2N, 2S	2.06	2.0	11
NiFe hydrogenase, all forms	3N, 2S	2.06	2.0	16
sulfite oxidase	2O, 3S	5.85	6.0	21
	2O, 3S	4.79	5.0	21
	2O, 3S	4.10	4.0	21
MoFe nitrogenase (Mo center)	1N, 2O, 3S	3.90	4.0	24
VFe nitrogenase (V center)	1N, 2O, 3S	3.21	3.0	26

[a] Number and type of ligands used to calculate the indicated BVS. [b] Oxidation state used to calculate the indicated BVS. [c] Source of bond lengths used for the analysis.

We discussed BVS values for a number of relevant $Mn_2O_2^{3+}$ complexes in our earlier paper.[5]

Nickel Enzymes. In the case of the CO dehydrogenase from *Clostridium thermoaceticum*, the nickel is coordinated only to sulfur and known to be paramagnetic.[9] Thus, a tetrahedral arrangement is strongly implied. EXAFS analysis gives a single Ni–S bond distance of 2.16 Å.[10] The BVS for a four-coordinate Ni(II) center is then 2.19, in good agreement with 2.0. In this case, the coordination number and type of donor atom are well defined, and the BVS analysis can be used to determine the oxidation state.

In the CO dehydrogenase from *Rhodospirillum rubrum*, EXAFS analysis gives a Ni–S distance of 2.22 Å and a Ni–N distance of 1.86 Å.[11] The fitting is consistent with two Ni–S bonds and either two or three Ni–N bonds. Here, analogy to the *C. thermoaceticum* enzyme strongly suggests an oxidation state of Ni^{2+}, but the coordination numbers are ambiguous. The BVS for the 2N + 2S coordination is 2.06, while that of the 3N + 2S geometry is 2.62. Thus, the BVS suggests that the metal center is four-coordinate, as in the *C. thermoaceticum* enzyme. Of course, the BVS would be consistent with the 3N + 2S model if the oxidation state were Ni^{3+}, but this can be ruled out from other measurements.[11] Relevant model complexes include the five-coordinate Ni(tpy)(S_2) complexes of Mascharak et al.,[12] which give BVS's in excellent agreement with the known oxidation states.

The hydrogenase enzyme from *Thiocapsa roseopersicina* can be isolated in three EPR-active forms: an oxidized form A, a catalytically inactive form B, and the active form C.[13] In addition, the enzyme can also be poised in two EPR-silent forms, one of which is highly reduced (R) and another that is intermediate (SI) between the oxidized A and B forms and the catalytically active form C. Recent magnetism studies on the enzyme from *Desulfovibrio baculatus* indicate that the nickel center in the SI form is a diamagnetic Ni(II).[14] This enzyme differs from the *T. roseopersicina* enzyme in that one of the ligands is a selenocysteine instead of a normal cysteine.[15] Nonetheless, the mechanism and oxidation state are thought to be analogous, and the SI form of the *T. roseopersicina* enzyme is therefore likely in the Ni^{2+} oxidation state.[16] Recent XAS studies studies of all five forms show no change in the absorption edge energy upon oxidation or reduction of the enzyme to any level.[16,17] In addition, the coordination environment indicated by EXAFS analysis of all five forms does not vary significantly from 3 ± 1 N or O ligands at 2.00 Å and 2 ± 1 S ligands at 2.23 Å.[16] These observations suggest that the nickel center remains in the same oxidation state in all five forms and that the oxidation state is Ni^{2+}.

The BVS analysis can be used to further test the proposal that the nickel center remains in the +2 oxidation state in all five forms. Here the analysis can be used to determine (a) if the absolute bond lengths are compatible with a Ni^{2+} and if so, what coordination model is appropriate, and (b) whether the changes in bond lengths in the five forms are consistent with no redox chemistry occurring at the nickel site. The XANES of the enzyme lacks features associated with planar four-coordinate nickel, which argues strongly for a five- or six-coordinate nickel site. If a coordination model of 3 nitrogens and 2 sulfurs is used, the average bond lengths for the five forms give a BVS of 2.06 when the site is modeled as Ni^{2+} and 2.64 when the site is modeled as Ni^{3+}. The site can be modeled as a six-coordinate Ni^{3+} (3N + 3S) to give a BVS of 3.24. Thus, the conclusion of the BVS analysis is that if the oxidation state is Ni^{2+}, the bond lengths are most compatible with a five-coordinate site, and if the oxidation state is Ni^{3+}, the bond lengths are most compatible with a six-coordinate site. Since the magnetism results strongly suggest an oxidation state of +2, the coordination model is likely 3N + 2S.

The analysis can also be used to determine if the changes in the bond lengths observed for the five forms are consistent with no change in the oxidation state of the nickel. The bond lengths for each individual form only vary by ±0.06 for the N or O distance and ±0.03 for the S distance.[16] These small changes clearly argue against changes in the oxidation state, since much larger contractions of bond lengths would be expected upon oxidation of the nickel center. Quantitatively, separate analyses of the bond lengths for each form gives a BVS of 2.06 ± 0.13 for all five forms. Clearly, these small variations in bond length are not compatible with redox chemistry at nickel.

Fits of the EXAFS of forms A, SI, and R are improved when an additional, longer Ni–S bond is included at 2.4–2.7 Å.[16] Such a bond would add only 0.13–0.2 to the BVS, because the distance is quite long compared to r_0. Thus, the presence of the longer bond does alter the analysis appreciably as it relates to the oxidation state. An additional consideration is the existence of a potential hydride ligand bound to nickel. Such a hydride ligand would not be detected by EXAFS, and there are not enough crystal structures to allow us to calculate an r_0 value for a nickel–hydride bond. Thus, the BVS analysis cannot provide additional information on the presence of a hydride ligand on nickel. Recent ENDOR experiments have detected a proton that originates from dihydrogen that interacts with the EPR-active center in form C.[18] This proton is dissociated upon photolysis, and XAS of the nickel center is unchanged by dissociation of the proton. This implies that there is not a hydride ligand bound to nickel, and the failure of the BVS analysis to account for such a ligand may not be problematic in this case.

Sulfite Oxidase. In the oxotransferase enzymes,[19] such as sulfite oxidase, oxidation occurs via transfer of an oxo ligand from a $Mo^{VI}O_2^{2+}$ functionality to the substrate to yield a $Mo^{IV}O^{2+}$ group. Reoxidation of the active site occurs one electron at a time, so that a discrete Mo(V) state can be observed. A broad substrate

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analogue system has recently reported and gives a BVS for $MoO_2(t\text{-BuL-NS})_2$ of 5.80, which is good agreement for this Mo^{6+} system.[20]

The EXAFS of sulfite oxidase in all three oxidation states have been reported recently by George and co-workers.[21] In the Mo^{6+} state, the results are consistent with two oxo ligands and either two or three sulfur ligands. The sulfite oxidase case is an excellent example of a system where the oxidation states are very well defined, but the number of sulfur ligands is not fully determined. The BVS analysis is in good agreement for the model with three sulfur ligands (BVS = 5.85) but far too low in the case of only two sulfur ligands (BVS = 5.05). In this case, the oxidation state of molybdenum is well established experimentally, and so the BVS method becomes a powerful tool for screening different structural models and coordination numbers. The results are similar for the Mo^{5+} state, where the EXAFS give three different bond distances: one short Mo–O distance, one long Mo–O bond distance, and two or three Mo–S distances. For the same model with three sulfur ligands, the BVS is 4.79. In the Mo^{4+} state, the same model gives a BVS of 4.10. Thus, the BVS analysis provides a compelling argument for the 3S + 2O coordination model, which is accommodated by the EXAFS data in all three oxidation states. We recently reported that all three oxidation states of methane monooxygenase could be successfully analyzed with this approach.[5]

MoFe Nitrogenase. The research on the MoFe protein of nitrogenase has been quite intense recently, culminating this year with a report of the crystal structure of the MoFe and Fe proteins.[22] Pulsed EPR results have been interpreted in terms of a Mo^{4+} oxidation state in the MoFe cluster, because the molybdenum center needs an even number of unpaired electrons,[23] but other proposals have been put forth. Since the crystal structure is known, this enzyme provides a case where the coordination model is well determined, and the BVS can be used to determine the oxidation state. Analysis of the molybdenum EXAFS gives distances for Mo–S of 2.37 Å and Mo–O/N of 2.12 Å.[24] Using the coordination model established by the crystal structure (three sulfide ligands from the iron cluster, two oxygen ligands from homocitrate, and one nitrogen ligand from histidine), a BVS of 3.90 is obtained. Thus, the BVS analysis is consistent with a +4 oxidation state for molybdenum, which had been assigned from pulsed EPR studies.[23] Interestingly, even though the EXAFS analysis cannot resolve the two oxygen ligands from the nitrogen ligand, the determined average distance is still useful in the BVS analysis.

VFe Nitrogenase. The VFe nitrogenase is thought to be analogous to the molybdenum enzyme, except that the molybdenum site is filled by vanadium.[25] The EXAFS of this protein from *Azotobacter chroococcum* gives a V–S distance of 2.33 Å and a V–N/O distance of 2.15 Å.[26] Using a model isostructural to the crystallographically characterized MoFe protein, a BVS of 3.21 is calculated. The V^{3+} state has been suggested on the basis of XANES analysis,[25] and the bond lengths are apparently consistent with that assignment.

Conclusions

In our initial studies, it was apparently fortuitous that metal ions were chosen for which Brown's initial r_0 values give reliable BVS's for coordination complexes. In the present cases of vanadium, molybdenum, and nickel, the initial values do not work nearly as well. We have now developed procedures for calculating r_0 distances for any set of crystal structures of a metal ion in a particular oxidation state (Tables I and II). As compared to the original method, which applies only to homoleptic systems, our program allows for up to three types of ligands to any given metal center. The comparison in Table II suggests that it is vitally important to use crystal structures that are relevant to the system of interest to determine r_0.

Analysis of bond lengths determined by EXAFS using the BVS approach appears to be a useful complement to XANES in determining the compatibility of a given oxidation state of a metal ion with a particular coordination model. In MoFe nitrogenase, where the structure is known from crystallography, the BVS analysis can be used to determine the oxidation state. When the oxidation state is known unambiguously, as in sulfite oxidase, the BVS method can be used to test structural models, especially coordination numbers.

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Supplementary Material Available: A complete set of references for the crystal structures from which the r_0 values were calculated (6 pages). Ordering information is given on any current masthead page.

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