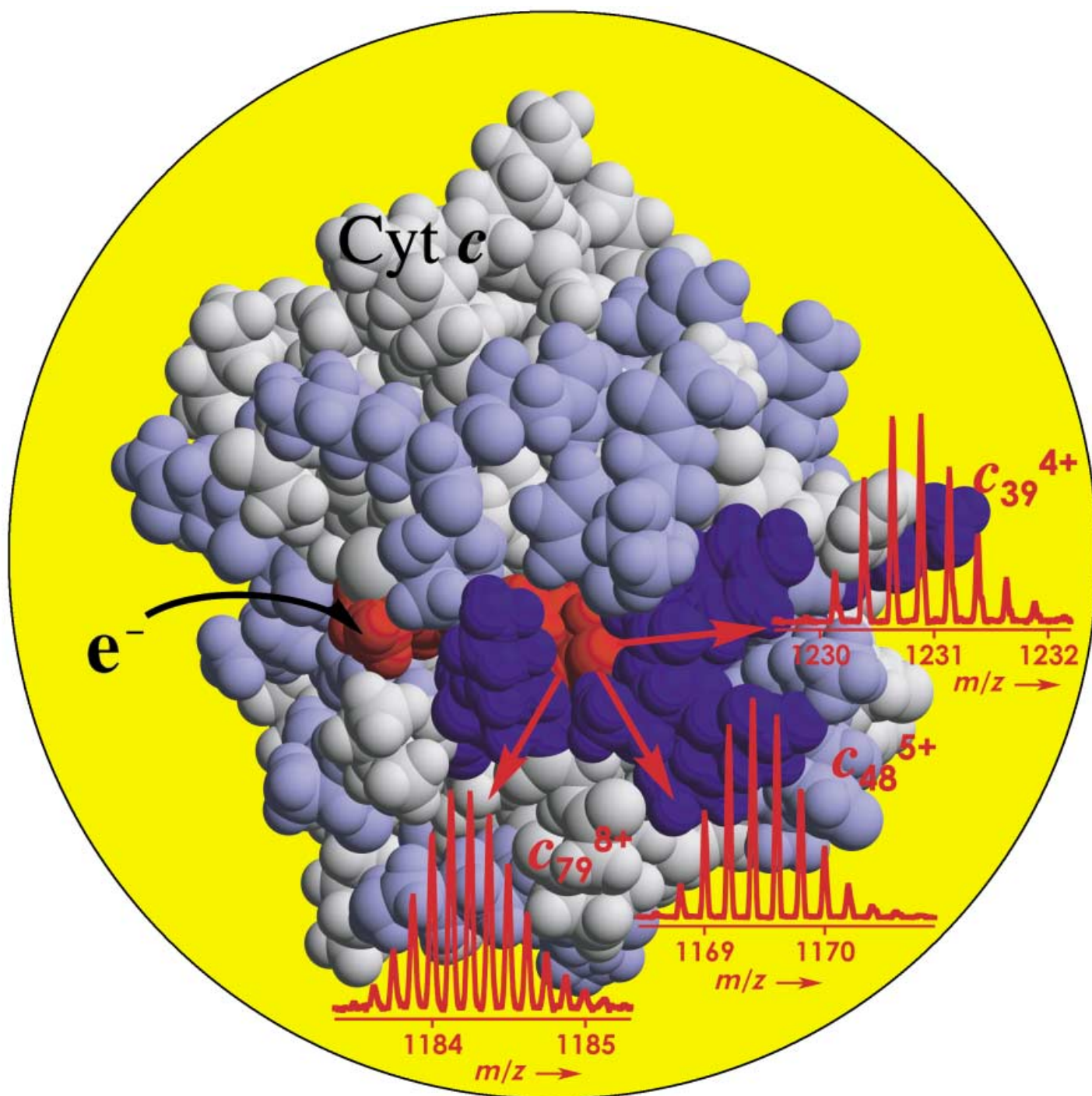# *Communications*

Native electron capture dissociation (NECD) is a new mass-spectrometric method for the structural investigation of noncovalent interactions in native proteins such as Cytochrome *c*. In contrast to conventional ECD techniques no external electrons are added because highly asymmetrical charge distribution (charge partitioning) occurs on dissociation of protein dimers and results in electron transfer.

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Communications

Native Electron Capture Dissociation for the Structural Characterization of Noncovalent Interactions in Native Cytochrome c**

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Recent efforts in biomolecular mass spectrometry (MS) are directed towards life-science problems beyond molecular-weight determination and protein identification,[1] for example, the study of noncovalent protein complexes[2] and protein folding.[2b,3] From electrospray ionization (ESI) MS experiments, the stoichiometry of binding partners within protein–protein, protein–ligand, and protein–nucleic acid complexes can be derived,[2] given that any changes in noncovalent bonding on entrance into the gas phase do not affect this stoichiometry. However, extensive studies show that hydrophobic and hydrogen bonds are weakened and strengthened, respectively, in the gas phase, and that gaseous tertiary structures can differ dramatically from those in solution.[4] The most detailed information on the structurally transformed gas-phase conformers from ESI has come recently from electron capture dissociation (ECD).[4d,g,h,5] We report herein what appear to be ECD mass spectra that instead reflect the *native* structure of the protein, formed *without* the experimental addition of electrons. We term this new technique "native ECD" (NECD).

Cytochrome c (Cyt c), a small electron-transfer protein with an almost spherical shape in its native state (Figure 1),[6] is among the proteins most thoroughly investigated with respect to structure and folding in the gas phase.[2c,3,4a–4f] After ESI of Cyt c solutions retaining the native structure (pH 3.5, 4.5) into a Fourier transform (FT) mass spectrometer, ECD of the

8+ and 9+ molecular ions (MS/MS) gave no product ions from backbone fragmentation; the nonergodic ECD cleaves the protein-ion backbone without rupture of the noncovalent bonding of the tertiary structure.[4d,g,h] After denaturation of the molecular ions by gentle collisional activation,[5d] the ECD spectrum demonstrates cleavage of 69 of the 103 inter-residue bonds in the Cyt c backbone (see Figure 2a). In agreement with the radical mechanism of ECD (Scheme 1) unique products *c*, *z*, and *a* (as well as *y* ions) are obtained; conventional ion-dissociation spectra from energetic cleavage (e.g., collisionally activated dissociation (CAD), or infrared multiphoton dissociation (IRMPD)) of protein ions instead show *b* ($RC\equiv O^+$) as well as *y* ions. Thus we were surprised to find *c* ions in an ESI spectrum of Cyt c (Figure 2b) run without added electrons under conditions of ion activation that produced no *b* ions. The other products in Figure 2b were *y* ions that generally arose by cleavages between the same amino acids that gave the *c* ions. Figure 2b and a show little resemblance; the most favored cleavage, between Lys79 and Met80, of Figure 2b is among the least favored in Figure 2a. Under the same conditions, but after rigorous stirring of the Cyt c solutions, spectra like those in Figure 2c were obtained;
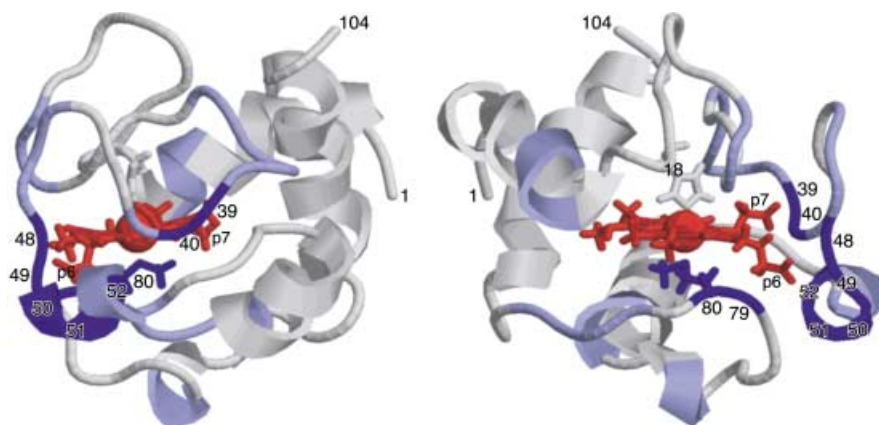

Figure 1. Band and stick representation of native (Fe^{III}) Cyt c (solution structure from the protein data base (PDB file 1AKK[11a]).[17] Red: heme; blue: residues 39, 40, 48–52, 79, 80 framing major NECD cleavages shown in Figure 2b,c; light blue: residues 11–13, 26–28, 33–38, 41, 45–47, 53–55, 68, 69, 72, 73, 82–85 framing minor NECD cleavages. The images differ by a 120° rotation around the vertical axis.

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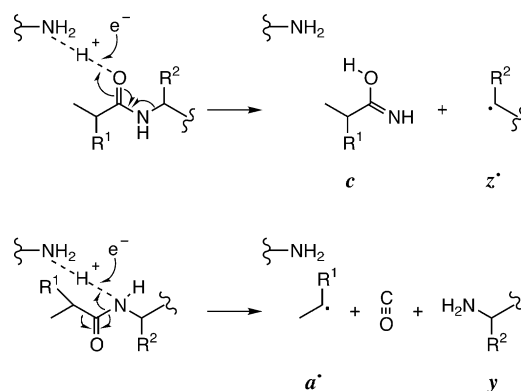
Scheme 1. Mechanism of ECD fragmentation, with the proton originally on an adjacent basic amino acid residue.
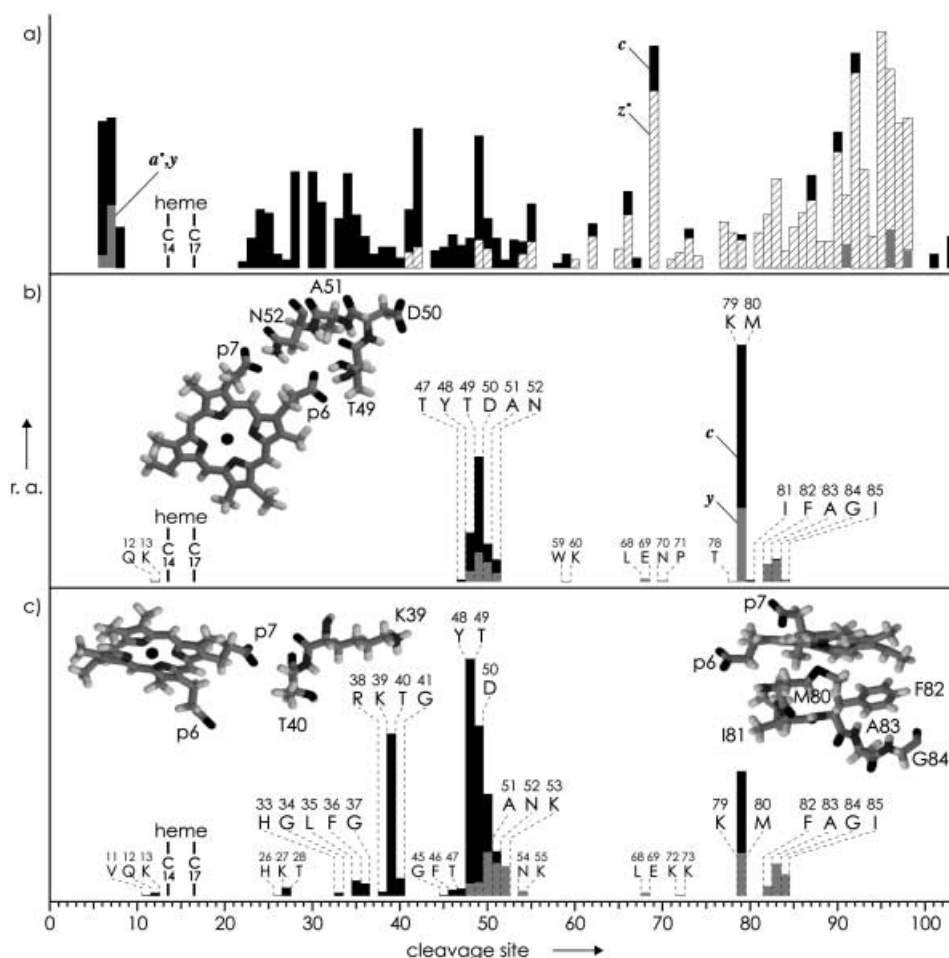
Figure 2. Relative abundances (r.a.) of Cyt c fragment ions: a) spectrum after conventional ECD of molecular ions from ESI of a 10 μM, pH 3.5 aqueous solution (in-beam collisional activation);[5d] b,c) ESI of a 75 μM, pH 4.5 aqueous solution without the external addition of electrons, both 36% NECD yield, with partial structures of heme and neighboring residues of native (Fe^{III}) Cyt c; c) spectrum after 1 h additional stirring of the ESI solution with a Vortex mixer. *c* ions: black bars, *y* ions (*a*· + *y* in (a)): gray bars, *z*· ions: striped bars.

these spectra appear to be the sum of the spectrum in Figure 2b plus a new one, whose formation is still under investigation. Although no extra electrons were added experimentally, the 36% yield of these *c, y* product ions is high compared to that of early ECD studies.[5a]

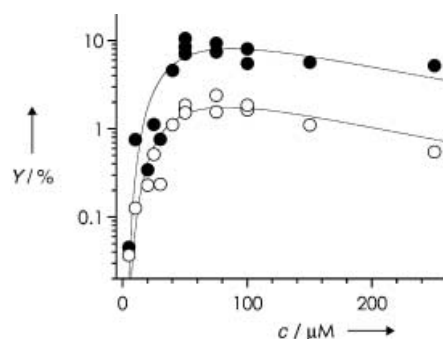The effect of the concentration of the ESI solution on fragment-ion yield is dramatic (Figure 3). In recent fluores-

Figure 3. Yield (Y) of *c* and *y* (●) and *c*$_{79}$ (○) ions from ESI of (Fe^{III}) Cyt c in H$_2$O as a function of solution concentration at pH 5.5. The solid line is to guide the eye.

cence studies, evidence for Cyt c dimerization in electrospray droplets was found for solution concentrations above 33 μM.[7] The corresponding increase shown in Figure 3 suggests that dimers are essential for the formation of *c* and *y* (although no dimer ions were detected in any of these spectra). The fragment yield decreases above 100 μM, presumably because of competitive formation of aggregates larger than dimers. Solution acidity also affects yield; for 100 μM aqueous solutions the following yields were obtained, pH 2.5, 5% (0% in 70% MeOH); pH 3.5, 13%; pH 4.5, 16%, and pH 5.5, 7% (adding 1% glycerol[8] did not affect the yield). These data are consistent with the decreasing denaturation of native Cyt c from pH 2.5 to 4.5,[9] with higher pH values (lower H$^+$ concentration) decreasing overall protonation during ESI. The yield was also strongly influenced by the temperature of the capillary through which the ESI ions enter the mass spectrometer. At 75 μM, pH 5, a reduction from 43 to 28°C decreased *c* and *y* formation from 21% to 2% (Figure 4) of the remaining molecular ions; lowering the pH to 3.5 at 30°C gave a yield of 31%. Formation of the *c* and *y* ions must occur in this capillary region, before extensive solvent removal (≈ 10^{-4} Torr), and requires significantly more energy than
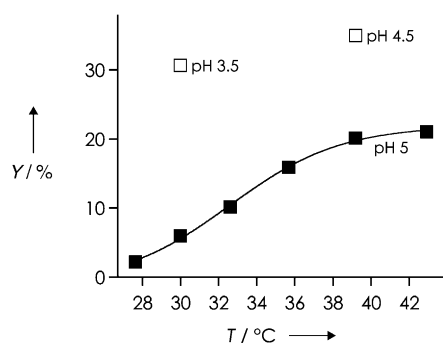
Figure 4. Yield (Y) of c and y ions from ESI of 75 μM (Fe^{III})Cyt$c$ aqueous solutions as a function of capillary temperature (measured at the capillary orifice where ions enter the mass spectrometer); temperatures inside the capillary are proportionally higher by an estimated factor of two. Solid line is to guide the eye.

Figure 5. Yield (Y) of molecular ions $[M+(n-1)H]^{n+}$ (MI, open bars), and $(c_{79}+nH)^{n+}$ and $(y_{25}+nH)^{n+}$ fragment ions (FI, black and gray bars, respectively) from ESI of aqueous (Fe^{III}) Cyt$c$ solutions (10 μM, pH 5.5 and 75 μMm, pH 4.5) versus charge state.

molecular-ion desolvation does. However, changing the instrumental parameters in lower pressure regions, such as the nozzle–skimmer voltage or related ion-lens potentials, had no effect.

The c, y fragmentation pattern of (Fe^{II})Cyt$c$[10] electrosprayed from 100 μM aqueous solution at pH 4.5 (data not shown) was, within experimental error, the same as that of (Fe^{III})Cyt$c$; the Fe^{III} and Fe^{II} native forms differ mainly in the reorientation of a few side chains.[11] Further, all the c products that include the heme unit covalently bound to Cys 14 and Cys 17, obtained from both Fe^{III} and Fe^{II} solutions, have m/z values that indicate reduction to Fe^{II}. This result suggests that there is facile electron transfer in forming the Fe^{II} products from the Fe^{III} precursors. This same source could also supply an electron to effect ECD and form the observed c and y products, although the spectra in Figure 2 b,c show none of the expected complementary $z^{\bullet}$ and $a^{\bullet}$ products (Scheme 1). However, these highly reactive and thermally labile radical species[4g] should undergo many energetic collisions in passage through the capillary region.

An unusual MS/MS dissociation of stable gaseous Cyt$c$ dimer ions electrosprayed from non-denaturing solutions and trapped in a FT-MS cell was recently elucidated by Williams and Jurchen.[12] Although with weak MS/MS (CAD) activation the dimer ions predominantly dissociate into monomers with equal charges, higher energies for activation result in asymmetric charge partitioning. This also appears to be the case for our dimer ions, which dissociate in the capillary region (Figure 5). ESI at 10 μM, a concentration at which little dimer or c and y ions are formed, produces almost exclusively 8+ monomer ions. ESI at 75 μM, a concentration favorable for formation of both dimers and c and y, instead shows asymmetric charge partitioning with monomer charge states as high as 12+. An even higher charge is indicated for the precursors of the c and y ions (Figure 5, below); the sum of the average charges of the quasi-complementary fragments c_{79} (+8.48) and y_{25} (+2.66), together with the two electrons for ECD and heme-iron-center reduction, is consistent with an approximate 13+ charge for their monomeric precursor. This precursor must have depleted the charge of the other monomer to 3+ or 4+, which indicates a huge charge
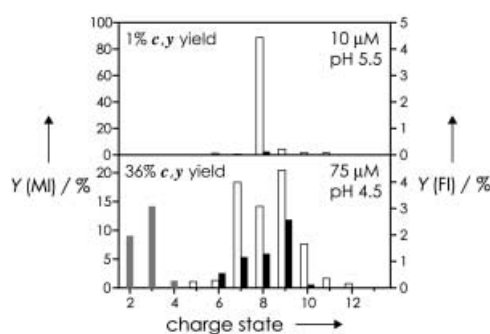
asymmetry prior to dimer dissociation that could have caused the electron transfers producing the c(Fe^{II}), y products. Williams and Jurchen give convincing evidence that this charge asymmetry in protein dimer ions following activation results from the unfolding of one of the monomers.[12] The increased separation of the basic amino acid residues greatly increases their proton affinity, which attracts protons from the other monomer.

Because the c and y ions are found only from ESI of the native Cyt$c$, does its structure[6] also provide a mechanistic rationalization for the highly specific c and y ion formation shown in Figure 2 b,c? At least one of the transferred electrons, that forming the Fe^{II} product, must go to the heme ligand. In both native Fe^{II} and Fe^{III} structures the heme is also noncovalently bound to amino acid residues at which a second electron transfer could cause protein-backbone cleavage (Figure 2 b,c).[11] These residues are all positioned close to the exposed edge of the heme (Figure 1), thought to be the site of biological electron transfer.[13] The most prominent fragments arise from cleavage on the N-terminal side of Met 80 whose sulfur atom distally coordinates the heme iron center (Figure 1 and 2). Other abundant products originate from cleavages next to Thr 40, Thr 49, and Asn 52, which are hydrogen bonded to the carboxylate units of heme propionates 7, 6, and 7, respectively, in (Fe^{III})Cyt$c$ (Asn 52 is hydrogen bonded to p6 in (Fe^{II})Cyt$c$).[11] The only other products, making up less than 1% of the total, are from cleavages close to the heme, such as near Lys 13 and in the 68–71 region. His 18, which is coordinated to the Fe center opposite to Met 80, exhibits no neighboring ECD. The proposal outlined in Scheme 2 supports the assumption that these cleavages, which occur during "gentle" ESI without external electron addition, actually involve the conventional ECD mechanism of Scheme 1. As the dimer passes through the inlet capillary, one of its monomers partially unfolds. The high-energy transition state in solution unfolding involves denaturation of the 3–14 and 90–101 terminal helices (Figure 1, right image, left side) by cleavage of the hydrophobic Phe 10–Leu 94 interhelix bond. These helices contain eight of the 21 basic residues in the monomer. Of the estimated eight protons on the monomer originally, around
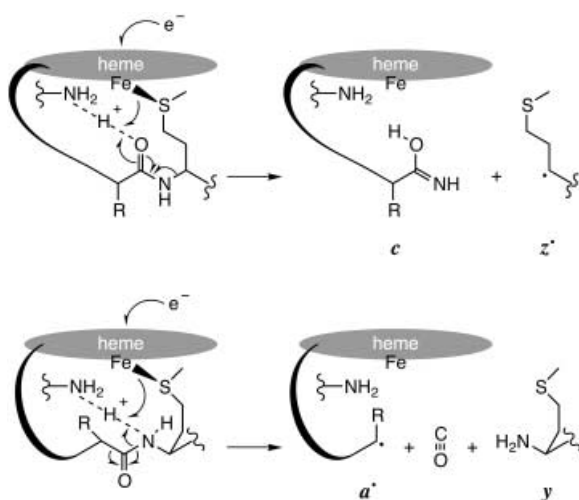
Scheme 2. Proposed mechanism of NECD fragmentation near Met 80.

three of them should be found on these helices. Partial denaturation attracts five protons from the other monomer, enough for protonation of the newly exposed basic sites and to give a total of eight protons on these terminal helices. In response to this huge charge asymmetry, the other monomer gives up electrons to the partially unfolded monomer, with one electron reducing the Fe^{III} center and another electron being transferred to the heme to effect the ECD process shown in Scheme 2.

Cyt c native molecular ions are stabilized significantly by hydrophobic bonding;[14] complete desolvation accompanying ESI causes their denaturation. Previous ECD experiments[4d] have shown that after denaturation gaseous Cyt c ions refold into more stable gaseous ion structures quite different from the native solution structure.[4g] Although ESI of native Cyt c solutions can give $\leq 8+$ molecular ions whose collision-cross-section values agree with those calculated for the native structure,[4e] our evidence indicates that they have unfolded and refolded during transfer to the gas phase. This possibility should be considered seriously before using conventional ESI mass spectra as evidence of noncovalent binding in solution.[2]

We have shown that NECD is a promising new MS technique for the structural probing of noncovalent interactions in native proteins. Charge partitioning in protein dimer dissociation can apparently be so asymmetric that it causes intermolecular electron transfer and ECD of the protein backbone. For Cyt c, the resulting fragment ions directly correlate with noncovalent protein–heme interaction sites in the native structure. Although this appears to be the most detailed evidence reported to date of native conformation retained in transfer to the gas phase, NECD also shows that this conformation is a transient structure even during "gentle" ESI.

Experimental Section

Experiments were carried out on a 6-Tesla FT mass spectrometer described elsewhere.[5c] Ions formed by ESI at atmospheric pressure entered the instrument through a heated capillary and are transferred into the FT-ICR cell ($< 10^{-9}$ torr, N_2 pulse trapping) through quadrupole ion guides. ESI utilized homemade emitters of $\approx 5\,\mu m$ tip internal diameter, flow 200–500 nL min^{-1}, and 1 kV spray potential. Horse heart Cyt c (Sigma, St. Louis, MO, USA) was dissolved in water (nanopure, pH 5.5), lower pH values were obtained by addition of acetic acid. For (Fe^{II}) Cyt c, ascorbic acid was added to give pH 4.5.[10,4c,15] Spectral interpretation utilized the automated THRASH program.[16]

[1] R. Aebersold, M. Mann, *Nature* **2003**, *422*, 198–207; F. Meng, B. J. Cargile, S. M. Patrie, J. R. Johnson, S. M. McLoughlin, N. L. Kelleher, *Anal. Chem.* **2002**, *74*, 2923–2929; G. E. Reid, S. A. McLuckey *J. Mass Spectrom.* **2002**, *37*, 663–675.

[2] a) J. A. Loo, *Mass Spectrom. Rev.* **1997**, *16*, 1–23; b) R. L. Winston, M. C. Fitzgerald, *Mass Spectrom. Rev.* **1997**, *16*, 165–179; c) B. N. Pramanik, P. L. Bartner, U. A. Mirza, Y.-H. Liu, A. K. Ganguly, *J. Mass Spectrom.* **1998**, *33*, 911–920; d) J. A. Loo, *Int. J. Mass Spectrom.* **2000**, *200*, 175–186; e) F. Sobott, C. V. Robinson, *Curr. Opin. Struct. Biol.* **2002**, *12*, 729–734.

[3] U. A. Mirza, S. L. Cohen, B. T. Chait, *Anal. Chem.* **1993**, *65*, 1–6; C. S. Hoaglund-Hyzer, A. E. Counterman, D. E. Clemmer, *Chem. Rev.* **1999**, *99*, 3037–3079; M. F. Jarrold, *Annu. Rev. Phys. Chem.* **2000**, *51*, 179–207; I. A. Kaltashov, S. J. Eyles, *Mass Spectrom. Rev.* **2002**, *21*, 37–71.

[4] a) D. Suckau, Y. Shi, S. C. Beu, M. W. Senko, J. P. Quinn, F. M. Wampler III, F. W. McLafferty, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 790–793; b) D. E. Clemmer, R. R. Hudgins, M. F. Jarrold, *J. Am. Chem. Soc.* **1995**, *117*, 10141–10142; c) F. W. McLafferty, Z. Guan, U. Haupts, T. D. Wood, N. L. Kelleher, *J. Am. Chem. Soc.* **1998**, *120*, 4732–4740; d) D. M. Horn, K. Breuker, A. J. Frank, F. W. McLafferty, *J. Am. Chem. Soc.* **2001**, *123*, 9792–9799; e) E. R. Badman, C. S. Hoaglund-Hyzer, D. E. Clemmer, *Anal. Chem.* **2001**, *73*, 6000–6007; f) R. Grandori, *Protein Sci.* **2002**, *11*, 453–458; g) K. Breuker, H.-B. Oh, D. M. Horn, B. A. Cerda, F. W. McLafferty, *J. Am. Chem. Soc.* **2002**, *124*, 6407–6420; h) H.-B. Oh, K. Breuker, S.-K. Sze, Y. Ge, B. K. Carpenter, F. W. McLafferty, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15863–15868.

[5] a) R. A. Zubarev, N. L. Kelleher, F. W. McLafferty, *J. Am. Chem. Soc.* **1998**, *120*, 3265–3266; b) R. A. Zubarev, N. A. Kruger, E. K. Fridriksson, M. A. Lewis, D. M. Horn, B. K. Carpenter, F. W. McLafferty, *J. Am. Chem. Soc.* **1999**, *121*, 2857–2862; c) R. A. Zubarev, D. M. Horn, E. K. Fridriksson, N. L. Kelleher, N. A. Kruger, M. A. Lewis, B. K. Carpenter, F. W. McLafferty, *Anal. Chem.* **2000**, *72*, 563–573; d) D. M. Horn, Y. Ge, F. W. McLafferty, *Anal. Chem.* **2000**, *72*, 4778–4784.

[6] L. Banci, M. Assfalg in *Handbook of Metalloproteins, Vol. 1* (Eds: A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt), Wiley, New York, **2002**, pp. 33–43.

[7] S. E. Rodriguez-Cruz, J. T. Khoury, J. H. Parks, *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 716–725.

[8] A. T. Iavarone, E. R. Williams, *J. Am. Chem. Soc.* **2003**, *125*, 2319–2327.

[9] Y. Goto, Y. Hagihara, D. Hamada, M. Hoshino, I. Nishii, *Biochemistry* **1993**, *32*, 11878–11885; Y. O. Kamatari, T. Konno, M. Kataoka, K. Akasaka, *J. Mol. Biol.* **1996**, *259*, 512–523.

[10] Cyt c molecular-ion m/z values from electrospray of reducing aqueous solutions (ascorbic acid, Cyt c oxidation state confirmed by UV/Vis spectroscopy) were $[M+nH]^{n+}$, consistent with Fe^{II}; ESI of (Fe^{III}) Cyt c gave $[M+(n-1)H]^{n+}$.

[11] a) L. Banci, I. Bertini, H. B. Gray, C. Luchinat, T. Reddig, A. Rosato, P. Turano, *Biochemistry* **1997**, *36*, 9867–9877; b) L. Banci, I. Bertini, J. G. Huber, G. A. Spyroulias, P. Turano, *J. Biol. Inorg. Chem.* **1999**, *4*, 21–31.

[12] J. C. Jurchen, E. R. Williams, *J. Am. Chem. Soc.* **2003**, *125*, 2817–2826.

[13] J. Rawlings, S. Wherland, H. B. Gray, *J. Am. Chem. Soc.* **1976**, *98*, 2177–2180; A. J. Ahmed, F. Millett, *J. Biol. Chem.* **1981**, *256*, 1611–1615; W. H. Koppenol, E. Margoliash, *J. Biol. Chem.* **1982**, *257*, 4426–4437; H. S. Pappa, T. L. Poulos, *Biochemistry* **1995**, *34*, 6573–6580.

[14] L. Hoang, S. Bédard, M. M. G. Krishna, Y. Lin, S. W. Englander, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12173–12178.

[15] F. He, C. L. Hendrickson, A. G. Marshall, *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 120–126; K. A. Johnson, B. A. Shira, J. L. Anderson, I. J. Amster, *Anal. Chem.* **2001**, *73*, 803–808.

[16] D. M. Horn, R. A. Zubarev, F. W. McLafferty, *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 320–332.

[17] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235–242.