IRON BINDING TO APOFERRITIN: A FLUORESCENCE SPECTROSCOPY STUDY

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1. Introduction

Iron is stored in animal tissues in the ferric state as the micellar core of ferritin; however it appears that a pre-requisite for both iron uptake and mobilization is its reduction to the ferrous state [1]. The currently accepted models of iron incorporation into apoferritin assume binding as Fe^{2+} and a subsequent oxidation catalysed by the protein [2]. While the catalytic action of apoferritin is well documented, no information is available regarding the metal binding step. Fluorescence spectroscopy seemed a very useful tool to investigate this aspect of the problem. In fact binding of the ferrous salt to the protein can be inferred from the quenching of the intrinsic fluorescence of apoferritin. During the course of the study information was also obtained on the structure of apoferritin and its isolated subunits with respect to the nature of the environment of the fluorescent residues.

2. Materials and methods

Horse spleen ferritin was obtained from OTI (Parma) Italy, or from Koch-Light Laboratories Ltd. (England). Apoferritin was prepared from ferritin by chemical reduction with $Na_2S_2O_4$ [3]. Apoferritin subunits were obtained with different procedures: by treatment of the polymer with 67% acetic acid in the cold followed by dialysis in dilute glycine buffer at pH 3.0 or by exposure to a KCl–HCl buffer of pH 1.6. [4]. The subunits prepared in this way were reassociated by dialysis in glycine–acetic acid buffer at pH 4.5. Alternatively the subunits were prepared by treatment of apoferritin with 6 M guanidine–HCl at pH 4.5, in this case reassociation was obtained after dialysis at pH 7.0 [5]. The state of association was analyzed in sedimentation velocity experiments carried out in a Spinco Model E analytical ultracentrifuge at 42040 rpm and at 10–12°C. The sedimentation coefficients were corrected to $S_{20,w}$ according to standard procedures. Protein concentrations were calculated from the absorption at 280 nm using the extinction coefficient $E^{1\,cm}_{1\%} = 9.0$ and from the areas in the ultracentrifuge experiments. Micellar iron formation was followed at 480 nm where the extinction coefficient is $E^{1\,cm}_{1\%} = 47$. The formation of a Fe^{2+} – α-α' dipyridyl complex was measured at 520 nm according to the procedure of Bothwell et al. [6].

Absorption spectra were taken with a Cary 14 or a Beckman DB-GT spectrophotometer. Fluorescence was measured with a FICA 55L spectrofluorimeter which corrects the spectra for inhomogeneities of the source and for the variable sensitivity of the photomultiplier.

The anaerobic absorption and fluorescence titrations with Fe^{2+} were performed in Thunberg-type fluorimeter cuvettes equipped with an air tight rubber cap and filled with prepurified oxygen-free argon. After

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recording the starting spectrum aliquots of oxygen-free ferrous ammonium sulphate solutions were added anaerobically with an air tight syringe. At the end of the titration air was admitted in the cuvette and the fluorescence intensity measured again. The results are plotted in terms of $(I_0\text{-}I)/I_0$ where I_0 is the initial fluorescence intensity and I is the intensity at any given stage of the titration.

3. Results

3.1. *Intrinsic fluorescence of apoferritin*

Ferritin, which contains an average of 2700 iron atoms/450 000, is completely non fluorescent due to the strong broad absorption of the metal below 500 nm. Removal of iron from the protein leads to the appearance of the intrinsic fluorescence (fig.1a). The maximum of emission is at 315 nm, a rather unusual wavelength for a tryptophan containing protein; furthermore the emission is only slightly affected by the presence of KI up to 0.5 M or 1 M CsCl. In contrast apoferritin subunits ($s_{20,w} \sim 4$ S) have a maximum of emission at the more common wavelength of 328 nm (fig.1b). The low wavelength of emission in the polymer could result from both tyrosine and tryptophans since a significant cut off in the spectrum is observed upon excitation at 295 nm where tyrosine absorption is negligible. The contribution of tyrosine is more evident in denatured apoferritin prepared by the combined action of 6 M guanidine hydrochloride and HCl (pH < 2). The spectrum in fig.2a was obtained by excitation at 280 nm, where both tyrosine and tryptophan absorb; that in fig.2b by excitation at 295 nm where only tryptophan is excited. In the latter spectrum the secondary peak at lower wavelength completely disappears, while that with a maximum at 355 nm is unchanged. Lowering the guanidine concentration from 6 to 1.5 M results in a shift of the maximum emission towards that of the native form.

Two preparations of reconstituted apoferritin, which showed 50% of polymeric material in the ultracentrifuge, were analysed; the fluorescence spectra were intermediate between those of the subunits and apoferritin.

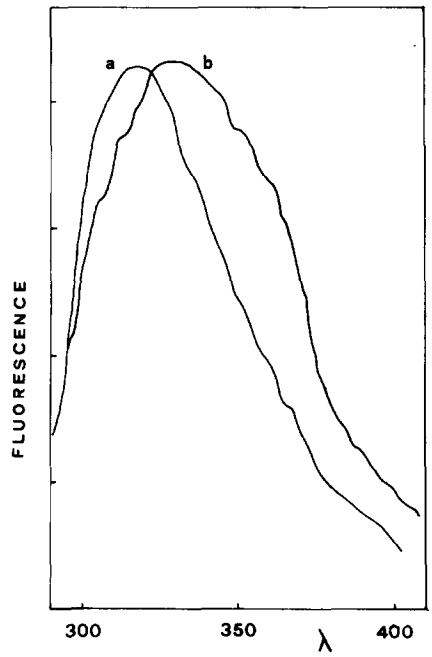

Fig.1. Fluorescence emission spectra of apoferritin (a) and apoferritin subunits (b). Excitation at 280 nm. Protence concentration: 3.78×10^{-7} M. Buffer: phosphate of I = 0.1 M at pH 7 (a); glycine–HCl of I = 0.05 M at pH 3 (b).

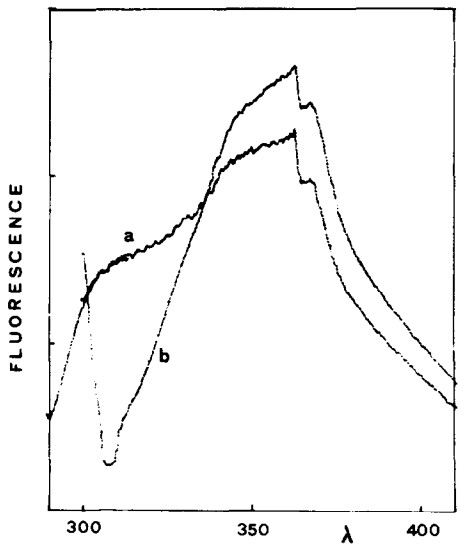

Fig.2. Fluorescence spectra of denatured apoferritin. Excitation at 280 nm (a) and at 295 nm (b). The abrupt change in fluorescence at 365 nm is due to a second order filter. Protein concentration: 4.45×10^{-7} M.

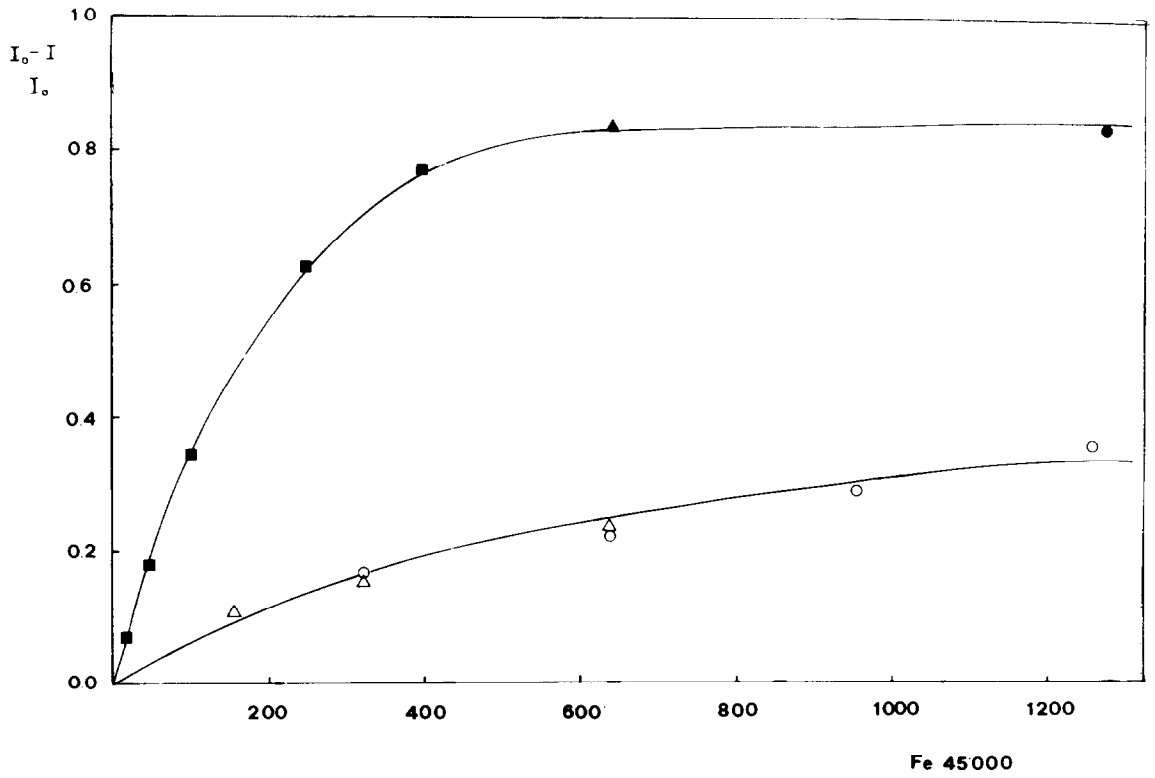

Fig.3. Fluorimetric titrations of apoferritin with ferrous ammonium sulphate in aerobiosis and anaerobiosis. Full symbols were obtained in the presence of air; different symbols refer to different titrations. Buffer: 200 mM $NaHCO_3$ at pH 8.2. Protein concentrations: 4.7×10^{-7} M.

3.2. *Titration of apoferritin with ferrous ammonium sulphate*

The aerobic addition of ferrous ions to apoferritin in bicarbonate buffer at pH 8.2 causes an immediate fluorescence quenching which is ~ 80% at a metal to protein ratio around 400 Fe atoms/450 000. A typical experiment is shown in fig.3a. In parallel spectrophotometric titrations there is the immediate appearance of a species absorbing at 480 nm, a wavelength characteristic of micellar ferric iron. Control experiments with $FeCl_3$ gave very little quenching entirely attributable to trivial inner filter effects; thus binding of iron is necessary in order to obtain considerable quenching.

Fluorimetric titrations with ferrous ammonium sulphate at the same pH values, but performed in anaerobiosis, give a much smaller fluorescence quenching at comparable Fe/protein ratios (fig.3b). Thus at a Fe/protein ratio of 640 atoms/450 000 only 25% quenching is obtained; upon opening the solution to air fluorescence is immediately quenched to 80%. In the spectrophotometric counterpart, addition of the ferrous salt gives a very small increase in optical density at 480 nm unless air is admitted; upon admission of air the increase in optical density occurs with a half time of the order of 30 sec. Thus under these experimental conditions the fast formation of micellar iron is in accord with the almost instantaneous quenching of fluorescence. In order to assign unequivocally the quenching of fluorescence obtained under anaerobic conditions to the binding of fluorescence obtained under anaerobic conditions to the binding of Fe^{2+} to apoferritin a few controls were performed. In a series of anaerobic and aerobic titrations the oxidation state of the iron was determined by measuring the amount of metal

capable of forming a complex with α-α' dipyridyl at the end of the experiment. In the anaerobic titrations all the iron added formed a complex and thus had remained in the form of Fe^{2+}; in contrast in the aerobic titrations only 15–18% of the iron added was in the form of Fe^{2+}. In another control experiment known amounts of oxygen were injected in the cuvette containing apoferritin and Fe^{2+} under anaerobiosis. The quenching obtained was proportional to the amount of iron added.

Anaerobic titrations with ferrous ammonium sulphate were also performed with apoferritin subunits. However in view of the reassociation occurring above pH 5, these experiments were carried out at pH 4.5. Only a slight degree of quenching was observed which did not increase significantly after admission of air. Parallel experiments with apoferritin gave essentially the same results; that the iron was not oxidized by the presence of the protein was shown by the fact that about 95% of the iron present formed a complex with α-α' dipyridyl. It should be recalled that autoxidation is an extremely slow process at these low pH values [7].

EPR experiments were performed under anaerobiosis at pH 8.2, but at higher concentrations; they did not show any detectable absorption. After introduction of air a very small signal of Fe^{3+} at $g = 4.3$ was observed which accounted for a few percent of the total iron present. This last finding is not surprising since in native ferritin Fe^{3+} is EPR undetectable due to strong magnetic coupling between ferric ions [8].

4. Discussion

The differences in the intrinsic fluorescence of apoferritin and its isolated subunits show peculiar features related to differences in the environment of the fluorescent species. It is well known that tryptophan residues which are primarily responsible for the intrinsic fluorescence in proteins are very sensitive probes of the dielectric constant of the environment [9]. A very accurate study of Burstein et al. [10] allows to classify all the emitting tryptophan residues inside the proteins into three classes on the basis of their emission maximum. The greater the hydrophobicity of their environment, the lower the wavelength of tryptophan emission down to 328 nm which corresponds to the fluorescence of *N*-acetyltryptophanamide in apolar solvents. Thus while the emission of apoferritin monomer is consistent with the scheme proposed by Burstein [10], the behaviour of the polymer requires a different rationale. A similar situation has been encountered only in two other proteins, namely Pseudomonas fluorescens azurin [11] and staphylococcal nuclease [9], where immobilization of the aromatic side chain besides hydrophobicity has been invoked to explain the blue shifted emissions (308 nm and 320 nm respectively). The change in fluorescence in apoferritin between subunits and polymer could be ascribed to a change in tryptophan environment. This interpretation is consistent with previous solvent perturbation experiments which showed that about half of the tryptophan residues undergo a change in their environment at acid pH just before dissociation occurs [12]. In this case the 315 nm fluorescence in the polymer would result from the emission of differently located tryptophans some of which could have a fluorescence maximum at very low wavelengths like the one contained in azurin [11]. None of the emitting tryptophans is however accessible to external quenching agents like iodide or cesium chloride. There is the possibility that tyrosine contributes to the blue shift in the emission of the polymer. In this case the increase in the emission wavelength from 315 nm to 328 nm upon dissociation could be attributed to a difference in the efficiency of energy transfer from tyrosine to tryptophan. The increase in emission wavelength would result from a lower contribution of the tyrosine to the overall fluorescence due to a greater efficiency of energy transfer between the aromatic amino acids. This situation is often found in tryptophan containing proteins where the emission of tyrosine is completely abolished by this mechanism [9].

The intrinsic fluorescence of apoferritin is quenched by the presence of ferric ions inside the molecule; thus no quenching is observed with $FeCl_3$, which is not incorporated into the molecule. The quenching produced by the micellar iron core is also almost certainly due to an intramolecular energy transfer mechanism. The fluorescence of the protein appears to be affected also by ferrous ions. A prerequisite to

obtain quenching however is binding to the protein. In fact at low pH (4.5), where iron is not bound, the fluorescence is not quenched upon addition of ferrous ions. The presence of oxygen leads to the oxidation and polymerization of iron; this process is very fast ($t_{1/2} \sim 30$ s) under the experimental conditions used as judged by the appearance of the typical ferritin absorption spectrum and the drop in fluorescence.

In conclusion the experiments reported give evidence of binding of Fe^{2+} to apoferritin. In addition they show that fluorescence spectroscopy can be used as a quick and simple tool for checking the state of polymerization of apoferritin.

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