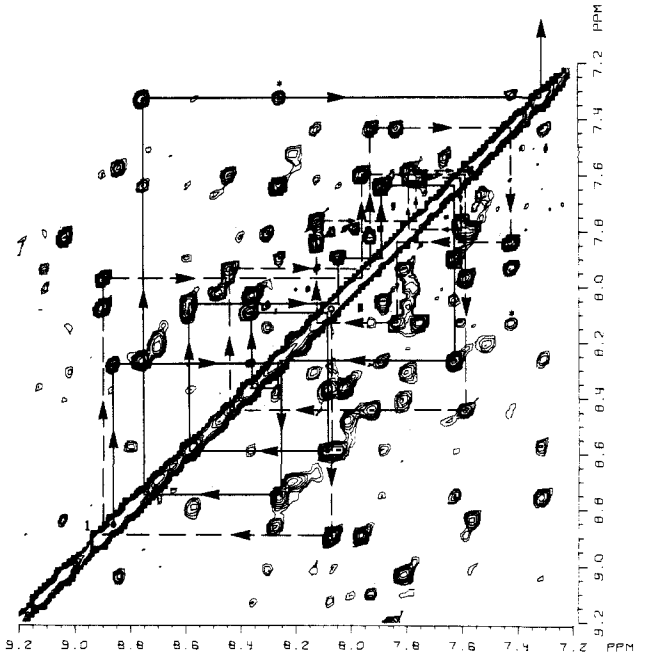

Figure 2. Two long sequences of d_{NN} connectivities in the 7.2–9.2-ppm region of the Nase NOESY spectrum. The sequence of d_{NN} connectivities traced by the solid line originates at the filled circle, terminates at 6.69 ppm, and links 12 residues. Two of the $d_{NN}(i,i+2)$ connectivities in this sequence are indicated by asterisks. The sequence of d_{NN} connectivities traced by the dashed line originates at the open circle, terminates at the open square, and links 13 residues. Experimental parameters as in Figure 1, except the mixing time was 0.3 s.

contrast the NOESY spectrum of the protiated protein obtained under the same conditions (not shown) shows strong cross-peaks mainly for the aromatic protons and for the nonequivalent sidechain amide protons. While the sidechain amide cross-peaks are seen in Figure 1, most of the cross-peaks in this spectrum are in sequences of d_{NN} connectivities. One such sequence, linking eight NH's, is indicated in the figure. The sequential assignments are given at the top of the figure.

Two longer sequences of d_{NN} connectivities are shown in the expanded NOESY spectrum, Figure 2, obtained by using a 0.3-s mixing time. The longer mixing time enables us to observe $d_{NN}(i,i+2)$ connectivities[1] as well. The observation of the $d_{NN}(i,i+2)$ connectivities removes possible ambiguities in a sequence of d_{NN} connectivities that can result from chemical shift degeneracy. Indeed, the $d_{NN}(i,i+2)$ connectivites permitted us to make an unambiguous assignment of the eight residue and thirteen residue d_{NN} sequences, in spite of a chemical shift degeneracy involving these sequences at 7.94 ppm. The $d_{NN}(i,i+2)$ connectivities are not normally found in the spectrum of a protiated protein because the large mixing time required to observe these signals generates many interfering second-order cross-peaks, and spin diffusion to nonexchangeable protons strongly attenuates the connectivity. We have not yet quantified the contributions of direct magnetization exchange and spin diffusion to the $d_{NN}(i,i+2)$ connectivities.

We have assigned residues in all three long sequences of d_{NN} connectivities by using the NOESY spectra and ^1H detected shift correlation spectra[13a-d] of $^{15}N/^{13}C$ labeled samples. These assignments, which will be presented elsewhere, show that each d_{NN} sequence corresponds to an α-helical domain[8c] of the protein.

The perdeuteriation approach presented here opens the way to investigate the effects of important variables such as site mutations, ligand interaction, etc. upon the helical domains of many proteins. Considering the fact that excellent spectra were obtained by using a protein concentration of 1.5 mM and physiological conditions, it is likely that the experiment will be effective on proteins much larger than Nase, particularly when performed on the higher sensitivity instruments now available.

It is clear that the present methodology can be extended by incorporating protiated or selectively deuteriated amino acids into an otherwise deuteriated protein. In this way it should be possible to link the assigned α-helical peptide protons to various sidechain and backbone protons within and outside of the α-helical domains.

After this manuscript was submitted, the sequential assignment of randomly deuteriated thioredoxin, MW = 11.7 kD, was reported by LeMaster and Richards.[14] Their spectra clearly show the benefits of protein deuteriation in applications of 2D NMR spectroscopy to proteins having molecular weights above 10 kD.

Note Added in Proof. The Nase solution used herein had a pH meter reading of 6.97.

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"Solid-State" Voltammetry of a Protein in a Polymer Solvent

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We report the first example of diffusion-controlled voltammetry of a redox protein dissolved in a biocompatible, semirigid polymer film. The experiment[1] is based upon humidifying or adding liquid microdroplets to thin, ionically conductive films of polyethylene oxide (PEO) or polyacrylamide to form thin, semirigid gel solutions resting on a coplanar set of three electrodes (Figure 1). "Solid-state" voltammetry in this electrochemical "gel-cell" can be used to study how the electron transfer and associated chemistry of proteinaceous materials respond to structured, viscous media. The gel-cell also offers a novel approach to protein "immobilization"[4] in the design of bioanalytical sensors.

The direct (unmediated) voltammetry[5] of cytochrome *c* was

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(1) The cell, Figure 1, consists of a 2.4-mm diameter EPG disk with concentric Pt foil (0.1 mm) ring auxiliary electrode and 0.35-mm diameter Ag wire tip reference electrode, all encapsulated in epoxy. The polymer films are cast from PEO_{18}·$LiCF_3SO_3$ solution[2] or for polyacrylamide by laying a disk of swollen or dry (ca. 50 μm) gel on the surface of the electrode assembly. Cytochrome *c*, $CpFeCpCH_2N(CH_3)_3^+PF_6^-$, and electrolyte are sorbed as solution microdroplets into N_2-dried gels, or by premixing with the PEO_{18}·$LiCF_3SO_3$ casting solution, or for polyacrylamide by gel-cell equilibration in a reagent solution. The polyethylene oxide and polyacrylamide contain $LiCF_3SO_3$ (or NaCl) and 0.1 M KCl electrolyte, respectively. Polyacrylamide gels (2% T, 2.6% C_{bis}, ca. 28% polymer by weight) were prepared[3] by room temperature, radical polymerization of 9.68 mL of a degassed, aqueous solution of acrylamide (9.68 mL) and *N,N'*-methylenebisacrylamide with 3-(dimethylamino)propionitrile (0.26 mL of 1% solution) and ammonium persulfate (0.1 mL of 1% solution) followed by thorough washing both before and after mounting on gel-cells to remove residual electroactive ammonium persulfate.

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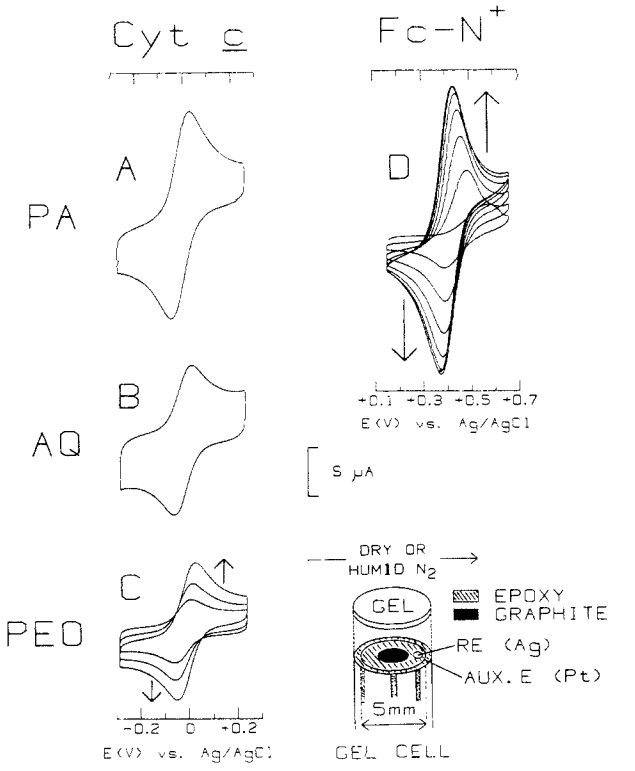

Figure 1. Cyclic voltammograms (20 mV/s) recorded in a gel-cell[1] (inset). (A) Cytochrome *c* in polyacrylamide. Cytochrome *c* (10 μL, 150 μM) in 0.1 M KCl was sorbed into a dry polyacrylamide gel-cell which was redried (N_2) and then equilibrated in humidified N_2. S = 0.27 μA. (B) Cytochrome *c* (150 μM) in aqueous solution, 20 mM Tris-HCl, 100 mM KCl, pH 7.5: S = 0.2 μA. (C) Cytochrome *c* in PEO. Cytochrome *c* (20 μL, 150 μM) in PEO_{18}-$LiCF_3SO_3$ was cast on the cell, and cyclic voltammograms were recorded at 10, 20, and 25 min during the 33-min evaporation of the casting film in N_2: S = 0.4 μA. (D) $CpFeCpCH_2N(CH_3)_3^+PF_6^-$ in polyacrylamide. 20 μL of 120 μM ferrocene derivative in 0.1 M KCl was sorbed into a dry polyacrylamide gel-cell which was redried (N_2) and then exposed to a humid N_2 stream, while consecutive cyclic voltammograms were recorded: S = 1 μA.

used to test the biological compatibility of the gel-cell and to investigate the electrochemical reaction of this redox protein in the semirigid polymers. We found that cytochrome *c* gives well defined cyclic voltammograms in polyacrylamide and PEO gel solutions by using an edge-plane pyrolytic graphite (EPG) working electrode (Figure 1 (parts A and C)). Both the formal potential ($E^{\circ\prime}$ = −20 mV versus Ag/AgCl) and heterogeneous electron transfer rate constant (k_s = 0.0027 cm/s) derived[6] from the protein voltammetry in polyacrylamide and in PEO gels are experimentally indistinguishable from values derived from aqueous solution voltammetry (Figure 1B) at naked EPG electrodes. We conclude that the polymer strands adjacent to the electrode surface in these gels do not significantly change either the thermodynamics or the kinetics of electron transfers of cytochrome *c* at EPG electrodes.

Voltammetric peak currents in gel solutions vary linearly with (potential scan rate)$^{1/2}$, which means that the protein electrode reaction is governed by diffusion through the polymer gel and not by adsorbed or precipitated material. This result is consistent with the high internal water content of PEO and polyacrylamide gels,[1,7] in which a protein molecule is likely to be surrounded by a linear (the percentage of cross-linking is low[1]), flexible, uncharged, hydrophilic polymer shell and its bound water. The diffusion of cytochrome *c* is slower, however, even in highly swollen[6b] polyacrylamide films (2.1×10^{-7} cm^2/s), than in aqueous solution (1.0×10^{-6} cm^2/s). The difference is not straightforwardly a viscosity effect since the small redox cation $CpFeCpCH_2N(CH_3)_3^+$ diffuses only 1.4× slower in the gel. Despite the high internal water content of the gel, diffusion of the protein seems limited by the network of polymer strands in whose interstitial spaces it is dissolved.

It is interesting to vary the quantity of gel-bound water since this variable is not usually addressed in gel polymer studies. Manipulating gel water contact via bathing gas humidity or by sorbing microdroplet solutions into dry gels[1] reveals two effects of changing the water content: diffusion rate and concentration.

Introducing sorbed water into dry gels increases diffusion rates of electroactive components as illustrated (Figure 1D) by the increasing currents in the sequence of $CpFeCpCH_2N(CH_3)_3^+PF_6^-$ cyclic voltammograms observed upon placing a dry polyacrylamide gel-cell, in which voltammetric currents are initially negligible, in humidified N_2. An equilibrated,[8] reversible, stable voltammogram (ΔE_p = 57 mV at 20 mV/s) is soon obtained. A similar response is obtained upon humidifying polyacrylamide gels containing cytochrome *c* (Figure 1A) and in PEO gels containing the ferrocene or cytochrome *c* (not shown). The stable protein electrochemistry in Figure 1A is typical and is unaffected by successive drying/humidification cycles in polyacrylamide, but voltammetry in PEO is somewhat degraded (ΔE_p increases) by drying/humidification cycles.

Currents are also increased when highly swollen gels are allowed to evaporate, which concentrates the electroactive solute as illustrated in Figure 1C for cytochrome *c* voltammetry. Evaporation of the droplet of aqueous casting solution is accompanied by increasing currents but no increase in ΔE_p in the electrochemical response until the PEO gel film is nearly dry, whereupon the current decreases (fairly abruptly) owing to the mobility effect described above.

These voltammetric experiments establish a basis for quantitative investigation of protein transport and electron-transfer properties in semirigid polymers containing a systematically variable solvent-to-polymer ratio. We anticipate that solid-state voltammetry with gels will contribute to miniaturized[2] biosensor design and to solid-state enzyme catalysis, topics under current investigation.[9] We further note that there is precedent[10] for using polyacrylamide gels in voltammetry and for the biocompatibility[3,11] of PEO and polyacrylamide, and other gel materials could be selected in the same light.

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Registry No. PEO, 25322-68-3; $LiCF_3SO_3$, 33454-82-9; NaCl, 7647-14-5; KCl, 7447-40-7; cytochrome *c*, 9007-43-6; acrylamide-*N*,*N*'-methylenebisacrylamide copolymer, 25034-58-6.

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