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# Characterisation of Non-covalent Complexes by Electrospray Mass Spectrometry

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***Abstract:*** *Electrospray Mass Spectrometry (ESMS) has been used to analyse protein/metal ion complexes directly in solution. A synthetic siderophore analogue and two sulphur-iron proteins have been used as models for the study of protein/iron interactions. These experiments were successfully extended to a protein/cofactor complex interaction model, myohemoglobin. Our results open the door to the characterization of weak interactions between large molecules by ESMS.*

ESMS has become a rapid and routine method for the measurement of the molecular mass of proteins up to 100 kDa with an accuracy of 0.01% [1]. The electrospray interface, producing gas-phase ions from a liquid surface via an electric field at atmospheric pressure [2], is ideal for the ionisation/volatilisation of thermally labile, non-volatile, polar compounds. At first it seemed that the ESMS ionisation/volatilisation process would not allow the study of non-covalent assemblies. However, under carefully controlled conditions, it was later shown in a few cases that it is possible to observe intact enzyme/inhibitor [3] or peptide/metal-ion complexes [4], but only to a small extent.

We would like to show here that if several parameters (pH, ionic strength, temperature, source voltage parameters) are carefully controlled, ESMS can be used as a general method for the study of non-covalent interactions. We believe that it is in fact possible to study non-covalent assemblies by ESMS provided that the conditions used before the MS study do not lead to a decomposition of the complexes. Most of the ESMS studies reported make use of MeOH/ $H_2O$ (1/1 ratio) as solvent. This solvent easily produces a stable electrospray but does not allow the pH to be controlled. The production of a stable electrospray in a buffered aqueous solution with control of pH is much more difficult to achieve owing to the presence of salts. Nonetheless, this approch would open the door to MS studies of the quaternary structure of proteins, of enzyme/substrate complexes, of ligand/metal complexes, which are usually performed by classical inaccurate methods (size exclusion chromatography, sedimentation equilibrium, ultra-centrifugation,...).

We have studied a series of model complexes to determine the ESMS conditions which preserve the integrity of the complexes during the ionisation process. First, metal complexation was tested with a synthetic analogue of the bacterial siderophore ferrichrome A (ligand L). This analogue is a ditopic trishydroxamate ligand having two ferric ion complexing sites [5]. Next, two sulphur-iron proteins (rubredoxin and ferredoxin) were selected to explore the possibilities of ESMS for the identification and the counting of the metal ions complexed by proteins. Finally, our studies were extended to cofactor/protein complexes using myohemoglobin as a model.

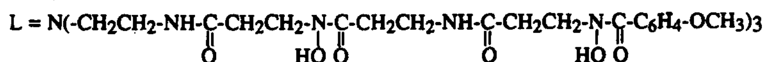
## Materials and Methods

Ligand L has been prepared as described elsewhere[5]. Horse heart myohemoglobin and *Porphyra umbilicalis* ferredoxin were purchased from Sigma (St Louis, USA). Rubredoxin was purified from *Clostridium thermosaccharolyticum* [6]. ESMS spectra were obtained by using a Bio-Q quadrupole mass spectrometer (Fisons, Manchester, UK). Values used for the extraction cone voltage (Vc) ranged from 30 to 250 volts. The source temperature was set at 50°C. In all cases, electrospray was pneumatically assisted. Ligand L and $FeCl_3$ were dissolved in methanol. Myohemoglobin was dissolved in a pyridinium acetate buffer (50 mM, pH = 5.0).

## Results and discussion

*Study of the ligand L/iron (III) complex*

Two experiments were carried out, using 0 or 2 iron (III) chloride equivalents. In all cases, intense doubly charged ions were produced. For the free ligand L (1284.5 Da) ionisation was mainly obtained by double proto-




nation $[M+2H]^{2+}$ (Fig. 1a). The peak at -16 Da corresponds to a synthetic impurity present in ligand L. Cationisation with $Na^+$ and $K^+$ was also observed, incidentally revealing an affinity of the ligand for these ions. With a $Fe^{3+}/L = 2/1$ ratio, all ligand molecules were shown to complex exclusively two ferric ions (Fig. 1b). The mass difference measured between the free ligand and the complex with two ferric ions is 105.5 Da and not 111.7 Da (2 x $Fe^{3+}$). This shows that the complexation of the two ferric ions is accompanied by the loss of six protons from the hydroxamate groups of the ligand L. When Vc was increased to 250 volts, no loss of iron was detected. This fits with the very high affinity of ligand L for iron ions[5]. We have already shown the possibilities of ESMS for the study of $Cu^+$/bipyridine containing catenates up to 6 kDa[7]. ESMS thus appears to be an easy and general method for ascertaining the number of metal ions in supramolecular chemistry.

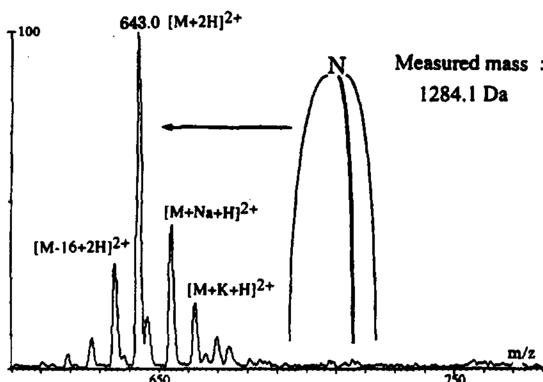



**Figure 1a**: ESMS spectrum of the free ligand L (Vc = 50 V), in MeOH.

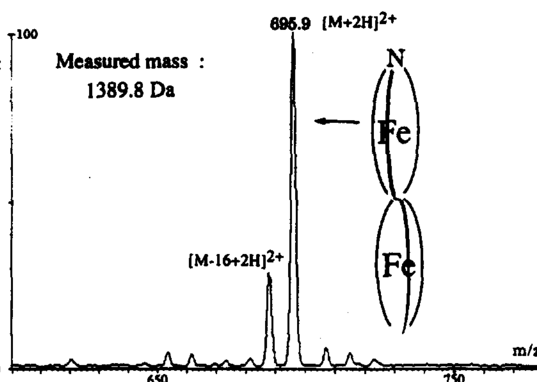



**Figure 1b**: ESMS spectrum of ligand L with an addition of two ferric ion equivalents, in MeOH.

*Study of the iron-sulphur proteins: rubredoxin and ferredoxin*

The results obtained on the small molecular weight ligand L were extended to natural sulphur-iron proteins of larger size. The rubredoxin active center consists of one ferric ion surrounded by 4 cysteine thiolates. The spectrum of rubredoxin (Fig. 2) recorded in $MeOH/H_2O$ (1/1) displayed three series of multiply charged ions

corresponding to aporubredoxin (no iron, A series, expected mass: 5936.5 Da), holorubredoxin (one iron, B series) and holorubredoxin with a $K^+$ adduct (C). For the major series (B), the net charge at the active center $[Fe^{3+}, 4Cys^-]^-$ is one negative charge. Thus the number of protons attached to the protein is expected to be one unit higher than the number of charges. This is actually observed (Fe - 4H + aporubredoxin + H = 5988.4 Da)[6].

Ferredoxins, proteins involved in photosynthesis, are known to carry two possible types of active centers (2Fe/2S or 4Fe/4S) [7]. We have used ESMS to count the number of iron and sulphur atoms. In $MeOH/H_2O$ (1/1, 1% AcOH), the ESMS analysis detected only the apoferredoxin (expected mass: 10827.8 Da). In contrast, when pure water was used as a solvent, several complexes were observed. Figure 3 shows the real mass scale spectrum (obtained after transformation of the m/z spectrum) of ferredoxin. The two major complexes (measured mass: 11232.7 Da and 11177.5 Da) correspond to the protein with a 4S/4Fe active center (apoferredoxin + 4S + 4Fe - 4H = 11176.6 Da) and a 4S/4Fe active centre plus one extra unexpected iron atom (apoferredoxin + 4S +4Fe - 4H + Fe =11232.5 Da). Two minor complexes, corresponding to proteins with a 2S/2Fe active center (11003.9 Da) and a 2S/2Fe active center plus one extra unexpected iron atom (11059.8 Da), were also detected. As for the case of rubredoxin, the measured masses fit with the expected calculated masses if it assumed that all ferric and sulphur ions are complexed by cysteine thiolates.

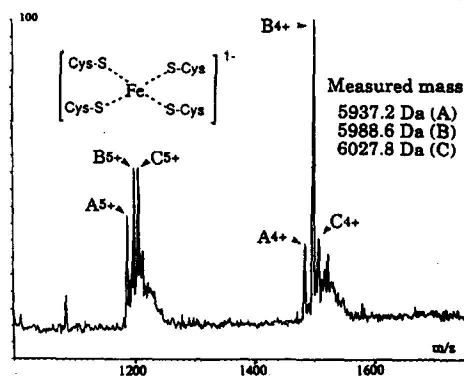



**Figure 2:** ESMS spectrum of rubredoxin (Vc = 50 V), in $MeOH/H_2O$ (1/1).

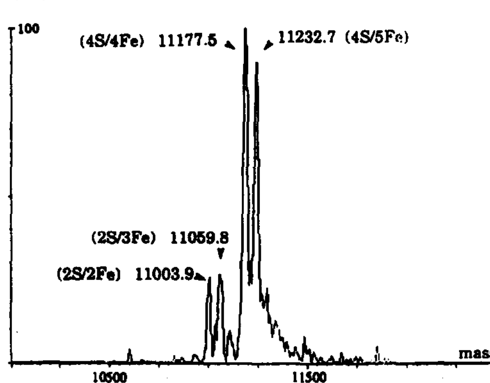



**Figure 3:** ESMS spectrum of ferredoxin (Vc =70 V), in 100% water.

*Study of a cofactor/protein complex: myohemoglobin*

Figure 4a shows the real mass scale ESMS spectrum of a non-covalent complex, myohemoglobin, recorded at pH = 5.0. At low Vc (40 volts), a single peak was detected at 17570.5 Da, corresponding to myohemoglobin (exp. mass: 17568.5Da). Interestingly, in these conditions the most abundant multiply charged ion carried 8 charges, while in $H_2O/MeOH$ it carried 24 charges. A small peak (5%) at 16950.5 Da reveals a minor loss of the heme (616.5 Da) which is normally attached to myoglobine by interaction of one ferric ion with the proximal histidine F8. At Vc = 65 volts (Fig. 4b), both species, myoglobin and myohemoglobin were present . The loss of the heme induced by collisions in the interface converts myohemoglobin to myoglobin. At Vc = 100 volts, the conversion is complete. Such conversion was also observed when using organic solvents or pH < 4.5; in both cases, the protein conformation is radically altered which induces the release of the heme. Similar results have been obtained on an $NADP^+$/aldose reductase complex [9].

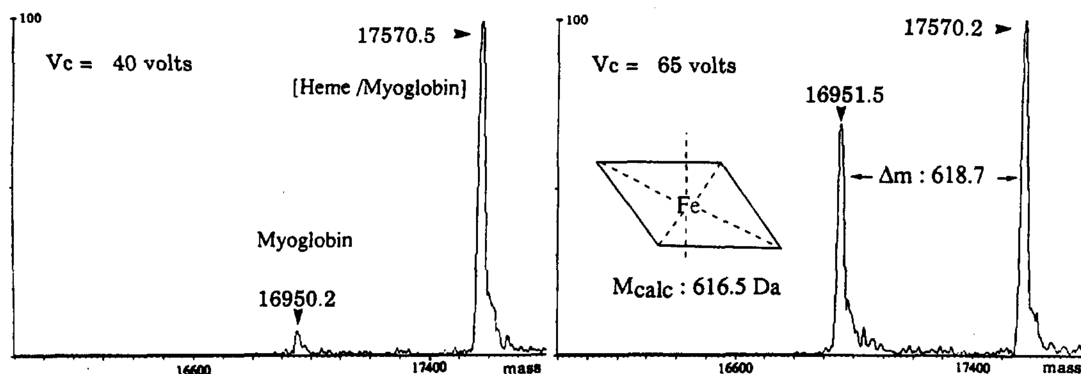



**Figure 4a:** ESMS spectrum of myohemoglobin (Vc = 40 V), in pyridinium acetate (pH = 5.0).

**Figure 4b:** ESMS spectrum of myohemoglobin (Vc = 65 V), in pyridinium acetate (pH = 5.0).

## Conclusion

In this work, ESMS has been shown to be a useful tool for the study of weak interactions. A non-covalent complex can resist the ESMS ionization step with only minor dissociation. The experimental conditions allowing such studies depend on the complex analysed. The solvent and the pH used must not decompose the complex and the ESMS interface conditions must also be adapted. ESMS could provide a new and rapid method to evaluate the relative affinity of several inhibitors for a protein.

## References


1. Smith R.D., Loo C.J., Edmonds C.G., Barinaga C.J. and Udseth H.R., *Anal. Chem.*, **1990**, *62*, 882-899; Van Dorsselaer A., Bitsch F., Green B., Jarvis S., Lepage P., Bischoff R., Kolbe H.V.J. and Roitsch C., *Biomed. Environ. Mass Spectrom.*, **1990**, *19*, 692-704.
2. Fenn J.B., Mann M., Meng C.K., Wong S. F. and Whitehouse C.M., *Mass Spectrom.Rev.*, **1990**, *9*, 37-70; Whitehouse C.M., Dreyer R.N., Yamashita M. and Fenn J.B., *Anal. Chem* , **1985**, *57*, 675-679; Bruins A.P., Covey T.R. and Henion J.D., *Anal. Chem.*, **1987**, *59*, 2642-2646.
3. Ganem B., Li Y.T. and Henion J.D., *J. Am. Chem. Soc.*, **1991**, *113*, 7818-7819; Baca M. and Kent S.B.H., *J. Am. Chem. Soc.*, **1992**, *114*, 3992-3993.
4. Allen M. H. and Hutchens T.W., *Rapid Commun. Mass Spectrom.*, **1992**, *6*, 308-312.
5. Libman J., Tor Y. and Shanzer A., *J. Am. Chem. Soc.*, **1987**, *109*, 5880-5881.
6. Meyer J., Gagnon J., Sieker L.C., Van Dorsselaer A. and Moulis J.M., *Biochem. J.*, **1990**, *271*, 839-841.
7. Bitsch F., Dietrich-Buchecker C., Khemiss A., Sauvage J.P. and Van Dorsselaer A., *J. Am. Chem. Soc.*, **1991**, *113*, 4023-4025.
8. Andrew P.W., Rogers L.J., Boulter D. and Haslett B.G., *Eur. J. Biochem.*, **1976**, *69*, 243-248.
9. Jaquinod M. and Van Dorsselaer A., *Analusis*, **1992**, *20*, 407-412.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