Chemical exchange in two dimensions in the 1H NMR assignment of cytochrome c

Yiqing Feng,* A.Joshua Wand,‡ Heinrich Roder,* and S. Walter Englander*
*Department of Biochemistry and Biophysics, University of Pennsylvania 19104; and ‡Institute for Cancer Research, Fox Chase Cancer Center, Fox Chase, Pennsylvania 19111 USA

ABSTRACT The important role played by chemical exchange in solving the proton assignment problem for oxidized and reduced horse cytochrome c is described. Some novel approaches for establishing oxidation-reduction exchange correlations in combinations of several two-dimensional spectra were used. Unambiguous chemical exchange correlations were established for 55 NH-C_αH resonances and all the aromatic and side chain methyl resonances. Consistent although not fully unambiguous main chain proton correlations were observed for 47 of the remaining 49 residues. The many exchange correlations found serve to multiply cross-connect the two extensive, individually self-consistent networks of assignments found for the oxidized and reduced forms, and thus help to confirm both sets of assignments.

INTRODUCTION

Cytochrome *c* has been used as a model protein in literally thousands of studies of protein structure, dynamics, interaction, and function. The recent solution of the proton resonance assignment problem for both reduced (1) and oxidized (2) horse cytochrome *c* now opens the possibility of applying the full power of two-dimensional nuclear magnetic resonance (2D NMR) methods to such studies. For that work, the reliability of the assignments becomes a central issue. The results described here address this issue.

The assignment of proton resonances to their parent protons in proteins rests on analyses of a variety of 2D and/or higher order NMR spectra. The level of confidence obtained in any particular case is determined by the ability to document consistent and diagnostic behavior of side chain spin systems in J-correlated spectra (e.g., reference 3) and by the density and self consistency of NOE connectivities among residues brought close together in secondary structural elements and tertiary folds (4). Because cytochrome *c* is a rather large protein by NMR standards, its 2D NMR spectra exhibit a high degree of cross-peak crowding, especially in the side chain region, and provide a relatively low density of NOE connectivities due to the protein's low content of regular secondary structure. With many proteins, complete confidence in proposed assignments and the resolution of outstanding ambiguities can often be achieved only by parallel studies such as selective isotopic enrichment combined with spectral editing (5–6).

Cytochrome *c* provides an alternative avenue. As an electron transport protein, cytochrome *c* can exist in both reduced and oxidized forms and can be made to interconvert between these forms by electron self exchange on the chemical shift time scale (7). Because the chemical shifts of many protons in cytochrome *c* change with the redox state, chemical exchange correlation can be used to generate cross-peaks in a 2D spectrum. Magnetization transfer in an exchanging system was among the earliest applications of 2D NMR spectroscopy (8). The utility of the exchange process has been demonstrated in NMR studies of the cytochromes *c* by a number of groups (7, 9–13), and it has more recently been used in studies of macromolecular equilibria such as ligand binding (e.g., reference 14) and protein folding transitions (15).

Here we show examples that illuminate the ways in which exchange effects were used to determine and establish the reliability of the proton assignments in cytochrome *c*. The combination of chemical exchange with several different types of 2D NMR experiment led to the assignment of difficult resonances in both redox states, allowed resonance assignments to be extended from one redox form to the other, and made it possible to extensively cross-check assignments made for given protons in the two different forms, thereby, greatly increasing the confidence level of the assignments.

MATERIALS AND METHODS

Sample preparation

Horse heart cytochrome *c* from Sigma Chemcal Co. (St. Louis, MO) (Type VI) was used without further purification. Dithionite ($Na_2S_2O_4$)

Dr. Y. Feng's present address is Department of Biochemistry, University of Illinois, Urbana, Illinois 61801.
Address correspondence to S. Walter Englander.

and potassium ferricyanide, added in minimal amounts to ensure full reduction or oxidation, respectively, were removed by filtering the cytochrome *c* solutions through Sephadex G25 columns equilibrated with buffer containing 150 mM NaCl and 100 mM phosphate at pH or pD 5.7 (uncorrected pH meter reading at 22°C). Half oxidized samples were prepared by mixing equal amounts of fully reduced and fully oxidized cytochrome *c* for a final protein concentration of 8–10 mM. NMR tubes were filled with argon gas, and the tubes were sealed with tight rubber stoppers. Under these conditions, the ratio of reduced to oxidized protein was found to remain stable for several days.

NMR spectroscopy

All proton NMR spectra were recorded at 20°C on a model AM-500 spectrometer (Bruker Instruments, Inc., Billerica MA). Two-dimensional chemical exchange spectra were recorded as described for NOESY experiments in Feng et al. (1) but with a mixing time of 50 ms. The exchange TOCSY experiment was described by Feng and Roder (12). Parameters used for double-quantum filtered COSY spectra were as given in Feng et al. (1).

RESULTS

Chemical exchange in cytochrome c

Fig. 1 is a 2D-chemical exchange spectrum recorded on a 1:1 mixture of oxidized and reduced cytochrome *c* with a mixing time of 50 ms. Each prominent cross-peak originates from the exchange correlation of individual proton resonances in the two different oxidation states. When the chemical shift of a proton is not significantly affected by the redox state, its exchange cross-peak cannot be resolved from the diagonal. The change in chemical shifts measured at over 400 proton positions has been compared with the shifts calculated for the various protons in oxidized cytochrome *c* on the basis of the paramagnetic g-tensor derived for the spin one-half heme iron (16).

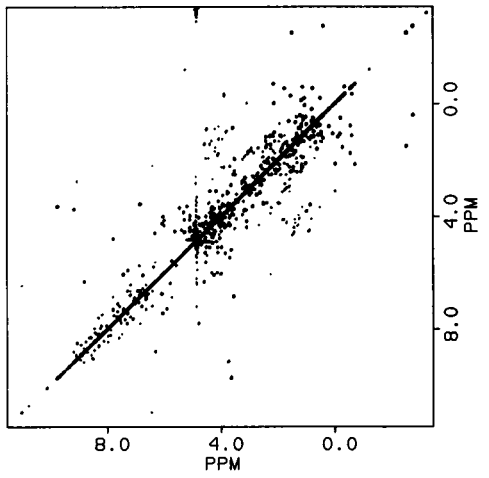

FIGURE 1 Two-dimensional chemical exchange spectrum of a 1:1 mixture of ferri- and ferrocytochrome *c* in D_2O.

The pulse sequence used for recording the chemical exchange spectrum is the same as that for a NOESY spectrum (8), so that cross-peaks in Fig. 1 may be produced not only by chemical exchange effects but also by the many intramolecular NOE effects in both oxidation states. However, the two processes are governed by different mechanisms, which can be utilized to selectively enhance one or the other (11). The moderately high salt concentration used in this study leads to an exchange rate that is fast compared to cross-relaxation rates (13, 17). This allows the use of a relatively short mixing time, which minimizes NOE effects. The fast chemical exchange further acts to reduce the intensity of nuclear Overhauser effects by competing for magnetization. As a result, essentially all the prominent cross-peaks in the 2D spectrum can be attributed to chemical exchange between the two functional forms. The NOE cross-peaks that do appear in Fig. 1, recognized by comparison with NOESY spectra for fully reduced or fully oxidized samples recorded with a longer mixing time that enhances NOESY cross-peak amplitudes, are typically much smaller than the exchange cross-peaks and can be clearly distinguished.

Double chemical exchange analysis

When the resonance of a proton is resolved in the 1D spectrum of either the reduced or oxidized protein, its exchange cross-peak can be unambiguously identified, because no other exchange cross-peak appears along the resolved frequency coordinate. However, when a proton is poorly resolved in both protein forms, by far the more usual case for a protein the size of cytochrome *c*, additional information is necessary to specify cross-peak identity. The information required may be obtained by correlating resolved peaks in 2D spectra.

Fig. 2 illustrates an analysis that uses the 2D exchange and COSY spectra to correlate the frequencies of a given NH-C_αH pair. The fingerprint region of the COSY spectrum is shown in Fig. 2 *A* for oxidized cytochrome *c* and in *D* for the reduced protein. Fig. 2, *B* and *C*, show matching spectral regions from the 2D-chemical exchange spectrum. Consider for example the NH-C_αH cross-peak in the oxidized COSY (Fig. 2 *A*) marked Ile95. At the NH and C_αH frequencies specified by the *J*-coupled cross-peak, one searches the exchange spectrum for matching NH–NH (Fig. 2 *C*) and C_αH-C_αH (Fig. 2 *B*) exchange-correlated cross-peaks. Candidate cross-peaks are tested for similar matching with NH-C_αH cross-peaks in the reduced COSY (Fig. 2 *D*). The presence of two COSY and two exchange cross-peaks that form a rectangular pattern, as indicated in Fig. 2 for

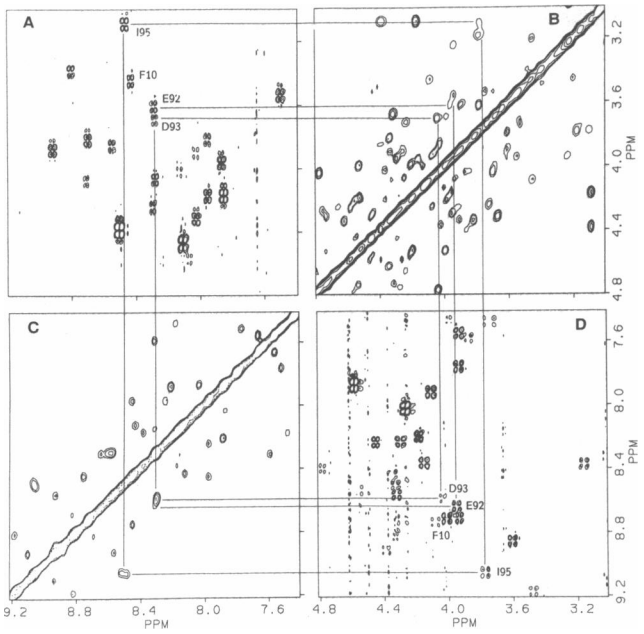

FIGURE 2 Sections of DQF-COSY spectra of ferricytochrome *c* (*A*) and ferrocytochrome *c* (*D*), in register with matching sections of the chemical exchange spectrum of a half oxidized:half reduced sample in D_2O solution (*B* and *C*).

Ile95 and several other residues, can establish a unique correlation between the NH-C_αH cross-peak in the oxidized COSY and the NH-C_αH cross-peak in the reduced COSY. This analysis provides both the NH_{ox}-NH_{red} frequency correlation and the $C_\alpha H_{ox}$-$C_\alpha H_{red}$ frequency correlation.

Exchange correlations can also be useful for resolving degeneracies. An example appears in Fig. 2 for Glu92 and Asp93. The two residues, sequence neighbors in the COOH-terminal helix, exhibit amide proton chemical shifts that are identical in oxidized cytochrome *c* (8.28 ppm), and very similar in the reduced protein. Although these residues have recognizably different spin systems, they could not be identified in *J*-correlated spectra due to spectral overlap, and the helical NOE connectivities in the NOESY spectrum of the oxidized protein were not visible, so that the main chain NOE connectivity search pursued in the assignment process (18) could not proceed unambiguously at this point. The problem was solved by use of the chemical exchange spectrum. Because the NH and C_αH assignments of Glu92 and Asp93 could be independently established in the reduced form (reference 2; see Fig. 2 *D*), their connection with the appropriate NH–NH and C_αH–C_αH exchange correlations (Fig. 2) led to straightforward assignments in the oxidized form. Similar examples occurred in both protein forms.

Another example is shown in Fig. 3 for the fast flipping Phe82 aromatic ring. Because COSY cross-peaks for aromatic ring protons generally fall near the diagonal, correlations that otherwise require four aligned panels, as in Fig. 2, can be obtained in a single overlapped representation (Fig. 3) that superimposes, onto the exchange spectrum, sections of phase sensitive COSY spectra for the oxidized protein (*upper triangle*) and the reduced protein (*lower triangle*). One can easily recognize the box pattern that interconnects the exchange cross-peak for the Phe 82 C_4H, the exchange cross-peak for $C_{3,5}H$, and their *J*-coupling cross-peaks in the oxidized (*upper right*) and reduced (*lower left*) spectra.

The $C_{2,6}H$ and $C_{3,5}H$ resonances of Phe82 occur at nearly the same frequency (6.07 and 6.04 ppm) in the oxidized state. Thus, the normally strong COSY cross-peak between them is not seen, the aromatic ring exhibits an apparent AX spin system (mimicking a fast flipping tyrosine), and one cannot define the correct spin system in the oxidized protein spectrum. However, these resonances are resolved in the reduced protein (6.70 and 7.49 ppm). Given the result just described for $C_{3,5}H$ and C_4H, one can then construct in Fig. 3 the box pattern that connects the exchange cross-peaks for $C_{3,5}H$ and

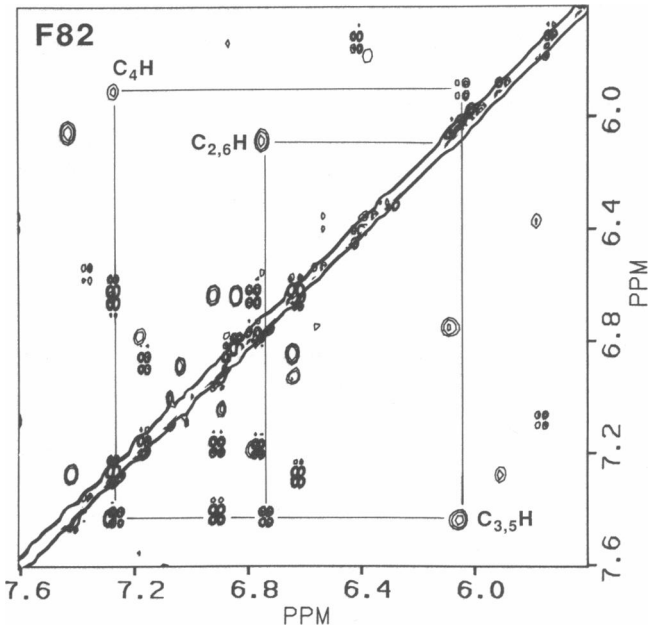

FIGURE 3 Superposition of phase sensitive COSY sections for ferri- (*A*, *upper triangle*) and ferrocytochrome *c* (*B*, *lower traingle*) onto the matching section of a chemical exchange spectrum in D_2O. The chemical exchange cross-peaks are labeled. COSY cross-peaks for aromatic CH protons fall near the diagonal, so that the correlations that require four aligned panels in Fig. 2 can be shown here in a single overlay.

$C_{2,6}H$ with their reduced COSY cross-peak (*lower left*) and identify the missing, near diagonal, oxidized COSY cross-peak. This comparison of the exchange and COSY spectra reveals the near degeneracy of the $C_{3,5}H$-$C_{2,6}H$ resonances in the oxidized form, and confirms the assignments for Phe82 in the oxidized state (19).

By use of these chemical exchange correlation methods, the main chain NH-$C_\alpha H$ resonance assignments of 53 residues, listed in Table 1, and all the methyl and ring proton resonances in both oxidation states of cytochrome *c* could be cross-correlated. For many residues, confidence in the assignments was further enhanced by the observation of relayed effects in TOCSY spectra, as discussed below.

Cross correlation in relayed TOCSY spectra

The crowding of many exchange cross-peaks close to the diagonal (Fig. 1) tends to obscure the useful information they carry, and even makes insecure some of the double chemical exchange correlations described above, involving e.g., the exchange correlated cross-peaks of *J*-coupled NH-$C_\alpha H$ to NH-$C_\alpha H$. Improved resolution can be obtained in relayed magnetization transfer experiments. Feng and Roder (12) have shown that relayed effects can be generated efficiently in TOCSY spectra without introducing an additional delay because both coherent and incoherent magnetization transfer occur simultaneously during the MLEV mixing sequence.

A relayed TOCSY experiment run on a mixture of chemically exchanging reduced and oxidized cytochrome *c* is shown in Fig. 4. The identity of the residue that gives rise to each rectangular cross-peak pattern is indicated next to the NH-$C_\alpha H$ cross-peak of the ferric form. At the corner opposite to the label is the NH-$C_\alpha H$ cross-peak of the ferrous state. These can be distinguished by comparison with TOCSY spectra recorded on fully oxidized or fully reduced samples. The two remaining cross-peaks in each rectangle are the relayed exchange cross-peaks, in this case one from NH(II) to $C_\alpha H$(III) and the other from NH(III) to $C_\alpha H$(II). (Oxidation states II and III correspond to reduced and oxidized forms, respectively.)

Relayed exchange effects in the TOCSY spectrum can move exchange information away from the diagonal into less congested spectral regions and reduce the assignment work to a pattern recognition problem. Each pair of *J*-coupled protons gives rise to an easily recognizable pattern, a rectangle with *J* cross-peaks at two corners and relay cross-peaks at the opposite corners. The exchange TOCSY approach also eliminates ambiguities in the recognition of NOESY cross-peaks because these have opposite phase relative to exchange and *J* cross-peaks. We found this feature especially useful when the intensity of exchange cross-peaks fell to the level of NOE cross-peaks due to relatively rapid relaxation in the paramagnetic oxidized form.

An analysis for Pro71 is shown in Fig. 5. Prolines are usually difficult to assign because their resonances appear in the most crowded region of the spectrum. However, Pro71 is readily recognized in the ferric form because the paramagnetic center shifts some of its resonances downfield into a less crowded region. Given the relayed connectivities shown in Fig. 5, the assignments of Pro71 in the reduced form, although poorly resolved, are unambiguous. With the $C_\alpha H$(II) identified from its NOE to Lys72 NH and exchange correlation to $C_\alpha H$(III), the two $C_\beta H$(II) and the $C_\gamma H$(II) resonances can be identified from the relayed cross-peaks to $C_\alpha H$(III)

TABLE 1 Main chain assignments confirmed unambiguously by exchange experiments

	1	2	3	4	5	6	7	8	9	10
01	Gly							Lys	Ile	Phe
11	Val		Lys	Cys	Ala	Gln	Cys	His	Thr	Val
21		Lys			Iys				Gly	
31	Asn	Leu	His				Gly			
41						Phe	Tyr		Thr	
51				Asn			Ile			
61	Glu	Glu		Leu	Met		Tyr	Leu		Asn
71	Pro	Lys	Lys		Ile	Pro	Gly	Thr		Met
81	Ile	Phe								Glu
91	Arg	Glu	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Lys
101	Ala			Glu						

The NH and $C_\alpha H$ resonance of residues listed and not underlined were documented by double chemical exchange experiments only. Residues underlined were confirmed by relayed exchange connectivities in the TOCSY experiments only. The double underline indicates confirmation by both exchange and relayed connectivities. Consistent although not fully ambiguous exchange correlations were found for the main chain protons of all but two of the remaining 49 residues.

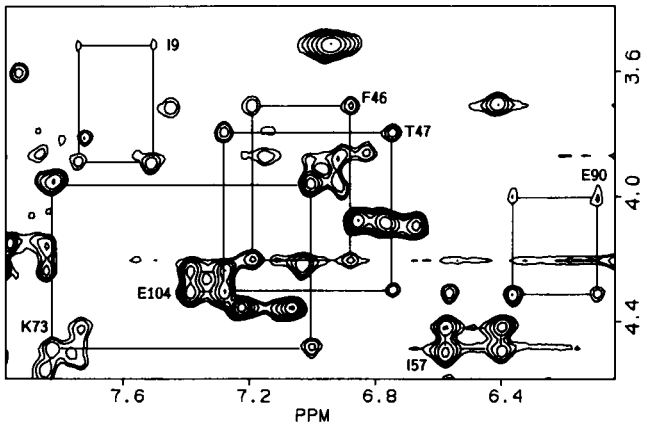

FIGURE 4 Section of TOCSY spectrum for a half oxidized sample in 90% H_2O/10% D_2O. Selected assignments are labeled at the NH-C_αH J-coupled cross-peaks in the oxidized form. The reduced form NH-C_αH cross-peak is at the opposite corner, and the pertinent exchange cross-peaks (NH(II)-NH(III) and C_αH(II)-C_αH(III)) complete the rectangular pattern.

and the two C_β(III), respectively. The rest of the spin system can be readily defined in a TOCSY spectrum of the fully reduced protein.

In exchange TOCSY spectra like these, unambiguous correlations were observed for main chain NH-C_αH proton assignments of 30 residues (Table 1), with 28 of them showing recognizable correlations in both chemical exchange and TOCSY spectra.

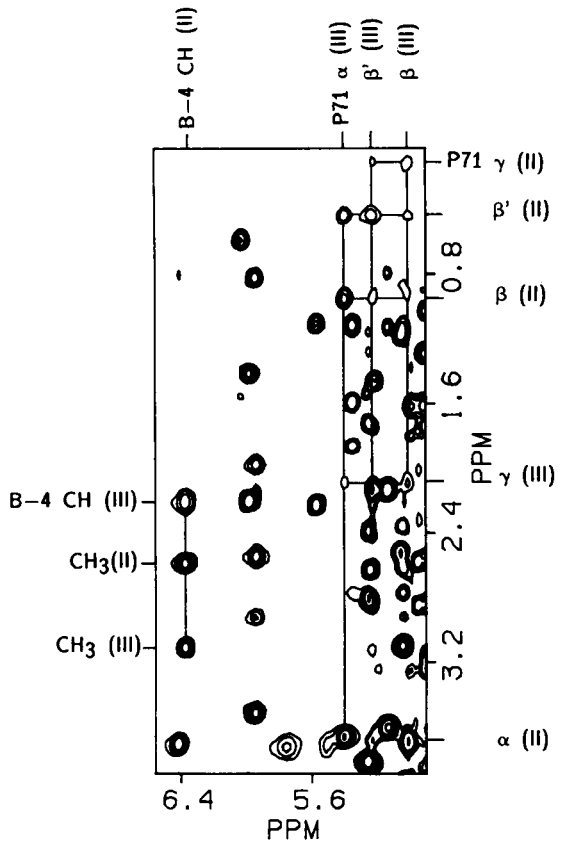

FIGURE 5 Section of exchange TOCSY spectrum of a mixture of ferri and ferrocytochrome *c* (mixing time 22 ms). Exchange cross-peaks are labeled for the Pro71 CH protons in oxidized (III) and reduced (II) states. The heme bridge-4 methyl and methenyl proton cross-peaks are also shown.

DISCUSSION

Our assignment work on cytochrome *c* (1, 2) was primarily based on the search for connectivities and certain predetermined patterns of connectivities involving NH, C_αH, and C_βH protons, as specified in the main chain directed (MCD) assignment strategy (18). The main chain resonances occur in the less congested regions of 2D spectra, an advantage that becomes increasingly necessary as larger proteins are studied. Another advantage of this approach is that the congestion that does occur among amide NH cross-peaks (in COSY, NOESY, or TOCSY spectra) can be reduced at will by taking advantage of the exchange between NH and solvent protons, which allows for H-D exchange editing of the spectra (1, 20, 21).

By use of the chemical exchange-based methods described here, both in D_2O and 90% H_2O solvent, main chain assignments in 55 of the 104 residues of cytochrome *c* could be cross-correlated in the reduced and oxidized forms (Table 1). For the remaining 49 residues, fully unambiguous exchange correlations among main chain protons could not be established due to the presence of degeneracies, but exchange cross-peaks consistent with the proposed assignments were found in 47 of the remaining 49 residues. The two residues for which neither directly confirming nor consistent exchange cross-peaks could be found are Glu21 and Lys79. However, examination of the 2D spectra for both the reduced and oxidized protein revealed no alternative to the assignments proposed for these residues. The C_αH protons of Glu21 and Lys79 resonate close to the solvent signal, and so tend to be bleached by the solvent saturation pulse, making observatin of their exchange cross-peaks difficult. The attenuation of amide proton resonances through nuclear Overhauser effects from the saturated C_α protons can further account for the lack of observable exchange correlations.

In respect to side chain protons, the methyl resonances of horse cytochrome *c* have been studied previously by use of 2D-chemical exchange correlations by other investigators (10, 11). No inconsistencies with

those results were found in the present study. In the methylene region, the number of exchange cross-peaks found (Fig. 1) was much less than expected (as calculated by Feng et al., reference 16). The failure to observe many of the expected exchange cross-peaks is presumably due to the competition between exchange and NOE processes for the magnetization. For methylene protons, leakage to NOE can proceed efficiently. In any case, the high resonance density in the aliphatic side chain region often renders chemical exchange correlations ambiguous. This situation is even worse in the relayed TOCSY experiment because J cross-peaks in both oxidation states are present. Note, however, that in the fingerprint region of the TOCSY spectrum, one can obtain greatly improved resolution by H-exchange editing of the amide NH (e.g., reference 21).

In our cytochrome *c* assignment work, the accumulation of exchange correlations served two important functions. The chemical exchange correlations in conjunction with other 2D-spectral information were used to extend assignments known in one redox form of cytochrome *c* to the alternative state (13 main chain cross-assignments were made). The cross-connections also provided valuable confirmatory information. In most cases, assignments for a given proton could be obtained for the two redox states independently, and the exchange connectivity then provided direct confirmation. More generally, the chemical exchange correlation of many individual protons in the two different redox forms provides added confidence in the assignments even when given protons have not been independently assigned. In solving the assignment problem for a given protein, one ultimately obtains many J-correlated and dipolar connectivities that appear to form an internally self-consistent network. When two different self-consistent connectivity networks, such as the reduced and oxidized forms of cytochrome *c*, can in addition be interconnected at many protons by exchange experiments, this greatly reduces the probability that errors exist among the assignments in either form.

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