Salt-Dependent Structure Change and Ion Binding in Cytochrome *c* Studied by Two-Dimensional Proton NMR†

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ABSTRACT: To search for salt-dependent structure changes that might help to explain physicochemical differences observed in previous solution studies, two-dimensional proton NMR spectra of reduced and oxidized cytochrome *c* were recorded at relatively high and low salt concentrations. The results rule out substantial ionic strength dependent structure change in either redox form over the salt concentrations tested (5 mM phosphate to 5 mM phosphate plus 200 mM NaCl, at pH 7). Chemical shift changes were found for several residues within a limited segment of the *oxidized* protein, most prominently in the sequence Lys-86, Lys-87, Lys-88, Thr-89. A salt-dependent binding of phosphate anion(s) at this site, as observed earlier by others, is indicated. The binding of one or two phosphates at the cytochrome *c* surface can explain earlier small-angle X-ray scattering observations of an increase in the calculated radius of gyration of the oxidized protein at the same low-salt condition used here. Other observations, by ultraviolet resonance Raman and 1D NMR spectroscopies, of salt-dependent changes could not be corroborated, but may depend on the still lower salt used and the absence of phosphate. The results obtained support the view that the absence of sizeable redox-dependent structure change observed in X-ray and NMR studies at varying salt conditions is characteristic of the protein at all salt conditions above the low millimolar range. Physicochemical differences between oxidized and reduced cytochrome *c* apparently represent differences in stability without patent structure change.

The possibility of structural differences between reduced and oxidized cytochrome *c* has received considerable study. Physical–chemical measurements have indicated substantial differences in stability (Margoliash & Schejter, 1966) and compressibility [Eden et al., 1982; though see also Kharakoz and Mkhitaryan (1986)] and widespread changes in hydrogen-exchange behavior (Ulmer & Kägi, 1968; Patel & Canuel, 1976; Liu et al., 1989; Wand et al., 1986; unpublished data from this laboratory). In all cases the oxidized form is less stable or rigid. In contrast, crystallographic results (Takano & Dickerson, 1981; A. Berghius and G. Brayer, personal communication) have found some subangstrom-level differences but overall impressive similarity in structure between the two oxidation states. NMR studies of cytochrome *c* in solution (Feng et al., 1990) are in good agreement with this view.

Small-angle X-ray scattering measurements of horse cytochrome *c* (Trewhella et al., 1988) suggest that a significant expansion of the *oxidized* protein occurs at low ionic strength but is suppressed at higher salt concentration. Low-salt-dependent structural effects have been reported also in resonance Raman (Liu et al., 1989) and 1D NMR (Rush et al., 1988) studies. These results raise the possibility that the failure to find serious redox-dependent structure changes in earlier work, especially in the X-ray studies at very high salt and possibily also in the NMR studies at more moderate salt, may be an artifact of the salt concentrations used. The experiments reported here were done to test this hypothesis.

The solution of the proton NMR assignment problem for reduced (Wand et al., 1989) and oxidized (Feng et al., 1989) equine cytochrome *c* now provides hundreds of identified protons that can serve as sensitive probe points throughout the protein. To search for salt-dependent changes in protein structure, we recorded two-dimensional ^{1}H NMR spectra for both redox forms of horse cytochrome *c* at 10 and 210 mM ionic strength at neutral pH in dilute phosphate buffer. Above this level, one does not expect ionic strength effects on protein structure to emerge. Protein structure changes are expected to produce measurable changes in the chemical shifts of protons on affected amino acid residues, insofar as the protons are displaced relative to the paramagnetic heme iron in the oxidized protein or experience even rather subtle environmental changes that can produce diamagnetic shifts in either the oxidized or the reduced form (Williams et al., 1985; Feng et al., 1990).

Methods

Horse heart cytochrome *c* was obtained from Sigma (grade VI) and used without further purification. The protein was studied at 20 °C in 90% H_2O/10% D_2O buffers containing either 5 mM phosphate (low salt) or 5 mM phosphate and 200 mM sodium chloride (high salt). These are the salt concentrations used in the small-angle X-ray scattering studies of Trewhella et al. (1988); low-salt condition was also used in the compressibility studies of Eden and co-workers (1982) and Kharakoz and Mkhitaryan (1986).

To ensure full oxidation of the oxidized samples, a minimal amount of potassium ferricyanide was added. Samples were dialyzed against these same buffers for 3 h (rapid microdialysis with three changes; Englander & Crowe, 1965) and then concentrated as necessary for 2D NMR by ultracentrifugal filtration (Centricon 10). For the reduced samples, ascorbic acid was added to maintain full reduction (~10 mM). The final pH of all samples was 7.0–7.2, and protein concentrations were 7–8 mM. It can be noted that the dialysis procedure used (Englander & Crowe, 1968) reequilibrates the protein solution against a large reservoir of salt (0.5 mL dialyzed against ~500 mL of buffer) so that free salt readjusts to, e.g., 5 mM in concentration, even though protein-bound ion concentration may exceed the free salt concentration.

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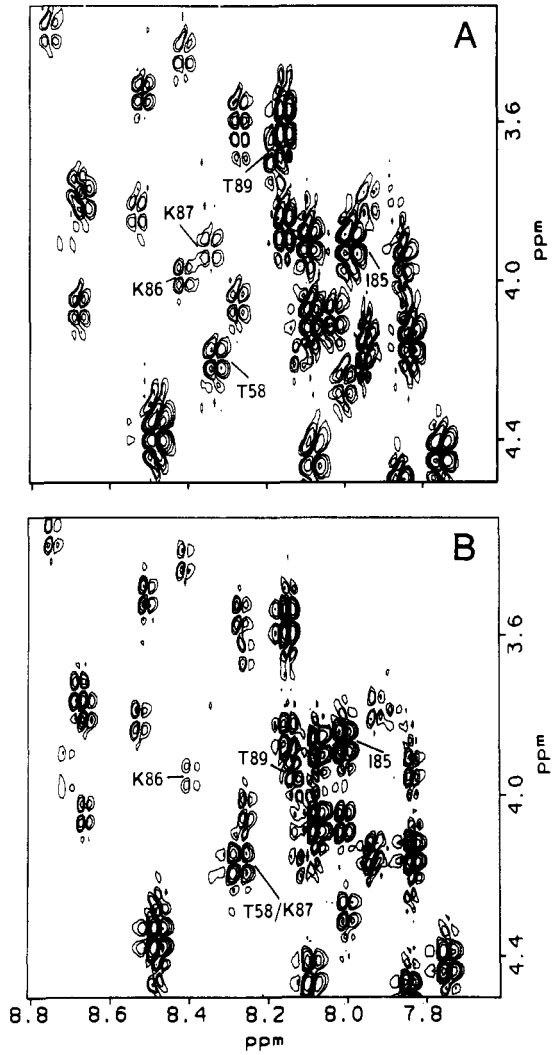

FIGURE 1: Section of the NH–C_αH (fingerprint) region from a 500-MHz ^{1}H NMR DQF-COSY spectrum of oxidized horse cytochrome *c*. Salt conditions are (A) 5 mM phosphate and (B) 5 mM phosphate and 200 mM NaCl. Resonances with salt-dependent chemical shift differences ≥0.05 ppm are labeled. Solution conditions were pH 7.0–7.2 in 90% H_2O/10% D_2O at 20 °C.

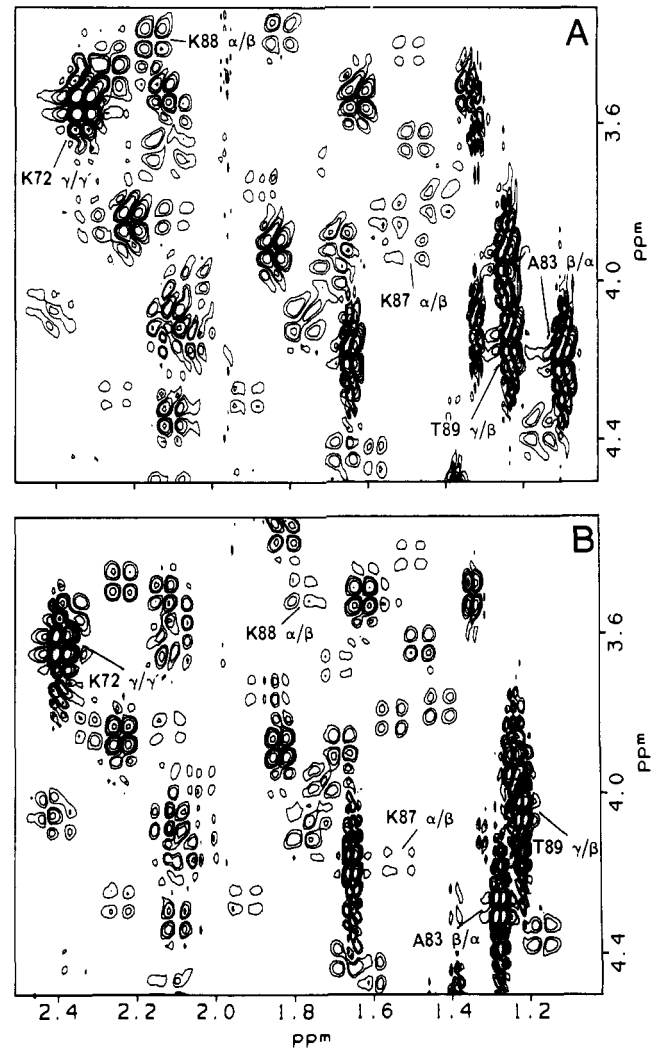

FIGURE 2: Section of the aliphatic region of the DQF-COSY spectrum of oxidized horse cytochrome *c*. Salt conditions are (A) 5 mM phosphate and (B) 5 mM phosphate and 200 mM NaCl. The labels mark cross peaks that exhibit salt-dependent change.

Double-quantum-filtered COSY (DQF-COSY) spectra were recorded and processed as described earlier (Feng et al., 1989). Spectroscopic parameters were as follows: 64 transients for each t_1; 650 t_1s; repetition time ≥1.47 s; spectral width 8.3 kHz in both dimensions; final apparent digital resolution 4.1 Hz/point; solvent suppression by direct saturation at all times except during acquisition.

Results

Sections of 2D NMR spectra at the low- and high-salt condition (5 mM phosphate and 5 mM phosphate plus 200 mM NaCl, respectively) are shown in the figures. The well-resolved NH–C_αH region for oxidized cytochrome *c* is shown in Figure 1 and part of the aliphatic region in Figure 2. Figure 3 shows the NH–C_αH region for the reduced protein. Cross peaks were identified with their parent protons in the protein at the high-salt condition by straightforward comparison with similar spectra used to assign both the reduced (Wand et al., 1989) and oxidized (Feng et al., 1989) proteins. A few ambiguities due to the pH difference (pH 7 here, pH 5.7 previously) were resolved by tracing out the side-chain *J* connectivitites.

Structure Change. If cytochrome *c* experiences significant internal packing changes on going from high to low ionic strength, major changes in the NMR spectrum would be expected, with numerous protons experiencing significant changes in chemical shift. *Neither the oxidized nor the reduced protein exhibits major salt-dependent changes.* Since chemical shift is quite sensitive to even subtle structure changes, this result indicates that cytochrome *c* structure is generally insensitive to ionic strength.

In a recent ultraviolet resonance Raman study, Liu et al. (1989) observed a subtle weakening of the 830-cm^{-1} tyrosine band in oxidized cytochrome *c* at low ionic strength (5 mM ionic strength in MOPS buffer compared to 1.5 M salt, adjusted with sodium sulfate). This together with other observations suggested the weakening or loss of a strong, internal tyrosine–OH H bond, pointing to tyrosine-48, which forms an internal H bond to the heme propionate.

Horse cytochrome *c* has four tyrosines, at positions 48, 67, 74, and 97. The ring CH resonances of Tyr-74 and -97 and the slowly exchanging Tyr-67 OH resonance have all been identified in the oxidized protein (Feng et al., 1989). None of these shift with salt. The resonances of the Tyr-48 ring protons in oxidized cytochrome *c* have not been found, perhaps due to an intermediate flip rate of the ring at room temperature. In this case, resonances of adjacent residues can provide a sensitive indication of changes in the Tyr-48 ring position. The $C_\alpha H_2$ resonances of Gly-41 are strongly ring current shifted by Tyr-48, but the C_αH–C_αH′ cross peak is unchanged

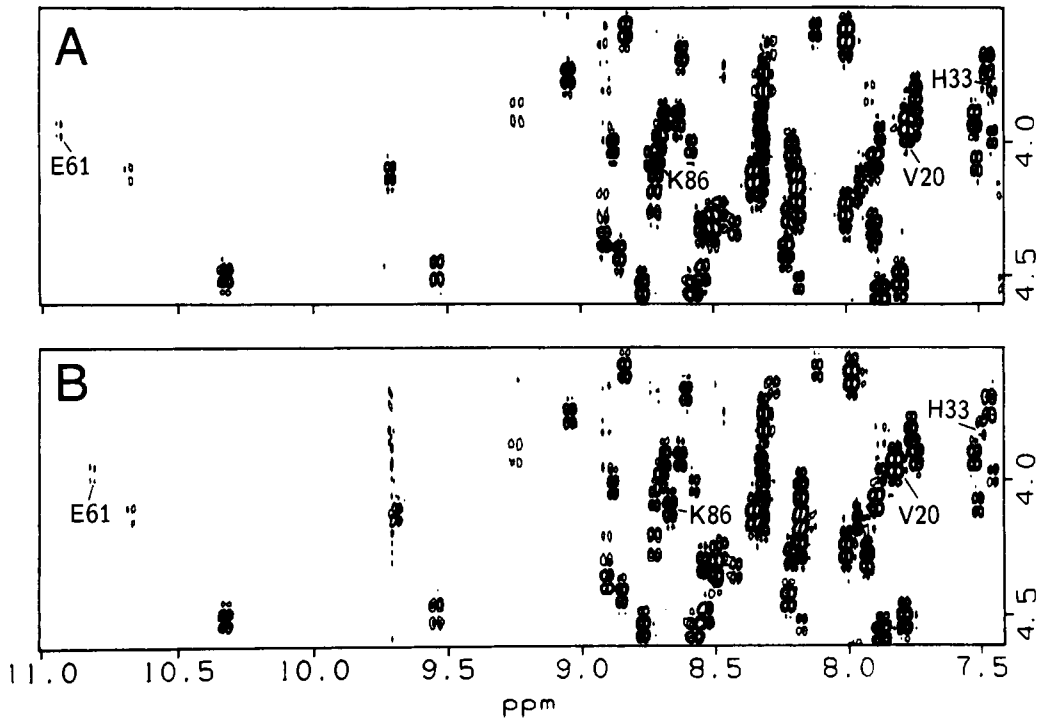

FIGURE 3: Section of the fingerprint region of reduced horse cytochrome c in (A) 5 mM phosphate and (B) 5 mM phosphate and 200 mM NaCl. All parameters are the same as for Figure 1.

Table I: Residues That Exhibit Salt-Dependent Resonance Shifts

	high salt				low salt				differences			
	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$
oxidized												
Glu-61	10.63	(3.64)	(2.09)		10.74	(3.68)	(2.13)		0.11			
Ala-83		4.28	1.28			4.20	1.10			−0.08	−0.18	
Lys-87	8.26	4.17	(1.53)		8.34	3.92	(1.49)		0.08	−0.25		
Lys-88		3.49	1.78			3.38	2.15			−0.11	0.37	
Thr-89	(8.13)	3.98	4.05	(1.22)	(8.17)	3.67	4.15	(1.23)		−0.31	0.10	
reduced												
Glu-61	10.81	(3.97)	(2.28)	(2.69)	10.93	(3.95)	(2.28)	(2.69)	0.12			

[a] Chemical shifts of protons that exhibit salt-dependent differences ≥0.10 ppm are listed. When the cutoff is reduced to 0.05 ppm, protons on a number of other residues enter, namely, Thr-58, Lys-72, and Lys-86 in oxidized and Val-20, His-33, and Lys-86 in reduced cytochrome c. Chemical shifts of assigned resonances in the tabulated residues that differ by less than 0.05 ppm, perhaps due to pH and temperature differences as well as salt effects, are given in parentheses. Cross peaks from the following resonances could not be unambiguously identified at both salt conditions due to either insufficient signal-to-noise ratio, insufficient resolution, or saturation of the C_α proton resonances along with the solvent signal. Oxidized: E21, H33, K39, K79. Reduced E21, K88.

at the two salt conditions tested. The Tyr-48 and Phe-46 rings are in direct steric contact; the clearly resolved Phe-46 C_2H–C_3H cross peak is unaltered under these salt conditions. (The other Phe-46 ring proton cross peaks are too close to water to be seen in the present spectra recorded in H_2O.)

Resonance Frequency Changes. A close comparison of cytochrome c spectra taken at the high- and low-salt conditions used here do reveal some limited salt-dependent proton resonance changes, especially in the oxidized protein (Table I). These are marked in the figures showing the fingerprint, NH–$C_\alpha H$ cross peak region (Figure 1), and part of the aliphatic region (Figure 2). In these comparisons of samples at different conditions, chemical shift changes ≥0.1 ppm were considered clearly significant and are listed in Table I. Some smaller differences are also noted in Table I, but those less than 0.05 ppm were discounted.

The identities of the salt-dependent resonances in the spectra taken at higher ionic strength (Figure 1B) are specified unambiguously by comparison with the published assignments. However, the identity of each shifted resonance in the low-salt spectrum remains somewhat ambiguous since several resonances are shifted at low salt. Assignments indicated in the figures at the low-salt condition (Figures 1A and 2A) simply assume the minimum perturbation for each shifted cross peak. Alternative possible assignments would produce shifts even larger than those listed in Table I. Some additional changes can be detected in the congested aliphatic side-chain region (spectral region between 1 and 3 ppm); these resonances are difficult to identify but perhaps represent the side chains of the lysines in Table I.

The clearest salt-sensitive changes, found in the oxidized protein, are largely localized to one segment of the protein, involving residues 83–89. Changes are especially evident for several protons in Lys-87, Lys-88, and Thr-89. Only smaller, scattered changes are found in the reduced protein (Figure 3 and Table I), involving Glu-61 and several other residues with marginal shift increments. Glu-61 and Lys-86 show some small effects in both protein forms.

The larger chemical shift perturbations observed in the oxidized protein (Table I) cannot be attributed to a special sensitivity to spatial displacement due to the presence of the paramagnetic center (i.e., pseudocontact shifts). For all the protons listed in Table I except those of Ala-83, a spatial displacement of 1 Å in the paramagnetic field would produce

a *maximum* hyperfine shift of less than 0.04 ppm, far less than many of the changes observed [calculated according to the method of Feng et al. (1990)].

Discussion

The NMR results obtained document the absence of major salt-dependent structure changes in cytochrome *c* at the conditions tested. Some salt-dependent chemical shift changes do occur, especially in the oxidized protein in the vicinity of residues 83–89. It is striking that residues 86, 87, and 88 are all lysines. This provides a compelling rationale for the salt effects observed. Both the high positive charge due to the Lys-86, Lys-87, Lys-88 sequence and the mobility of the long lysine side chains could contribute to a binding site for phosphate anion (present at 5 mM in these experiments), and phosphate binding can be expected to decrease when NaCl is added (NaCl = 200 mM at the high-salt condition). The binding of phosphate in this way could produce the observed local proton chemical shift changes due to protein structural displacements and/or changes in local electrostatic field (Clayden & Williams, 1982).

Phosphate Binding. The results cited here indicate that *oxidized* cytochrome *c*, at the low-salt condition tested, experiences a net binding of one or more phosphate anions at the lysine-rich site (residues 86–88). The binding of anions to cytochrome *c* in this and other regions has been noted before by many workers [e.g., Barlow and Margoliash (1966), Stellwagen and Shulman (1973), Margalit and Schejter (1973), Anderson et al. (1979), and Arean et al. (1988)]. Especially pertinent is the evidence pointing to a relatively strong phosphate binding site in the immediate vicinity of Lys-86 and/or -87 (Brautigan et al., 1978; Osheroff et al., 1980) and Thr-89 (Taborsky & McCollum, 1979). In agreement with the present results, these workers found the affinity for phosphate to be greater for the oxidized than for the reduced protein. Phosphate bound at the strong site was not displaced by added chloride in the experiments of Osheroff et al. (1980), but in that case only relatively low chloride (Cl^- <35 mM; P_i at 8 mM) was tested, compared with the 200 mM NaCl and 5 mM phosphate used here. Brautigan et al. (1978) found indications of a second phosphate binding site involving Lys-27. Our NMR results are ambiguous in respect to possible salt-dependent changes at Lys-27 in oxidized cytochrome *c*, since the $C_\alpha H$ resonance is close to the water resonance and the NH–$C_\alpha H$ cross peak is overlapped by other cross peaks. In the reduced protein, the NH, $C_\alpha H$, and $C_\beta H$ resonances of Lys-27 are known and can be seen to be unaffected by the salt condition. Brautigan et al. (1978) also considered the possibility of Lys-8 involvement in phosphate binding. The NMR results show no change in the NH, $C_\alpha H$, or $C_\beta H$ resonances of Lys-8 in either the oxidized or reduced forms.

Molecular Dimension by X-ray Scattering. Trewhella et al. (1988), in small-angle X-ray scattering studies at the same low-salt condition studied here, observed an 8% increase in radius of gyration, selective for the oxidized form of cytochrome *c*, in the low-salt 5 mM phosphate buffer. The binding of a single phosphate anion at the surface of cytochrome *c* will increase its apparent radius of gyration by about 4%. This can be appreciated by a simplified calculation that considers the effect on the radius of gyration of adding a phosphate group (95 Da, mass density ~ X-ray scattering density ~ 2) at the periphery of the protein approximated as a sphere (12 500 Da, ~15-Å radius, scattering density 1.4 compared to water at 1). Binding farther from the center will have an even larger effect. The present NMR observations were made at the same salt conditions used in the X-ray scattering work. It therefore appears that the X-ray scattering results can be explained in terms of the binding of phosphate anion(s) at the lysine-rich site and possibly another site elsewhere in either a site-specific or a more distributed manner [see Lattman (1989)].

Other Salt-Dependent Changes. In ultraviolet resonance Raman (UVRR) studies of cytochrome *c* as a function of salt concentration, Liu et al. (1989) found no change in the amide bands, either as a function of redox state or ionic strength (5 mM MOPS buffer at pH 7.6 with sodium sulfate between 0 and 1.5 M), consistent with the absence of significant change observed in the present study and others. A salt-dependent change in H-exchange rate of the deeply buried, internally H bonded ring NH of Trp-59 was found in both redox forms, in spite of the absence of changes in several tryptophan UVRR bands that are sensitive to structure change. This is consistent with the prior observation of the other H-exchange changes in cytochrome *c* in the absence of structure change (see the introduction) and the conclusion that H-exchange rate keys to structural stability (dynamics) rather than structure per se (Englander & Kallenbach, 1984). However, some change was observed in the 830/850-cm^{-1} tyrosine doublet, at low salt (5 mM MOPS buffer, no phosphate) in the oxidized protein, thought to signal a change in the Tyr-48 H bond to the heme propionate 7 carboxyl oxygen, although UVRR bands due to Trp-59, which is H bonded to the same carboxylate (other oxygen), showed no change. The present observations provide clear evidence for the absence of change at Tyr-48. (It can be noted that the strong phosphate binding site measured here is very remote from Tyr-48.)

In other work, Rush et al. (1988), in studies on cytochrome *c* oxidation rate as a function of salt concentration, noted changes in the 1D NMR spectrum of the oxidized protein at very low salt (3.5 mM KCl, no phosphate), indicating that approximately 11% of the protein was present in a nonnative "open" form. The new peaks, apparent in a resolved part of the 1D spectrum, do not appear in the 5 mM phosphate solutions used in our work. It can be noted that the solution ionic strength in the work of Rush et al. (1988) was 3-fold lower than our low-salt condition. Also, a possible effect of the phosphate binding, present in our work but not used by Rush et al. (1988), might be considered.

Structure Change and Cytochrome c Behavior. In summary, the results presented here show that major salt-dependent structure change does not occur in either oxidized or reduced cytochrome *c* through the salt concentrations tested. Only localized chemical shift differences are seen, more prominently in the oxidized form, and these relate to phosphate binding. Selective phosphate binding to the oxidized protein at low ambient counterion concentration is able to explain the apparent salt-dependent change in molecular size observed by small-angle X-ray scattering (Trewhella et al., 1988). The results obtained do not support the suggestion of a change in oxidized cytochrome *c* structure as a basis for reported changes in a tyrosine resonance Raman peak or of fractional structure changes observed by 1D NMR. However, since phosphate anion, found in our work to bind selectively to the protein in low salt, was absent in both these cases, the possibility that phosphate binding pulls a structural equilibrium toward the native state characteristic of the higher salt condition must be considered.

The present results are consistent with the view that the general absence of redox-dependent structure change found in X-ray (Takano & Dickerson, 1981; A. Berghius and G. Brayer, personal communication) and NMR (Feng et al.,

1990) studies is not an artifact of the high salt concentration used. The various physicochemical differences seen between oxidized and reduced cytochrome *c* in solution at moderate salt appear to reflect differences in stability, and the conjugate parameter, structural dynamics, which occur without substantial changes in the three-dimensional structure of the average native state. The selective salt binding observed here and in prior work suggests that electrostatic rather than structural difference should be considered.

Registry No. Cytochrome *c*, 9007-43-6; phosphate, 14265-44-2.

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NMR Study of the Phosphoryl Binding Loop in Purine Nucleotide Proteins: Evidence for Strong Hydrogen Bonding in Human N-ras p21†

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ABSTRACT: The structure of the phosphoryl binding region of human N-ras p21 was probed by using heteronuclear proton-observed NMR methods. Normal protein and a Gly-12 → Asp-12 mutant protein were prepared with two amino acids labeled with ^{15}N at their amide positions: valine and glycine, aspartic acid and glycine, and lysine and glycine. We completed the identification of amide ^{15}NH resonances from Gly-12 and Asp-12 to the end of the phosphoryl binding domain consensus sequence (Lys-16) in protein complexed with GDP and have made tentative amide identifications from Val-9 to Ser-17. The methods used, together with initial identifications of the Gly-12 and -13 amide resonances, were described previously [Campbell-Burk, S. (1989) *Biochemistry 28*, 9478–9484]. The amide resonances of both Gly-13 and Lys-16 are shifted downfield below 10.4 ppm in both the normal and mutant proteins. These downfield shifts are presumed to be due to strong hydrogen bonds with the β-phosphate oxygens of GDP.

There is a large class of purine nucleotide binding proteins having a consensus sequence Gly-X-X-X-X-Gly-Lys at the phosphoryl binding site (Walker et al., 1982; Higgins et al., 1986). We have been studying members of an important subclass of these proteins, the 21-kilodalton products of the human N-ras gene, human N-ras p21 (henceforth p21) and an oncogenic mutant thereof, using NMR (Campbell-Burk et al., 1989; Campbell-Burk, 1989). For N-ras p21 protein, the version of the consensus sequence starts at Gly-10 and is Gly-Ala-Gly-Gly-Val-Gly-Lys, while the mutant has aspartic acid substituted for glycine at position 12 (Barbacid, 1987).

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