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PROTON RESONANCE ASSIGNMENTS FOR PSEUDOMONAS AERUGINOSA FERROCYTOCHROME c-551

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A comparison between two sets of resonance assignments for ferrocytochrome c-551 from Pseudomonas aeruginosa reveals that major differences can be explained by pH effects. In turn, these reveal side chain protonation events in c-551 that markedly influence spectra. The behavior of resonances in a homologous protein from Pseudomonas stutzeri help to clarify ambiguities in the P. aeruginosa case. A corrected and completed set of proline assignments is presented. Labile side chain protons in residue 47, which hydrogen bonds to the inner heme propionate, appear to be in fast exchange with the solvent.

Cytochrome c-551 is an electron transport protein found in bacteria where it performs a function analogous to cytochrome c in mitochondria. With about 82 amino acids, it is a simpler version of mitochondrial cytochrome, although it shares many structural and functional aspects (1). During 1990 two reports (2,3) appeared with extensive ^{1}H-NMR assignments for ferrocytochrome c-551 from Pseudomonas aeruginosa, but under different conditions of temperature and pH. There was substantial agreement for the majority of main and side chain assignments, but the later report noted some discrepancies. It is useful to resolve these points for two reasons. A consistent set of assignments in the literature would be available for future studies on homologous proteins or derivatives. It also turns out that the basis for the differences reveals some interesting aspects of cyt c-551 structure and behavior. In this communication, we will attempt to resolve

Abreviations Used: PA, Pseudomonas aeruginosa; PS, Pseudomonas stutzeri; ATCC, American Type Culture Collection; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; HOHAHA; homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; pD, pH measured by a glass electrode in deuterium oxide solution but not corrected for the isotope effect.

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differences in assignments and/or chemical shifts reported for Ps. aeruginosa (PA) cyt c-551. This is possible because of spectra obtained for PA c-551 at a variety of conditions, but also because assignments are now available for a homologous protein, ferrocytochrome c-551 from Pseudomonas stutzeri ATCC 17588 (PS). This protein has 27 different residues from PA, but the sequence and now NMR spectroscopy indicate that it is highly homologous in structure. The assignments for PS help to confirm the homologous set in PA.

Materials and Methods

Spectroscopic conditions and methods (2), and purification of PA c-551 and PS c-551 (4) have been described. Full details for resonance assignments for PS c-551 will be published in a later report. For PA c-551, one dimensional spectra with internal dioxane (3.74 ppm) indicated that the 20 meso proton had a chemical shift of 9.22 ppm largely independent of pH and temperature, and so this resonance was used as a reference for two-dimensional data sets. In a similar manner for PS c-551, the Met 61 S-methyl protons were found to be constant at -2.91 ppm, and this resonance was used as a convenient reference. The homologous S-methyl resonance in PA c-551 shifts from -2.87 to -2.94 ppm with increasing pH with an apparent pKa around 7. This may reflect the ionization of the heme proprionate described previously (2). Why the resonance in PS c-551 is insensitive to pH remains to be explained, but, even for PA c-551, the shift is small.

Results and Discussion

Detlefsen et al. (3) reported that the side chain of Tyr 27 passed from rapid rotation at high temperature to hindered at low, with the evidence being the disappearance of DQF-COSY and TOCSY cross peaks between the degenerate 2,6 protons and the 3,5 protons at temperatures below 60 °C. Chau et al. (2) reported fast or intermediate ring flipping for all benzoid residues from 4-60 °C and pH 3.5-10.6. We also observed the disappearance of the Tyr 27 cross peak, although as shown in Fig. 2 broad, overlapping resonances are still observable in one dimensional spectra. For PS c-551, all the benzoid side chains (Phe 7, Phe 27, and Tyr 34 in PS, while in PA, Phe 7, Tyr 27, and Phe 34) appear to be rapidly flipping, because all 2,6 and 3,5 protons appear to be degenerate, and are observable in one dimensional, DQF-COSY, HOHAHA, or NOESY spectra from 27 to 60 °C and pH 4.5 to 10.0.

There are two sets of main chain amides that were reported with significantly different chemical shifts; residues Ser 67 to Asp 69, and Asp 2 and Glu 4 (residue 3 is a proline with no amide proton). The changes are substantial, but are due to pH

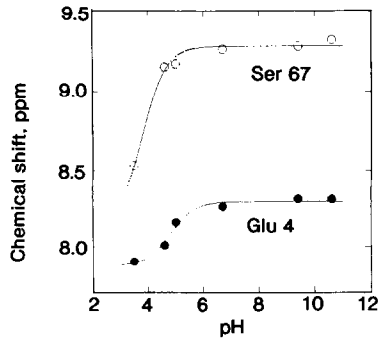

Fig. 1. Titration curves for the main chain amides of Ser 67 and Glu 4 in PA c-551. Chemical shifts were taken from the fingerprint region of HOHAHA spectra at 32 °C. The solid curves correspond to theoretical titration curves with pKa's of 3.79 (Ser 67 data) and 4.80 (Glu 4 data).

differences for the data sets as illustrated in Fig. 1. In the crystal structure of PA c-551 (5), the amide of Ser 67 is hydrogen bonded to the side chain carboxylate of Glu 70, and we believe that as Glu 70 protonates there is a perturbation felt mostly by 67, but also by 68 and 69. Their amides show similar titration curves with a similar pKa. The chemical shift of the amide of Glu 4 may be sensitive to the protonation of the side chain carboxylate of either Glu 4 or Asp 2. The Asp 2 amide is also sensitive to pH, but its chemical shift (and to a lesser extent those of the alpha and beta protons) is further sensitive also to temperature and to a structural heterogeneity noted by Chau et al. as a splitting of residues. The Asp 2 amide is marked by some clear cross peaks in two dimensional spectra that enable it to be readily followed under different conditions. In TOCSY or HOHAHA spectra the spin system can be traced in the fingerprint region through the alpha proton at 4.73 to 4.84 ppm and the beta protons at 2.6 to 2.78 ppm. In the fingerprint region of NOESY spectra there are distinctive crosspeaks from the amide to the alpha and beta protons of Glu 1 and the delta protons of Pro 3.

The tentative assignments Chau et al. for some proline residues were incorrect. The problem arose from an unfortunate degeneracy of proline delta residues and led to incorrectly mixing some alpha and beta protons among prolines. This became evident upon examining the spectra of PS c-551 which has many homologous prolines, with similar, but not identical shifts. Prolines for both proteins were assigned by first using inter-residue NOE's to determine alpha and/or delta protons, then using HOHAHA spectra of progressively longer spin lock times to build up relayed

Table 1. Proline residue assignments for P. aeruginosa cyt c-551 [a]

Residue	alpha	beta	gamma	delta
Pro 3	3.78	0.81, 1.38	1.65, 1.95	3.63, 3.88
Pro 25	3.52	0.77, 0.50	0.13, 0.30	2.85, 2.08
Pro 58	4.77	2.25	2.29, 2.05	3.93, 3.70
Pro 60	4.86	1.99, 1.53	2.09, 2.01	3.90, 4.13
Pro 62	4.22	2.18, 1.55	1.80, 1.67	2.97, 3.78
Pro 63	3.23	1.52, 2.05	1.75, 1.63	3.28, 3.22

[a]Chemical shifts in ppm were taken from crosspeaks in HOHAHA spectra recorded in deuterium oxide at pD 8 and 60 °C. These conditions represent a good compromise for observing the proline resonances wherein the linewidths are favorable for sharp peaks, there is minimal interference from the residual water signal, and there is good resolution from non-proline crosspeaks. The low values of Pro 25 are due to the heme ring current as discussed in ref. 2. The low values for Pro 3 are due to a ring current effect from Trp 77 which is close in space.

magnetization transfers from the alpha protons out to the delta's, and, in a parallel fashion, from the delta chemical shifts to chemical shifts of alpha. Table 1 is a set of revised proline assignments for PA c-551 that now agrees substantially with those of Detlefsen et al., and slightly extends their results.

Residue 47 is a critical side chain in c-551's because of its proximity to the heme. From the crystal structure of PA c-551 it has been reported to hydrogen bond to the inner heme propionic acid and this interaction has been implicated as crucial for moderating the reduction potential of the iron (6). Detlefsen et al. reported at least one NH(eta) for PA Arg 47 at 6.49 ppm. We have been unable to locate this residue in our spectra. Fig. 2 is an expansion of the 6.5 ppm region of normal one-dimensional spectra at two different tmeperatures and pH values. The region is fairly resolved, and the only resonance that we can resolve at 6.5 ppm is the main chain amide of Thr 20. It can be assigned very firmly in HOHAHA spectra by scalar connectivity to alpha, beta, and gamma protons of a typical threonine spin system. In two-dimensional spectra, especially NOESY and ^{1}H-^{15}N correlation spectra (7), we have been unable to locate any cross peaks ascrible to Arg 47 eta protons.

It is always unsettling to report failures or negative evidence, but, this situation has also extended to the case of His 47 in the analog PS c-551. The C2 and C5 protons of His 47 were readily assigned by their sharp singlet appearance in 1D spectra, by inter-residue NOE's to neighbors, by intra-residue NOE's from C5 to beta protons, and even by a weak HOHAHA cross peak between C2 and C5 at long spin lock times. Note that in protic buffers,

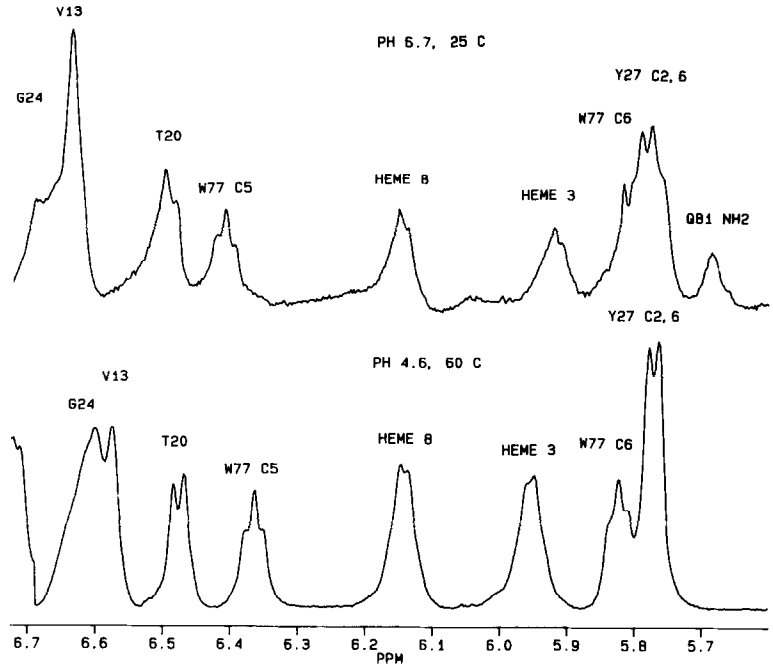

Fig. 2. Portions of one dimensional $^{1}_{2}$H-NMR spectra of PA c-551 in 50 mM phosphate buffer, 90% ^{1}H, 10% ^{2}H-water. The pH 4.6 sample was 10 mM in protein, while the pH 6.7 sample was 0.8 mM, which accounts for the difference in noise levels. The baselines have been corrected with a polynomial curve to remove a gradual slope due to the residual water resonance, which was suppressed by presaturation. The observed resonances have been assigned to the main chain amides of Gly 24, Val 13, and Thr 20; the aromatic protons of Trp 77 C5 and C6 and the degenerate protons C2 and C6 of Tyr 27; the thioether methine protons of the heme at the 3 and 8 positions (IUB-IUPAC tetrapyrrole nomenclature); and one of the side chain amide protons of Gln 81 which moves to lower frequency outside the window shown for the lower spectrum.

C2 and C5 for the free amino acid histidine will give a cross peak even though the ring nitrogen protons do not give observable resonances because they are in fast exchange with the solvent. But we have been unable to obtain any evidence for NH(pi) and NH(tele) in His 47 at a pH values of 4.7, 8.2, and 10.0 and temperatures of 27 and 47 °C. If these protons are in slow exchange with the solvent, then spectra taken in protic water should show observable cross peaks in NOESY and HOHAHA spectra, especially representing correlations to C2 and C5. So we know where to look, but have been unable to find the peaks. It is certainly possible that the signal to noise ratios in our spectra have hidden the signals, but it seems then that they must be weak. Spectra have been recorded using echo techniques (8) rather than presaturation, and candidate cross peaks still cannot be observed, so it is possible to rule out saturation transfer as a mechanism for rendering the resonances

unobservable. We conclude that the exchangable peaks for Arg 47 and His 47 are in fast or intermediate exchange with the solvent. Looking at the crystal structure of PA c-551, it is dififcult to catergorize the environment around residue 47 as either "freely accessible" or totally "inaccessible" to solvent. The implications of the NMR data are that even though a hydrogen bond may be present, the 47 protons exchange with solvent and so there must be some degree of conformational flexibility. The interaction of 47 and the propionate has been a prime candidate for rationalizations of the pH dependence of the heme redox potential (6,9-12), and this may indeed be the case, but, it seems important to note that there is solvent accessibilty and some conformational freedom between the pair.

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