Cobalt(II) as a Probe of the Metal Binding Sites of Transferrins

LUIGI MESSORI* and MARIO PICCIOLI

Department of Chemistry, University of Florence, via Gino Capponi 7, 50121 Florence (Italy)

(Received January 5, 1990; revised March 12, 1990)

Abstract

The cobalt(II) derivative of human serum transferrin has been prepared and investigated through electronic and ^{1}H NMR spectroscopy. Cobalt(II) binds the protein quantitatively in a 2:1 ratio; metal binding requires invariantly the presence of the synergistic anion and occurs at the same binding sites as iron(III). Intersite differences are very slight; on the other hand, there are marked differences in the absorption and CD spectra of $Co(II)_2Tf$ with respect to $Co(II)_2Otf$ which could be explained on the ground of differences in the coordination polyhedron and in the chirality. ^{1}H NMR spectroscopy of the cobalt(II) derivatives allows selective detection of the isotropically shifted signals from protons of the metal coordinated residues and can be used at a first stage as an extremely sensitive conformational fingerprint.

Introduction

It has been previously reported that cobalt(II) binds apo ovotransferrin (Otf hereafter) in a 2:1 ratio at the same sites as iron(III) requiring the presence of bicarbonate or oxalate as synergistic anion [1, 2]. The interaction of cobalt(II) with the protein has been characterized through electronic and ^{1}H NMR spectroscopy [1, 2]; such studies resulted in (i) the determination of hexacoordination around the metal; (ii) the assessment of the substantial equivalence of the two sites; (iii) a tentative assignment of the ^{1}H NMR signals to the protons of the metal binding residues and (iv) the analysis of the conformational states and their sensitivity to the nature of the synergistic anion. The recent appearance of the X-ray structures of both iron lactoferrin [3] and iron human serum transferrin [4] at a fairly good resolution has raised new interest into the structural analysis of the metal binding sites of transferrins. We have extended the spectral analysis to the case of the cobalt(II) derivative of human serum transferrin (Tf hereafter). Significant differences have been found in the electronic spectra with respect to $Co(II)_2Otf$; in particular the intensity of the visible d–d transitions is markedly higher suggesting some relevant change in the coordination geometry of the cobalt(II) ion in the two proteins.

Experimental

Human serum transferrin, in the apo form, was purchased from Sigma Chemical Company and purified according to the reported procedure [5]. Protein concentration was determined through UV analysis by monitoring the intensity of the 280 nm band (ϵ = 92 000). Cobalt(II) derivatives were prepared by addition of aliquots of cobalt(II) chloride water solutions to apoprotein solutions. All sample handling was performed under exclusion of air, in order to avoid cobalt(II) oxidation. Sample deuteration was accomplished by adding deuterium oxide to the lyophilized protein and repeating the procedure at least twice.

The UV and the visible spectra were recorded on a Cary 17 D spectrophotometer operating at room temperature; the circular dichroism (CD) spectra in the visible region were obtained on a Jasco J500C spectropolarimeter.

The ^{1}H NMR spectra at 90 and 200 MHz were performed on a CXP 90 and a MSL 200 Bruker instrument, respectively. The spectra were recorded in quadrature detection; both MODEFT [6] and SuperWEFT [7] pulse sequences were used to suppress the solvent signal. The concentration of the NMR samples was 1–2 mM in protein. The reported spectra typically consisted of 10 000–30 000 scans for the experiments run at 200 MHz and of 50 000–200 000 scans for those run at 90 MHz. Chemical shifts are reported in parts per million, referenced to $(CH_3)_4Si$, with downfield shifts taken as positive.

Results and Discussion

Absorption and CD Spectra

Titration of apotransferrin with increasing amounts of cobalt(II), in the presence of 20 mM sodium bicarbonate, at pH 8, has been followed through visible absorption spectroscopy; formation

*Author to whom correspondence should be addressed.

0020-1693/90/$3.50

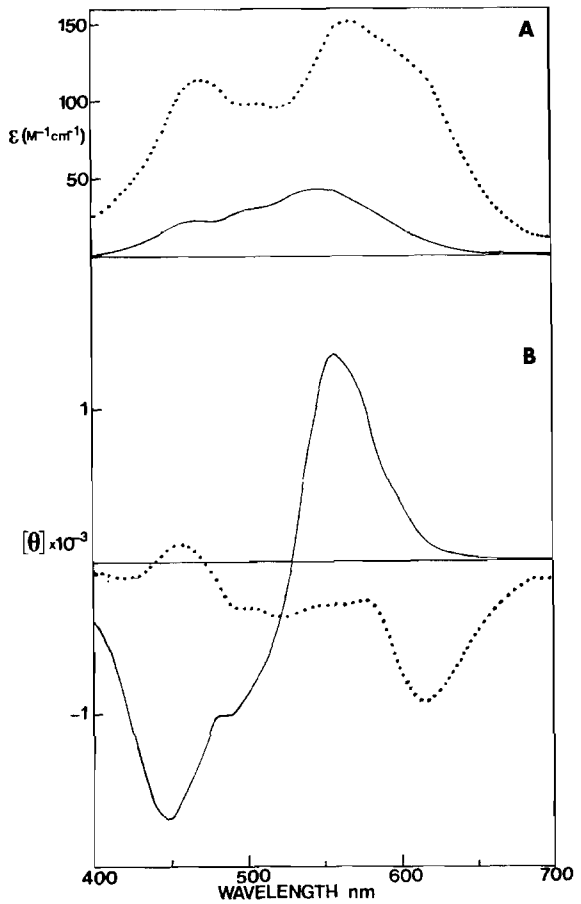

Fig. 1. (A) Visible absorption spectra of $Co(II)_2Tf$ (·····) and $Co(II)_2Otf$ (——). (B) Visible circular dichroism spectra of $Co(II)_2Tf$ (·····) and $Co(II)_2Otf$ (——). Experimental conditions: 1 mM protein concentration, 20 mM sodium bicarbonate, Tris buffer 0.05 M, pH 8.

of a metal protein adduct is witnessed by the appearance of some typical d–d transitions in the 400–700 nm range, whose intensity increases monotonically with increasing cobalt(II) concentration, up to a 2:1 metal to protein stoichiometry. The pattern of the titration is continuous suggesting essential equivalence between the two sites. The final spectrum, shown in Fig. 1(A) is characterized by four d–d bands respectively centered at 460, 510, 570 and 615 nm. The electronic spectra of high spin cobalt(II) derivatives have been extensively discussed in the case of cobalt(II) substituted enzymes [8]. Six-coordinated chromophores give rise to absorption which is about one order of magnitude less intense than for four- or five-coordinated complexes. Hence, the molar extinction coefficient is extremely sensitive to coordination and symmetry distortions. The molar extinction coefficient per cobalt of the 570 nm band is about 75 M^{-1} cm^{-1}; this value is significantly higher than that previously found for cobalt(II) ovotransferrin

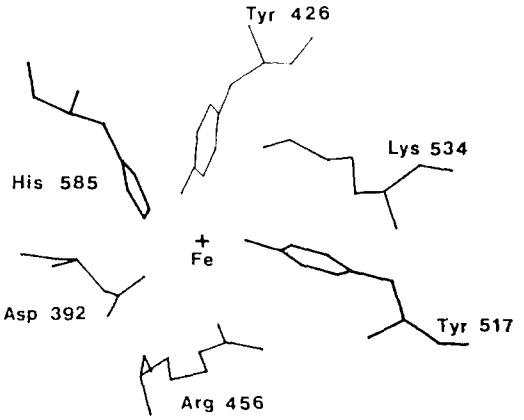

Fig. 2. Schematic drawing of the metal binding site of rabbit serum transferrin as reported by Lindley and coworkers [4]. Residue numbering refers to the N-terminal lobe.

($\epsilon_{550} = 20$ M^{-1} cm^{-1}) (Fig. 1(A)) [2]. Whereas the latter value is nicely consistent with hexacoordination, the former value is indicative of an overall lower symmetry [8].

Figure 2 shows the putative structure of the metal site of transferrins as it results from the recent X-ray studies [4]; the metal is surrounded by four donor atoms from the protein (Tyr-95, Tyr-188, Asp-63, His-249; residue numbering refers to the N-terminal lobe of rabbit serum transferrin) which are disposed at the vertices of a strongly distorted octahedron. This scheme is of general validity for all the transferrins since the four protein ligands directly coordinated to the high spin iron(III) center are conserved throughout both lobes of human serum transferrin [9], human lactoferrin [10] and chicken egg ovotransferrin [11].

At the actual stage of refinement of the X-ray data it is not yet clear which is the nature of the additional metal ligands; independent spectroscopic evidences suggest that either the synergistic anion [12, 13] arranged in a bidentate fashion or the monodentate synergistic plus a trapped solvent molecule [14–16] may occupy the two remaining positions of the coordination polyhedron. Alternatively, the chromophore could just be distorted pentacoordinated with the monodentate synergistic as fifth ligand. In the case of lactoferrin, it has been recently proposed that the synergistic anion acts as bidentate [17].

Since it is highly probable that the four protein ligands to the iron(III) center are invariant and monodentate, a change in the coordination environment from distorted octahedral to distorted pentacoordinated could only arise either from a different arrangement of the synergistic anion or from a weakening of the bond between the metal and the hypothetically coordinated solvent molecule. Due to the fact that carbonate can act both as a bidentate or a monodentate, it is tempting to assume that just a

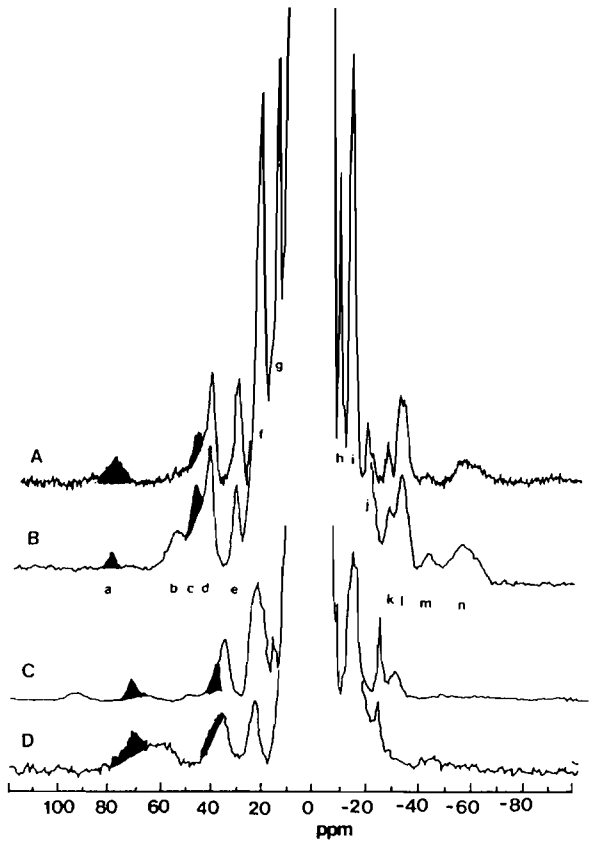

Fig. 3. ^{1}H NMR spectra of the $Co(II)_2Tf$ complex at 200 (A) and 90 (B) MHz (298 K) and of the $Co(II)_2OTf$ complex at 200 (C) and 90 (D) MHz. Shaded signals disappear upon deuteration.

slight difference in its arrangement could originate the observed variation in the intensity of the d–d transitions.

Titration of apo-Tf with cobalt(II), followed via circular dichroism, gives analogous results. Again, the shape of the final spectrum, which is reached after the addition of two equivalents of cobalt(II) (Fig. 1(B)), is strikingly distinct from that previously reported for $Co(II)_2Otf$, indicating the presence of relevant differences in the chirality of the two chromophores. In particular it should be observed that the Cotton effects centered at 460 and 615 nm exhibit opposite sign with respect to those observed for $Co(II)_2Otf$.

^{1}H NMR Spectra

The ^{1}H NMR spectra of $Co(II)_2Tf$, at pH 8, Tris buffer 0.05 M, recorded at both 200 and 90 MHz, are reported in Fig. 3(A) and (B), respectively. These spectra exhibit several isotropically shifted resonances spread over the chemical shift interval between +100/−60 ppm. The general shape of the spectrum as well as signal linewidths and T_1 values are similar to those found for the analogous spectra of $Co(II)_2Otf$ run both at 200 and 90 MHz which are reported in Fig. 3(C) and (D), respectively. The shaded signals are due to exchangeable protons since they disappear when the spectra are recorded in D_2O. It should be observed that increasing the magnetic field results in an improved observation of the spectral region near to the diamagnetic part and in a further broadening of the far shifted resonances, probably due to Curie relaxation effects [18]. Indeed, the 60 MHz ^{1}H NMR spectrum of $Co(II)_2Otf$, previously reported, allowed a better detection of the broad upfield shifted signals [1].

The resolution of the ^{1}H NMR spectra of the present system does not allow differentiation of the N- and C-terminal sites in either $Co(II)_2Tf$ or $Co(II)_2$-Otf. The spectra of the mono-cobalt(II) derivatives have been recorded in both cases and no differences have been monitored with respect to the bis-cobalt(II) derivatives; this is a clear evidence of the extreme structural similarity of the two sites, despite spectral inequivalences previously detected through ^{205}Tl NMR on $Tl(III)_2Tf$ [19] and through EPR of iron(III) [20], oxovanadium(IV) [21, 22] and copper(II) derivatives [23, 24].

On the other hand, the ^{1}H NMR spectrum of cobalt(II) transferrin differs from that of cobalt(II) ovotransferrin, even if the differences are apparently minor. Indeed, slight but significant differences between Tf and Otf had been previously observed in the EPR spectra of the iron(III) [25], oxovanadium(IV) [21, 22] and copper(II) derivatives [23, 24]. It remains to be understood to what extent the different metals enhance the intrinsic structural inequivalences.

The assignment of the isotropically shifted signals to the protons of the metal liganded protein residues relies on qualitative criteria [1, 2]. The four *ortho* protons of the two coordinated tyrosines experience large paramagnetic effects of contact nature and probably give rise to the broad upfield shifted signals (signal m and n); on the other hand, the four *meta* protons experience smaller paramagnetic effects and are expected to originate narrower signals located near to the diamagnetic region. The β-CH_2 protons of Asp-63 and the two *ortho*-like CH protons of His-249 are expected to give rise to downfield shifted signals falling in the 100–30 ppm range (possibly signals b, d, e) [8].

The exchangeable signals deserve a particular comment; two such signals are observed in both $Co(II)_2Tf$ and $Co(II)_2Otf$ in the downfield region exhibiting quite different chemical shift values (see Fig. 3). In the light of the recent X-ray data it is tempting to assign the farthest shifted one to the NH of the coordinated histidine. The second exchangeable proton could be assigned to a non-coordinated proximal NH experiencing a sizeable dipolar effect from the

metal ion; inspection of the X-ray data through computer graphics suggests that the most probable candidate is a NH of Arg-124, the residue involved in the binding of the synergistic anion.

In conclusion slight but meaningful geometrical changes have been revealed by electronic spectroscopy in the frame of two essentially similar chromophores, as suggested by the ^{1}H NMR spectra.

Acknowledgements

We are grateful to Professor Ivano Bertini for his continuous advice and support. We would like to acknowledge the discussion with Professor Peter F. Lindley and his kindness for allowing us to use his refined X-ray coordinates.

References

1 I. Bertini, C. Luchinat, L. Messori and A. Scozzafava, *Eur. J. Biochem., 141* (1984) 375.
2 I. Bertini, C. Luchinat, L. Messori, R. Monnanni and A. Scozzafava, *J. Biol. Chem., 261* (1986) 1139.
3 B. F. Anderson, H. M. Baker, E. J. Dodson, G. E. Norris, S. V. Rumball, J. M. Waters and E. N. Baker, *Proc. Natl. Acad. Sci. U.S.A., 84* (1987) 1.
4 S. Bailey, R. W. Evans, R. C. Garratt, B. Gorinsky, S. Hasnain, C. Horsburh, H. Jhoti, P. F. Lindley, A. Mydin, R. Sarra and J. L. Watson, *Biochemistry, 27* (1988) 5804.
5 G. W. Bates and M. R. Schlabach, *J. Biol. Chem., 248* (1973) 3228.
6 J. Hochmann and H. P. Kellerhals, *J. Magn. Reson., 38* (1980) 23.
7 T. Inubushi and E. D. Becker, *J. Magn. Reson., 51* (1983) 128.
8 I. Bertini and C. Luchinat, *Adv. Inorg. Biochem., 6* (1984) 71.
9 R. T. A. MacGillivray, E. Mendez, J. G. Shewale, S. K. Sinha, J. Lineback-Zins and K. Brew, *J. Biol. Chem., 258* (1983) 3543.
10 J. Williams, T. C. Elleman, I. B. Kingston, A. G. Wilkins and K. A. Kuhn, *Eur. J. Biochem., 122* (1982) 297.
11 M.-H. Metz-Boutigue, J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik and P. Jolles, *Eur. J. Biochem., 145* (1984) 659.
12 I. Bertini, C. Luchinat, L. Messori, A. Scozzafava, G. Pellacani and M. Sola, *Inorg. Chem., 25* (1986) 1782.
13 I. Bertini, L. Messori, G. C. Pellacani and M. Sola, *Inorg. Chem., 27* (1988) 761.
14 S. H. Koenig and W. E. Schillinger, *J. Biol. Chem., 244* (1969) 6250.
15 I. Bertini, F. Briganti, S. H. Koenig and C. Luchinat, *Biochemistry, 24* (1985) 6287.
16 V. L. Pecoraro, W. R. Harris, C. J. Carrano and K. N. Raymond, *Biochemistry, 20* (1981) 7033.
17 P. F. Lindley, personal communication.
18 I. Bertini and C. Luchinat, *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin/Cummings, Menlo Park, CA, 1986.
19 I. Bertini, C. Luchinat and L. Messori, *J. Am. Chem. Soc., 105* (1983) 1347.
20 C. P. Thompson, B. M. McCarty and D. N. Chasteen, *Biochim. Biophys. Acta*, (1986) 530.
21 N. D. Chasteen, L. K. White and R. F. Campbell, *Biochemistry, 16* (1977) 363.
22 J. D. Casey and N. D. Chasteen, *J. Inorg. Biochem., 13* (1980) 111.
23 J. L. Zweier and P. Aisen, *J. Biol. Chem., 252* (1977) 6090.
24 J. L. Zweier, *J. Biol. Chem., 255* (1980) 2782.
25 P. Aisen, R. A. Pinkowitz and A. Leibman, *Ann. NY Acad. Sci., 222* (1973) 337.