Bromine K-edge EXAFS studies of bromide binding to bromoperoxidase from *Ascophyllum nodosum*

Holger Dau[a], Jens Dittmer[a], Matthias Epple[b], Jan Hanss[c], Erzsébet Kiss[b], Dieter Rehder[b,*], Carola Schulzke[b], Hans Vilter[d]

[a] *Fachbereich Biologie, Universität Marburg, D-35032 Marburg, Germany*
[b] *Institut für Anorganische und Angewandte Chemie, Universität Hamburg, D-20146 Hamburg, Germany*
[c] *Institut für Physik, Universität Augsburg, D-86159 Augsburg, Germany*
[d] *Zurmaienstraße 16, D-54292 Trier, Germany*

Received 4 June 1999; received in revised form 30 July 1999

Abstract Bromine K-edge EXAFS studies have been carried out for bromide/peroxidase samples in Tris buffer at pH 8. The results are compared with those of aqueous (Tris-buffered) bromide and vanadium model compounds containing Br-V, Br-C(aliphatic) and Br-C(aromatic) bonds. It is found that bromide does not coordinate to the vanadium centre. Rather, bromine binds covalently to carbon. A possible candidate is active site serine.

Key words: Bromoperoxidase; Bromine K-edge EXAFS; Bromovanadium complex; Aqueous rubidium bromide

1. Introduction

Vanadate-dependent peroxidases have been isolated from sea-weeds such as the brown marine alga *Ascophyllum nodosum* (AnPO), from a lichen and from the fungus *Curvularia inaequalis* (CiPO) [1]. While the CiPO is a chloroperoxidase, AnPO and other peroxidases from sea-weeds predominantly are bromo- (and iodo-)peroxidases, which catalyse the two-electron oxidation of bromide by peroxide to a Br^+ species. From the thermodynamic point of view, the most likely candidate for Br^+ is hypobromous acid, HBrO, which non-enzymatically brominates organic substrates or, in the absence of such substrates, reacts with peroxide to produce singlet oxygen. Many of the halogenated hydrocarbons found in algae exhibit anti-fungal and anti-bacterial properties. The halogenating activities of the haloperoxidases may thus be related to a protection mechanism.

The CiPO [2,3] and AnPO [4] have been crystallised and structurally characterised (Fig. 1). CiPO is a monomer of a 67.5 kDa molecular weight, while AnPO is a homo-dimer, comprising two ca. 60 kDa subunits containing one vanadate each. The active site structures (Fig. 1) are practically the same in the two enzymes, with the exception that Phe-397 in CiPO is replaced by His in AnPO. Vanadium, in its +V state in the active form, is in a trigonal-bipyramidal environment, covalently bonded to Nε of His-496 and to four oxo/hydroxo and/or water ligands. The axial OH in the native form of the CiPO is hydrogen-bonded to His-404, the protonation and deprotonation of which may play an important role in the mechanisms of bromide oxidation [5,6]. Several other amino acid side chains (cf. Fig. 1) are involved in a complex hydrogen bonding network to the vanadate moiety. Particularly noteworthy in the context of the following discussion is Ser-402, which is at a distance to the vanadium centre of 4–5 Å.

While an X-ray diffraction analysis [2] and ^{17}O NMR measurements [7] of the peroxo forms of the peroxidases have revealed direct coordination of peroxide to the vanadium centre, well in accord with the known ability of V(V) to form peroxo complexes, information on the binding of bromide to the active centre is scarce. The affinity of AnPO for Br^- as related to the Michaelis-Menten constant, K_m amounts to 18 mM (pH = 8) [8], but nothing is known about the binding mode. Although bromovanadium complexes again are well-established, direct binding of bromide to vanadium is being considered less probable for this enzyme. In order to elucidate the question of the nature of bromide binding, we have now undertaken an EXAFS analysis at the bromine edge of bromide-enzyme samples of different Br^-:AnPO ratios, including aqueous bromide, bovine serum albumin (BSA) and model compounds with bromine covalently bonded to vanadium, aromatic or aliphatic carbon. There are a few reports available on XANES studies of AnPO and model compounds [9–12], all carried out at the vanadium K-edge. One of these studies directed towards substrate binding to the enzyme supports direct peroxide binding but disfavours bromide binding to vanadium [10].

2. Materials and methods

AnPO was prepared and purified as described [13]. The effective (calculated for $M = 60$ kDa) final c(AnPO) was 1.3 mM in all samples. BSA (fraction V, Merck) was employed as a reference protein due to its similar molecular weight and isoelectric point. A 26 mM stock solution of high purity RbBr (Kawecki Berylco Industries) in deionised water and a 0.1 M Tris buffer of pH 8.0, prepared from tris(hydroxymethyl)aminomethane (Serva) and HCl, were employed in preparing the protein essays. The final chloride concentrations (due to the chloride contents of the buffer) were 29 mM and hence well below the concentration where AnPO also exhibits a chloroperoxidase activity (ca. 1 M [14]). The ratio Cl^-/Br^- was about twice that in sea water. The following samples were investigated: (1) 2.9 mM RbBr in Tris buffer, (2) 22 mM RbBr in Tris, (3) 0.95 mM RbBr+1.3 mM BSA in Tris, (4a) 1.3 mM RbBr+1.3 mM AnPO in Tris, (4b) 0.8 mM RbBr+1.3 mM AnPO in Tris, (4c) 0.6 mM RbBr+1.3 mM AnPO in Tris. The following model compounds (preparative details and characteristics of the model compounds and model reactions will be reported elsewhere) were employed in the form of microcrystalline powders: *trans*-(VBr_2(mal)$_2$) (mal = maltolate(1-)) (I), $VO(CBr_3)(OtBu)_2$ (II) and $VO(H_2O)$(3-Br,5-Cl-sal-gly) (sal-gly is the Schiff base from salicylaldehyde and glycine) (III) (see Scheme 1).

X-ray absorption spectroscopy at the Br K-edge (ca. 13 470 eV) was carried out at the Hamburger Synchrotonstrahlungslabor (HASY-

*Corresponding author. Fax: (49) (040) 4123 2893.

PII: S0014-5793(99)01045-5

LAB) at the Deutsche Elektronen-Synchroton (DESY), Hamburg, Germany.

The spectra for the model compounds I, II and III were taken at beamline X (ROEMO II). The incoming synchrotron beam was monochromatised by a Si(111) double-crystal monochromator. Experiments were performed in transmission mode at room temperature. The ground samples, kept under inert gas atmosphere, were put onto adhesive tape that was wrapped to exclude air as far as possible. Prior to the experiment, the samples were stored under dry nitrogen. The EXAFS experiments themselves were carried out in air. For quantitative data evaluation, the program packages AUTOBK and FEFFIT of the University of Washington were employed [15]. Theoretical standards were computed with the program FEFF 5.04 [16], using the crystal structures of the pure compounds as input data. All fits were carried out with k^3-weighted data in R-space. The following fit parameters were used and obtained for the model compounds. I: fit range in k: 2–9.5 Å, fit range in R: 1–5.9 Å, $S_0^2 = 0.56$, $\Delta E_0 = 6.8$ eV, coordination numbers fixed to 1, 4 and 1, R(Br-Br) set to R(Br-V). II: fit range in k: 2–13 Å, fit range in R: 1–3.4 Å, $S_0^2 = 0.66$, $\Delta E_0 = +6.8$ eV, coordination numbers fixed to 1, 1 and 2. III: fit range in k: 2–11 Å, fit range in R: 1–3.0 Å, $S_0^2 = 1.04$, $\Delta E_0 = +2.9$ eV, coordination numbers fixed to 1 and 2. In general, the amplitude reduction factor S_0^2, the zero-energy shift ΔE_0, the distances R and the Debye-Waller factors (σ^2) were allowed to vary in the fits, unless specifically mentioned. See also supplementary material, available from the authors on request, encompasses the unfiltered k-spectra of samples (1) and (4c), the experimental spectra superimposed to the model functions for samples (2), (4a–c) and I–III.

For samples 1–4, spectra were collected at beamline D2 of the EMBL Hamburg outstation. The liquid samples were injected into 1 mm cuvettes with capton foil windows. Fluorescence-detected X-ray absorption spectra were measured at 18 K as described elsewhere [17]. The energy scale of all collected EXAFS spectra was converted to a k-scale using an E_0 of 13 470 eV, k^3-weighted spectra were used for curve-fitting and calculation of Fourier transforms. For the Fourier transform shown in Fig. 2, the data corresponding to the energy range 13 480–13 740 eV were multiplied by a fractional cosine window (10% cosine fraction at low and high R-side). For curve-fittings, the energy range was 13 480–13 940 eV. See also supplementary material, available from the authors on request, encompasses the unfiltered k-spectra of samples (1) and (4c), the experimental spectra superimposed to the model functions for samples (2), (4a–c) and I–III.

Bromine EXAFS spectra are complicated by multiple excitation effects [18] which give rise to peaks in the Fourier transforms below 1.2 Å on the apparent distance scale (see spectrum of aqueous bromide in Fig. 2). The multiple excitation peaks of the aqueous bromide were extracted by Fourier isolation (window ranging from 0 to 1.5 Å, fractional cosine window with a 2 and 10% cosine fraction at the low and high R-side, respectively) and subtracted from all four k^3-weighted spectra before curve-fitting of spectra was approached. For simulation of k^3-weighted spectra, complex backscattering amplitudes were calculated using the FEFF 7 software [19]. Using the program SimX (Jens Dittmer, unpublished), the four k^3-weighted spectra were simulated simultaneously (joint-fit approach) in such a way that corresponding bromine-backscatterer distances and Debye-Waller parameters of the four spectra were 'coupled' (i.e. constrained to a common value). In contrast to the coupled bromine-backscatterer distances (r) and Debye-Waller parameters (σ_i), the coordination number parameter (N_i) remained unconstrained (independent variation of the N_i parameters of all four spectra by the fit algorithm). The obtained N_i values are apparent or relative coordination numbers which correspond to the product of the 'real' coordination number and the relative concentration of either aqueous bromide (for Br-O and Br-H) or protein-bound bromine (for Br-C and Br···(C)-C).

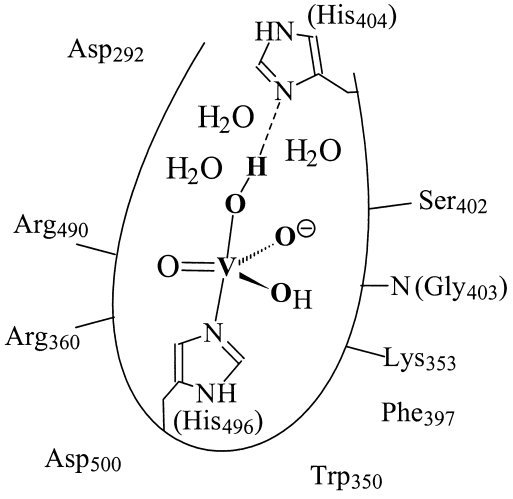


Fig. 1. Schematic representation of the active site of the chloroperoxidase CiPO, based on X-ray diffraction analysis adapted from [2,3]. In the bromoperoxidase AnPO, Phe-397 is replaced by His [4]. Amino acid residues connected to the protein pocket by dashes are in direct hydrogen bonding interaction with the oxovanadate(V) centre.

3. Results and discussion

The EXAFS results are collated in Table 1, Fourier transformations of the experimental spectra for the samples (2), (4a–c) are shown in Fig. 2, those for the model compounds I, II and III in Fig. 3.

Table 1
EXAFS parameters[a]

Sample[b]	Assignment	N	r/Å	σ^2/Å$^2 \times 10^3$
(2)[c] Br^- 22 mM	Br-(H)-O	4.9	3.23	25
	Br-H(-O)	4.9[d]	2.38	9
(4)[c] Br^- 0.6–1.3 mM+AnPO 1.3 mM	Br-C	1.03[e]	1.88	1[d]
	Br-(C)-C	1.09[e]	2.89	1[d]
I	Br-V	1[d]	2.39	5.2
	Br-(V)-O	4[d]	3.15	16.6
	Br-(V)-Br	1[d]	4.79[f]	22.4
II	Br-C_{aliph}	1[d]	1.97	2.8
	Br-(C)-V	1[d]	3.40	8.7
	Br-(C)-Br	2[d]	3.16	5.1
III	Br-C_{arom}	1[d]	1.89	2.5
	Br-(C)-C	2[d]	2.84	6.8

[a]Coordination number N, effective distance r and Debye-Waller factor σ^2.
[b]For sample assignments, see also Section 2 and Scheme 1.
[c]Curve-fit results from a joint-fit of the samples (2) and (4a–c) (cf. Section 2).
[d]Fixed parameters.
[e]Obtained from an extrapolation of N_{rel} to $d = -1.94$, where d is the normalised difference between bromide and enzyme concentrations, see text.
[f]Fixed to $2r$(Br-V).

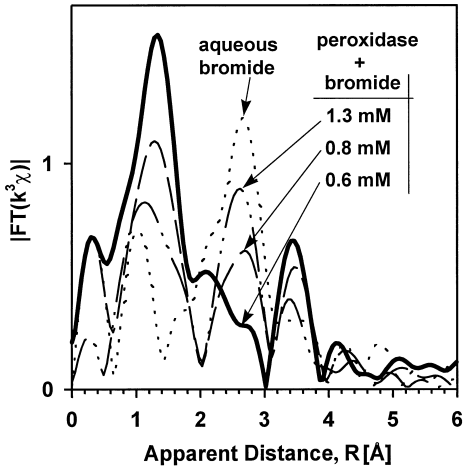


Fig. 2. Fourier transformations of samples (2) (22 mM bromide, dotted line) and (4a–c) (1.3/0.8/0.6 mM bromide+1.3 mM AnPO, broken and solid lines).

EXAFS features characteristic of the hydration sphere of bromide and its possible interaction with the Tris buffer have been obtained for aqueous RbBr solutions at low concentrations (2.9 and 22 mM) in 0.1 M Tris buffer of pH 8.0. The main peak in the Fourier-transformed spectrum (cf. Fig. 2, dotted line) at an effective distance of 3.23 Å can be interpreted in terms of an oxygen shell and compares with distances previously reported for the Br···O of the hydration shell of bromide in concentrations of 1 M up to saturation [20,20]. The Br···O coordination number found in our study, 4.9, exceeds that in previous reports (average of ca. 2.6 in 1 M solutions [20]). These differences may be due to a more pronounced hydration at lower concentrations or due to the participation of alcoholic functions of the buffer in outer sphere coordination. Apart from the Br···O peak, there is a distinct

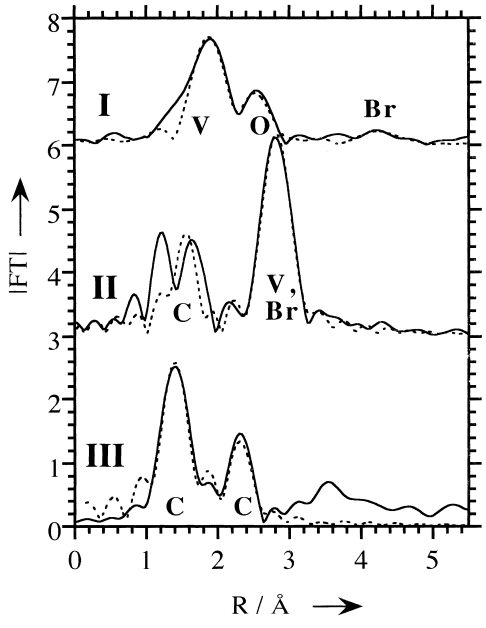


Fig. 3. Fourier transformations of model samples I (V-Br bond), II (V-$C_{aliphat}$ bond) and III (V-C_{arom} bond). See Scheme 1 for the model compounds. The solid lines represent experimental spectra, dotted lines represent model functions.

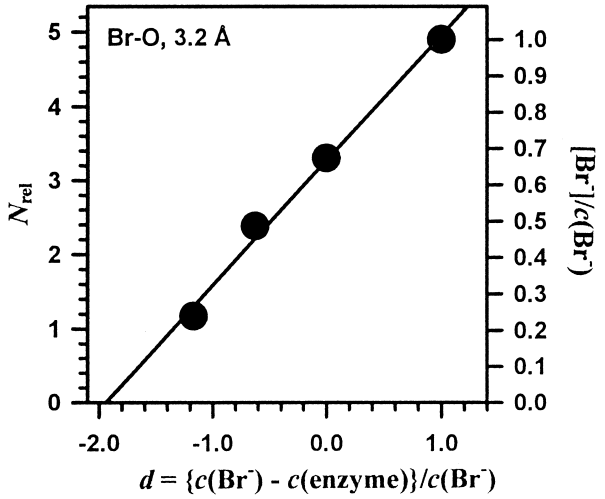
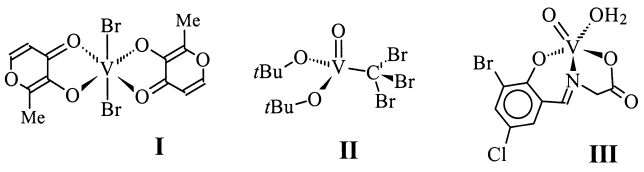


Fig. 4. Plot of the normalised concentration of free bromide, $[Br^-]/c(Br^-)$, versus the normalised difference (d) of the concentrations of overall bromide and enzyme. $[Br^-]/c(Br^-)$ is related to the relative coordination number N_{rel} for the hydration shell of free bromide by $[Br^-]/c(Br^-) = N_{rel}/4.9$.

feature at 2.38 Å, which was successfully modelled by five hydrogens and which we therefore tentatively assign to hydrogens in hydrogen bonding contact with bromide. Additional characteristics in the spectra are assigned to photoelectron backscattering at the bromide electron shell (1.0 Å [22]), double-electron excitations [18,23] and higher solvation spheres.

In order to minimise the possibility that EXAFS patterns in the enzyme-bromide samples are due to unspecific bromide binding to the protein, we have also measured a sample containing BSA and bromide under the same concentration and pH conditions (cf. sample (3) in Section 2) as with the enzyme samples. No differences between bromide solutions with and without the serum albumin were detected.

EXAFS spectra of AnPO+bromide in Tris buffer (samples 4a–c) were obtained for the concentration ratios 1.3/1.3, 1.3/0.8 and 1.3/0.6 mM. The Fourier transformations have been included in Fig. 2, the experimental spectra are provided in the supplementary material. For EXAFS parameters, cf. Table 1. With a decreasing bromide concentration (increasing the ratio c(enzyme)/c(bromide)), the peak due to the hydration shell loses intensity. Concomitantly, a peak arises at an effective distance of 1.88 Å. Comparison with bond parameters from diffraction studies and with the model compounds I–III (Fig. 3) excludes coordination of the bromide to vanadium and suggests covalent binding to a light atom, the closest candidate being an sp^2-hybridised (e.g. aromatic) carbon. The covalent character of the bond is also revealed by the position of the K-edge, which moves to higher energies as hydrated bromide is increasingly replaced by active site-bound bromine. Hence, our results indicate that an organic residue is



Scheme 1.

brominated. Assignment of the 1.88 Å feature to the Br-C_{arom} bond (a possible candidate is the imidazole of a histidine), however, requires an electrophilic attack, i.e. a Br^+ species, the generation of which in the assay used is unlikely to occur. A candidate for nucleophilic substitution, on the other hand, is the active site serine. EXAFS distances Br-C_{aliph} of bromoalkanes [23,23] and bromoacetates [22,25,26] range from 1.93 to 1.96 Å. A search for Br-C bond lengths of Br-C bonds in the vicinity (β position) of an amide bond revealed 1.94 Å as an average value (the range is 1.87–1.99 Å). Geometry-optimised simulation of the distances Br-Cβ and Br···Cα in the serinyl residue with HyperChem affords 1.91 and 2.82 Å, respectively. Hence, although from a chemical point of view, bromination of serine, possibly by a short-lived bromovanadium(V) intermediate (in vitro studies have shown that the methyl ester of serine is brominated by $VOBr_3$ and type I complexes (cf. Scheme I) in acetonitrile), is a reasonable assumption, the short EXAFS parameter of 1.88 Å is not fully explained on such a basis. If serine is the site of bromination, one would expect, according to the X-ray diffraction results on CiPO and AnPO, the metal centre at a distance of 4–5 Å. Fig. 2 shows a feature at about this distance. At the present stage of experimental evidence, an unambiguous assignment can, however, not be carried out.

We have finally evaluated the EXAFS results with respect to the extent of saturation of the bromine binding site, using Br-C and Br-O coordination numbers. The overall bromide concentration $c_0(Br^-)$ in the bromide-enzyme samples is distributed between the equilibrium concentrations of free bromide, [Br^-], and enzyme-bound bromide. If the normalised equilibrium concentration of free bromide, $[Br^-]/c(Br^-)$, is plotted against $d = (c(Br^-) - c(\text{enzyme}))/c(Br^-)$, the normalised difference between the concentrations of bromide and enzyme, a linear relation is obtained (Fig. 4). The intercept with the d axis at -1.94 corresponds to $c(Br^-) = 0.44$ mM, the situation where all of the bromide is bound to the enzyme (c(enzyme) = 1.3 mM). The linear regression is indicative of a high affinity of the enzyme for the bromide. The concentration-dependence, however, implies that a maximum of 40% of the enzyme actually binds bromine covalently. There are several reasons for this deficiency. For one thing, the vanadium contents may be less than one vanadate per enzyme subunit (or part of the vanadium is bound unspecifically). For another thing, admixtures of iodide, which competes with bromide for the binding site, may be present. We have earlier provided evidence for the presence of up to 0.5 mol iodine per mol enzyme [27] and also for the unspecific binding of vanadate [28].

Acknowledgements: This work was financially supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. Further, we wish to thank Dr. Wolfram Meyer-Klaucke and Larc Tröger from EMBL/DESY for support.

References

[1] Vilter, H. (1995) in: Metal Ions in Biological Systems (Sigel, H. and Sigel, A., Eds.), Vol. 31, Chapter 10, Marcel Dekker, New York.
[2] Messerschmidt, A., Prade, L. and Wever, R. (1997) Biol. Chem. 378, 309–315.
[3] Macedo-Ribeiro, S., Hemrika, W., Renirie, R., Wever, R. and Messerschmidt, A. (1999) J. Biol. Inorg. Chem. 4, 209–219.
[4] Weyand, M. (1999), private communication.
[5] Butler, A. and Baldwin, A.H. (1997) Struct. Bond. 89, 109–132.
[6] Colpas, G.J., Hamstra, B.J., Kampf, J.W. and Pecoraro, V. (1996) J. Am. Chem. Soc. 118, 3469–3478.
[7] Rehder, D. (1999) The ^{17}O NMR of An-PO in Tris buffer (pH 8), treated with ^{17}O-enriched H_2O_2 shows three resonances tentatively assigned to free $HVO_2(O_2)^{2-}$, active site ($V(\eta^2-O_2)$) and active site ($V(\eta^1-O_2H)$), unpublished.
[8] de Boer, E. (1988) J. Biol. Chem. 263, 12326–12332.
[9] Arber, J.M., de Boer, E., Garner, C.D., Hasnain, S.S. and Wever, R. (1989) Biochemistry 28, 7968–7973.
[10] Küsthardt, U., Hedman, B., Hodgson, K.O., Hahn, R. and Vilter, H. (1993) FEBS Lett. 329, 5–8.
[11] Hormes, J., Kuetgens, R., Chauvistre, R., Schreiber, W., Anders, H., Vilter, H., Rehder, D. and Weidemann, C. (1988) Biochim. Biophys. Acta 956, 293–299.
[12] Weidemann, C., Rehder, D., Kuetgens, U., Hormes, J. and Vilter, H. (1989) Chem. Phys. 136, 405–412.
[13] Vilter, H. (1994) in: Methods in Enzymology (Walter, H. and Johansson, G., Eds), Vol. 228, pp. 665–672, Academic Press.
[14] Soedjak, H.S. and Butler, A. (1990) Inorg. Chem. 29, 5015–5017.
[15] Stern, E.A., Newville, M., Ravel, B., Yacoby, Y. and Haskel, D. (1995) Physica B117, 208–209.
[16] Rehr, J.J., Albers, R.C. and Zabinsky, S.I. (1992) Phys. Rev. Lett. 69, 3397.
[17] Schiller, H., Dittmer, J., Iuzzolino, L., Dörner, W., Meyer-Klaucke, W., Sole, V.A., Nolting, H.-F. and Dau, H. (1998) Biochemistry 37, 7340–7350.
[18] D'Angelo, P., Di Cicco, A., Filipponi, A. and Pavel, N.V. (1993) Phys. Rev. A47, 2055–2063.
[19] Zabinsky, S.I., Rehr, J.J., Ankudinov, A., Albers, R.C. and Eller, M.J. (1995) Phys. Rev. B52, 2995.
[20] Bertagnolli, H., Ertel, T.S., Hoffmann, M. and Frahm, R. (1991) Ber. Bunsenges. Phys. Chem. 95, 704–709.
[21] Ludwig Jr., K.F., Warburton, W. and Fontaine, A. (1987) J. Chem. Phys. 87, 620–629.
[22] Epple, M., Kirschnick, H., Greaves, G.N., Sankar, G. and Thomas, J.M. (1996) J. Chem. Soc. Faraday Trans. 92, 5035–5042.
[23] Burattini, E., D'Angelo, P., Di Cicco, A., Filipponi, A. and Pavel, N.V. (1993) J. Phys. Chem. 97, 5486–5494.
[24] Epple, M., Tröger, L. and Hilbrandt, L. (1997) J. Chem. Soc. Faraday Trans. 93, 3035–3037.
[25] Epple, M., Sazama, U., Reller, A., Hilbrandt, L. and Tröger, J. (1996) J. Chem. Soc. Chem. Commun., pp. 1755–1756.
[26] Epple, M., Sankar, G. and Thomas, J.M. (1997) Chem. Mater. 9, 3127.
[27] Knüttel, K., Müller, A., Rehder, D., Vilter, H. and Wittneben, V. (1992) FEBS Lett. 302, 11–14.
[28] Rehder, D., Holst, H., Priebsch, W. and Vilter, H. (1991) J. Inorg. Biochem. 41, 171–185.