Probing Protein Conformation by a Combination of Electrospray Mass Spectrometry and Molecular Modelling

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Electrospray-ionisation mass spectrometry and molecular modelling have been used to probe two conformations of tuna cytochrome c; the number of protons which could be exchanged by deuterons was in remarkable agreement with that calculated using an algorithm for the determination of solvent-accessible surface.

Until recently, the most common techniques used to probe the three-dimensional conformation of proteins in solution have been nuclear magnetic resonance[1] and spectrophotometric techniques[2,3] such as fluorescence, IR, optical rotary dispersion and circular dichroism. Mass spectrometry has usually been restricted to the determination of the primary structure of peptides and proteins.[4,5] With the advent of electrospray-ionisation mass spectrometry, analysis of increasingly higher molecular weight proteins has become possible.[6] It has also been demonstrated that electrospray-ionisation mass spectrometry can be effective[7] in probing conformation of proteins in solution, especially when hydrogen exchange experiments are carried out.[8] However, in these experiments the nature of these conformations was not fully investigated. Knowledge of this conformation is essential both for drug design and for the identity of mechanisms that lead to protein denaturation. To achieve this we have obtained electrospray-ionisation mass spectra of tuna cytochrome c in acidic aqueous solutions and

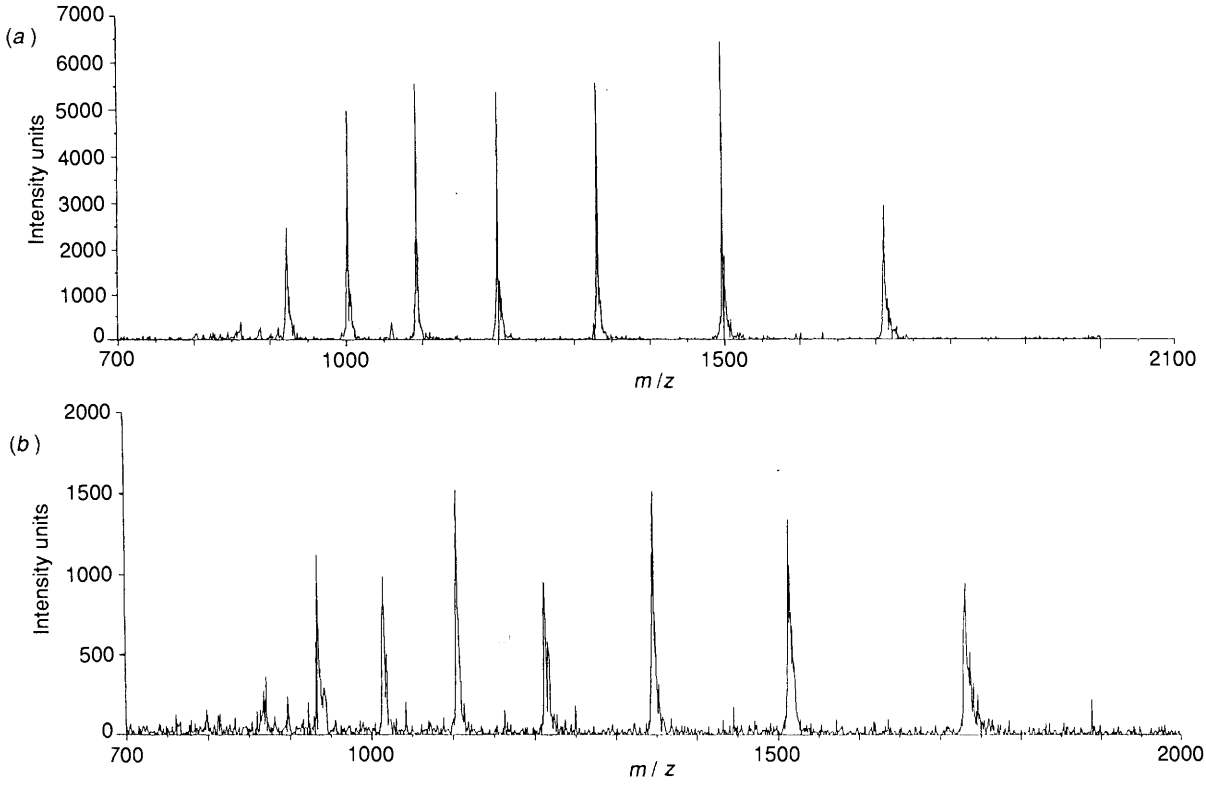

Fig. 1 Electrospray-ionisation mass spectra of tuna cytochrome c obtained in (*a*) 60% H_2O, 35% CH_3OH and 5% CH_3CO_2H; and (*b*) 60% D_2O, 35% CH_3OD and 5% CH_3CO_2D. The concentration of the protein was 0.6 mg ml^{-1}.

Table 1 Estimated molecular weights for tuna cytochrome c

	Conformer I	Conformer II
H_2O	11 986	11 986
D_2O	12 102	12 139

have interpreted the results with the aid of molecular modelling.

The mass spectra for tuna cytochrome c in H_2O- and D_2O-based solutions are shown in Fig. 1(*a*) and (*b*) respectively. The peaks correspond to different levels of protonation of the protein and, as expected, all peaks are shifted to a higher molecular weight in the deuteriated solvent. There are 25 basic groups in cytochrome c. However, the maximum number of protonated residues seen in the mass spectrum is only 18, indicating that seven basic residues are in some way shielded from solvent. As in the case of bovine cytochrome c[7] the distribution of the multi-protonated ions has a biphasic, rather than a Gaussian distribution, both in H_2O and D_2O solution. These maxima relate to two conformations of tuna cytochrome c. The maximum with the higher mass/charge ratio belongs to a conformational state (conformer I) which is tighter than that related to the second maximum (conformer II). The number of exchangeable protons was measured experimentally from the mass spectra as 116 and 153 for the tight and loose conformations respectively. Estimated molecular weights for the two conformers are given in Table 1. It must be appreciated that only in D_2O will a molecular weight change be observed.

Algorithms for determination of solvent-accessible surface area are well known.[9,10] For the present study we used the INSIGHT molecular modelling package[11] to examine the albacore tuna cytochrome c, available in the Brookhaven Protein Data Bank[11,12] and to generate the solvent-accessible surface using the algorithm of Connolly. Examination of the output file allowed us to determine which of the protons attached to nitrogen or oxygen were solvent-accessible.

Calculations were performed with the ionisation state of each residue being that expected in acidic aqueous solution (that is, all lysines, arginines and histidines ionised and aspartates and glutamates neutral). Fig. 2(*a*) shows the 3-D structure of tuna cytochrome c and the various protons on the surface of this protein accessible to solvent. We calculated that of the potentially 209 exchangeable protons only 115 are accessible to proton exchange. This value is in good agreement with that obtained by mass spectrometry for one of the conformations.

A closer examination of the 3-D structure of cytochrome c reveals that this is characterised by N- and C-terminal α-helices with the central portion of the protein being predominantly a series of loops in close contact with the haem group. The N-terminal helix lies over the top of the C-terminal helix apparently holding it in place. In an attempt to relate our experimental observations with the denaturation of cytochrome c we 'partially denatured' the computer model of the protein by separating the two α-helices, as shown in Fig. 2(*b*). This process involves initially the N-terminal helix moving into solvent and the C-terminal helix subsequently detaching from the central portion of the protein. Both steps are quite simply achieved by changes in backbone torsion angles with no loss in secondary structure. We have found that manipulation of residues 1–13 (cys 14 is bound covalently to the haem group) so that they project out into solvent allows a further two exchangeable protons to come into contact with solvent, and

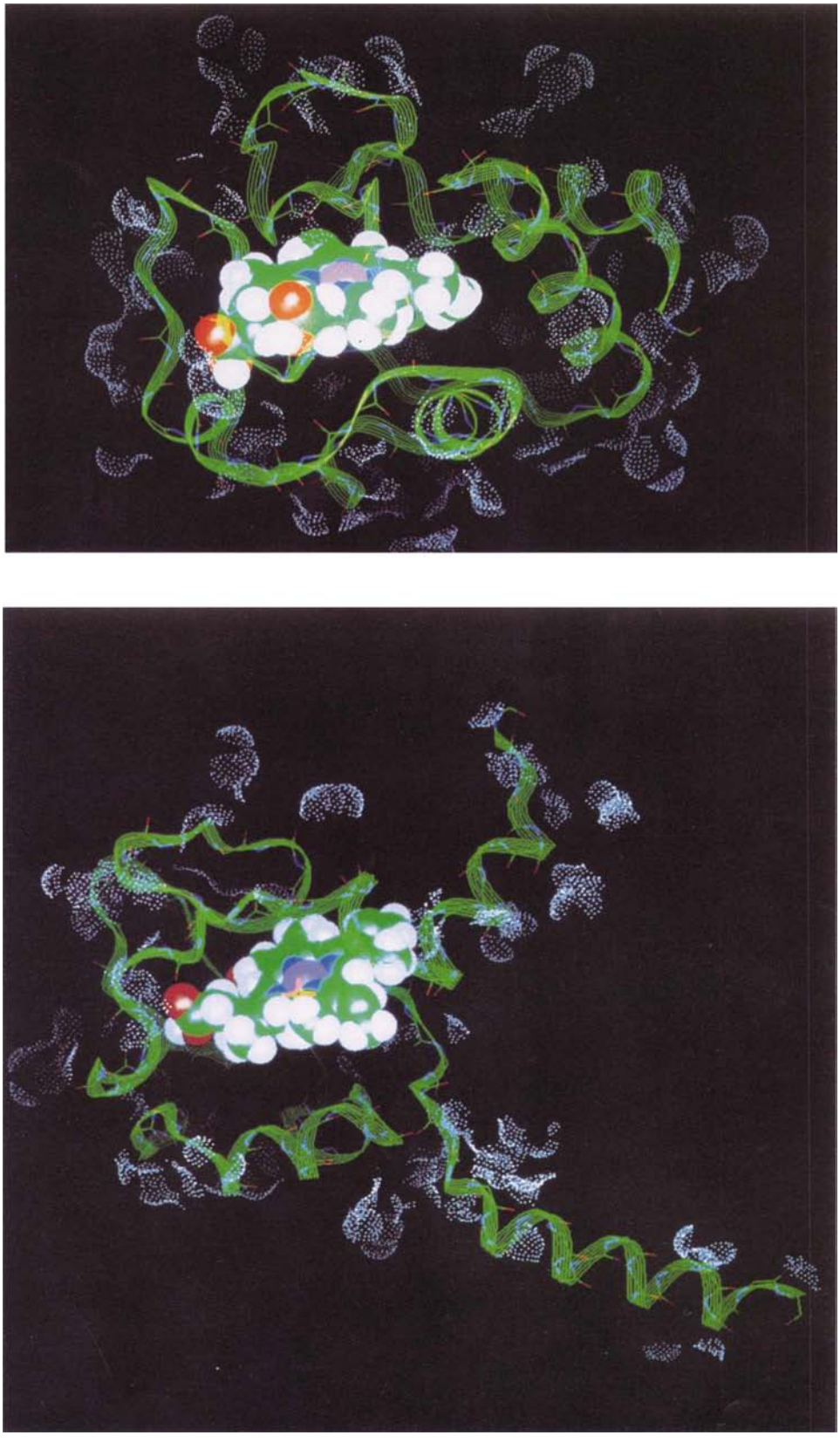

Fig. 2 (*a*) Peptide backbone of tuna cytochrome c as obtained from X-ray structure and (*b*) backbone after separation of the two helices containing the N- and C-terminals

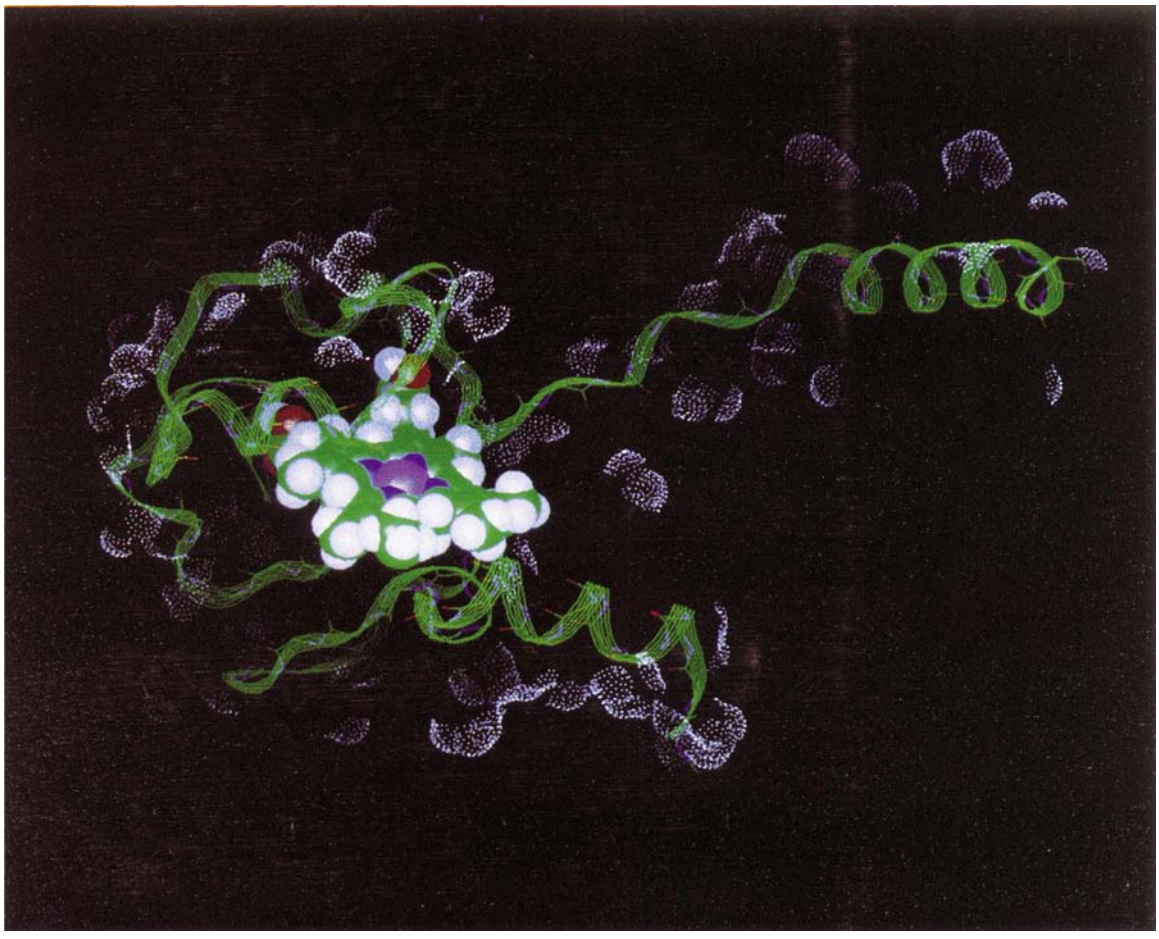

Fig. 3 Peptide backbone of tuna cytochrome c after further unwinding of the protein from residue 81 onwards (α-helices have been kept intact for clarity)

doing the same for the C-terminal helix residues 87 onwards reveals a further five exchangeable protons. This gives a total of 122, a value which is substantially lower than the experimentally observed value of 153. We therefore conclude that further denaturation is taking place under acidic conditions and involves the loss of the helical structure of both the N and C-terminal helices. If this is done to the extent that all possible exchangeable protons in this portion of the molecule become solvent-exposed, this adds a further 19 protons to give a total of 141. Finally, assuming (*i*) that the structure of cytochrome c unwinds from residue 81 onwards (met 80 is coordinated to the iron in the haem moiety), giving a structure (Fig. 3) with residues 14–80 intact and (*ii*) that all exchangeable protons in the N- and C-terminal helix are solvent-exposed at some time, a total of 151 exchangeable protons is obtained (30 from the 13 N-terminal residues, 50 from the 23 C-terminal residues and 71 from the 'undisturbed' central portion of the protein). This value is very close to the experimental value of 153. Calculated values should, of course, be regarded as a lower limit because they are performed on a single static conformer, whereas in solution thermal motions in the protein may cause additional exchangeable protons to become exposed to solvent. However, the present findings are in good agreement with recent reports[3,14] showing that cytochrome c loses much of its α-helical structure under acidic conditions (near pH 2) at low ionic strength.

Biphasic distributions have been seen by us for other proteins when studied by electrospray mass spectrometry. We think that this behaviour may be due either to protein unfolding or to oligomer formation. We are applying molecular modelling to interpret these phenomena.

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