
FOR THE RECORD

Backbone and side-chain heteronuclear resonance assignments and hyperfine NMR shifts in horse cytochrome *c*

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Abstract

The [H26N, H33N] mutant of horse heart cytochrome *c* was expressed in *E. coli* during growth on isotopically enriched minimal media. Complete resonance assignments of both the diamagnetic reduced (spin zero) and paramagnetic oxidized (spin 1/2) states of the protein were obtained using standard triple resonance and total correlation spectroscopy using the previously determined ¹H chemical shifts of the wild-type protein as a guide. The correspondence of chemical shifts between the wild type and the mutant protein is excellent, indicating that they have nearly identical structures. The expanded library of chemical shifts for both redox states in both proteins allowed the refinement of the electron spin g-tensor of the oxidized states. The g-tensors of the oxidized states of the wild-type and [H26N, H33N] mutant proteins are closely similar, indicating that the subtle details of the ligand fields are nearly identical. The refined g-tensors were then used to probe for redox-dependent structure change in the two proteins.

Keywords: NMR resonance assignments; labeling hemeproteins; g-tensor; hyperfine shifts; paramagnetic shifts

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Horse cytochrome *c* ($M_r \sim 12.36$ kD) functions as a soluble mediator of electron transfer between redox proteins in an electron transfer cascade and contains a covalently attached heme prosthetic group. Cytochrome *c* has emerged as a paradigm for a range of biophysical studies, particularly for issues in protein folding and stability (Englander 2001). NMR spectroscopy has played a significant role in these works and has been aided by an extensive library of proton resonance assignments of the wild-type protein in both redox states (Feng et al. 1989; Wand et al. 1989). Unfortunately, the utility of cytochrome *c* as a model system has been hindered by an inability to prepare biophysical quantities of isotopically enriched protein (Pollock et al. 1998),

which is necessary for use in modern NMR-based investigations. Expression of eukaryotic cytochrome *c* has been particularly problematic. Fortunately, this barrier has now largely been overcome (Dolgikh et al. 1998; Pollock et al. 1998; Patel et al. 2001; Jeng et al. 2002; Martin et al. 2002; Rumbley et al. 2002).

Here we examined the [H26N, H33N] double mutant of horse cytochrome *c*, which has superior expression properties but is otherwise largely indistinguishable from the wild-type protein (Rumbley et al. 2002 and below). The [H26N, H33N] cytochrome *c* and mutants thereof are being used as model systems for a variety of studies of the folding, stability, and dynamics of c-type cytochromes. Standard heteronuclear triple resonance methods were employed to obtain essentially complete resonance assignments of the reduced (diamagnetic, spin 0) and oxidized (paramagnetic, spin 1/2) states of the holoprotein. Comparison to natural abundance ¹³C- and ¹⁵N-HSQC spectra led to extensive cross-assignments of the natural wild-type horse heart cy-

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tochrome *c* in its two redox states. These data then allowed the electronic *g*-tensors of the oxidized state of both the wild-type and [H26N, H33N] mutant to be determined and compared. Knowledge of the *g*-tensor also allowed for redox-dependent changes in both proteins to be detected.

Results and Discussion

NMR assignments of ferro- and ferricytochrome c

Sequence-specific assignments of the backbone ^1H , $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, ^{13}CO , and ^{15}N resonances were obtained using the HNCACB (Wittekind and Mueller 1993), CBCA(CO)NH (Wittekind and Mueller 1993), and CT-HNCO (Grzesiek and Bax 1992; Wittekind and Mueller 1993) three-dimensional NMR spectra. Starting from the published proton assignments of the wild-type protein (Feng et al. 1989; Wand et al. 1989), backbone $^1\text{H}_\text{N}$, $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, ^{13}CO , and ^{15}N resonance assignments were obtained for all residues except Gly1 and Gly56 in the reduced state, and Gly1 and Thr28 in the oxidized state. Side-chain methine, methylene, and methyl resonances were assigned using the three-dimensional C(CO)NH (Montelione et al. 1992), HC(CO)NH (Bax et al. 1990), and HCCH₃-TOCSY (Uhrin et al. 2000) experiments. Stereospecific assignments of the methyl groups of leucine and valine were obtained using the limited glucose labeling approach of Neri et al. (1989). The resonance assignments for the [H26N, H33N]-horse cytochrome *c* have been deposited with BioMagResBank under accession 5827 (reduced form) and 5828 (oxidized form).

Wild-type horse cytochrome *c* is commercially available. Natural abundance ^{15}N -HSQC and ^{13}C -HSQC spectra were obtained at 750 MHz (^1H) and compared to the resonance assignments obtained for the recombinant mutant protein. Because of the close correspondence of the spectra of the two proteins, essentially complete cross-assignment of the natural horse cytochrome *c* in both redox states could be achieved (BMRB accession 5829 and 5830). The high degree of correspondence of the solution structures of the two proteins is illustrated by the high degree of correlation of chemical shifts of corresponding resonances as shown in Figure 1, panels a–d for the reduced state and in Figure 1, panels e–h for the oxidized protein. Significant deviations are found to be highly localized to the sites of the two mutations.

Electronic g-tensor parameters

The paramagnetic iron center of ferricytochrome *c* contributes directly to the chemical shift of neighboring nuclei via hyperfine interactions termed the Fermi contact (δ_c) and the through-space pseudocontact (δ_pc) shifts (Bertini et al. 2002). The difference of the chemical shift of a nucleus in the two oxidation states may also reflect diamagnetic shift effects due to structural change (δ_str). For a given nucleus,

the observed redox-dependent change in chemical shift, $\Delta\delta_\text{obs}$ can then be expressed as:

$$\Delta\delta_\text{obs} = \delta_\text{ox} - \delta_\text{red} = \delta_\text{c} + \delta_\text{pc} + \delta_\text{str}$$

The pseudocontact shifts are determined by the electronic *g*-tensor (Kurland and McGarvey 1970; Horrocks Jr. and Greenberg 1973).

$$\delta_\text{pc} = [\beta^2 S(S+1)/9kT\text{r}^3] \times [g_\text{ax} (3\cos^2\theta - 1) + 1.5 g_\text{eq} (\sin^2\theta \cos 2\phi)]$$

$$g_\text{ax} = g_z^2 - 1/2 (g_x^2 + g_y^2)$$

$$g_\text{eq} = g_x^2 - g_y^2$$

where β is the Bohr magneton, *S* is the electron spin quantum number ($1/2$ for ferricytochrome *c*), and *T* is the absolute temperature. The position of each proton is defined by its polar coordinates (*r*, θ , ϕ) in the reference system of the electron spin *g* tensor. The principal *g*-tensor component g_ax , g_eq , and three Euler angles, α , β , and γ for horse cytochrome *c* are obtained using a least-squares fitting method (Feng et al. 1990).

In the past, reference to the crystal structure and knowledge of the chemical shifts of parent nuclei in the paramagnetic oxidized and diamagnetic reduced states have been used to determine the *g*-tensor of the oxidized heme (e.g. Feng et al. 1990; Boyd et al. 1999). In that case, a consensus is sought for those sites for which $\delta_\text{c} + \delta_\text{str}$ is negligible and can therefore be used to determine the parameters defining the *g*-tensor (Feng et al. 1990). We have repeated this procedure using the crystal structure of oxidized horse cytochrome *c* (Berghuis and Brayer 1992; PDB code 1HRC) and the expanded chemical shift library of the wild-type protein (Fig. 1, panels i–l) and the [H26N, H33N] mutant (Fig. 1, panels m–p). Excellent convergence of the fit was observed in both cases. The obtained *g*-tensor parameters for both proteins are essentially identical (Table 1). As pointed out by Boyd et al. (1999), the amide nitrogen sites show considerable variance (Fig. 1, panels l and p), which can be attributed to the exquisite sensitivity of this nucleus to small changes in the electrostatic environment (Ubbink et al. 2002).

Redox-dependent structure change

Having determined the parameters defining the electronic *g*-tensors of both the wild-type and mutant proteins, one can begin to assess the degree of true structure change upon a change in redox state (Feng et al. 1990). The optimized *g*-tensor parameters were used together with spatial coordinates for the oxidized form to calculate the pseudocontact shift contribution (δ_pc) to all nuclei. The distributions of an

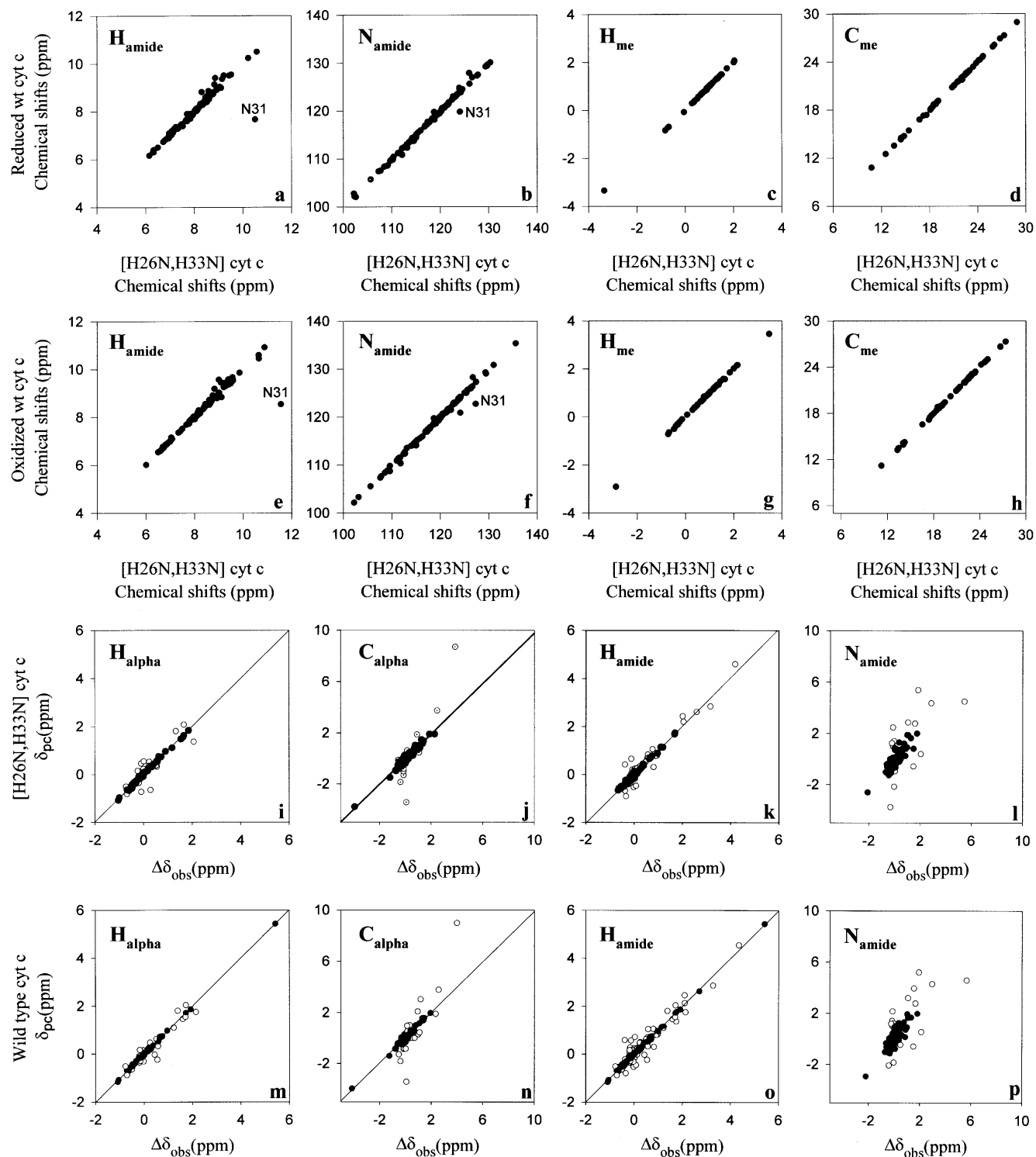


Figure 1. Comparison of the solution structures of natural wild-type horse cytochrome *c* and recombinant [H26N, H33N] horse cytochrome *c*. Correlation of the chemical shifts of reduced natural wild-type horse cytochrome *c* with those of reduced recombinant [H26N, H33N] horse cytochrome *c* (panel a, amide hydrogens; b, amide nitrogens; c, methyl protons; d, methyl carbons). Correlation of the chemical shifts of oxidized natural wild-type horse cytochrome *c* with those of oxidized recombinant [H26N, H33N] horse cytochrome *c* (panel e, amide hydrogens; f, amide nitrogens; g, methyl protons; h, methyl carbons). Refinement of the electronic g-tensor parameters for recombinant [H26N, H33N] horse cytochrome *c* (panels i–l) and natural wild-type horse cytochrome *c* (panels m–p). Solid circles correspond to those sites used to determine the parameters of the g-tensor by minimizing the difference between the observed and calculated pseudocontact shifts essentially as described by Feng et al. (1990). A similar plot is shown for recombinant [H26N, H33N] horse cytochrome *c* in panels m–p.

Table 1. Determined *g*-tensor parameters for wild-type and [H26N, H33N]-recombinant cytochrome *c*

Number of sites	g_x	g_y	g_z	α	β	γ	χ^2_{red}
[H26N,H33N] 300	2.22	2.64	2.31	106	13	251	0.002
[Wild type] 296	2.23	2.61	2.32	106	13	251	0.002
[Wild type] 64 ^a	2.25	2.59	2.32	106	13	251	0.004

^a Reference set of Feng et al. (1990).

apparent redox-dependent chemical shift discrepancy ($\Delta\delta_{\text{obs}} - \delta_{\text{pc}}$) could then be evaluated, and for the most part, the residual chemical shift discrepancies are negligible. Exceptions include the loops containing residues 27–33, 37–43, and 50–60, and regions localized near the axial heme ligands Met80 and His18. The latter class is anticipated to have a large Fermi contact contribution (δ_c). The remaining regions are consistent with structural changes and correspond closely to those previously found for the wild-type protein (Feng et al. 1990).

In summary, these data strongly indicate that the recombinant [H26N, H33N] mutant of horse cytochrome *c* is structurally highly similar to the wild-type protein in both redox states. The close correspondence of the *g*-tensors of the two proteins reinforces the notion that the structural details of heme ligation are nearly identical. Accordingly, the [H26N,H33N] mutant of horse cytochrome *c* should be considered a highly suitable biophysical model of wild-type cytochrome *c*.

Materials and methods

The expression vector for [H26N,H33N] horse cytochrome *c* has been described elsewhere (Rumbley et al. 2002). Cultures were inoculated from glycerol stocks and grown overnight in LB broth (30°C). Cells were pelleted by centrifugation and resuspended in fresh 20 mL minimal media and used to inoculate 1L of minimal media (Morar et al. 1999) containing 1 g of ¹⁵NH₄Cl and 2 g of ¹³C-glucose. Cells were grown for 60 h at 30°C and harvested by centrifugation. The cell pellet was suspended in 25 mM potassium phosphate, pH 7, containing PMSF, benzamide, and DNase, and broken with an Aminco French press. Ammonium sulfate was added to the whole-cell lysate to 25% saturation, and the precipitate was removed by centrifugation. The supernatant was dialyzed against 25 mM potassium phosphate, pH 7. Cytochrome *c* was oxidized with potassium ferricyanide and isolated on a CM-Sephrose column using a NaCl gradient. The pure cytochrome *c* was concentrated by pressure filtration. NMR samples (650 μ L) of uniformly ¹⁵N-/¹³C-labeled cytochrome *c* (1 mM) were prepared in 50 mM potassium phosphate buffer, pH* 5.8 (95% H₂O/5% D₂O). The yield was generally 6–8 mg per liter of minimal media.

NMR spectra were recorded at 293 K on a Varian INOVA 750 MHz and 500 MHz NMR spectrometers equipped with four RF channels and pulsed field gradients. Triple resonance and total correlation experiments were carried out, and the data were processed essentially as described elsewhere (Liu et al. 2001). ¹⁵N and ¹³C chemical shifts were referenced indirectly using the consensus

X_i ratios of gyromagnetic ratios, 0.101329118 and 0.251449528 for ¹⁵N/¹H and ¹³C/¹H, respectively (Wishart et al. 1995).

Electronic supplemental material

¹⁵N- and ¹³C-HSQC spectra annotated with resonance assignments are given as electronic supplemental material.

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