Ytterbium(III) as a CD Probe for the Investigation of the Metal Binding Sites of Transferrins

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(Received December 23, 1985; revised March 17, 1986)

Since the pioneering work by Luk (1971) [1], it is well known that almost all the lanthanides are able to specifically bind transferrins up to a metal-to-protein ratio of 2:1. The binding occurs at the same sites which are involved in iron binding, as shown by the typical pattern of the UV difference spectra [1, 2]. Only in the case of neodymium(III) and praseodymium(III) was the binding stoichiometry shown to be less than 2:1, and this behavior was interpreted as due to size restriction requirements of the *N*-terminal binding site [1–3].

In principle the binding of lanthanides to transferrins could be studied through the analysis of the f–f transitions; in practice the low intensity of such electric dipole forbidden transitions and the relatively low concentration of the biological samples makes absorption spectroscopy unavailable for this kind of investigation; so the studies performed up to date have mainly utilized luminescence techniques [1, 4–8]. The recent appearance of a report on near IR CD spectroscopy of ytterbium(III) bound to chiral ligands [9] prompted us to utilize this technique for studying ytterbium(III) binding to transferrins.

Two equivalents of ytterbium(III) chloride were added to millimolar solutions of both human serum transferrin (Tf hereafter) and chicken egg ovotransferrin (Otf hereafter) at pH 8 (Tris 0.1 M buffer) in presence of 20 mM bicarbonate. The resulting near IR CD spectra of these samples are reported in Fig. 1. Such spectra show several relatively narrow and intense transitions in the region between 900 and 1000 nm. These transitions originate from the splitting of the free ion $^2F_{7/2} \rightarrow {}^2F_{5/2}$ electronic transition in presence of crystal field perturbations [10, 11]. The relatively high intensity of the CD spectra with respect to optical absorption spectra arises from the fact that the above transition is magnetically allowed. Furthermore, it appears that negatively charged oxygen donor atoms (tyrosinate oxygens in transferrins) are able to enhance the intensity of these transitions [9]. The CD spectra are sensitive to the intrinsic chirality of the electronic ground state of the chromophore [12] and can provide structural information on the metal environment in metalloproteins [13]. Unfortunately up to this date we lack literature data regarding the possibility of correlating the sign and the intensity of near IR CD bands of ytterbium(III) derivatives with specific structural information on the chromophore. Nevertheless, due to the nice resolution of the above CD spectra, we could use them as a useful fingerprint of transferrin binding sites.

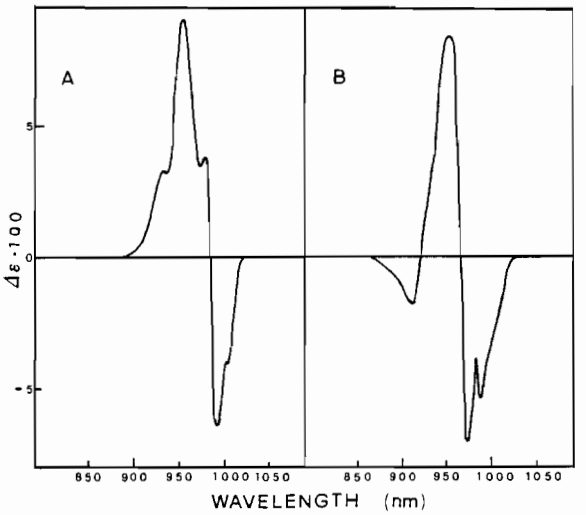

Fig. 1. Near IR CD spectra of Yb_2–Otf (a) and Yb_2–Tf (b) complexes. Protein concentration 1 mM, sodium bicarbonate 20 mM, tris buffer 0.1 M, pH 8. $\Delta\epsilon$ values are referred to protein concentration. Room temperature CD spectra were obtained on a Jasco J 500C spectropolarimeter, using 0.1 dm pathlength cells.

To get more information on the binding properties, we performed further experiments on the ovotransferrin derivative. The titration of Otf at pH 8.5 by stepwise additions of ytterbium(III) confirmed the above 2:1 stoichiometry. The pattern of the titration, reported in Fig. 2a, shows that the spectrum arising from the addition of the first equivalent is almost superimposable to that resulting from the addition of the second equivalent, obtained through computer subtraction of the 1:1 spectrum from the final spectrum; this suggests that either the two sites are indistinguishable or the affinity constants of the sites are comparable (or both).

Performing the same titration at pH 7 we obtained the spectra reported in Fig. 2b. In this case the stoichiometry of the binding is still 2:1, the position of the transitions and their relative intensity is almost unchanged, but the absolute intensity of the transitions is significantly decreased. Furthermore, the spectrum relative to the first equivalent is now considerably different from that relative to the second equivalent, the former exhibiting mainly the negative band at 985 nm, and the latter the positive

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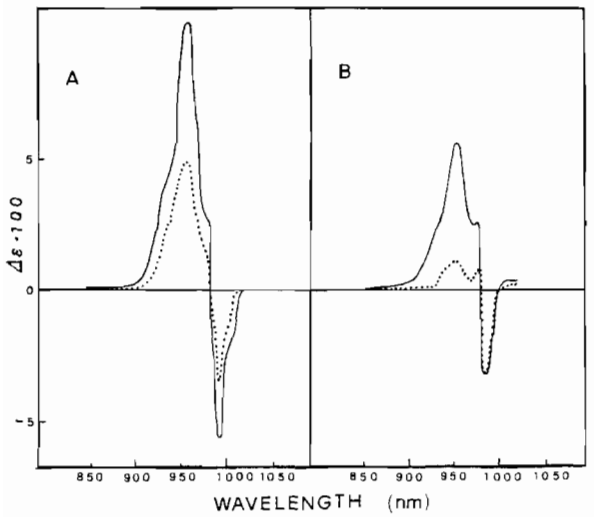

Fig. 2. (a) Near IR CD spectra of Yb–Otf at pH 8.5 after addition of one (·····) and two (——) equivalents of ytterbium(III) chloride. (b) Near IR CD spectra of Yb–Otf at pH 7 after addition of one (·····) and two (——) equivalents of ytterbium(III) chloride. Solution conditions are the same as in Fig. 1.

band at 955 nm; this spectral behavior could indicate that at pH 7 the affinity constants of the sites are different, that binding of ytterbium is sequential and that the spectral features of the sites are deeply dissimilar. Up to now we do not have any explanation for the pH dependent change in intensity of the spectra; a preliminary suggestion is that this change could be due to deprotonation of solvent molecules bound to the metal ion.

By further lowering the pH, the adduct between ytterbium and Otf breaks down; at pH 6 no near IR CD transition is detectable. On the other hand, raising the pH to a value of about 10 does not significantly alter the spectrum of Fig. 1.

UV difference measurements performed at different pH values on Yb–Otf according to the method reported in the literature [1, 2] showed that in the pH range between 7 and 10 the stoichiometry of ytterbium(III) binding is always 2:1, in agreement with the above CD results.

The final experiment we performed on the Yb–Otf derivative was the study of the salt effect on the CD spectrum. It is well known that transferrins undergo marked spectral changes upon addition of 'chaotropic agents', *i.e.*, perchlorate, chloride, etc. [14, 15]. Indeed, near IR CD spectra appear to be significantly sensitive to high concentrations of sodium chloride; addition of sodium chloride to the sample of Fig. 1 up to 1 M final concentration causes a marked decrease of the positive band at 955 nm (Fig. 3), closely reflecting the conformational state of the protein. But we do not have evidence of shift in the position of the bands, possibly suggesting that

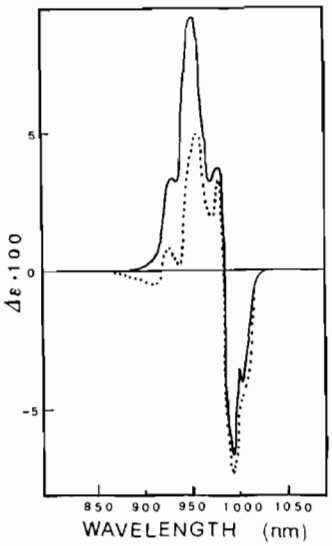

Fig. 3. Near IR CD spectra of Yb_2–Otf complexes at pH 8 before (——) and after (·····) addition of sodium chloride up to 1 M final concentration.

the stereochemistry remains unchanged. This indicates that our technique is an appropriate tool for the detection of anion induced conformational transitions.

In conclusion, on the basis of these preliminary data, we can state that near IR CD spectroscopy of ytterbium(III) derivatives appears to be a useful technique for the investigation of metal binding sites of metalloproteins, and particularly suitable for the study of conformational effects. Further experimental and theoretical work on model complexes would be desirable in order to get a more detailed and possibly quantitative interpretation of the spectral data.

Acknowledgements

Thanks are expressed to Prof. P. Salvadori for valuable discussions concerning the use of lanthanides as CD probes and to Prof. I. Bertini for his encouragement and support. This work has been performed with a contribution of the 'Progetto Finalizzato Chimica Fine e Secondaria' of the Italian CNR.

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