Metal binding sites of oxovanadium(IV) in native and modified soluble collagen*

Rosa Pia Ferrari

Dipartimento di Chimica Inorganica, Chimica Fisica e Chimica dei Materiali, Università di Torino, via P. Giuria 7, 10125 Turin (Italy)

(Received April 20, 1990)

Abstract

Oxovanadium(IV) coordination to native and modified soluble collagen from calf skin, has been studied by EPR spectroscopy on aqueous and lyophilized samples at physiological pH. At room temperature, the isotropic spectrum $g_0=1.967$, $A_0=95.61\times10^{-4}$ cm^{-1} of a VO(IV)–collagen complex, weakly immobilized in solution has been seen, with vanadyl rotational correlation time $\tau_r\approx6\times10^{-9}$ s shorter than rotational tumbling time $\tau_r\approx10^{-7}$ s of the whole protein. This is probing a local environment for the VO(IV) site and vanadyl monocoordination to the protein. Simultaneously, an anisotropic spectrum with axial symmetry has been detected from VO(IV)–native collagen and VO(IV)–derivative collagen complexes in solution at 77 K and, in the solid fibrous state at both r.t. and 77 K. The apparent g_0 and A_0 values calculated for the powder spectra are comparable with those of solution spectra at r.t. of VO(IV)–insulin in the A site and VO(IV)–(N_2O_2) model compounds. However, oxovanadium(IV) seems to interact with two nitrogens and two water molecules on the equatorial plane. The two protein nitrogen ligands could be two imidazole nitrogens, or one imidazole nitrogen and one amino group, or two amino groups. By increasing the vanadyl concentration, the metal-ion interaction with tropocollagen molecules loses its specificity, to give a mixture of VO(IV)–collagen compounds.

Introduction

Studies on Cu(II)–collagen and Cu(II)–modified collagen using different kinds of collagen have been made in our laboratory [1, 2]. The N_{Him}(histidine) and the ϵ NH_2 (lysine and hydroxolysine) sites on the protein, as potential binding sites for the copper present in lysiloxidase, have been investigated. Intermolecular cross-links in collagen seem to be the ϵ NH_2 groups close to the histidine residue at the end of the tropocollagen molecule [3, 4].

On this basis, structural and conformational studies were made on native collagen, and on its derivatives (deuterated collagen, collagen with methylated carboxylate groups and collagen with dinitrophenylated amino groups) using VO(IV) as an EPR probe. We chose to operate on calf skin collagen which is the most similar to human collagen, and to use the vanadyl as interacting metal-ion because vanadium is present in living beings and has been identified *in vivo* from tissues as VO(IV) by EPR [5].

Furthermore as is well known, EPR studies on VO(IV)–globular proteins (e.g. insulin and carbonic anidrase), VO(IV)–nucleic acid and VO(IV)–model compounds, gave some important structural informations on their metal active sites [6] and VO(IV) is a very convenient EPR probe because it provides very narrow EPR spectra observable at room temperature.

Materials and methods

Native soluble calf·skin collagen, freshly made, was purchased from Sigma and purified from metal-ion impurities by dialysis against EDTA 0.1 M on magnetic stirring, for 24 h at 4°C. Deuterated-collagen, CH_3O-collagen and DNP-collagen were made in our laboratory according to published procedures [1]; $VOSO_4\cdot5H_2O$ and the other reagents were analytical grade from Merck.

The protein solutions were purged with nitrogen gas before addition of vanadyl ion to avoid oxidation, then 0.05, 0.75 and 5.00 ml (5.0×10^{-2} M) of $VOSO_4\cdot5H_2O$ were added to 50.0 ml of protein

*Abbrevations: D-collagen, deuterated collagen; CH_3O-collagen, collagen with methylated carboxylate groups; DNP-collagen, collagen with dinitrophenylated amino groups.

0020-1693/90/$3.50 © Elsevier Sequoia/Printed in Switzerland

aqueous solution (5.0×10^{-5} M) to have VO(IV)–protein molar ratios of 1:1, 15:1 and 100:1, respectively. All samples were adjusted to pH $\approx$ 7 using NaOH (0.1 N). Equivalent solutions were made in NaCl (0.1 M) and Hepes (0.1 M).

To make solid samples, the solutions were lyophilized and held in a vacuum drying apparatus.

Vanadium contents for all samples were evaluated by atomic absorption spectroscopy and by EPR spectroscopy using $VOSO_4\cdot5H_2O$ as a standard. In this case we considered the intensity for the central peak of the spectral pattern of the complexes based on the signal-to-gain ratio.

Tripsin assay and viscosity measurements on the treated and untreated samples assure that they are undenatured [2]. The vanadyl ion was removed from the VO(IV) complexes by EDTA 0.1 M. EPR spectra were made on solutions and on lyophilized samples at room temperature and at 77 K, using a Varian E 109 X band spectrometer equipped with AQM, Auriga/XT (Stelar) data system.

Results and discussion

The VO(IV)–collagen solution 1:1 molar ratio at r.t. and physiological pH, possesses the vanadyl isotropic pattern typical of oxovanadium(IV) complexes weakly immobilized in solution, $\tau_r\approx6\times10^{-9}$ s, as estimated from an empirical formula reported in ref. 7, and $g_0=1.967, A_0=95.61\times10^{-4}$ cm^{-1} (Fig. 1(A)), which exclude the presence of the polymeric or oligomeric species $[VO(OH)_3]^-$, EPR undetectable, and of $[VO(H_2O)_5]^{2+}$, $g_0=1.964$, $A_0=106.30\times10^{-4}$ cm^{-1} [6].

The rotational correlation time $\tau_r\approx6\times10^{-9}$ s of the vanadyl site is much shorter than the overall tumbling time $\tau_r\approx10^{-7}$ s, estimated from the Debye relationship for the whole protein. This is thus probing a local environment for the metal-ion, and suggests its apical monocoordination to the protein. An analogous type of coordination has been found for the weak sites of carboxypeptidase A [6].

Furthermore, this is in good agreement with the hypothesis that oxovanadium(IV), due to its steric hindrance, can interact easier with collagen in the non-triple-helical regions, at the end of the chains, made of 9–50 amino acid residues [8]. The time scale τ_r estimated for these regions falls between $\tau_r\approx2.7\times10^{-10}$ s and $\tau_r\approx1.5\times10^{-9}$ s values, close to the vanadyl time scale $\tau_r\approx6\times10^{-9}$ s. The EPR parameters g_0 and A_0, and the presence of a histidine and an ϵ NH_2 residue at the end of the chain, could suggest nitrogen coordination to the protein.

Up to about 15 mol VO(IV)/mol protein the apparent A_0 value of the spectrum appears slightly

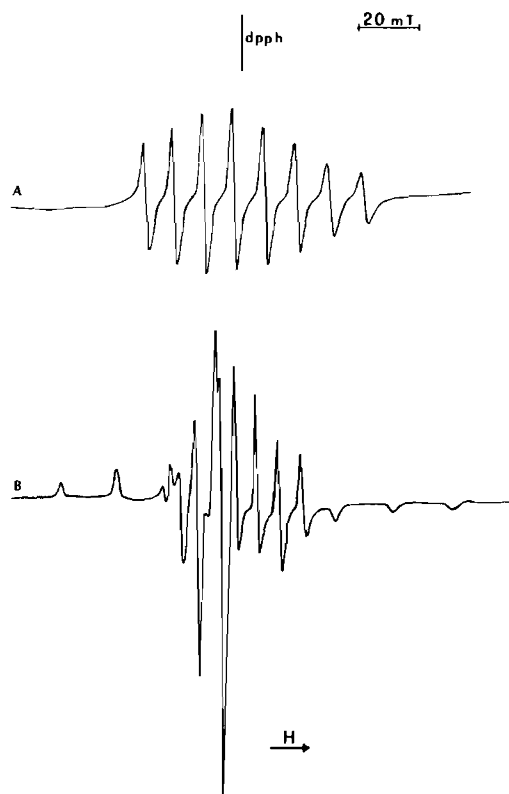

Fig. 1. (A) Isotropic EPR spectrum of VO(IV)–collagen in solution at r.t., (B) anisotropic EPR spectrum of the complex in a rigid matrix.

modified. Since one tropocollagen molecule (calf skin, rat skin and human placenta) contains fifteen histidine residues, one mole of collagen can fix a number of vanadyl ions equal to the histidine residues, to give possibly more VO(IV)–N(histidine) equivalent species, as happens for collagen–copper interaction [1].

When concentration increases to 100 mol VO(IV)/mol protein, the EPR apparent parameter $A_0=102.02\times10^{-4}$ cm^{-1} becomes higher, and is indicative of VO^{2+} binding to carboxylate-groups, that are weakly coordinating sites [9].

More generally, in the last two cases, because the line intensities of the isotropic spectra are enhanced regularly, we can argue that more metal-ions can interact with more tropocollagen molecules on different metal binding sites.

The VO(IV)–collagen samples (1:1) in NaCl 0.1 M and Hepes 0.1 M, present analogous EPR patterns and parameters, which suggest that, also in these conditions, only one major species is present in solution at physiological pH. The presence of Na^+ in solution does not influence the VO(IV)–collagen coordination and the buffer does not appreciably interact with vanadyl.

TABLE 1. EPR parameters of VO(IV)–native collagen and VO(IV)–derivative collagen complexes[a,b]

Protein	$g_{\parallel}$	$g_{\perp}$	g_0	$A_{\parallel}$	$A_{\perp}$ ($\times 10^{-4}$ cm^{-1})	A_0
Collagen	1.934	1.965	1.955	168.40	61.80	97.33
DNP-collagen	1.931	1.969	1.956	168.01	61.71	97.14
CH_3O-collagen	1.926	1.963	1.951	167.25	60.66	96.19
Collagen (NaCl)	1.926	1.962	1.950	167.09	61.11	96.44
Collagen (Hepes)	1.924	1.965	1.951	167.20	60.80	96.27
D-collagen	1.921	1.956	1.944	166.10	60.30	95.57

[a]VO(IV)–collagen solution at r.t. molar ratio 1:1 gives: $g_0 = 1.967$; $A_0 = 95.61 \times 10^{-4}$ cm^{-1}. [b]VO(IV)–collagen lyophilized at r.t. molar ratio 1:1 gives: $g_{\parallel} = 1.922$; $g_{\perp} = 1.960$; $A_{\parallel} = 166.50 \times 10^{-4}$ cm^{-1}; $A_{\perp} = 61.88 \times 10^{-4}$ cm^{-1}; $g_0 = 1.947$; $A_0 = 96.75 \times 10^{-4}$ cm^{-1}. VO(IV)–collagen lyophilized at 77 K, molar ratio 1:1 gives: $g_{\parallel} = 1.926$; $g_{\perp} = 1.962$; $A_{\parallel} = 167.09 \times 10^{-4}$ cm^{-1}; $A_{\perp} = 61.11 \times 10^{-4}$ cm^{-1}; $g_0 = 1.950$; $A_0 = 96.44 \times 10^{-4}$ cm^{-1}.

With elapsing contact time, partially at room temperature, and completely with decreasing the temperature to 77 K, an anisotropic EPR pattern appears, showing that the metal-ion has been immobilized on the macromolecular proteic matrix (Fig. 1(B)).

Besides, for the correspondent lyophilized samples the anisotropic spectral pattern is already evident at room temperature. In the fibrous solid state the compact collagen conformation can easily immobilize the coordinated vanadyl ion. The anisotropy is typical of axial symmetry and can be described by the spin Hamiltonian

$$\hat{\mathcal{H}} = \beta g_{\parallel} H_z\hat{S}_z + \beta g_{\perp}(H_x\hat{S}_x + H_y\hat{S}_y) + A_{\parallel}\hat{S}_z\hat{I}_z + A_{\perp}(\hat{S}_x\hat{I}_x + \hat{S}_y\hat{I}_y)$$

with $g_{\parallel} < g_{\perp} < 2$. The spin Hamiltonian parameters (Table 1) were calculated following an iterative procedure using the set of equations reported in refs. 6 and 10.

The apparent mean values $g_0 = 1.953$ and $A_0 = 96.87 \times 10^{-4}$ cm^{-1}, calculated for collagen frozen solutions at 77 K, are in good agreement with those of VO(IV)–insulin in the A site, where the donor atoms are two imidazole nitrogens and two water molecules [11] ($g_0 = 1.965$ and $A_0 = 96.80 \times 10^{-4}$ cm^{-1}) and those of the model compounds [$VO(N_2O_2)$] ($g_0 = 1.968$ and $A_0 = 96.95 \times 10^{-4}$ cm^{-1}, $2N_{Him}/2H_2O$); ($g_0 = 1.968$ and $A_0 = 95.05 \times 10^{-4}$ cm^{-1}, $2NH_2/2H_2O$); ($g_0 = 1.968$ and $A_0 = 96.00 \times 10^{-4}$ cm^{-1}, $1N_{Him}/1NH_2$) [12] (Table 2). In particular, we notice that the A_0 parameter reflects the ligand environment around vanadyl on the equatorial plane [6]. The slight differences are due to the temperature effects on the rotational correlation time τ_r [6]. The EPR parameters of lyophilized samples both at r.t. and 77 K are similar and comparable with those of solution samples. The $g_{\parallel}$ and $g_{\perp}$ values are close to those of globular proteins and slightly less than those of model compounds. This could be correlated to the increase of the viscosity coefficient, going from simple model compounds to protein.

TABLE 2. EPR parameters g_0 (calc.) and A_0 (calc.) for $VO(N_xO_y)$ model sytems in the equatorial plane[a,b]

Model systems on the equatorial plane	g_0 (calc.)	A_0 (calc.) ($\times 10^{-4}$ cm^{-1})
[$VO(O_4)$]	1.964	108.30
[$VO(O^-O_3)$]	1.965	104.15
[$VO(O_2{}^-O_2)$]	1.965	102.47
[$VO(N_{Him}O^-O_2)$]	1.966	99.47
[$VO(N_{NH_2}O^-O_2)$]	1.967	98.52
[$VO(N_{(Him)2}O_2)$]	1.968	96.95
[$VO(N_{(NH2)2}O_2)$]	1.968	95.05
[$VO(N_{Him}N_{NH_2}O_2)$]	1.968	96.00
[$VO(N_4)$]	1.980	77.40

[a]The g_0 and A_0 values for additivity calculations were taken from ref. 12. [b]$O = H_2O$, O^- = carboxylate group oxygen, N_{NH_2} = amino group nitrogen, N_{Him} = imidazole nitrogen.

VO(IV)–collagen in D_2O and VO(IV)–deuterated collagen samples show analogous EPR patterns and parameters. D_2O improves the spectral resolution, reducing the linewidth [6] and confirms the coordination of water molecules [13].

VO(IV)–DNP–collagen and VO(IV)–CH_3O–collagen present very similar EPR parameters for all the samples analyzed in the different physical states.

However, when carboxylate groups are blocked, the vanadyl coordination is probably addressed to N_{Him} and N_{NH2}, when amino groups are blocked, it is addressed to two N_{Him} (Table 1).

Because of the symmetry of the ground state, superhyperfine splitting is rarely observed with vanadyl complexes. Rather, nitrogen coordination in frozen solution shows up as a broadening of the line [14].

In the vanadyl complexes with axial symmetry, the ligand opposite to the vanadyl oxygen has a very small effect on the EPR parameters with respect to the equatorial ligands. Indeed vanadium is displaced 0.5 Å out of the equatorial plane and this results in the weak coordination of the sixth ligand. From our data, hoowever, it is not possible to draw an exact conclusion about this ligand. The sixth position could be occupied by one carboxylate group of the tropocollagen molecule or by one water molecule.

Conclusions

The oxovanadium(IV) at nearly physiological pH seems to interact with tropocollagen at room temperature, to give preferentially nitrogen monocoordinated complexes in aqueous solution, and chelate complexes of the VO(IV)–(N_2O_2) type when the protein is in a rigid matrix state.

The nitrogen and oxygen donor atoms bound to the metal-ion could be the N_{Him}(histidine) and/or the N_{NH2} (lysine or hydroxolysine) and the oxygens of the water molecules, respectively. Indeed, interaction with nitrogen atoms can occur, in spite of metal-ion nitrogen binding being longer and therefore less stable [6]. As for copper, one can thus say that, when vanadyl interacts with the imidazole nitrogen and/or the amino group, it can occupy one of the potential sites for cross-link formation in the mature collagen [1, 2].

Furthermore, it is evident that oxovanadium(IV) because of its steric hindrance can only bind the amino acid side chains at the end, or on the outside of the tropocollagen molecule.

In conclusion we suggest that the nature of the collagen and VO(IV) interaction, in addition to being intermediate between that of model compounds [12] and that of VO(IV)–globular proteins [6], confirms that the non-helical end regions are very important sites for the formation of intermolecular bonds.

Acknowledgements

The author is grateful to Professor Ivano Bertini for useful suggestions and constructive criticism. Effective help by Cristina Tonello during measurements is acknowledged. The work was supported by the Ministero Italiano della Pubblica Istruzione.

References

1 M. Marzona and R. P. Ferrari, *Inorg. Chim. Acta, 93* (1984) 1.
2 R. P. Ferrari and M. Marzona, *Inorg. Chim. Acta, 136* (1987) 123.
3 D. R. Eyre, M. A. Paz and P. M. Gallop, *Ann. Rev. Biochem., 53* (1984) 717.
4 T. Housley, M. L. Tanzer, E. Henson and P. M. Gallop, *Biochem. Biophys. Res. Commun., 67* (1975) 824.
5 H. Sakurai, S. Shimamura, K. Fukuzawa and K. Ishizu, *Biochem. Biophys. Res. Commun., 96* (1980) 293.
6 N. D. Chasteen, in L. J. Berliner and J. Reuben (eds.), *Biological Magnetic Resonance*, Vol. 3, Plenum, New York, 1981, p. 53.
7 I. D. Campbell and R. A. Dweek, in *Biological Spectroscopy*, Benjamin/Cummings, Menlo Park, CA, 1984, p. 193.
8 K. Kuhn, in H. Furthmayir (ed.), *Immunochemistry of the Extracellular Matrix*, Vol. 1, CRC Press, Boca Raton, FL, 1982, p. 1.
9 N. D. Chasteen and J. Francavilla, *J. Phys. Chem., 80* (1976) 867.
10 L. Casella, M. Gullotti, A. Pintor, S. Colonna and A. Manfredi, *Inorg. Chim. Acta, 144* (1988) 89.
11 N. D. Chasteen, R. J. Dekoch, B. L. Rogers and M. W. Hanna, *J. Am. Chem. Soc., 95* (1973) 1301.
12 N. F. Albanese and N. D. Chasteen, *J. Phys. Chem., 82* (1978) 2758.
13 N. D. Chasteen, L. K. White and R. F. Campbell, *Biochemistry, 16* (1977) 363.
14 R. J. Dekoch, D. J. West, J. C. Cannon and N. D. Chasteen, *Biochemistry, 13* (1974) 4347.