& Kraut, J. (1973) *J. Biol. Chem. 248*, 3910–3921.
Spiro, T. G., Strong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.
Strekas, T. C., & Spiro, T. G. (1974) *Biochim. Biophys. Acta 351*, 237–245.
Sundberg, R. J., & Gupta, G. (1973) *Bioinorg. Chem. 3*, 39–48.
Tasaki, A., Otsuka, J., & Kotani, M. (1967) *Biochim. Biophys. Acta 140*, 284–290.
Valentine, J. S., Sheridan, R. P., Allen, L. C., & Kahn, P. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1009–1013.
Weber, P. C., Bartsch, R. G., Cusanovich, M. A., Hamlin, R. C., Howard, A., Jordan, S. R., Kamen, M. D., Meyer, T. E., Weatherford, D. W., Xuong, Ng. H., & Salemme, F. R. (1980) *Nature (London) 286*, 302–304.
Weber, P. C., Howard, A., Xuong, Ng. H., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399–424.
Yagil, G. (1967) *Tetrahedron 23*, 2855–2859.

# Proton Nuclear Magnetic Resonance Spectra of *Pseudomonas aeruginosa* Ferricytochrome $cd_1$†

Russell Timkovich* and Margaret S. Cork

ABSTRACT: Proton nuclear magnetic resonance spectra of ferricytochrome $cd_1$ from the denitrifying bacterium *Pseudomonas aeruginosa* have been obtained. The normal 0–10-ppm chemical shift range shows many overlapping and nonresolvable peaks, as would be expected for a dimeric protein of molecular weight approximately 120 000. In the downfield region between 10 and 50 ppm, and in the upfield region between 0 and −20 ppm, resolvable resonances corresponding to a small number of protons are observed. The temperature and pH behavior of these resonances have been examined. For some of the resolved resonances, the pH behavior of chemical shifts and intensities indicates that the oxidized form of the enzyme undergoes a structural transition with a p$K$ of 5.8 ± 0.3. On the basis of several lines of evidence, some assignments are proposed in which resolvable resonances are assigned as originating from either the heme $c$ or the heme $d_1$ prosthetic groups of the enzyme.

Cytochrome $cd_1$ is a dissimilatory nitrite reductase found in facultative denitrifying bacteria. In its oxidation–reduction cycle, it accepts reducing equivalents from donor cytochromes $c$ and transfers these to nitrite, reducing it to nitric oxide predominantly (Wharton & Wintraub, 1980). In a nonphysiological reaction, it also may reduce oxygen to water (Timkovich & Robinson, 1979). The native enzyme is a dimer composed of two identical subunits. Each subunit has a molecular weight of approximately 60 000 and contains one covalently bonded heme $c$ and one noncovalently bonded heme $d_1$ (Kuronen et al., 1975). The structure and characteristics of this enzyme, especially as isolated from *Pseudomonas aeruginosa*, have been extensively explored by a variety of spectroscopic techniques [see Cotton et al. (1981) and references cited therein]. The purpose of this report is to present the application of proton nuclear magnetic resonance (NMR) to structural studies of *Pseudomonas* cytochrome $cd_1$.

Proton NMR has yielded important structural information on heme proteins, most notably on the environment of the heme prosthetic groups. For cytochrome $cd_1$, the application of NMR presents formidable problems arising from the large molecular weight of the native enzyme. The spectral region between 0 and 10 ppm has so many overlapping resonances from the amino acid residues that no useful spectroscopic information has been obtained to date. However, hyperfine chemical shifts from the heme iron are expected to place some resonances outside of the main protein envelope. Although there are four hemes per native unit, the NMR line widths are influenced by paramagnetic broadening as well as by the correlation time for the enzyme as a whole. This correlation time corresponds to a molecular weight unit of 120 000. Very broad resonances are therefore expected and have been observed in the ensuing spectra. Nevertheless, the hyperfine-shifted resonances cover a large shift range and are extremely sensitive to heme environment so that useful structural and mechanistic information is obtainable.

## Materials and Methods

*Pseudomonas aeruginosa* (ATCC 19429) was cultured in 501 lots, and cytochrome $cd_1$ was purified as described by Parr et al. (1976) with the following exception. The first ammonium sulfate fraction precipitating between 40 and 90% saturation from the crude extract was redissolved in pH 7.5 phosphate buffer and applied to a gel permeation column (5.0 × 90 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals). On the basis of molecular weight, the green cytochrome $cd_1$ was well resolved from cytochrome $c$-551 and azurin in this step. Thereafter, ion-exchange chromatographic steps were performed as described by Parr et al. (1976). The final material was typically ≥95% pure as judged by the Soret to 280-nm spectroscopic ratio index. In some initial experiments, protein only 80% pure was used. At this level, the cytochrome has been purified from other heme proteins, and the spectral regions outside the 0–10-ppm range are indistinguishable from those for pure protein. Protein was stored frozen in liquid nitrogen or as a suspension in 80% saturated ammonium sulfate at 4 °C. Protein was concentrated for NMR studies by the following procedure. Solutions were brought to 80%

† From the Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616. *Received April 28, 1982.* Financial support was provided by Grant GM-23869 from the National Institutes of Health. The NMR facility was provided by a grant from the NIGMS Shared Instrumentation Program of the National Institutes of Health (GM-26071-02S1).

0006-2960/82/0421-5119$01.25/0 

saturation in ammonium sulfate by the slow addition of solid. The precipitated protein was collected by centrifugation, redissolved in a minimal volume of 50 mM ammonium bicarbonate, pH 7.8, and dialyzed overnight at 4 °C against the same buffer. Samples were lyophilized and redissolved in 99.7% $D_2O$ that was 50 mM in potassium phosphate at the appropriate pH for the spectrum desired. Redissolving the lyophilized protein did produce a small insoluble residue that was removed by centrifugation. The soluble protein after lyophilization gave oxidized and reduced visible spectra in agreement with the literature (Yamanaka & Okunuki, 1973; Barber et al., 1976).

Samples for spectroscopy were 0.5 mL in 5-mm tubes containing between 0.2 and 1 mM protein, expressed as the subunit concentration. Samples were kept at 0–4 °C until just prior to the NMR experiment. Chemical shifts were referenced against internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), 0.3 mg/tube, with downfield shifts assigned positive values. Because of large peak widths, chemical shift accuracy was on the order of ±0.05 ppm. Visible spectra of samples in the NMR tube were obtained through use of a rapid scan array detector spectrophotometer (Update Instruments) by tipping the concentrated protein solution onto the walls of the tube. Absolute intensities were not obtainable because of the arbitrary path length, but band wavelengths and relative intensities were valuable in assuring the redox state of the NMR sample and checking against protein degradation. Other visible spectra used in this study were obtained on a Cary Model 210 spectrophotometer in conventional 1-cm cells.

For titration experiments, protein samples in NMR tubes were adjusted to high pH values with 0.5 M NaOD or to low values with 0.1 M phosphoric acid in 99.7% $D_2O$. The pH was measured after adjustment and again after data collection with an Ingold micro-pH electrode and is reported as the uncorrected meter reading. During acid titration to low values, a small amount of precipitate would occasionally form which was attributed to incomplete initial mixing. When this occurred, the residue was removed by centrifugation. Spectral titration data were analyzed by regression analysis employing the algorithm of Dwek (1973).

Spectra were obtained on a Nicolet spectrometer operating at 300 MHz for protons in the Fourier-transform mode. Transients were accumulated at 132-ms intervals over a 60-kHz spectral bandwidth by employing quadrature phase detection and a solvent HDO presaturation pulse sequence. Useful spectra required on the order of 6–16 h of accumulation per spectrum. The large spectral widths necessary to observe the wide range of shifts that are present introduced severe nonlinearity into the spectral base line. This base-line roll is aggravated by the scale expansions necessary to see something on the order of a dozen hyperfine-shifted protons out of a protein of molecular weight 120000. The base line was straightened by application of a polynomial smoothing function which is a standard Nicolet software feature. Unfortunately, this introduced artifactual features into some spectra which will be labeled and discussed when they appear.

Temperature was controlled to ±1 °C by a Nicolet variable temperature unit that had been calibrated with ethylene glycol. Peak areas were measured by Nicolet software or by a cut and weigh technique with no significant difference in precision.

## Results

The upfield and downfield portions of the spectrum of oxidized cytochrome $cd_1$ are displayed in Figure 1. The observed resonances may arise from paramagnetic shifts that could contain both contact and pseudocontact contributions. Since

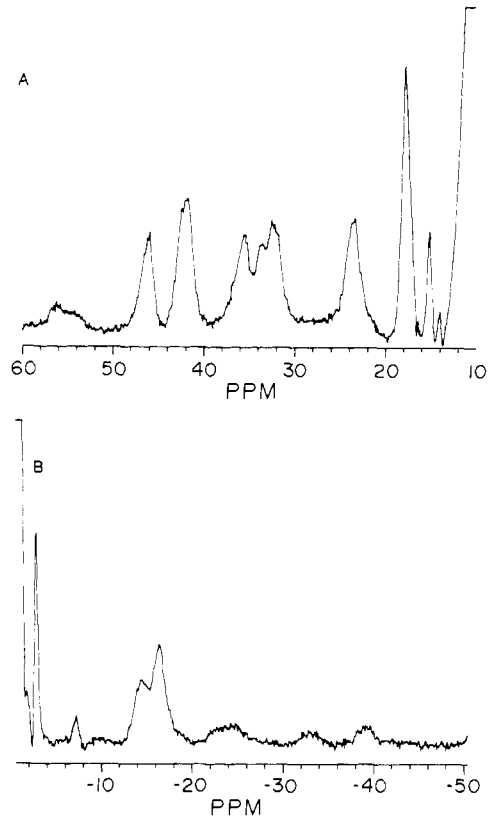


FIGURE 1: (A) Downfield region of the proton NMR spectrum of ferricytochrome $cd_1$. The protein was 0.8 mM (subunit concentration) in 99.7% $D_2O$ in a phosphate buffer, 50 mM, pH 7.5, 22 °C. This particular spectrum required 400000 transients obtained over 16 h. The base line has been adjusted as described in the text. (B) Upfield region of the spectrum of ferricytochrome $cd_1$. Conditions were as described for (A).

both effects are very short ranged, the resonances reflect protons directly bonded to the porphyrin ring or to the axial ligands or on amino acid residues in the heme pocket. By analogy with other heme proteins and model complexes (La-Mar, 1979), porphyrin methyl substituents would be expected to appear in the downfield region between 10 and 60 ppm. Axial ligand protons may be either upfield or downfield. Resonances in the near-upfield region, between approximately 0 and −5 ppm, may result from ring-current shifts with aromatic amino acid residues, the porphyrin $\pi$ system, or a combination of both.

In the downfield portion of the spectrum, broad but clear peaks are observed at 46.0, 42, 35.4, 33, 23.3, 17.5, 15.0, and 14.0 ppm. The very broad feature around 56 ppm has not been seen in all samples. The resonances at 33 and 42 ppm show a partial resolution into pairs at 42.5/41.8 ppm and 33.7/32.5 ppm. These splittings are very close to the present limits of detectability, but they have been consistently reproducible in all spectra where the signal to noise ratio was comparable to Figure 1. All downfield peaks are within a chemical shift range consistent with both hemes $c$ and $d_1$ being in the low-spin ferric form. The far-downfield range up to 110 ppm was examined, but no further peaks were observed. In the upfield region, a narrow resonance is obvious at −2.9 ppm with broader peaks at −7.5, −13, and −16 ppm. The very weak features at −24, −33, and −39 ppm are not always observable and could be artifactual. Upfield peaks (not shown) were observable at approximately 0.15 and −0.25 ppm in samples without DSS.

An absolute proton count of observed resonances has been difficult to obtain because internal standards are not fully relaxed under the rapid cycle time conditions that are necessary

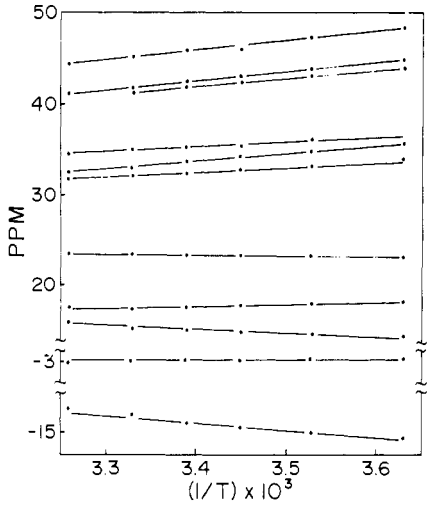


FIGURE 2: Temperature dependence of chemical shifts of resolved resonances of ferricytochrome $cd_1$. Shifts were measured with respect to internal DSS. The protein was in 50 mM phosphate buffer, pH 7.5.

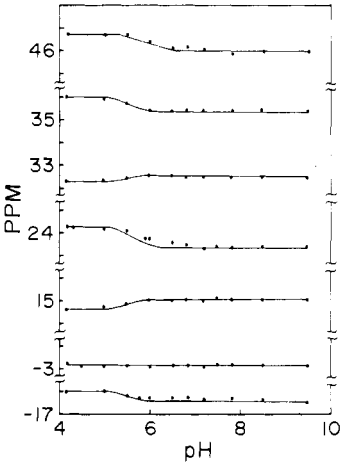


FIGURE 3: Effects of pH on resonances of ferricytochrome $cd_1$. The protein was in 50 mM phosphate, 22 °C, with the pH adjusted as described under Materials and Methods. The ordinate has been subdivided into sections for a concise display, but a constant vertical scale of 1 ppm per division has been maintained. The solid lines are drawn to illustrate the trends in the data points. The data points themselves were fit by statistical analysis to one proton titration curve, yielding a common transition p$K$ of 5.8 ± 0.3.

to accumulate sufficient transients to observe cytochrome $cd_1$ in a realistic total time. By analogy to many heme proteins, many of the downfield resonances are expected to arise from heme methyl substituents. On the basis of relative peak areas averaged over spectra obtained on different samples, a relative proton count may be obtained. Peaks at 46.0, 35.4, 23.3, 17.5, and −16 ppm correspond to three protons. The total areas of the possible doublets at 42 and 33 ppm each correspond to three protons. Peaks at 15.0, −2.9, −7.5, and −13 ppm correspond to one proton each. Peak areas at 15.0 and −7.5 ppm are very difficult to quantitate because the base-line correction algorithm tends to distort peak shapes at these shifts. The stated areas at 15 and −7.5 ppm must be viewed as tentative and subject to possibly large error.

Certain of the downfield resonances have shown intensity variability from sample to sample. The peaks at 46.0, 35.4, and 17.5 ppm in some samples have shown decreased intensity as low as one-fourth of their normal values. In the course of this study, more than ten independently prepared batches of enzyme have been examined, albeit all preparations have employed the same general method of isolation. This has provided a qualitative correlation between peak intensities and sample history. The variability is not related to the exact purity of a batch. Freshly isolated enzyme that has been prepared for NMR without lyophilization shows intense peaks at 46.0, 35.4, and 17.5 ppm. Samples that have been repeatedly lyophilized, stored in liquid form for more than 3 weeks, and exposed to high (>9) or low (<5) pH for more than a day may show decreased intensity at the aforementioned shift values, although we cannot quantitatively predict or control the extent of decrease. It should be noted that NMR samples that show decreased intensity at these shifts still demonstrate the temperature and pH behavior discussed in subsequent paragraphs. Within our experimental accuracy, the decrease appears to be concerted; that is, if any of the peaks at 46.0, 35.4, or 17.5 ppm is low in intensity, then the other two peaks are also decreased by the same extent.

The temperature dependence of the chemical shifts of resolved lines was studied between 2 and 34 °C. There was no sign of heat damage to the protein at 34 °C, but heat denaturation as evidenced by precipitate formation was observed at 39 °C after several hours of data collection. Chemical shifts are plotted vs. $1/T$ in Figure 2. Resonances downfield of 30 ppm, at +17.5 ppm, and upfield of −10 ppm show the expected Curie law behavior, but it should be noted that because of the small temperature range covered and the error limits on measuring chemical shifts, subtle deviations would not be detected. The resonance at −2.9 ppm shows no temperature dependence which is consistent with (but not proof of) a ring-current origin for the upfield shift. Resonances at 23.3 and +15.0 ppm show unexpected behavior in that the former shows no change and the latter a slight dependence of opposite slope to a Curie law effect. In mitochondrial cytochrome *c* (Wutrich, 1971), in cytochrome *c*-551 from *Pseudomonas aeruginosa* (Chao et al., 1979), and in cytochrome $c_2$ from *Rhodospirillum rubrum* (Smith, 1979), the two heme methyl substituents with the smallest downfield shifts also show an anomalous temperature dependence. Since $cd_1$ contains the same type of covalently bonded heme as these small cytochromes, it is tempting to assign these resonances to heme *c* methyl substituents.

The pH dependence of the oxidized spectrum was examined between pH 4 and 9.5. Below pH 4, there exists the possibility of dissociating the noncovalently bonded heme $d_1$ from the protein (Walsh et al., 1980). There was a pH dependence that was manifested in two ways. First, the chemical shift of several of the downfield resonances varied with pH in a systematic fashion, as plotted in Figure 3. The shift changes were subtle, comprising at most a net change on the order of 0.5 ppm, but were consistent and reproducible among three independent batches of enzyme. The more dramatic effect was the appearance and intensity increase of the resonance at +17.5 ppm on going from pH 4.2 to 9.5. This behavior is evident in the spectra of Figure 4 and the plot of Figure 5. The shift and intensity changes were fit by regression analysis to one-proton titration curves that predicted a common transition p$K$ value of 5.8 with a standard deviation of 0.3.

A correlation was sought between this transition and the visible optical spectrum of the enzyme. The visible spectrum of oxidized cytochrome $cd_1$ was originally reported to be pH invariant (Yamanaka & Okunuki, 1963). However, careful examination of the heme $d_1$ band at 635 nm revealed a gradual shift of the band maximum toward higher wavelengths with increasing pH. This shift, plotted in Figure 6, shows a slight inflection near pH 6 that correlates with the NMR observed transition. It should be noted that the inflection is a very subtle

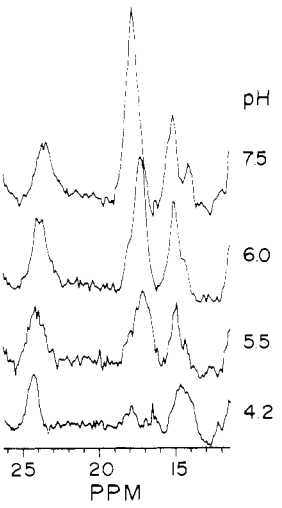


FIGURE 4: Effect of pH on the intensity of the downfield resonance at 17.5 ppm of ferricytochrome $cd_1$. Values of pH are given to the right of the corresponding spectrum.

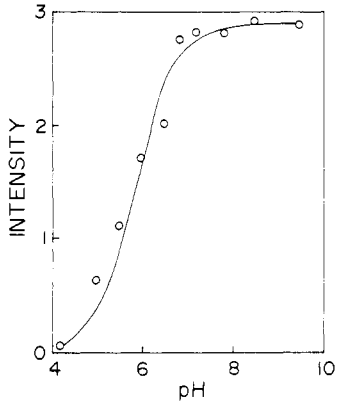


FIGURE 5: Plot of the peak intensity for the 17.5-ppm resonance as a function of pH. Open circles are the observed intensities, and the solid line is the theoretical titration curve for a p$K$ of 5.8. The vertical scale is in units of protons on a relative basis as discussed in the text.

phenomenon at the limits of detectability; its existence was acknowledged by us only after repeated optical titrations on different samples and should require confirmation by an independent laboratory. No changes could be detected in the heme $c$ or Soret regions of the spectrum.

## Discussion

Data from electron paramagnetic resonance (Gudat et al., 1973, Walsh et al., 1979) and magnetic circular dichroism (Walsh et al., 1979) studies are in agreement that oxidized cytochrome $cd_1$ is a low-spin ($S = {}^1/_2$) ferric protein. The chemical shifts of the hyperfine resonances are also typical of a low-spin ferric form. The number of resonances seen in the downfield region is larger than what is observed for monoheme cytochromes but is reasonable in view of the multiheme nature of cytochrome $cd_1$. The exact chemical structure of the porphyrin skeleton of heme $d_1$ has not been determined, although it is believed to be similar to the heme $d$ from *Micrococcus aerogenes* characterized by Barrett (1956). Kinetic (Parr et al., 1975) and visible spectra data (Blatt & Pecht, 1979) have demonstrated the existence of subunit cooperativity. Local environmental differences between subunits may therefore split resonances as has been observed for hemoglobins (LaMar, 1979).

In light of these complications, resonance assignments may require the accumulation of different lines of evidence, so it

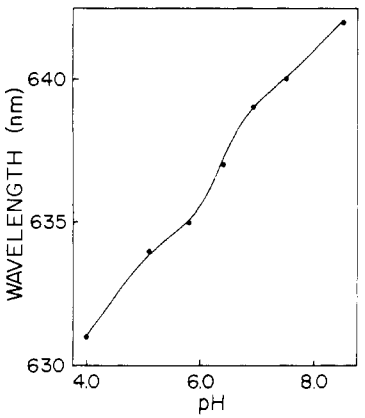


FIGURE 6: Effect of pH on the wavelength maximum of a heme $d_1$ absorption band in ferricytochrome $cd_1$. Spectra were obtained on 5 $\mu$M protein solutions in 50 mM phosphate in standard 1-cm cells. This band, assigned to the heme $d_1$ chromophore, is broad in the visible spectrum. The computer interfaced to the spectrophotometer was used to store data and replot portions of spectra with large-scale expansions so that the peak maximum could be more readily seen. The monochromator wavelength scale was calibrated with a holmium oxide filter provided by the manufacturer. The solid line through the data points has no theoretical significance and is intended to illustrate the general trend.

is useful to speculate on what may be gleamed from these initial experiments. One might expect that the pH behavior could aid in making assignments with respect to heme $c$ or $d_1$, because it appears that in the optical spectrum, heme $d_1$ predominantly reflects the transition. The upfield resonance at −2.9 ppm was pH as well as temperature invariant, suggesting assignment to neither heme. However, because of broad lines and subtle changes, it is difficult to claim pH invariance for any of the downfield resonances. Those showing the largest changes with pH were at 46.0, 35.4, 23.3, and 17.5 ppm. In was also these resonances that showed the intensity variability discussed under Results. This could reflect a lability of heme $d_1$ not shown by heme $c$. Potential sources of such lability could be orientation in the noncovalent heme pocket or axial ligand coordination differences, such as an open sixth ligand or a water sixth ligand. Resonances with smaller pH variation were at 42, 33, and 15.0 ppm. Comparisons may be made to heme methyl resonances assigned in small cytochromes $c$. For mitochondrial cytochrome $c$, heme methyls have been assigned at 34.0, 31.3, 7.2, and 10.3 ppm with corresponding methyls in *R. rubrum* $c_2$ at 33.9, 30.1, 15.1, and 10.8 ppm (Smith, 1979), and in *Pseudomonas* $c$-551 at 31.9, 27.4, 15.4, and 13.4 ppm (Chao et al., 1979). Methyl groups in microsomal cytochrome $b_5$ occur at 21.9, 14.3, and 12.1 ppm (Keller et al., 1976). Therefore, in cytochrome $cd_1$, the resonances at 33 and 15.0 ppm are consistent candidates for an assignment involving heme $c$. The inverted temperature dependence of the 15.0-ppm resonance also matches the behavior of the corresponding 15-ppm resonances in cytochromes $c$-551 and $c_2$.

The transition p$K$ of 5.8 observed in the proton spectrum of oxidized $cd_1$ correlates with the pH activity profile of the enzyme. The enzymatic activity of *Pseudomonas* cytochrome $cd_1$ was originally reported to possess a maximum in the range of 5.1–6.4, with activity decreasing at higher and lower pH values (Horio et al., 1961). This profile has been confirmed for *Pseudomonas* $cd_1$ (Yamanaka et al., 1961; Silvestrini et al., 1979) and similar behavior found for $cd_1$ in *Paracoccus denitrificans* (Timkovich et al., 1982). The NMR and optical data of this report implicate the oxidized form of $cd_1$ as the form responsible for the pH activity behavior. The optical

spectrum of the reduced $cd_1$ shows a very strong dependence on pH (Yamanaka & Okunuki, 1963). A transition p$K$ has not been reported, but from the raw data [see Figure 2 of Yamanaka & Okunuki (1963)], one can estimate that any possible p$K$ must lie to higher values than 5.8 by at least 1 unit. A recent $^{15}N$ NMR study (Timkovich & Cork, 1982) has proposed that the sequence of events in nitrite reduction is first reduction of cytochrome $cd_1$ by an appropriate donor and then interaction of reduced $cd_1$ with substrate nitrite. If correct, then the identification of the activity pH maximum as a property of oxidized $cd_1$ suggests that the activity vs. pH behavior involves the rate of reduction of $cd_1$ by donor. This interpretation is equivocable and contrary to other mechanistic hypotheses (Shimada & Orii, 1975) but warrants consideration on the basis of the new data provided by proton NMR.

It is tempting to attribute the observed transition p$K$ of 5.8 to a protein amino acid residue such as a histidine, but the heme $d_1$ prosthetic group possesses some remarkable properties that may relate to this issue. Heme $d_1$ extracted from the native enzyme has been shown to undergo in the ferric form a visible spectral transition with a p$K$ of 6.5 (Walsh et al., 1980). The chemical nature of this transition remains unidentified. One must consider the possibility that the transition that occurs in isolated heme $d_1$ with a p$K$ of 6.5 is the same as the transition with a p$K$ of 5.8 in the intact enzyme. A shift of 0.7 unit could be reasonable for a change in environment such as free solution to a protein heme pocket.

Proton NMR studies on cytochrome $cd_1$ remain far from completed, but these results illustrate the potential applicability of NMR toward unraveling the mechanistic complexities of this class of denitrifying enzymes.

Acknowledgments

Bacterial cultivation was accomplished with the technical assistance of In S. Rhee. We gratefully acknowledge Professor Kenneth D. Kopple, director of the NMR facility, for technical assistance and advice in obtaining spectra.

References

Barber, D., Parr, S. R., & Greenwood, C. (1976) *Biochem. J. 157*, 431–438.

Barrett, J. (1956) *Biochem. J. 64*, 626–639.

Blatt, Y., & Pecht, I. (1979) *Biochemistry 18*, 2917–2922.

Chao, Y. H., Bersohn, R., & Aisen, P. (1979) *Biochemistry 18*, 774–779.

Cotton, T. M., Timkovich, R., & Cork, M. S. (1981) *FEBS Lett. 133*, 39–44.

Dwek, R. A. (1973) in *NMR in Biochemistry*, pp 107–108, Clarendon Press, Oxford.

Gudat, J. C., Singh, J., & Wharton, D. C. (1973) *Biochim. Biophys. Acta 292*, 376–390.

Horio, T., Higashi, T., Yamanaka, T., Matsubara, H., & Okunuki, K. (1961) *J. Biol. Chem. 236*, 944–951.

Keller, R., Groudinsky, O., & Wutrich, K. (1976) *Biochim. Biophys. Acta 427*, 497–511.

Kuronen, T., Saraste, M., & Ellfolk, N. (1975) *Biochim. Biophys. Acta 393*, 48–54.

LaMar, G. N. (1979) in *Biological Applications of NMR* (Sulman, R. G., Ed.) pp 305–343, Academic Press, New York.

Parr, S. R., Wilson, M. T., & Greenwood, C. (1975) *Biochem. J. 151*, 51–59.

Parr, S. R., Barber, D., & Greenwood, C. (1976) *Biochem. J. 157*, 423–430.

Shimada, H., & Orii, Y. (1975) *FEBS Lett. 54*, 237–240.

Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., & Greenwood, C. (1979) *Biochem. J. 183*, 701–709.

Smith, G. M. (1979) *Biochemistry 18*, 1628–1634.

Timkovich, R., & Robinson, M. K. (1979) *Biochem. Biophys. Res. Commun. 88*, 649–655.

Timkovich, R., & Cork, M. S. (1982) *Biochemistry 21*, 3794–3797.

Timkovich, R., Dhesi, R., Martinkus, K. J., Robinson, M. K., & Rea, T. (1982) *Arch. Biochem. Biophys. 215*, 47–58.

Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, C., Springall, J. P., & Thomson, A. J. (1979) *Biochem. J. 177*, 29–39.

Walsh, T. A., Johnson, M. K., Barber, D., Thomson, A. J., & Greenwood, C. (1980) *J. Inorg. Biochem. 14*, 15–31.

Wharton, D. C., & Wintraub, S. T. (1980) *Biochem. Biophys. Res. Commun. 97*, 236–242.

Wutrich, K. (1979) in *Probes of Structure and Function of Macromolecules and Membranes*, Vol. II, Academic Press, New York.

Yamanaka, T., & Okunuki, K. (1963) *Biochim. Biophys. Acta 67*, 394–406.

Yamanaka, T., Ota, A., & Okunuki, K. (1961) *Biochim. Biophys. Acta 53*, 294–308.