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Article

The Correlation of Redox Potential, HOMO Energy, and Oxidation State in Metal Sulfide Clusters and Its Application to Determine the Redox Level of the FeMo-co Active-Site Cluster of Nitrogenase

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This paper describes a procedure that permits the total charge state (i.e., oxidation state) of a complex molecule to be obtained from its redox potential data by comparison with good data (both charge state and redox potential) for reference compounds that are chemically similar. The link between the reference data and the unknown compound is made by the calculated energies of the Fermi level or highest occupied molecular orbital (HOMO). The HOMO energies are calculated by unrestricted density functional methods (DMol) for the reference compounds in their known charge states, and a graphical correlation of HOMO energy and redox potential for oxidation (corresponding to loss of an electron from the HOMO) is constructed. The measured redox potential of the unknown is then applied to the correlation to yield the HOMO energy of the unknown, against which the calculated HOMO energies for various charge states of the unknown are assessed. This method is generally applicable. Using 26 reference data, the method is used here to determine the resting redox state, $[NFe_6MoS_9]^0$, of the core of the FeMo cofactor (FeMo-co, bound to the MoFe protein) which is the active site of nitrogen-fixing enzymes. The analysis also shows that if the atom at the center of FeMo-co is C rather than N, then FeMo-co must be protonated in its resting state, but if FeMo-co is N-centered, it would not be protonated in the resting state.

Introduction

For a polymetallic compound with variable oxidation states, the redox potential measured electrochemically correlates with the oxidation state: the reduction potential becomes more positive for more positive oxidation states. In the majority of transition metal clusters, the overall oxidation state is measurable (usually as net charge) and the correlation of oxidation state and electrochemical potential is fully determined. Throughout this paper, the term oxidation state is used for the net molecular oxidation state (net charge), avoiding artificial apportionment of oxidation state to individual atoms.

When the oxidation state of a compound is not measurable, it becomes desirable to be able to use this oxidation state−electrochemical potential correlation to determine the unknown or ambiguous oxidation state. To illustrate the problem, a polymetallic cluster at the active site of a redox enzyme may not be separable from the protein without change, and therefore its net charge in each of the relevant turnover redox states may not be directly measurable, but

its thermodynamic potential for redox change may be measurable electrochemically: how then are the net oxidation states of the cluster to be determined from the redox potential data? This paper shows how this can be done, with application to the FeMo-cofactor (FeMo-co, Figure 1) which is the active site of the nitrogen-fixing enzymes.[1−6]

The method involves comparison of the unknown system with known systems that are chemically similar. Comparison is made via the energy of the highest occupied molecular orbital (HOMO). The energy of the HOMO is related to the potential for oxidation, that is, removal of an electron from the HOMO, and the HOMO energy is also dependent on the oxidation state of the compound. Therefore, a correlation between HOMO energy and oxidation potential is developed for chemically comparable reference compounds, and from

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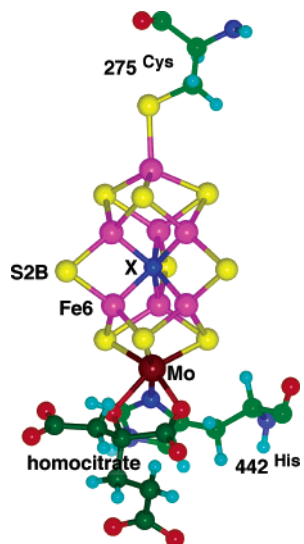(6) Smith, B. E. *Adv. Inorg. Chem.* **1999**, *47*, 159−218.

Figure 1. FeMo-cofactor, Fe$_7$MoS$_9$X(homocitrate)(cys)(his), the catalytic site of nitrogenase enzymes, linked to the surrounding MoFe protein via 275^{Cys} and 442^{His} (PDB structure 1M1N): Fe, magenta; Mo, brown; S, yellow; N, blue; O, red; C, green (homocitrate C atoms dark green). The unproven identity of the central atom X, probably N, is discussed in the text.

this correlation, the oxidation potential for the unknown is used to determine the HOMO energy in the unknown. HOMO energies are then calculated for possible oxidation states of the unknown compound, and these, by comparison with the 'measured' HOMO energy, allow determination of its most probable oxidation state.

The redox thermodynamics and orbital energies for a metal cluster are influenced by other factors which need to be recognized and controlled in this comparative methodology. Major influences are the ligand bonding types and the nature of the environment surrounding the cluster. Lesser influences are the spin and electronic states. All of these effects are illustrated in the results presented here.

The outcome for the oxidation state of the resting FeMo-co of nitrogenase has been communicated briefly previously,[7] without details of the considerations and calculations. In this paper, more accurate and more extensive calculations are reported in detail, so that the efficacy of the method can be assessed. In addition, the uncertainty about the identity of the atom X at the center of FeMo-co is addressed. There is still no positive identification of X:[8-10] various theoretical calculations[7,11-14] have concluded that X could be N or C but not O or F.

Methods

The metal clusters included in this investigation are listed in Table 1 and pictured in the chart. Structures and electrochemical

potentials were taken from the literature cited. Only electrochemical potentials that are reported as reversible or almost reversible are used, to avoid kinetic effects (at the electrode and/or in solution) which shift potentials from their thermodynamic values. Any small variations in potential from measurements in different solvents are averaged. All potentials (V) have been re-referenced to the normal hydrogen electrode (E_{NHE}), using the shifts $E_{NHE} = E_{SCE} + 0.25 = E_{Fc} + 0.63 = E_{Ag/AgCl} + 0.2$ (SCE is the saturated calomel reference electrode, Fc is the ferrocene/ferrocinium couple, and Ag/AgCl is the Ag/AgCl reference electrode).[15,16] The structures of the reference clusters are either taken directly from the literature or constructed from closely related literature structures: all structures have been optimized using the density functional theoretical methods described below.

The model used to calculate FeMo-co is **1**, in which 442^{His} is truncated to imidazole, 275^{Cys} is truncated to SCH$_3$, and homocitrate is truncated to glycollate, $-OCH_2COO-$: this retains the native coordination of all metal atoms. Calculated structures are denoted **1**(X)^z to identify the central atom, X, and the overall charge, z.

Density functional calculations using the program DMol3 (version 3.2, 2005)[28-31] have been described previously.[32] Numerical basis sets (dnp in DMol3) included polarization functions and were calculated to a distance cutoff of 9 au. Relativistic corrections are not needed for the atoms involved here. The functional is BLYP. The calculations are all-electron and unrestricted. Detailed validations of this methodology for FeMo-co and related systems have been reported.[32] All structures were optimized, finally in point group C_1. Calculations were performed on isolated complexes (gas-phase model), and in some cases on complexes in simulated condensed phases using a continuum solvation model, in which the charge distribution of the solute polarizes the dielectric medium and generates electrostatic energies. These calculations used the con-

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Table 1. Compounds and Data Used in the Correlation[a]

compound type and calcd species	exptl system	couple	midpoint potential (V) (solvent), redox reference electrode	ref
Fe(SR)₄	[Fe(S-cysteine)₄] in protein	−2/−1	−0.06, SHE	17
[Fe(SMe)₄]	[Fe(SEt)₄]	−2/−1	−1.08, SCE	18
	[Fe(S₂-o-xylyl)₂]	−2/−1	−0.79, SHE	17
Fe₂S₂(SR)₄	[Fe₂S₂(S-cysteine)₄] in protein	−3/−2	−0.24 to −0.43, SHE	17
[Fe₂S₂(SMe)₄]	[Fe₂S₂(SMe)₄]	−3/−2	−1.05, SHE	17
	[Fe₂S₂(SEt)₄]	−3/−2	−1.31, SCE	18
	[Fe₂S₂(SPh)₄]	−3/−2	−0.85, SHE	17
Fe₃S₄(SR)₃	[Fe₃S₄(S-cysteine)₃]	−3/−2	−0.13, NHE	19
[Fe₃S₄(SMe)₃]				
Fe₄S₄(SR)₄	[Fe₄S₄(S-cysteine)₄]	−3/−2	−0.400, NHE (*D. africanus* Fd III)	19
[Fe₄S₄(SMe)₄]			−0.420, NHE (*D. gigas* Fd II)	
			−0.345, NHE (*D. furiosus* Fd)	
		−2/−1	+0.34, SHE (HIPIP)	17
	[Fe₄S₄(SMe)₄]	−3/−2	−1.05, SHE	17
	[Fe₄S₄(SBuᵗ)₄]	−3/−2	−1.18, SHE	17
	[Fe₄S₄(SBuᵗ)₄]	−2/−1	+0.10, +0.34, SHE	17
Fe₄S₄(SAr)₄	[Fe₄S₄(SPh)₄]	−4/−3	−1.96 (MeCN), Fc	20
[Fe₄S₄(SPh)₄]			−2.06 (DMF), Fc	
			−2.08, −2.15 (NMP), Fc	
	[Fe₄S₄(SPh)₄]	−3/−2	−1.25, −1.31 (MeCN), Fc	20
			−1.35, −1.45 (DMF), Fc	
			−1.44, −1.45 (NMP), Fc	
Fe₄S₄(OAr)₄	[Fe₄S₄(OPh)₄]	−4/−3	−2.20 (NMP), Fc	20
[Fe₄S₄(OPh)₄]			−2.18 (MeCN), Fc	
	[Fe₄S₄(OPh)₄]	−3/−2	−1.55 (NMP), Fc	20
			−1.46 (MeCN), Fc	
Fe₄S₄Cl₄	[Fe₄S₄Cl₄]	−4/−3	−1.99 (MeCN), Fc	20
[Fe₄S₄Cl₄]	[Fe₄S₄Cl₄]	−3/−2	−1.04 (MeCN), Fc	20
			−1.10 (MeCN), Fc	
			−1.25 (NMP), Fc	
			−1.25 (DMF), Fc	
Fe₄S₄(NO)₄	[Fe₄S₄(NO)₄]	−2/−1	−0.65, SCE	21
[Fe₄S₄(NO)₄]	[Fe₄S₄(NO)₄]	−1/0	+0.13, SCE	21
Fe₄S₃(NO)₇	[Fe₄S₃(NO)₇]	−4/−3	−1.75, −1.81, SCE	21
[Fe₄S₃(NO)₇]	[Fe₄S₃(NO)₇]	−3/−2	−1.26, −1.35, SCE	21
	[Fe₄S₃(NO)₇]	−2/−1	−0.68, −0.77, SCE	21
(RS)₄W₂FeS₆	[(edt)₂W₂FeS₆]	−3/−2	−0.34 (DMSO), SCE	22
[(edt)₂W₂FeS₆]				
Fe₆S₉(SR)₂	[Fe₆S₉(SEt)₂]	−5/−4	−2.00 (MeCN), Fc	20
[Fe₆S₉(SMe)₂]			−1.75, SCE	18
	[Fe₆S₉(SEt)₂]	−4/−3	−0.88 (MeCN), Fc	20
			−0.63, SCE	18
(RS)₄Mo₂Fe₄S₉	[(edt)₂Mo₂Fe₄S₉]	−4/−3	−0.76 (DMSO, SCE	22
[(edt)₂Mo₂Fe₄S₉]	[(edt)₂Mo₂Fe₄S₉]	−3/−2	−0.30 (DMSO), SCE	22
[(EtSFe)₃S₄Mo(allyl₂cat)(CN)]	[(EtSFe)₃S₄Mo(allyl₂cat)(CN)]	−4/−3	−1.60, SCE	23
[(EtSFe)₃S₄Mo(allyl₂cat)(CN)]	[(EtSFe)₃S₄Mo(allyl₂cat)(CN)]	−3/−2	−0.54, SCE	23
[(EtSFe)₃S₄Mo(allyl₂cat)(DMSO)]	[(EtSFe)₃S₄Mo(allyl₂cat)(DMSO)]	−3/−2	−1.33, SCE	23
[(EtSFe)₃S₄Mo(allyl₂cat)(DMSO)]	[(EtSFe)₃S₄Mo(allyl₂cat)(DMSO)]	−2/−1	−0.35, SCE	23
[MeN(CH₂COO)₂MoS₄(FeCl)₃]	[MeN(CH₂COO)₂MoS₄(FeCl)₃]	−3/−2	−0.83, Ag/AgCl	24
[MeN(CH₂COO)₂MoS₄(FeCl)₃]				
[(ClFe)₃S₄Mo(C1₄cat)(pyrazine)]	[(ClFe)₃S₄(C1₄cat)Mo(pyrazine)]	−3/−2	−0.89, Ag/AgCl	25
[(ClFe)₃S₄(C1₄cat)Mo(pyrazine)]				
[(ClFe)₃S₄Mo(−SR)₃Fe(CNCR₃)₃]	[(ClFe)₃S₄Mo(−SEt)₃Fe(CNCMe₃)₃]	−2/−1	−1.32, Fc	26
[(ClFe)₃S₄Mo(−SEt)₃Fe(CNCH₃)₃]	[(ClFe)₃S₄Mo(−SEt)₃Fe(CNCMe₃)₃]	−1/0	−0.07, Fc	26
[VFe₄S₆(PR₃)₄SR]	[VFe₄S₆(PEt₃)₄SEt]	−1/0	−0.91, SCE	27
[VFe₄S₆(PMe₃)₄SMe]	[VFe₄S₆(PEt₃)₄SEt]	0/+1	−0.05, SCE	27
[MoFe₄S₆(PR₃)₄SR]	[MoFe₄S₆(PEt₃)₄SEt]	−1/0	−0.99, SCE	27
[MoFe₄S₆(PMe₃)₄SMe]		0/+1	−0.20, SCE	27
[(R₃P)₆Fe₆S₈]	[(Et₃P)₆Fe₆S₈]	0/+1	−1.05, −1.03, SCE	21
[(Me₃P)₆Fe₆S₈]		+1/+2	−0.06, +0.06, SCE	21
		+2/+3	+0.59, +0.80, SCE	21
		+3/+4	+1.25, +1.36, SCE	21

[a] Bold formulas refer to the structural diagrams. Formulas are given for the species calculated theoretically (column 1) and for the species providing the electrochemical data (column 2). R, alkyl; Ar, aryl; Ph, phenyl

ductorlike screening model (COSMO) implemented in DMol,[33,34] with the van der Waals radii increased to S, 2.1; Fe, 2.5; Mo, 2.8 Å.

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Results

I first describe the construction of the correlation between HOMO energy and electrochemical potential for oxidation. The structures of the reference compounds listed in Table 1 were optimized (as gas-phase systems) in the charge states

Chart 1. Structures of the Reference Compounds.
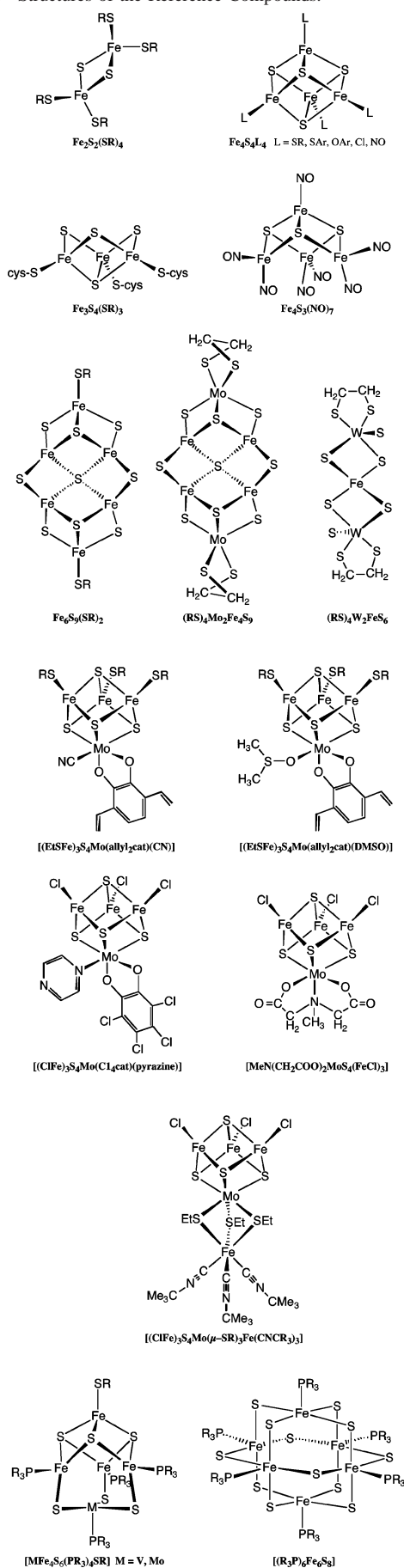
corresponding to the reduced member of each of the couples listed. All optimized structures were geometrically close to the crystallographically determined structures. One possible reference compound $[(PhS)_2Fe(\mu\text{-}S)_2Fe(\mu\text{-}S)_2Fe(SPh)_2]^{4-}$, with tetrahedral coordination at each of the Fe atoms,[18] was found to optimize to a lower-energy structure with near planar stereochemistry at the central Fe atom: because this is inconsistent with the experimental data, this compound was excluded from the correlation. Where the spin state of the reference system is not known experimentally, some investigation of the variation in HOMO energy with spin state was made, but this variation is relatively small (<0.1 eV) and insignificant in the context of the results to be correlated.

For the majority of the reference compounds (particularly the anions), the calculated energy of the HOMO is positive, which is not physically realistic. These positive HOMO energies arise in the gas-phase model calculations because the compounds are devoid of their condensed-phase surroundings. Calculation with a continuum solvation model (COSMO[33,34]), simulating the surroundings as a polarizable dielectric, yields realistic negative energies for the HOMO. To illustrate, the cluster with the most positive HOMO energy, $[Fe_6S_9(SMe)_2]^{5-}$, has calculated values of the HOMO energy of $+10.3$ eV in the gas phase, $+1.6$ eV for COSMO with dielectric permittivity 4, -0.95 eV with dielectric permittivity 10, -2.0 eV with dielectric permittivity 20, and -2.6 eV with dielectric permittivity 80. In addition, the dispersion of HOMO energies for the various clusters and charge states is largest in the gas phase and ameliorated in the solvated models, as expected. For this reason and also because the continuum solvation model requires assumptions about the dielectric permittivity of the medium and is sensitive to the van der Waals radii used to generate the solute cavity in the solvent, the gas-phase HOMO energies were used for the correlation with redox potentials. While this correlation of gas-phase results with condensed phase data is artificial, it does not introduce significant errors, and it does amplify the qualities and deviations of the correlation.

Figure 2 shows the results for all of the reference compounds, many of which occur in electron-transfer series represented by the connecting lines. For the systems $[Fe(SR)_4]$, $[Fe_2S_2(SR)_4]$, $[Fe_3S_4(SR)_3]$, and $[Fe_4S_4(SR)_4]$, there are electrochemical data when they are embedded in proteins (marked black on Figure 2), as well as data from synthetic systems (marked red): some differences between protein potentials and chemical solution potentials have been described and discussed previously.[35,36] The results are plotted as the energy of the HOMO in the reduced member of a couple vs the redox (reduction) potential, E, for the couple. This correlation has negative gradient because the energy of the HOMO is related to electron loss and therefore to the energy for oxidation, i.e., $-E$.

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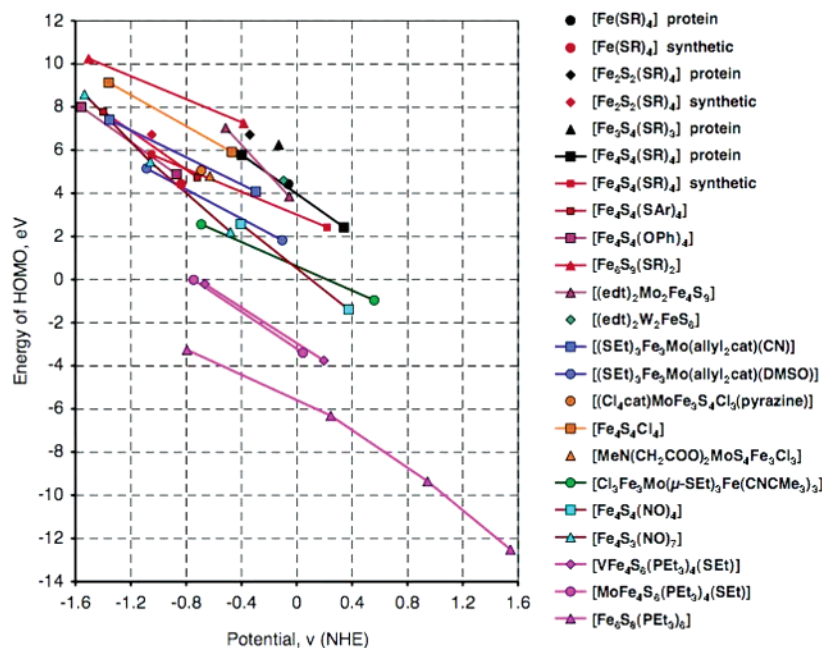
Figure 2. Correlation, for reduced members of the couples in Table 1, of the calculated gas-phase HOMO energy (eV) and the electrochemical potential (V).
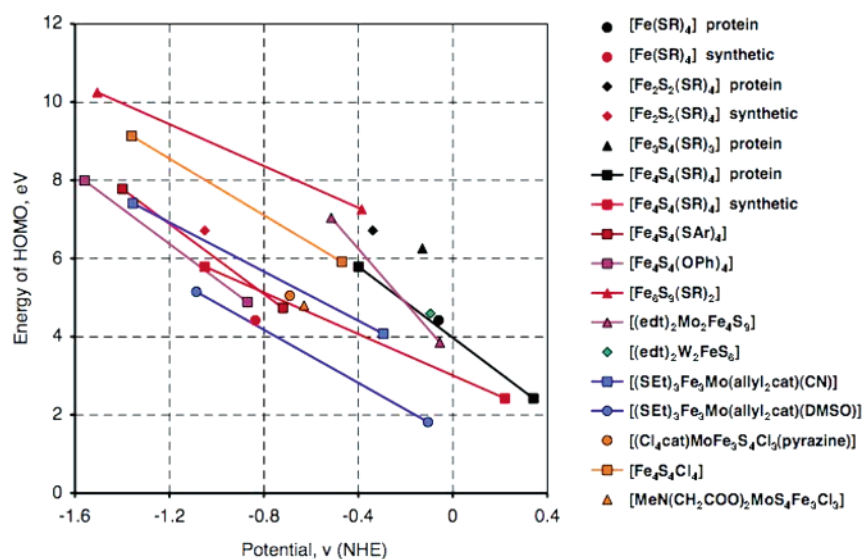

Figure 3. Subset of data from Figure 2 used for the application to the resting state of FeMo-co.

Before proceeding, it is necessary to assess the relevance of the reference compounds and to refine the reference set to be used in the correlation by culling the less-relevant systems. In Figure 2, notice that the reference compounds with terminal phosphine ligands are well displaced below the reference compounds with terminal thiolate ligands, reflecting the fundamentally different bonding characteristics of phosphine ligands: accordingly, the compounds with phosphine ligands are to be excluded from the reference set. Further, the reference compound [(ClFe)$_3$S$_4$Mo(μ-SEt)$_3$Fe-(CNCR$_3$)$_3$], with three isocyanide ligands (π-accepting like phosphines), has HOMO energies more negative than the remainder, and the [Fe$_4$S$_4$(NO)$_4$] and [Fe$_4$S$_3$(NO)$_7$] compounds with non-innocent nitrosyl terminal ligands have slightly lower HOMO energies: these compound types are

therefore also excluded from further consideration in the correlation.

Figure 3 shows detail of the 26 data points for the reference compounds that can be used to assess the redox state of FeMo-co. The gradients for the HOMO energy vs electrochemical potential are relatively constant for the nine series of electron-transfer processes: the steeper gradient for the [(edt-Mo)$_2$Fe$_4$S$_9$] series may be due to the less-reversible (large $E_p^a - E_p^c$ difference) character of its electrochemical data.[22] Figure 3 demonstrates the difference between the energy scales for the HOMO energy and the redox potential [Δ(HOMO energy)/Δ(redox potential) $\approx$ 3], which is, as described previously, mainly a consequence of the calculation of HOMO energies for species in the gas phase.

Table 2. HOMO Energies Calculated for FeMo-co $1(N)^z$ in the Gas Phase, as Dependent on Its Total Charge, z, Spin State, and in Some Cases Alternative Electronic States[a]

total charge z	spin	relative energy of the spin/electronic state (kcal mol^{-1})	HOMO energy eV
−4	1	+8.0	7.09
−4	1	+2.4	7.05
−4	0	+1.4	7.17
−4	2	0	7.06
−3	3/2	+7.4	4.10
−3	1/2	+6.6	4.16
−3	3/2	+6.1	4.08
−3	3/2	+3.2	3.80
−3	1/2	+0.5	4.11
−3	3/2	+0.3	3.82
−3	5/2	0	4.07
−2	2	+12.5	0.90
−2	0	+3.6	0.95
−2	1	0	0.91
−1	5/2	+5.6	−2.14
−1	3/2	+4.9	−2.06
−1	1/2	0	−2.18
0	2	+1.5	−5.18
0	0	+1.2	−5.14
0	1	0	−5.18
+1	5/2	+5.9	−8.26
+1	3/2	+2.2	−8.24
+1	1/2	0	−8.34

[a] The third column contains the relative energies (kcal mol^{-1}) of the different spin/electronic states for each total charge.

Table 3. HOMO Energies Calculated for C-Centered FeMo-co $1(C)^z$ in the Gas Phase, as Dependent on Its Total Charge Spin State, and in Some Cases Alternative Electronic States

total charge z	spin	relative energy of the spin/electronic state (kcal mol^{-1})	HOMO energy eV
−4	1/2	+10.7	7.26
−4	3/2	+4.9	7.23
−4	5/2	+0.8	7.07
−4	1/2	0	7.24
−3	0	+9.0	4.21
−3	2	+7.1	4.15
−3	1	+6.4	4.12
−3	0	0	3.96
−2	1/2	+6.4	1.17
−2	5/2	+4.2	1.05
−2	3/2	+3.8	1.03
−2	1/2	0	0.97

Application to the FeMo-co Active Site of Nitrogenase.

The reference compounds portrayed in Figure 3 have local coordination of Fe, Mo, and S that closely mimics the coordination of Fe, Mo, and S in FeMo-co (Figure 1). Therefore, the next step is to locate on the reference correlation the relevant redox potential data for the resting state of FeMo-co (also denoted M^N) in its protein and thereby to estimate the corresponding HOMO energy for FeMo-co. I emphasize that this analysis is made for FeMo-co as it is bound within the MoFe protein, not the isolated cofactor.[37] The enzyme does not exchange electrons with electrodes, and the potentials for oxidation of the resting state have been determined by mediated potentiometric titrations: species dependent values between 0 and −0.18 V (vs NHE) have been reported.[6,38−40] Superimposing this potential range on the reference correlation (Figure 3), leads to the conclusion that the energy of the HOMO for the resting state of FeMo-co is in the range 2−6 eV. Since the data for FeMo-co are in protein, the reference data taken from proteins (marked black in Figure 3) should be emphasized in reading from the correlation chart, and so it is concluded that the (gas-phase) energy of the HOMO of the resting state of FeMo-co lies in the range 3.5−6 eV.

Next, the energies of the HOMO of FeMo-co in various charge states are calculated to determine which best fits this conclusion. Table 2 lists the HOMO energy for FeMo-co

calculated in various spin and electronic states for net charges ranging from +1 to −4. (The results in Table 2 differ slightly from those previously published[7] because the basis set cutoff distance used throughout the present work is set for maximum accuracy.) The conclusion is obvious: only the −3 net charge on FeMo-co provides agreement between the calculated HOMO energy (3.8−4.2 eV) and that derived from the correlation with pertinent reference compounds (3.5−6 eV). Since it is known that the resting state of FeMo-co has an odd electron count ($S = 3/2$), the nearest alternative charge states would be −1 or −5, both of which have calculated HOMO energies that are >5 eV away from that derived from the reference correlation.

Central Atom of FeMo-co. The previous analysis assumed that the central atom X of FeMo-co is nitrogen, but this has not been proven. Crystallographic diffraction analysis indicated only that this central atom has an atomic number <10.[41] A number of theoretical investigations have shown that C and N are the only feasible possibilities: elements O or F as X cause the Fe−X distances to enlarge, unsymmetrically, in a manner that is inconsistent with accurately measured Fe−X distances.[7,13] Careful spectroscopic investigations involving labeled FeMo-co both isolated and in protein, have not been able to detect any signal corresponding to X = N, and currently there is no positive evidence for the identity of X.[8,10]

I have calculated the HOMO energies for C-centered FeMo-co and compared them with the HOMO energy obtained from the reference correlation. The results (Table 3) show that the calculated HOMO energies are very similar to those for X = N (Table 1) for the same total charge, although the electron counts differ by one. Since the resting state of FeMo-co has an odd electron count ($S = 3/2$), the total charge state, −3, that agrees well with the reference HOMO energy for X = N is not possible for X = C. The closest possibilities for X = C consistent with the electron count are total charge −4 (calculated HOMO energy ca. 7.1 eV) or total charge −2 (calculated HOMO energy ca. 1.1 eV), but both of these HOMO energies are well outside the 'experimental' reference range of 3.5−6 eV. Therefore, it

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Table 4. Gas-Phase Calculated HOMO Energies for Protonated FeMo-co, Model [**1**(X) + H]^z (X = N or C)

species	total charge, z	spin state	HOMO energy eV
[**1**(N) + S2BH]	−2	3/2	0.99
[**1**(N) + S2BH]	−3	0	4.30
[**1**(N) + exo-Fe6H]	−2	1/2	0.91
[**1**(N) + exo-Fe6H]	−3	0	3.84
[**1**(C) + S2BH]	−2	0	1.06
[**1**(C) + S2BH]	−3	1/2	4.26
[**1**(C) + S2BH]	−3	3/2	4.08
[**1**(C) + exo-Fe6H]	−2	0	1.02
[**1**(C) + exo-Fe6H]	−3	1/2	4.37
[**1**(C) + exo-Fe6H]	−3	3/2	4.12

is concluded that this C-centered model of FeMo-co is less likely than the N-centered model.

Protonation of Resting FeMo-co? Another issue needs to be considered, and that is the possibility that the resting state of FeMo-co is protonated or hydrogenated. Protonation is a formal concept for a process that is potentially detectable via the pH dependence of properties. The polarization of an S−H or Fe−H bond in FeMo-co is such that the atom charge on H is close to zero, not +1,[32] and so 'protonated FeMo-co' would in effect contain a bound H atom. An H atom could be detected by unusually accurate diffraction studies, or via spectroscopic coupling data, but there is no such datum for FeMo-co in its resting state: there is spectroscopic evidence for H bound to reduced states of FeMo-co in a modified protein during turnover.[42]

In the sense that protonation affects overall charge, it causes a shift in the HOMO energies. Here species will be described as [**1**(X) + H]^z, emphasizing that the net charge is important. As shown in Table 4, formal addition of a proton to **1**(N)$^{-3}$ at S2B (a likely position[32]) generates a species [**1**(N) + H]$^{-2}$ and a calculated gas-phase HOMO energy of +0.99 eV, while formal protonation at the exo-Fe6 coordination position[32] yields a calculated gas-phase HOMO energy of +0.91 eV. These are clearly different from the calculated HOMO energy of ca. +4 eV for unprotonated **1**(N)$^{-3}$. In contrast, formal hydrogenation rather than protonation at S2B or the exo-Fe6 position (i.e., [**1**(N) + H]$^{-3}$) yields calculated gas-phase HOMO energies of 4.3 or 3.8 eV, respectively, both close to the result from **1**(N)$^{-3}$. Similar results are obtained for [**1**(C) + H]^z (Table 4): when the net charge is −3, the HOMO energies are consistent with the 'measured' value from the correlation, but they are not when $z = -2$.

What are the consequences of these results? If N-centered FeMo-co in the resting state does contain an H atom bound to S or Fe, then the calculated HOMO energy results in Table 4, compared with the reference results, indicates that the net charge would be −3, but for such species, the total number of electrons is even, in conflict with the ESR data. Stated differently, there is an intrinsic discrepancy in the data on hypothetical [**1**(N) + H]: in order to retain agreement in the HOMO energies, the equivalent of an uncharged H atom

(or e$^-$ + H$^+$) needs to be added, but addition of one electron cannot be consistent with the measured odd spin for resting FeMo-co. Therefore, it is concluded that N-centered FeMo-co in the resting state does not harbor an undetected H atom. However, the results in Table 4 show that [**1**(C) + H]$^{-3}$ models have the correct HOMO energy, electron parity, and spin (3/2), and so it is possible that the resting state of the MoFe protein could contain C-centered FeMo-co, bearing one H atom and with total charge −3.

Discussion

This paper has described a procedure that permits the total charge state (or oxidation state) of a complex molecule to be obtained from its redox potential data, provided that good data (both charge state and redox potential) are available for reference compounds that are chemically similar. The link between the reference data and the unknown compound is made by the calculated energies of the Fermi level or HOMO. The HOMO energies are calculated for the reference compounds in their known charge states, and a graphical correlation of HOMO energy and redox potential for oxidation (corresponding to loss of an electron in the HOMO) is constructed. The measured redox potential of the unknown is then applied to the correlation to yield the HOMO energy of the unknown, against which the calculated HOMO energies for various charge states of the unknown are assessed. The experimental data are from condensed phases: the calculations could be made for HOMO energies in simulated condensed phases, but these simulations introduce uncertainties, and calculations for isolated molecules (reference and unknown) in the gas phase, although unrealistic, are more reliable and amplify the effects. In this procedure, it is more important to ensure that there is close chemical similarity between the reference systems and the unknown compound, such as the presence of ligands with similar bonding characteristics. Variations in spin and electronic state do not have significant influences in the systems discussed in this paper. It is worth noting that for systems such as FeMo-co in the gas phase the HOMO energy varies by ca. 3 eV per unit charge change, but because the parity of the electron count must be maintained in the charge states to be considered, the choice of HOMO energies involves jumps of ca. 6 eV.

This method is generally applicable. In addition to use with metal clusters in proteins, it could be applied to systems such as the polyoxometalate clusters, where there are extended electron-transfer series of clusters in which non-isolable species are characterized by electrochemical and ESR data[43,44] and for which there is a considerable volume of reference electrochemical data from which the correlation could be constructed.

The application to FeMo-co of the nitrogenase enzymes is specifically for the cofactor in the MoFe protein, not the partially deligated cluster that can be extracted from the

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protein, and the result applies to the resting state of FeMo-co, also known as M^N. The conclusion is that the resting state of FeMo-co has net charge (for model **1**) of -3 if the central atom is N, but if the central atom is C, then the model must also contain a hydrogen atom and have net charge of -3.

For the reasons outlined below, I believe that the central atom is more likely to be N, and so I will discuss here the interpretation of the preferred model **1**(N)$^{-3}$. To translate this charge to the core of FeMo-co, the charges of the $-SCH_3$ (equivalent to negatively charged cysteine) and the $-OCH_2COO-$ (equivalent to the coordinated section of homocitrate) are subtracted, leaving a core $[NFe_6MoS_9]^0$. This, $[NFe_6MoS_9]^0$, is the sustainable expression of the result and of the oxidation level of resting FeMo-co. Any further decomposition into atomic oxidation states is artificial and arbitrary because all four elements in this core can have variable formal oxidation states. There is evidence that Mo in FeMo-co has properties attributable to Mo(IV),[45,46] and if formal oxidation states N($-$III) and S($-$II) are assumed, then the iron atoms could be described as Fe(II)$_4$Fe(III)$_3$. However, this is an arbitrary assignment. I note that Pauling's electroneutrality principle is seriously contravened if the FeMo-co core is described as $[(N^{3-})(Fe^{3+})_3(Fe^{2+})_4(S^{2-})_9(Mo^{4+})]$. The calculated atom partial charges (mean of Mulliken, Hirshfeld, and electrostatic potential methods of calculation) are N, -0.44; Fe, $+0.33$; S, -0.46; Mo, 0.77.

Nevertheless, to focus on the important result, there is now consensus that $[NFe_6MoS_9]^0$ is the redox state of resting FeMo-co.[7,12,13,47]

A factor that can affect analyses such as these is protonation, which changes charge without changing the electron count or redox level of the cluster. It is concluded that N-centered FeMo-co in its resting state could not be protonated. This is consistent with the Thorneley−Lowe kinetic data and general mechanistic scheme for the nitrogenase which ascribes protonation only to reduced FeMo-co.[48] However, experiments show that the potential for the oxidation of resting FeMo-co in the MoFe protein of *Klebsiella pneumoniae* is pH dependent and consistent a protonatable site with a pK_a of 7.0 in the oxidized form of the protein.[38] Further experiments using deuterium indicated that this protonation does not occur at FeMo-co but probably at a surrounding residue, which is consistent with the conclusions of the present analysis.

The possibility remains that FeMo-co is C-centered and protonated in its resting state. As just discussed, there is no evidence for this proton. My reservations about C-centering arise also from consideration of the chemistry that would be involved in the biosynthesis of FeMo-co.[49] Established metal clusters containing an interstitial C atom are generated with reactants and reactions that are quite unbiological,[50−52] and it is not obvious what biological reactant could introduce a C atom into FeMo-co. A mechanism for irreversible insertion of an N atom into empty FeMo-co, using N$_2$ as reactant, has been proposed.[7]

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