Volume 287, number 1,2, 10–14 FEBS 09955 August 1991

ADONIS 001457939100665L

Iron entry route in horse spleen apoferritin

Involvement of the three-fold channels as probed by selective reaction of cysteine-126 with the spin label 4-maleimido-tempo

A. Desideri[1], S. Stefanini[3], F. Polizio[2], R. Petruzzelli[2] and E. Chiancone[3]

[1] *Department of Organic and Biological Chemistry University of Messina, 98166 Messina, Italy,* [2] *Department of Biology, University of Rome 'Tor Vergata', 00173 Rome, Italy* and [3] *Department of Biochemical Sciences, CNR Center of Molecular Biology, University of Rome 'La Sapienza', 00185 Rome, Italy*

Received 25 April 1991; revised version received 27 May 1991

Apoferritin has been selectively labeled with a maleimide nitroxide derivative at Cys-126, located in the hydrophilic 3-fold channels. Titration of this derivative with Fe(II), which gives rise to the initial Fe(III)–apoferritin complex, produces, at low metal-to-protein ratios, a decrease of the intensity of the label EPR signal due to the occurrence of a magnetic dipolar interaction. A label-metal distance ranging between 8–12 Å can be estimated from titrations performed with VO(IV), which is known to bind in the 3-fold channels, and likewise produces a decrease in the label EPR signal. The present findings indicate that iron binds in the hydrophilic channels in its higher oxidation state and that these channels represent the metal entry route at least at low metal-to-protein ratios.

Apoferritin; Iron binding site; Spin label; EPR spectroscopy

1. INTRODUCTION

Ferritin is the iron storage protein utilized by most living cells to maintain iron in a form available for specific and crucial uses such as oxygen transport and activation, electron transfer, and nitrogen reduction. Despite intensive studies the molecular mechanism by which iron is reversibly acquired, stored and released by the protein has not been elucidated fully [1,2].

The ferritin molecule consists of a spherical protein shell of 432 symmetry produced by the assemblage of 24 subunits; it is characterized by the presence of 8 hydrophilic and 6 hydrophobic channels which lie around the 3-fold and the 4-fold axes of the protein molecule, respectively [3,4]. The hydrophilic channels have been proposed as possible avenues through which iron enters into the protein and the carboxylate residues of Asp-127 and Glu-130 as the sites at which the initial binding and oxidation of Fe(II) occurs yielding the initial Fe(III)–apoferritin complex. Thus, X-ray diffraction studies of horse spleen apoferritin have located two Cd(II) binding sites in each of the eight hydrophilic channels, the ligands being provided by the carboxylates of the Asp-127 and Glu-130 residues [3]. In line with this finding, chemical modification of carboxyl residues and the binding of Cd(II) inhibit iron incorporation [5,6]; in turn, the binding of Fe(II) competes with that of Cd(II) as indicated by ^{113}Cd NMR spectroscopy [7]. Other metal probes, like VO(IV) and Mn(II), which bind in the 3-fold channels [8,9] likewise compete with iron binding on the basis of EPR experiments. In addition, selective modification with *p*-chloromercuribenzoate (PMB) of Cys-126, located on the outer surface of the hydrophilic channels, has a specific effect on the iron uptake kinetics and on its spectroscopic properties [7]. However, in a recent work on human H-chain ferritins bearing sequence changes in the 3-fold channels, Treffry et al. [10] suggested that, although iron may enter the molecule through such channels, the metal-binding carboxylates are not required for iron oxidation and that this process may occur inside the molecule.

The present paper reports data obtained on horse spleen apoferritin reacted at Cys-126 with a maleimide nitroxide derivative, 4-maleimido-2,2,6,6-tetramethyl-piperidino-1-oxyl (Mal-6), and titrated with iron or VO(IV) at low metal-to-protein ratios. Under these conditions when Fe(II) is employed in air the initial Fe(III)–apoferritin complex is formed and is stabilized with respect to the polynuclear site, the likely precursor of the ferrihydrite core crystallite [11]. Formation of the Fe(III)–apoferritin complex produces a decrease in the EPR signal of the probe and thus provides evidence for the occurrence of a magnetic dipolar interaction between the maleimido spin label and the Fe(III). This finding implies that in horse spleen apoferritin iron

Correspondence address: E. Chiancone, Department of Biochemical Sciences, University of Rome 'La Sapienza', Piazza A. Moro, 00185 Rome, Italy. Fax: (39) (6) 4440062

Published by Elsevier Science Publishers B.V.

binds in the hydrophilic channels in its paramagnetic oxidized state before traversing the protein shell and entering into the storage cavity.

2. MATERIALS AND METHODS

Horse spleen ferritin was prepared as described previously [12]; apoferritin was obtained by reduction of iron with thioglycolic acid and chelation with α,α'-bipyridyl [8]. The quality of apoferritin preparations was checked by means of their CD spectra in the near UV region [13]; their iron content was determined as the α,α'-bipyridyl complex at 520 nm and was found to correspond to less than 0.2 iron atoms per subunit. The apoferritin concentration was calculated from the absorbance at 280 nm by using the extinction coefficient $E^{1\%}_{1\,cm} = 9.0$ [14] or by the Bradford method [15] and is expressed on the basis of the subunit molecular mass.

The spin label 4-maleimido-2,2,6,6-tetramethylpiperidino-1-oxyl-(Mal-6) was obtained from Aldrich Co. Spin labeled horse spleen apoferritin was obtained by incubating 3×10^{-4} M protein in 10 mM Tris-HCl buffer at pH 8 with 10 mM Mal-6 for 12 h at 4°C. The unreacted spin labeling reagent was removed by exhaustive dialysis first against 10 mM Tris-HCl buffer at pH 8.0 and thereafter against 20 mM MOPS buffer at pH 6.5. The specific targeting of Cys-126 was established by sequencing the chymotryptic peptide containing the spin label. The following procedure was used: spin labeled horse spleen apoferritin (3 mg) was dialyzed against 0.1 M ammonium bicarbonate (pH 8.0) in the presence of 1% SDS and digested with chymotrypsin (1:50 w/w) at 37°C for 30 min. The fragments were purified by HPLC using a large pore column (Aquapore RP 300, 4.6 × 250 mm, Brownlee Laboratories) developed in 60 min with a linear gradient of acetonitrile (from 0% to 60%) generated in a Beckman model 340 instrument at a flow rate of 1.0 ml/min. The absorbance of the effluent was monitored at 220 and at 250 nm. The fractions absorbing at both wavelengths were checked for the presence of the spin label EPR signal. The fraction showing the EPR signal was loaded onto an Applied Biosystem model 470 A gas phase sequencer equipped with an Applied Biosystem model 120A PTH-analyzer for the on-line detection of PTH-amino acids.

The sequence obtained corresponds to that of the peptide Asp-112–Phe-128 of horse spleen apoferritin [16] clearly indicating the specific labeling of Cys-126.

The extent of reaction with Mal-6 was estimated by double integration of the EPR signal before and after denaturation of the protein upon addition of concentrated NaOH. The resulting numerical value was compared to that obtained from a standard solution; it was found to correspond to 0.6–0.8 reacted groups per subunit. In line with this finding, upon titration of the spin-labeled sample with PMB 0.4–0.2 sulfhydryl groups per molecule were modified. This result indicates a direct competition between the two sulfhydryl reagents for the same binding site. The spin-labeled protein was found to incorporate iron fully and with the same kinetics of the control as verified by monitoring the change in absorbance at 420 nm [7].

The iron titrations with Fe(II) were performed at room temperature in air by addition of appropriate aliquots of a Fe(II) ammonium sulfate stock solution to the protein. The Fe(II) ammonium sulfate stock solution was prepared in water and was kept anaerobically under a nitrogen atmosphere; its concentration was determined by the bipyridyl method before and after reduction with dithionite in order to verify the oxidation state of the metal (the amount of Fe(III) was always below 5%). After addition of Fe(II) to the protein and before running the EPR spectra the solution was allowed to stand in air for 2–3 min, namely for the time required to obtain complete oxidation of the metal by the protein under the experimental conditions employed. The progress of the titration was measured by following the amplitude of the spin label signal.

The VO(IV) titrations were followed either at room or at liquid nitrogen temperature. Vanadyl solutions were prepared by dissolving anaerobically the appropriate amount of $VOSO_4$ in water. The titrations at liquid nitrogen temperature were performed in an EPR tube sealed to a Thunberg-type apparatus. VO(IV) solutions de-aerated by three cycles of vacuum-flushing with purified argon, were added to a de-aerated protein solution through serological caps by means of a microsyringe. For the room temperature experiments the de-aerated solutions were transferred to a quartz capillary flushed with N_2. The progress of the titration was measured by following the amplitude of the spin label signal. The EPR signal of the label was not distorted by that of the vanadyl due to the very low metal concentration and the low modulation amplitude and microwave power used. Titration of the unlabeled apoferritin with VO(IV) gave a stoichiometry of 0.3 in line with the results of Wardeska et al. [9].

X-band EPR spectra were recorded at room and liquid nitrogen temperature with a Bruker ESP 300 spectrometer; normally eight scans were accumulated.

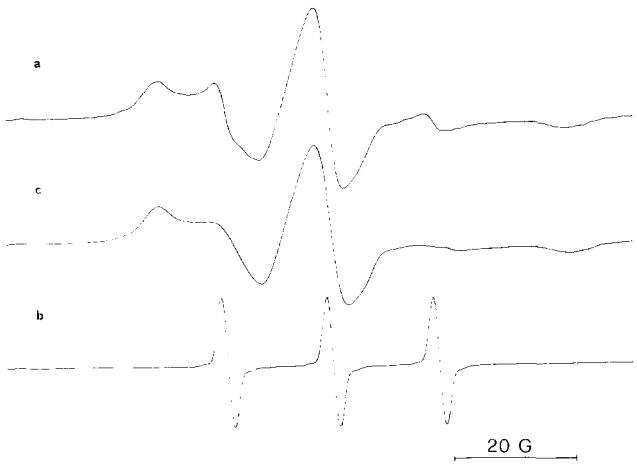

Fig. 1. EPR spectra of: (a) free maleimido tempo (Mal-6) spin-labelled apoferritin; (b) free Mal-6; (c) a-0.005b. EPR conditions: power 5 mW, modulation amplitude 1.5 G, frequency 9.8 GHz, temperature 25°C. Buffer: 20 mM MOPS, pH 6.5. Concentration: (a) 1.5×10^{-4} M; (b) 3×10^{-5} M.

3. RESULTS

3.1. *Addition of Fe(II) and formation of the Fe(III)-spin-labeled apoferritin complex*

Fig. 1 shows the EPR spectrum of apoferritin specifically labeled at Cys-126 with Mal-6. The spectrum is characterized by two components, a strongly immobilized one ($2T_{11} = 65$ gauss), which accounts for a great part of the signal, and a second one, characteristic of the 'free' label, whose amount is always lower than 5% of the total signal, and which probably arises from protein denaturation.

The spin-labeled apoferritin was titrated with Fe(II) and spectra were taken as a function of the time elapsed after addition of the metal. At any given iron-to-subunit ratio the final signal intensity is reached within 2–3 min when all the iron is present as the Fe(III)–apoferritin complex. Fig. 2 brings out that upon increase in the iron to subunit ratio the intensity of the EPR signal of the label decreases without changes in line-shape. A plot of the EPR intensity against the Fe-to-subunit ratio is reported in Fig. 3. In another set of experiments, the diamagnetic Cd(II) ion (at 1

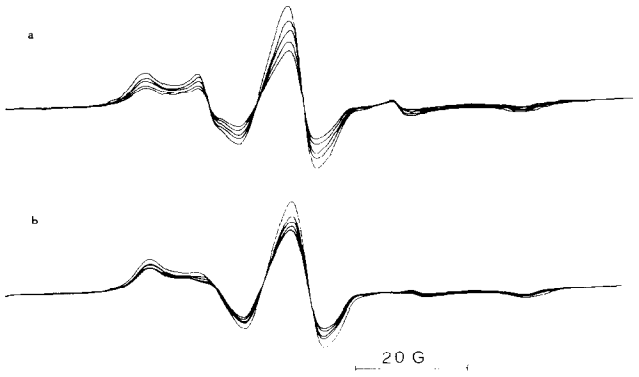

Fig. 2. EPR spectra of Mal-6 spin-labeled apoferritin as a function of iron added as Fe(II) in air and forming the Fe(III)–apoferritin complex. (a) Spectra with decreasing amplitude corresponding to the following iron/subunit ratios: 0, 0.07, 0.33, 0.66, 1.33. (b) Same as (a) but after subtraction of the free label component. Conditions as in Fig. 1.

equivalent/subunit) or H_2O_2 (at 3 equivalents/subunit) were added to spin-labeled apoferritin containing 0.2 Fe(III)/subunit; in neither case was the original EPR signal restored. The H_2O_2 experiment excluded that the quenching of Mal-6 is caused by reduction of the nitroxide moiety with ferrous ion, whereas the Cd(II) experiment pointed to a much lower affinity for apoferritin of Cd(II) with respect to Fe(III). The use of higher Cd(II) concentrations, however, was precluded by the low solubility of the metal.

3.2. *Addition of VO(IV) to spin-labeled apoferritin*

The room temperature titration of spin-labeled apoferritin with VO(IV) is reported in Fig. 4 which shows the relative intensity of the label EPR spectrum as a function of the VO(IV)/subunit ratio. Addition of further VO(IV) did not affect the EPR intensity of the label in line with the results of Wardeska et al. [9] which show that unbound VO(IV) (in an excess of 0.6 VO(IV)/subunit) is EPR silent. Titrations were followed also at liquid nitrogen temperature because a strict anaerobiosis could be achieved better. The same decrease in the intensity of the label EPR signal was observed at both temperatures. Addition of a 3-fold excess of diamagnetic Cd(II) at the end of the titration restores the original EPR label signal almost completely, thus ensuring that the observed decrease cannot be ascribed to chemical reduction of nitroxide.

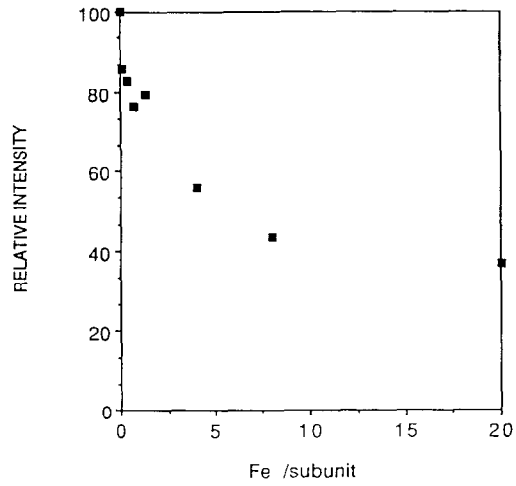

Fig. 3. Relative intensity of the EPR spectra of Mal-6 spin-labeled apoferritin as a function of iron added as Fe(II) in air and forming the Fe(III)–apoferritin complex. Protein concentration 1.5×10^{-4} M; buffer: 20 mM MOPS, pH 6.5. The intensities were evaluated by double integration of either the experimental spectra or of the immobilized label signal after subtraction of the free label component. The relative intensities obtained by the two methods are identical due to the low and essentially constant contribution of the free label component.

4. DISCUSSION

The present data provide direct evidence for the binding and oxidation of Fe(II) at the hydrophilic channels of the apoferritin molecule. The observed decrease of the Mal-6 signal intensity (Figs. 2 and 3) as a function of the concentration of Fe(III) oxidized in the channels to yield the initial Fe(III)–apoferritin complex points to a strong dipolar interaction between the two spins which hence must be located in close proximity. In fact it can be excluded that the quenching of the Mal-6 intensity is due to reduction by ferrous ion since addition of H_2O_2, which is known to oxidize reduced spin labels [17], does not perturb at all the EPR signal.

The theory developed by Leigh [18] for two interacting spins can be applied when the rotational correlation time of the macromolecule is long but the correlation time, characterizing the dipolar interaction bet-

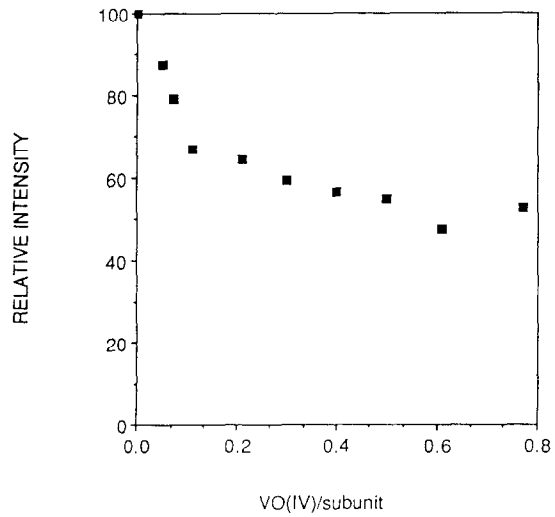

Fig. 4. Relative intensity of the EPR spectra of Mal-6 spin labeled apoferritin as a function of added VO(IV). Protein concentration 1.5×10^{-4} M; buffer: 20 mM MOPS, pH 6.5. In this sample the extent of reaction with Mal-6 corresponded to 0.65 SH per subunit. For the evaluation of the intensities see Fig. 3.

ween the two spins, is short. In this case the EPR line-width of the label is

$$\partial H = C(1 - 3\cos^2\Theta)^2 + \partial H_0$$

where ∂H_0 is the natural EPR line-width of the label in the absence of the paramagnetic ion, Θ is the angle between the applied magnetic field and the vector joining the two spins and

$$C = g\beta\mu^2\tau/hr^6$$

where r is the distance between the two spins, μ is the effective magnetic moment of the paramagnetic atom, g is $1/3(2g_1 + g_{11})$ and τ is the correlation time for the modulation of the dipolar interaction. For a system in a rigid geometry, τ is dominated by the electron spin relaxation time T_1 of the spin which creates the relaxing field. After evaluation of the coefficient C from the relative amplitude of the label signal [18] the distance r can be calculated. Leigh's theory can be used confidently only when a single paramagnetic metal atom interacts with a single label. In apoferritin the analysis is complicated by the presence of 3 cysteines (Cys-126) at the opening of the 3-fold channels. Thus only a semiquantitative estimate of the value r can be obtained.

In the case of the titration with Fe(II), with subsequent formation of the initial Fe(III)–apoferritin complex, there is an additional complication which prevents the evaluation of the spin–spin distance due to the presence in the hydrophilic channels of at least three different Fe(III) species, with different magnetic characteristics at low iron/subunit ratios [19]. As a result a relatively large number of iron atoms is bound to the protein and in the titration of spin-labeled apoferritin with iron the EPR signal reaches a constant value at a Fe/subunit ratio of 10 (Fig. 3).

The VO(IV) ion has been shown to occupy the same binding site of iron by means of competition experiments [9]; moreover, an exact stoichiometry of binding has been established and no evidence for a vanadium–vanadium interaction (i.e. cluster formation) has been detected [8,9]. In the experiments of Fig. 4, a linear decrease of the label signal is observed up to approximately VO(IV) to subunit ratios of 0.2, the intensity being fairly constant upon further additions. Hence the different endpoints of the titrations given in Figs. 3 and 4 are accounted for by the different behaviour of VO(IV) and Fe(III) with respect to cluster formation.

The difference in the binding stoichiometry of VO(IV) for the unlabeled and the labeled apoferritin can be attributed to the incomplete labeling of the protein (70 ± 5%). The observed decrease of the spin label EPR signal at such low VO(IV)/subunit ratios is undoubtedly due to a dipolar interaction between the label and a mononuclear VO(IV) atom diagnostic of a close proximity between the two centers. In fact, a decrease due to a redox reaction can be excluded by the reappearance of the nitroxide signal upon displacement of the paramagnetic atom by the diamagnetic Cd(II). The metal–label distance can be estimated from the signal decrease, using Leigh's theory, as the magnetic properties of the mononuclear VO(IV) atom are known [8]. By calculating C from the signal decrease at the VO(IV)/subunit ratio corresponding to 0.2 and by letting the relaxation time of the vanadyl ion fluctuate between 10^{-9} s and 10^{-8} s [20], a spin–spin distance ranging from 8 to 12 Å is obtained. These values are in good agreement with the distances of 6.54 Å and 10.8 Å found by X-ray diffraction between the 3-fold axis related Hg attached to Cys-126 and the Cd ions bound to the three Glu-130 residues and to the Asp-127 site, respectively (Harrison, private communication). Although these numbers have to be considered with caution, the observed decrease in the EPR signal of Mal-6 is in line with the presence of the Fe(II) oxidation sites in the hydrophilic channels.

In conclusion, labeling of apoferritin with the 4-maleimido tempo spin probe has provided further evidence that the 3-fold channels are involved in the process of metal entry into the protein shell and that Fe(II) is oxidized by the protein and is bound in the 3-fold channels in its higher oxidation state before traversing the shell to reach the storage cavity.

Acknowledgements: We wish to acknowledge the help of Mr. Stefano Cavallo in the preparation of the protein. This work was partly supported by CNR Grant 89.01057.02.

REFERENCES

[1] Harrison, P.M. and Lilley, T.H. (1989) in: Iron Carriers and Iron Proteins (Loehr, T.M. ed.) pp. 133–238, VCH Verlagsgesellschaft Weinheim.
[2] Crichton, R.R. and Charloteaux-Wauters, M. (1987) Eur. J. Biochem. 164, 485–506.
[3] Rice, D.W., Ford, G.L., White, J.L., Smith, J.M.A. and Harrison, P.M. (1983) Adv. Inorg. Biochem. 5, 39–50.
[4] Harrison, P.M., Ford, G.G., Rice, R.W., Smith, J.M.A., Treffry, A. and White, J.L. (1986) in: Frontiers in Bioinorganic Chemistry (Xavier, A.V. ed.) pp. 268–277, VCH Verlagsgesellschaft, Weinheim.
[5] Niederer, W. (1970) Experientia 26, 218–220.
[6] Macara, I.G., Hoy, T.G. and Harrison, P.M. (1973) Biochem. J. 135, 785–789.
[7] Stefanini, S., Desideri, A., Vecchini, P., Drakenberg, T. and Chiancone, E. (1989) Biochemistry 28, 378–382.
[8] Chasteen, N.D. and Theil, E.C. (1982) J. Biol. Chem. 257, 7672–7677.
[9] Wardeska, J.G., Viglione, B. and Chasteen, N.D. (1986) J. Biol. Chem. 261, 6677–6683.
[10] Treffry, A., Harrison, P.M., Luzzago, A. and Cesareni, G. (1989) FEBS Lett. 247, 268–272.
[11] Yang, C.Y., Meagher, A., Huynh, B.H., Dale Sayers, E. and Theil, E.C. (1987) Biochemistry 26, 497–503.
[12] Stefanini, S., Chiancone, E., Arosio, P., Finazzi Agró, A. and Antonini, E. (1982) Biochemistry 21, 2293–2299.
[13] Leach, B.S., May, M.E. and Fish, W. (1976) J. Biol. Chem. 251, 3856–3861.
[14] Bryce, C.F.A. and Crichton, R.R. (1973) Biochem. J. 133, 301–309.

[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Heusterspreute, M. and Crichton, R.R. (1981) FEBS Lett. 129, 322–327.
[17] Gaffney, B.J. (1976) in: Spin Labeling, vol. I (Berliner, L.J. ed.) Academic Press, New York.
[18] Leigh, J.S. (1970) J. Chem. Phys. 52, 2608–2612.
[19] Bauminger, E.R., Harrison, P.M., Nowik, I. and Treffry, A. (1989) Biochemistry 28, 5486–5493.
[20] Bertini, I. and Luchinat, C. (1986) NMR of Paramagnetic Molecules in Biological System, Benjamin Cummings Press.