Inorganica Chimica Acta, *106* (1985) 193–196

Absorption of X-Radiation by Single Crystals of Proteins Containing Labile Metal Components: the Determination of the Number of Iron Atoms within the Central Core of Ferritin

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Received October 19, 1984

Abstract

The number of metal atoms contained within a displaceable inorganic component of a metalloprotein was determined by considering X-ray absorption by single crystal samples of holo- and apo-proteins. Since this method is non-destructive, it can be used to determine the number of metal atoms associated with the molecules forming the crystal actually used for X-ray diffraction data collection and subsequent structure solution. The method has been applied to the iron storage protein ferritin, isolated from horse spleen, to give a reliable estimate of the average iron content of the ferritin molecules within the crystal. This value, of around 2000 iron atoms per molecule is consistent with that found for a typical ferritin preparation in solution and suggests non-selectivity of the crystallisation process for ferritin in terms of molecular iron content.

Introduction

The iron storage protein, ferritin, has a thin, roughly spherical protein shell which surrounds a central inorganic iron core [1]. In a typical sample, the size of the individual iron core varies [2], ranging from natural iron-free and low iron fractions to high iron ferritins containing up to 4500 iron atoms [3]. The structure of the iron core complex is not well determined although its composition is that of hydrated ferric oxide-phosphate [4], $(FeOOH)_8$-$(FeOOPO_3H_2)$, and evidence suggests that it is closely related to the mineral ferrihydrite [5]. The Fe(III) iron core can be removed from ferritin by reduction and dialysis to give the iron free protein, apoferritin [6]. Isolated from horse spleen, ferritin and apoferritin form isomorphous octahedral crystals from cadmium sulphate solutions [6, 7]. The structure of horse spleen apoferritin has been solved at 2.8 Å resolution [8] and the amino acid sequence determined [9].

It is possible to isolate different iron content fractions from a single ferritin preparation by density gradient centrifugation [10] and to determine the average molecular iron content by spectroscopic methods [11]. For crystalline samples, the measurement of the attenuation of a collimated X-ray beam by a single crystal contained within a sealed glass capillary provides a non-destructive method by which the average molecular iron content actually within that crystal can be determined.

Theory

It is assumed that the attenuation of a collimated X-ray beam of intensity I_0 on passing through a sample of thickness, t, is given by:

$$I = I_0 \exp(-\mu t) \tag{1}$$

where I is the attenuated beam intensity and μ the total linear absorption coefficient of the attenuator. A necessary requirement for this equation to hold is that the sample is larger than the total beam cross section [12]. In the experimental set up described below, the total attenuation of the beam by the crystal sample arises not only from absorption by the crystal, but has components from air absorption and from absorption by the glass capillary. These are eliminated by subtracting a control measurement taken with the crystal just removed from the X-ray beam:

$$\ln\left(\frac{I_0}{I}\right)_{\text{crystal}} - \ln\left(\frac{I_0}{I}\right)_{\text{control}} = \mu_{\text{crystal}} t_{\text{crystal}} \tag{2}$$

Since the thickness of the crystal, t_{crystal}, can be measured, the linear absorption coefficient, μ_{crystal},

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0020-1693/85/$3.30

can be determined. This may also be expressed in terms of a summation of the products of the mass fractions, g_i, corresponding to atomic elements i contained within the crystal having mass absorption coefficients $(\mu/\rho)_i$:

$$\mu_{crystal} = \rho_{crystal} \sum_i g_i \left(\frac{\mu}{\rho}\right)_i \quad (3)$$

where $\rho_{crystal}$ is the density of the crystal.

Since apoferritin and ferritin can be regarded as two and three phase systems respectively, eqn. 3 may be written:

$$\mu_{apo} = \rho_{apo}\left[g_{apo}\left(\frac{\mu}{\rho}\right)_{apo} + g_{sol}\left(\frac{\mu}{\rho}\right)_{sol}\right] \quad (4)$$

for apoferritin, and:

$$\mu_{fer} = \rho_{fer}\left[g'_{apo}\left(\frac{\mu}{\rho}\right)_{apo} + g'_{sol}\left(\frac{\mu}{\rho}\right)_{sol} + g'_{fe}\left(\frac{\mu}{\rho}\right)_{fe}\right] \quad (5)$$

for ferritin. The linear absorption coefficients for apoferritin, μ_{apo}, and ferritin, μ_{fer}, are considered to have components arising from the protein shell, apo, the associated solvent, sol, and in the latter case, the iron core complex, fe. The mass fractions, g_i, may be re-written in terms of the mass of each component, m_i, relative to the total mass, m_{tot}. Equations 4 and 5 then become:

$$\mu_{apo} = \rho_{apo}\left[\left(1 - \frac{m_{sol}}{m_{tot}}\right)\left(\frac{\mu}{\rho}\right)_{apo} + \frac{m_{sol}}{m_{tot}}\left(\frac{\mu}{\rho}\right)_{sol}\right] \quad (6)$$

and:

$$\mu_{fer} = \rho_{fer}\left[\left(1 - \frac{m'_{fe}}{m'_{tot}} - \frac{m'_{sol}}{m'_{tot}}\right)\left(\frac{\mu}{\rho}\right)_{apo} + \frac{m'_{sol}}{m'_{tot}}\left(\frac{\mu}{\rho}\right)_{sol} + \frac{m'_{fe}}{m'_{tot}}\left(\frac{\mu}{\rho}\right)_{fe}\right] \quad (7)$$

Since the protein component is identical in ferritin and apoferritin, its absorption will be the same in each case and $\mu_{fer} - \mu_{apo}$ will, therefore, give the absorption due to the iron core and solvent displaced only. Equating the terms in eqns. 6 and 7 associated with the protein component:

$$\rho_{fer}\left(1 - \frac{m'_{fe}}{m'_{tot}} - \frac{m'_{sol}}{m'_{tot}}\right)\left(\frac{\mu}{\rho}\right)_{apo} = \rho_{apo}\left(1 - \frac{m_{sol}}{m_{tot}}\right)\left(\frac{\mu}{\rho}\right)_{apo} \quad (8)$$

allows the expression of the mass of solvent associated with the ferritin molecule, m'_{sol}, in terms of known quantities.

$$m'_{sol} = m'_{tot}\left[1 - \frac{m'_{fe}}{m'_{tot}} - \frac{\rho_{apo}}{\rho_{fer}}\left(1 - \frac{m_{sol}}{m_{tot}}\right)\right] \quad (9)$$

Combination of eqns. 6 and 7 with the equality given in eqn. 8 gives:

$$\mu_{fer} - \mu_{apo} = \rho_{fer}\left[\frac{m'_{sol}}{m'_{tot}}\left(\frac{\mu}{\rho}\right)_{sol} + \frac{m'_{fe}}{m'_{tot}}\left(\frac{\mu}{\rho}\right)_{fe}\right] - \rho_{apo}\left[\frac{m_{sol}}{m_{tot}}\left(\frac{\mu}{\rho}\right)_{sol}\right] \quad (10)$$

Eqn. 10 contains the desired mass fraction of the iron core complex in ferritin, m'_{fe}/m'_{tot}, and substituting for m'_{sol} from eqn. 9 gives:

$$\frac{m'_{fe}}{m'_{tot}} = \frac{\mu_{fer} - \mu_{apo} - \left[\left(\frac{\mu}{\rho}\right)_{sol}(\rho_{fer} - \rho_{apo})\right]}{\rho_{fer}\left[\left(\frac{\mu}{\rho}\right)_{fe} - \left(\frac{\mu}{\rho}\right)_{sol}\right]} \quad (11)$$

which expresses the mass fraction in terms of known or measured quantities.

Experimental

Large crystals of horse spleen ferritin and apoferritin were supplied from a common preparation by Professor P. M. Harrison and co-workers. No attempt was made to isolate any particular ferritin iron fraction prior to crystallisation.

The attenuation measurements were made on ferritin and apoferritin crystals mounted in sealed glass capillaries on an Arndt-Wonacott oscillation camera at station PX7.2 at the Daresbury Synchrotron Radiation Source (SRS) [13] with a sensitive ionisation chamber [14] mounted in place of the film cassette and a small detector to monitor fluctuations in incident beam intensity.

The square pyramidal (half octahedral) morphology of the crystals enabled an orientation in which the collimated X-ray beam and pyramidal crystal axis were coincident for the attenuation measurements. In this orientation, the effective thickness of a square pyramidal crystal, t_{eff}, representing the mean path through the crystal traversed by the X-ray beam, is given by:

$$t_{eff} = p\left[1 - \frac{8d}{(3\sqrt{2})\pi a}\right] \quad (12)$$

where d is the diameter of the collimator, p the perpendicular height and a the basal edge of the crystal.

The attenuation measurement data are summarized in Table I.

TABLE I. Experimental Attenuation Measurement Data.

	t_{eff} (mm)	$\ln\left(\frac{I_0}{I}\right)_{crystal}$	$\ln\left(\frac{I_0}{I}\right)_{control}$	μt	μ (mm^{-1})
Apoferritin	0.385	−1.7627	−2.0978	0.335	0.8704
Ferritin	0.156	−1.5034	−2.2303	0.727	4.6596

TABLE II. Mass Absorption Coefficients for the Protein, Solvent and Iron Core Complex Components.

	Mass absorption coefficient at λ = 1.488 Å $(\mu/\rho)_i$ [15] (cm^2 g^{-1})	Mass fractions of elemental composition		
		Protein component apo	Solvent component sol	Iron core component fe
C	3.5	0.532		
N	6.0	0.173		
O	9.5	0.216	0.889	0.382
P	66.2			0.035
S	82.2	0.010		
Fe	300.1			0.571
$(\mu/\rho)_x$ (cm^2 g^{-1})		5.75	8.44	177.45

SRS Beam Parameters

2 GeV, 93.4 mA, wavelength, λ = 1.488 Å (calibrated with a pure nickel foil), collimator diameter = 0.15 mm.

Crystal Parameters [7, 8]

Apoferritin (Horse Spleen)

F432, a = 184.8 Å, $V = 6.33 \times 10^6$ Å^3, Z = 4, ρ = 1.134 g cm^{-3} (by flotation), MW = 476616 (from amino acid sequence [9]).

Ferritin

Isomorphous with apoferritin, ρ = 1.302 g cm^{-3}, iron core complex stoichiometry $Fe_9P_1O_{21}H_{10}$ [4], MW = 879.62.

Results

The application of eqn. 2 to the attenuation measurements enables the determination of the respective linear absorption coefficients, as summarized in Table I.

$\mu_{fer} = 46.596$ cm^{-1}

$\mu_{apo} = 8.704$ cm^{-1}

The total mass associated with the ferritin molecule, m'_{tot}, is given by:

$$m'_{tot} = \frac{\rho_{fer} N V}{Z} \tag{13}$$

where ρ_{fer} is the density of the ferritin crystal, N Avogadro's number, V the unit cell volume and Z the number of molecules in the unit cell. Similarly for apoferritin.

The mass absorption coefficients for each of the three components, $(\mu/\rho)_x$ are calculated from the summation over the i constituent atomic elements and are listed in Table II.

$$\left(\frac{\mu}{\rho}\right)_x = \sum_i g_i \left(\frac{\mu}{\rho}\right)_i \tag{14}$$

The individual mass absorption coefficients are dependent upon the wavelength and providing the wavelength used is distant from an absorption edge, their values can be well-determined from the Cromer approximation [15].

Substitution of all these quantities into eqn. 11 gives the mass fraction of the iron core complex.

$$g'_{fe} = \frac{m'_{fe}}{m'_{tot}} = 0.166$$

The total number of iron atoms, N_{Fe}, can be calculated from this mass fraction by:

$$N_{Fe} = g'_{fe} \times m'_{tot} \times \left(\frac{Z_{comp}}{M_{comp}}\right) \tag{15}$$

where Z_{comp} = 9, the number of iron atoms in the iron core complex of overall molecular weight,

M_{comp}. Substitution into eqn. 15 of the calculated values gives:

N_{Fe} = 2106 iron atoms.

The errors associated with the determination of N_{Fe} arise from experimental errors in the measurements and from approximations in the calculations. In eqn. 11 the largest errors occur in the determination of the linear absorption coefficients from the measured thickness and observed absorption. These values are probably determined to within 3%. Errors arising from the approximation of $(\mu/\rho)_{sol}$ are relatively unimportant since this term is only a small modification of the expression. An error of around 7% in the determination of N_{Fe} would therefore be predicted.

N_{Fe} = 2100 ± 150 iron atoms

Discussion

The average molecular iron content within the crystal of 2100 ± 150 iron atoms calculated by the method described here is comparable with spectrophotometric determination [16] of around 2000 iron atoms per molecule in solution for a typical ferritin preparation. This value represents a statistically half-full molecule as would be predicted for a crystallisation that does not differentiate between iron content of molecules. Indeed, such a differentiation would not be expected as the iron component is contained within the protein shell and has little effect on the regions of intermolecular contact in the crystal [17], this is corroborated by co-crystallisation of ferritin and apoferritin [18].

The method referred to here has allowed a reliable *in situ* estimate of the average number of iron atoms to be made on the crystal actually used to study the molecular structure of ferritin [19], and the interactions of the protein with the iron core complex.

Acknowledgements

We thank the SERC, Daresbury Laboratory and the University of Keele for support and Professor P. M. Harrison and co-workers for the provision of horse spleen ferritin and apoferritin single crystal samples.

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