Volume 311, number 3, 213–216 FEBS 11654 October 1992

Ionization of the heme propionate substituents in pseudomonad cytochromes *c*-551

Mengli Cai and Russell Timkovich

Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487-0336, USA

Received 10 July 1992; revised version received 6 August 1992

The heme propionate substituents in *Pseudomonas* cytochrome *c*-551 are partially buried by folds of polypeptide in the structure of the protein, and are involved in several hydrogen bonds. The ionization behavior of these groups has been of interest because the oxidation potential of the heme changes with pH in a manner that may parallel ionization of a propionate. The ionization pK_a's of these groups have been determined by following the NMR chemical shifts of nearby protons acting as probes of the ionization state of the propionates. In *Pseudomonas aeruginosa* *c*-551 the *13*-propionate (IUB-IUPAC porphyrin nomenclature) has been assigned a pK_a of 3.1, and the *17*-propionate a pK_a of 7.2. In the homologous *Pseudomonas stutzeri* *c*-551, the respective propionates both have pK_a values of 3.0.

Pseudomonas cytochrome; Propionate ionization; Dissociation constant

1. INTRODUCTION

Cytochrome (cyt) *c*-551 is a member of the cytochrome family found widely distributed among prokaryotes, with high concentrations especially in pseudomonads [1–3]. With approximately 82 residues, it is a smaller version of mitochondrial cytochrome *c* (approximately 103 residues). In spite of the deletions, it has a highly similar protein folding structure, including characteristic features like ligand geometry, partial burying of the heme propionic acid groups, and a network of hydrogen bonds involving the propionates [4–6]. In spite of many similar functional properties to mitochondrial cyt *c*, one outstanding difference is the fact that in cyt *c*-551 the oxidation potential of the heme is sensitive to pH, while in mitochondrial cyt *c* it is insensitive [7–9]. NMR resonances in cyt *c*-551 show chemical shift titration behavior that closely follow the oxidation potential change with pH, and so NMR has been used extensively to probe the question of what specific site in cyt *c*-551 is ionizing in concert with the oxidation potential change. Although there have been other proposals [6,10], recent multi-dimensional NMR studies [11] have confirmed the earlier conclusions [8,9] that in *Pseudomonas aeruginosa* cyt *c*-551 it is the inner or buried propionic acid, the *17*-propionate in IUB-IUPAC porphyrin nomenclature, that ionizes with a pK_a similar to the transition pK for the oxidation potential change.

The inner propionic acid is hydrogen bonded to a sequence invariant, Trp-56, and to residue 47. This is arginine in cyt *c*-551 from *Ps. aeruginosa* and many other strains, but substitutions do occur, such as replacement by a histidine in *Ps. stutzeri* and *Ps. mendocina*. In these latter cases, the sensitive ionization has still been assigned to the inner propionate, albeit with a perturbation in pK_a value due to the Arg/His change [9]. The assigned pK_a's were 7.3 in *Ps. aeruginosa* and 8.3 in *Ps. stutzeri* cyt *c*-551. A solution structure conformation has recently been determined for ferrocytochrome *c*-551 from *Ps. stutzeri*, based upon extensive proton assignments and NMR-derived structure constraints [12]. With the assignments in hand for protons structurally close to the propionates, it became possible to probe their chemical shifts with pH. In spite of extremely high structure homology between cyt *c*-551 from *Ps. aeruginosa* and *Ps. stutzeri*, the evidence indicates that the ionization events for the inner propionates are very different.

Correspondence address: R. Timkovich, Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487-0336, USA. Fax: (1) (205) 348-9104.

2. EXPERIMENTAL

References to isolation procedures and NMR techniques have been given [13]. The strain of *Ps. stutzeri* was obtained from the American Type Culture Collection (ATCC 17588). For comparison to the earlier literature, this strain was originally deposited there as the Stanier strain 221. The chemical shifts of resonances to be discussed were determined in resolved cases in one-dimensional spectra, or otherwise in two-dimensional NOESY or homonuclear Hartmann–Hahn spectra.

3. RESULTS AND DISCUSSION

Fig. 1 displays the chemical shift titration behavior of His-47 imidazole protons in *Ps. stutzeri*. The shifts to lower frequency at alkaline pH and the magnitude of the jump are virtually textbook examples of an imidazolium-to-imidazole deprotonation monitored by the

Published by Elsevier Science Publishers B.V.

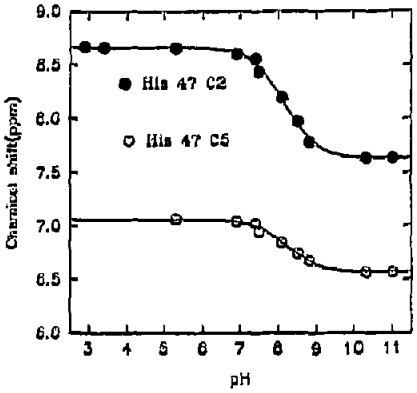

Fig. 1. Dependence on pH of the chemical shifts of His-47 C2 and C5 in *Ps. stutzeri* ferrocytochrome c-551. Theoretical curves correspond to a pK_a of 8.2.

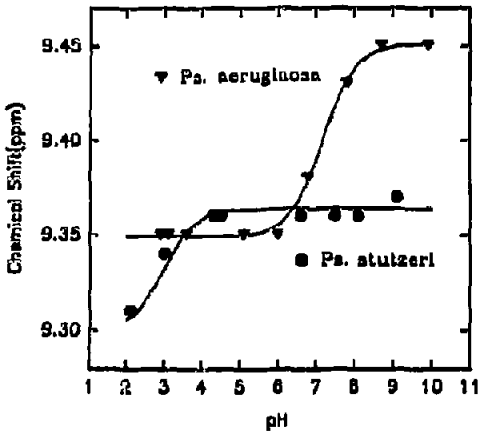

Fig. 3. Dependence on pH of the chemical shift of the *15* meso proton in *Ps. stutzeri* and *Ps. aeruginosa* ferrocytochrome c-551. Theoretical curves correspond to a pK_a of 3.0 for *Ps. stutzeri* and 7.2 for *Ps. aeruginosa*.

non-labile carbon-bound protons [14]. The pK_a observed of 8.2 matches very closely the reported midpoint oxidation potential pK of 8.3 [9]. The -COOH proton of the propionic acid has never been observed and presumably is in fast exchange with the solvent. In principle, one might expect that the ionization of the carboxylate could be monitored by the propionate alpha- and beta-methylene protons. However, it has been shown that these are relatively insensitive to pH, and that better probes are provided by the protons hydrogen bonded to propionate carbonyl oxygens [11]. The indole NH of Trp-56 was determined to be hydrogen bonded to an inner propionate carbonyl oxygen in both the crystal structure of *Ps. aeruginosa* and the solution structure of *Ps. stutzeri* cyt *c*-551. Fig. 2 displays the titration behavior of this resonance in both proteins. The major transition with pK_a of 7.2 in *Ps. aeruginosa* cyt *c*-551 is attributed to the ionization of the inner propionate. The minor transition with pK_a of 12 is ascribed to deprotonation of the Arg-47 side chain. In *Ps. stutzeri* cyt *c*-551, the major transition occurs with a pK_a of 3.0, indicating that in this protein the inner propionate is much more acidic than in *Ps. aeruginosa*. A minor transition at a pK_a of 8.2 reflects His-47 deprotonation. These transitions in the two proteins are reflected by other protons near to the propionates, even if not hydrogen bonded. For example, the *15* meso proton (IUB-IUPAC porphyrin nomenclature) and the gamma protons of the ligand Met-61 are in spatial proximity to the inner propionate. Both show pH dependences (Figs. 3 and 4) that follow the assigned pK_a's of the inner propionate.

The outer propionate (*13*-propionate) in both proteins is hydrogen bonded to the main chain amide proton of Val-55. Fig. 5 displays the titration behavior of this resonance in both cases. The major transition is nearly identical for both, indicating that the exposed outer propionate ionizes in both proteins with a typical

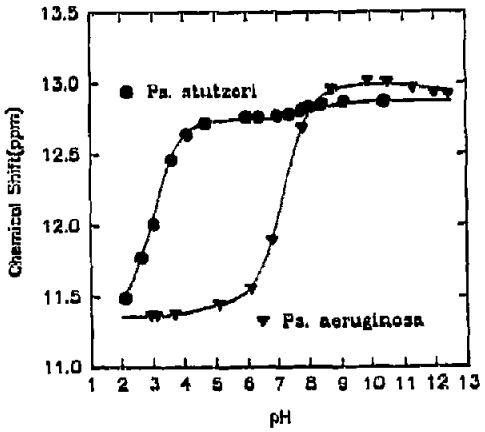

Fig. 2. Dependence on pH of the chemical shift of the Trp-56 indole proton in *Ps. stutzeri* and *Ps. aeruginosa* ferrocytochrome c-551. Theoretical curves correspond to pK_a's of 3.0 and 8.2 for *Ps. stutzeri*, and 7.2 and 12.0 for *Ps. aeruginosa*.

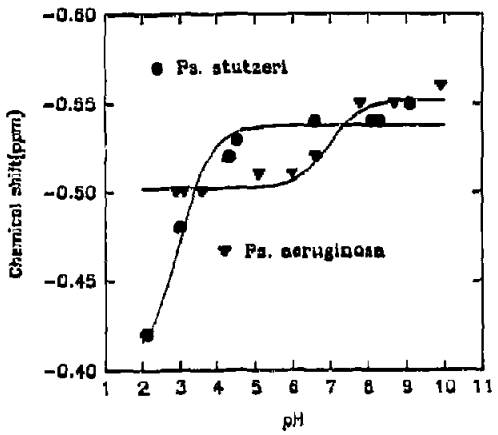

Fig. 4. Dependence on pH of the chemical shift for the Met-61 gamma proton in *Ps. stutzeri* and *Ps. aeruginosa* ferrocytochrome c-551. Theoretical curves correspond to respective pK_a's of 3.0 and 7.2.

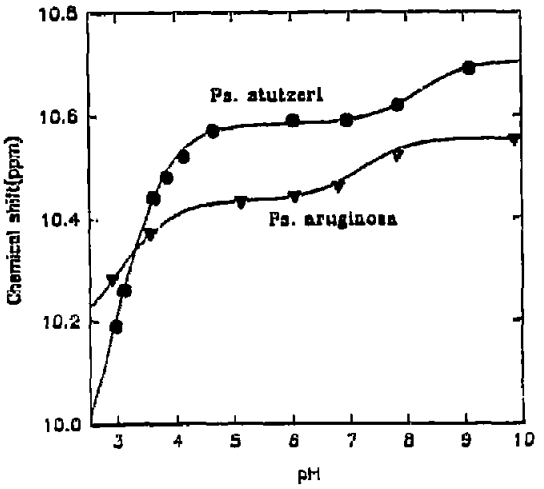

Fig. 5. Dependence on pH of the chemical shift of the amide of Val-55 in *Ps. stutzeri* and *Ps. aeruginosa* ferrocytochrome *c*-551. Theoretical curves correspond to pK_a's of 3.0 and 8.2 for *Ps. stutzeri*, and 3.1 and 7.2 for *Ps. aeruginosa*.

carboxylate pK_a near 3. In the case of *Ps. stutzeri*, the chemical shift perturbation is especially large and this is ascribed to a sensitivity of the Val-55 amide to the charge state on the inner propionate whose pK_a is also near 3.

The scheme in Fig. 6 schematically summarizes the proposed formal protonation states in the two proteins. Why is the inner propionic acid such a stronger acid in *Ps. stutzeri* given the highly homologous structures? We hypothesize with the following simple physical picture, which is easy to grasp even if it lacks rigor. The weaker base in *Ps. stutzeri*, His-47, binds the imidazolium proton less strongly than Arg-47, the stronger base, binds the guanidinium proton. This allows a stronger hydrogen bond to the oxygen of the inner propionate. In turn this weakens the bond to the non-shared proton and allows it to dissociate more readily in *Ps. stutzeri* than in *Ps. aeruginosa*.

The symbolic picture in Fig. 6 is an over-simplification. Because of tautomerization, the precise location of the acidic proton is problematic. The precise charge distribution over the groups involved would depend upon the actual polarization of any hydrogen bond, and for clarity the figure displays pure donor and acceptor characteristics. As reported here and in previous work [11,13,15], the Trp-56 indole proton is non-labile and readily observed in spectra, but neither the propionate -COOH, the His-47 imidazole, nor the Arg-47 terminal NH protons have been observed. We have sought evidence for these protons in terms of one-dimensional peaks or two-dimensional cross-peaks in NOESY or homonuclear Hartmann–Hahn spectra at acidic, neutral, and basic pH values and have not been able to find any indication of their presence. Solvent exchange appears to be taking place rapidly, even though the inner propionate environment is relatively buried. It is perhaps most appropriate to think of this region in terms of an interlocking network of hydrogen bonds, including interactions with solvent. There may be differences in the polarization of bond strengths between two proteins, such as in *Ps. stutzeri* and *Ps. aeruginosa*, and these may allow a formal assignment of respective pK_a's. However, the total behavior within the network may be the most important factor. It is not generally true that the ionization of the inner propionate is linked to the pH dependence of oxidation potential, for in *Ps. aeruginosa* the oxidation potential midpoint matches that of the propionate pK_a while in *Ps. stutzeri*, it matches the His-47 imidazole. This work does not answer the mechanistic question of how behavior in the network is related to the oxidation potential. Is it a cause or merely a symptom of other factors? This work does provide insight into some of the characteristics of

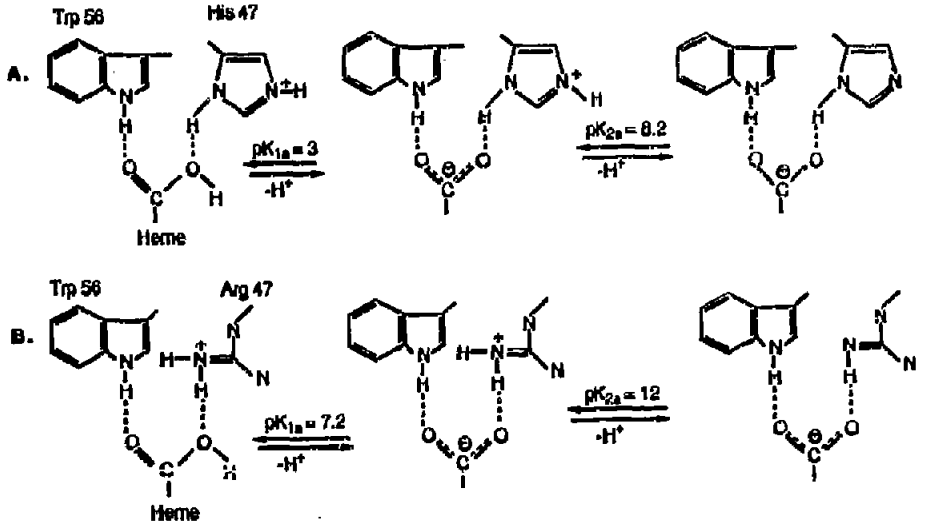

Fig. 6. Proposed ionization of the inner propionic acid and residue 47 in ferrocytochromes. (A) *Ps. stutzeri*. (B) *Ps. aeruginosa*.

a hydrogen bonding pattern that is invariant in cytochromes *c*-551.

Acknowledgements: Partial support was provided by Grant GM-43292 from the National Institutes of Health.

REFERENCES

[1] Ambler, R.P. (1973) Syst. Zool. 22, 554–564.
[2] Ambler, R.P. (1982) in: From Cyclotrons to Cytochromes (N.O. Kaplan and A. Robinson, eds.) pp. 263–280, Academic Press, San Diego.
[3] Meyer, T.E. and Kamen, M.D. (1982) Adv. Protein Chem. 35, 105–212.
[4] Dickerson, R.E., Timkovich, R. and Almassay, R.J. (1976) J. Mol. Biol. 100, 473–491.
[5] Almassay, R.J. and Dickerson, R.E. (1978) Proc. Natl. Acad. Sci. USA 75, 2674–2678.
[6] Matsuura, Y., Takano, T. and Dickerson, R.E. (1982) J. Mol. Biol. 156, 389–409.
[7] Moore, G.R. and Williams, R.J.P. (1977) FEBS Lett. 79, 229–232.
[8] Moore, G.R., Pettigrew, G.W., Pitt, R.C. and Williams, R.J.P. (1980) Biochim. Biophys. Acta 590, 261–271.
[9] Leitch, F.A., Moore, G.R. and Pettigrew, G.W. (1984) Biochemistry 23, 1831–1838.
[10] Chao, Y.H., Bersohn and Aisen, P. (1979) Biochemistry 18, 774–779.
[11] Chau, M.H., Cai, M. and Timkovich, R. (1990) Biochemistry 29, 5076–5087.
[12] Cai, M., Bradford, E.G. and Timkovich, R. (1992) Biochemistry (in press).
[13] Cai, M. and Timkovich, R. (1991) Biochem. Biophys. Res. Commun. 178, 309–314.
[14] Dwek, R.A. (1973) NMR in Biochemistry, pp. 85–94, Clarendon Press, Oxford.
[15] Timkovich, R., Walker, L.A. and Cai, M. (1992) Biochim. Biophys. Acta 1121, 8–15.