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pH Dependence of Structural and Functional Properties of Oxidized Cytochrome *c″* from *Methylophilus methylotrophus**

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Cytochrome *c″* from *Methylophilus methylotrophus* is an unusual monoheme protein that undergoes a major redox-linked change in the heme arrangement: one of the two axial histidines bound to the iron in the oxidized form is detached upon reduction and a proton is taken up. The kinetics of reduction by sodium dithionite and the spectroscopic properties of the oxidized cytochrome *c″* have been investigated over the pH range between 1.4 and 10.0. The rate of reduction displays proton-linked transitions of pK_a ≅ 5.5 and 2.4, and a spectroscopic transition with a pK_a ≅ 2.4 is also observed. The protein displays a complete reversibility after exposure to low pH, and both electronic absorption and resonance Raman spectroscopic properties suggest that the transition at lower pH brings about a drastic change in the heme coordination geometry. Circular dichroism spectra indicate that over the same proton-linked transition, the protein undergoes a marked decrease (~60%) of the α-helical content toward a random coil arrangement, which is recovered upon increasing the ionic strength. The structural change at low pH is linked to a concerted two-proton transition, suggesting the detachment and protonation of axial histidine(s). Such kinetic and spectroscopic features along with the remarkable capacity of this protein to recover its native structure after exposure to extremely low pH values makes it a promising model for studying folding processes and stability in heme proteins.

Cytochrome *c″* from the obligate methylotroph *Methylophilus methylotrophus* is a soluble monoheme protein of ~15 kDa, which displays a redox-linked spin state transition from a low spin state in the oxidized form to a high spin state in the reduced form (1). The two axial ligands are histidines in the oxidized form, one of which is detached from heme upon reduction of the iron atom (2). Cytochrome *c″* is a unique example of a heme-c protein with bis-histidine coordination and spectroscopic features similar to those observed in model compounds where axial ligand planes are forced into a perpendicular orientation by steric constraints (3). NMR studies of the heme pocket have shown that it is quite stable at neutral pH, with low amide proton exchange rates and one of the heme propionates largely exposed at the surface, whereas the other one is buried in the protein (1). Attempts to crystallize the protein have been unsuccessful to date. The N-terminal half of the amino acid sequence has been determined, and it displays no significant similarity with sequences from any other protein (4).

The midpoint redox potential of cytochrome *c″* has a strong pH dependence (*i.e.* a redox Bohr effect) over the range between pH 4.0 and 10.0 (4). NMR spectroscopy shows that some of the methyl resonances in the oxidized form display a shift as large as 3 ppm over the same range. The pH dependence of the midpoint redox potential has been analyzed in terms of a model that considers two ionizing groups with pK_a values that change with the redox state of the protein (1, 4). It was concluded that the group mainly responsible for the redox Bohr effect exhibited by cytochrome *c″* is one of the axial histidines that becomes detached in the reduced state with a pK_a ≅ 8.1 for the ϵ_2-NH in the reduced form and a pK_a < 2 in the oxidized form (1, 4). However, this very low pK_a could not be determined accurately, because its value was far outside the range investigated by NMR and redox potentiometry.

Cytochrome *c″* shows a surprising resistance to acid, allowing us to extend the kinetic and spectroscopic investigation of its redox behavior to a more acid pH range and to characterize a reversible conformational transition in the oxidized form, which accounts for the whole proton-linked redox behavior. It is therefore an excellent model system of a proton/electron coupling device working over a very large pH range.

EXPERIMENTAL PROCEDURES

Cytochrome *c″* from *M. methylotrophus* was purified as previously reported (4), dialyzed *versus* H_2O, and kept frozen in small aliquots until employed for the experiment.

Kinetics of reduction was measured in a Gibson-Durrum stopped flow apparatus equipped with a 2-cm path length observation cell and interfaced to a desk-top computer for fast data acquisition (On Line Instrument Service, Jefferson, GA). The system was carefully degassed, and oxidized cytochrome *c″* (in a degassed buffer at the desired pH value) was mixed with a solution of sodium dithionite (at pH 7.0 in a very low ionic strength, *i.e.* I = 2 mM). In this way, exploiting the stability of cytochrome *c″* from *M. methylotrophus* at every pH investigated, we were able to keep the sodium dithionite solution in a stable situation, exposing it to acid pH values only for a few milliseconds. It must be remembered that the observed rate constant for reduction by sodium dithionite cannot necessarily be described only by the second order reduction rate constant k, because the overall rate is

$$k_{obs} = k \cdot \sqrt{([SO_4^{2-}]/K)} \qquad \text{(Eq. 1)}$$

where k is the intrinsic reduction rate constant and $K = 7.1 \times 10^8$ M^{-1} (5) is the equilibrium constant for the association of sodium dithionite

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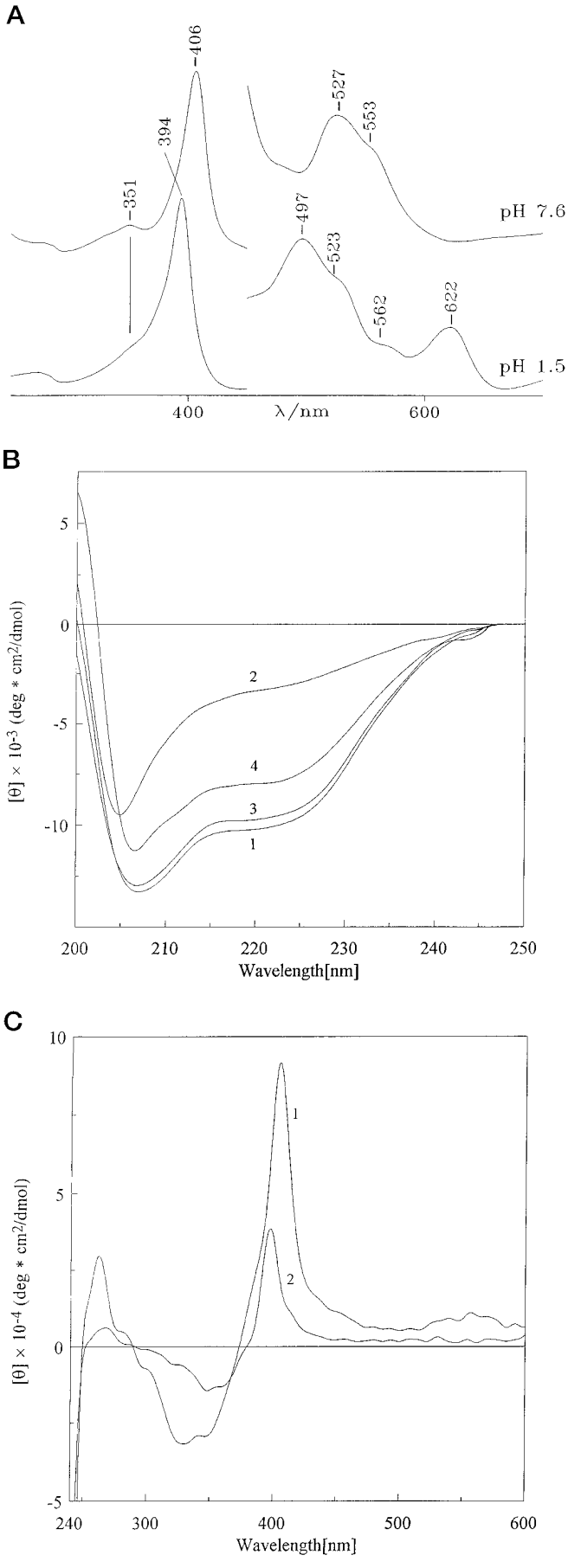

FIG. 1. *A*, absorption spectra of ferricytochrome *c″* from *M. methylotrophus* at pH 7.6 (*upper spectrum*) and at pH 1.5 (*lower spectrum*). The buffer used was 0.3 M phosphate. The temperature was 20 °C. The *right portion* of the spectrum is 10-fold amplified. For further details, see text. *B*, circular dichroism spectra between 200 and 250 nm of oxidized cytochrome *c″* from *M. methylotrophus* at pH 8.2 (*spectrum 1*), at pH 1.4 in 0.3 M phosphate (*spectrum 2*), brought back to pH 7.8 after exposure to acid pH for 30 min (*spectrum 3*), and at pH 1.4 after the addition of 1 M NaCl (*spectrum 4*). Ellipticity is expressed as degree·cm^2/dmol of residue. *C*, circular dichroism spectra between 240 and 600 nm of oxidized cytochrome *c″* from *M. methylotrophus* at pH 8.2 (*spectrum 1*) and at pH 1.4 in 0.3 M phosphate (*spectrum 2*). Ellipticity is expressed as degree·cm^2/mol of protein.

$2SO_2^- \leftrightharpoons S_2O_4^{2-}$, because SO_2^- is the actual reducing agent (see below and Ref. 5). Therefore, according to Equation 1 a variation of k_{obs} may be attributed to several different sources, such as (i) a variation of k, (ii) a modification of the sodium dithionite dissociation constant K, or (iii) an alteration of sodium dithionite concentration as a consequence of the multiple equilibria present (6). Different buffers (all at 0.3 M) were employed according to the pH range, namely potassium phosphate/HCl between pH 1.5 and 3.5, sodium acetate between pH 3.5 and 5.5, potassium phosphate, MES[1] and HEPES between pH 5.5 and 7.5, Tris/HCl between pH 7.5 and 9.0, and sodium bicarbonate between pH 9.0 and pH 10.0. No buffer-dependent variation for the kinetic behavior was observed at overlapping pH values.

The resonance Raman spectra were obtained by excitation with the 413.1-nm line of a Kr^+ laser (Coherent, Innova 90/5). The back-scattered light from a slowly rotating NMR tube was collected and focussed into a computer-controlled double monochromator (Jobin-Yvon HG 2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. The resonance Raman spectra were calibrated with indene as a standard to an accuracy of 1 cm^{-1} for intense isolated bands.

Circular dichroism spectra were obtained employing a Jasco J-700 spectropolarimeter at 20 °C. The results are expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100 \cdot \theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in mol of residue per liter, and l is the observation path length in cm. The CD spectra were obtained at a protein concentration of 3 μM with a 0.2-cm path length cell. Below 200 nm the signal-to-noise ratio of the spectrum as well as the quality of the base line was too low to obtain any reasonable information; therefore spectra were collected only in the 200–250 nm range. Absorption spectra were obtained employing a Cary 5 spectrophotometer.

RESULTS AND DISCUSSION

At neutral pH the absorption spectrum of oxidized cytochrome *c″* from *M. methylotrophus* is characterized by an α band at 553 nm, by a β band at 527 nm, and by an absorption in the Soret region at 406 nm (Fig. 1*A*), all of which are typical features of an hexa-coordinated low spin (6-coordinated low spin) heme iron, as confirmed by the resonance Raman spectrum (Fig. 2), characterized by core-size marker bands (7) at 1375 (ν_4), 1504 (ν_3), 1587 (ν_2), and 1639 cm^{-1} (ν_{10}).

Upon pH lowering, the absorption spectrum remains unchanged until pH 3.5. Further acidification brings about a transition from pH 3.0 to pH 1.5, which is fully reversible. This is a remarkable feature, because it indicates that the conformation of cytochrome *c″* at pH 1.5 is a stable one, never going beyond the threshold of a fully reversible structural change. In this respect, circular dichroism spectra have been obtained for oxidized cytochrome *c″* at pH 8.2 and 1.4 (Fig. 1, *B* and *C*). The α-helical content of cytochrome *c″* from *M. methylotrophus*, as indicated by the ellipticity at 222 nm (see *spectrum 1* in Fig. 1*B*), does not differ significantly from that reported for cytochrome *c* from horse heart (8). On the other hand, the circular dichroism spectrum of oxidized cytochrome *c″* from *M. methylotrophus* in the visible, Soret, and near UV region is markedly different from that observed for horse heart cytochrome *c* (Fig. 1*C*, *spectrum 1*). This difference, which mainly consists in the absence of a negative Cotton effect in cytochrome *c″* from *M.*

[1] The abbreviation used is: MES, 4-morpholineethanesulfonic acid.

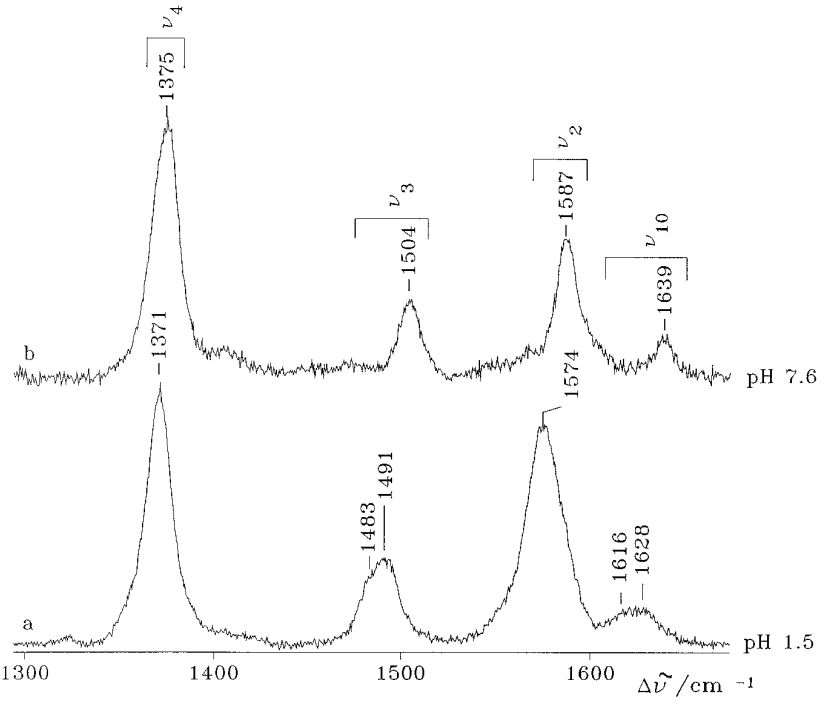

FIG. 2. **Resonance Raman spectra of ferricytochrome *c″* from *M. methylotrophus* at pH 7.6 (*b*) and at pH 1.5 (*a*) in 0.3 M phosphate buffer at 20 °C, taken with the 413.1 nm excitation wavelength.** Experimental conditions were 5 cm^{-1} resolution, 20 mW laser power at the sample. *a*, 8 s/0.5 cm^{-1} collection interval; *b*, 5 s/0.5 cm^{-1} collection interval. For further details, see text.

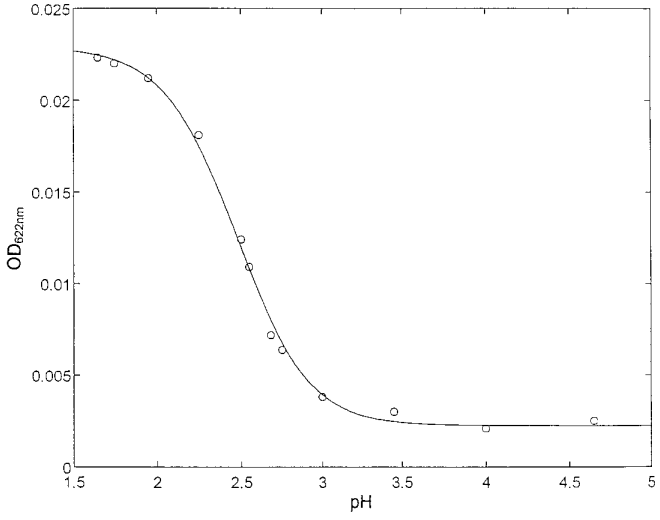

FIG. 3. **Absorption change at 622 nm of ferricytochrome *c″* from *M. methylotrophus* as a function of pH in 0.3 M phosphate buffer.** At 20 °C the continuous line corresponds to the least square fit of data according to the following equation.

$$OD_{622} = OD_{alk}/(1 + K[H^+]^2) + OD_{ac} \cdot K[H^+]^2/(1 + K[H^+]^2) \qquad \text{(Eq. 2)}$$

where OD_{622} is the observed absorption at 622 nm, OD_{alk} and OD_{ac} are the optical density at 622 nm of the sample before and after the pH-linked transition, $[H^+]$ is the proton concentration, and K is the proton binding affinity. The continuous curve corresponds to OD_{alk} = 0.002, OD_{ac} = 0.0229, and $K = 8.7 \times 10^4$ M^{-2}. For further details, see text.

methylotrophus, is not unexpected in view of the fact that in this cytochrome the iron atom is coordinated by two histidines (2). It is evident that the pH lowering in *M. methylotrophus* is accompanied by a marked decrease of the α-helical content (as indicated by the decrease of ellipticity at 222 nm for *spectrum 2* in Fig. 1*B*) and by an increase of the random coil arrangement (from the increase of ellipticity below 200 nm, see *spectrum 2* in Fig. 1*B*). Further, the circular dichroic spectrum also shows a change in the Soret and near UV region, mainly represented by a decreased ellipticity, by a blue shift of the maximum value of θ in the Soret region, and by a red-shift in the near-UV region (Fig. 1*C*, *spectrum 2*). A similar change can be observed in the far UV range for horse heart cytochrome *c*, and this species has been identified as an acid-denatured form (8). These spectral changes are all fully reversed if the protein is returned to neutral pH (see *spectrum 3* in Fig. 1*B*) or transferred at pH 1.4 into a high ionic strength medium (1 M NaCl, see *spectrum 4* in Fig. 1*B*). Therefore, exposure to low pH reduces the α-helical content of cytochrome *c″* toward an unfolded conformation (closely similar to what has been called U_A; see Ref. 9), which reverts to a fully native structure upon raising pH above 3.5 (see *spectrum 2* in Fig. 1*B*), or it undergoes a transition toward a "state A" (9) upon increasing the ionic strength (see *spectrum 4* in Fig. 1*B*). Such a state has been described before for cytochrome *c* from horse heart, and in that case it was proposed that the molecule is characterized by a high level of secondary structure and the absence of tertiary structure most probably because of the charge respulsion related to the widespread protonation of residues at this low pH (10). On the other hand, recent observations indeed suggest that helix-helix interactions stabilize the state A of horse heart cytochrome *c* (8), indicating that the tertiary structure as well is relevant for the stabilization energy of this intermediate conformation.

It is outside the purpose of the present study to dissect the contribution arising from the secondary and the tertiary structural arrangement to the stability of the conformation assumed at acid pH by cytochrome *c″* from *M. methylotrophus*. However, we can outline that in the absence of NaCl the absorption spectrum of the acid-denatured species at pH 1.5 is characterized by a charge transfer band at 622 nm, an α band at 523 nm, a β band at 497 nm (all corresponding to a high spin species) and an absorption band in the Soret region at 394 nm (Fig. 1*A*). The corresponding resonance Raman spectrum (Fig. 2) indicates the presence of at least two high spin species (labeled 5-coordinated high spin and 6-coordinated high spin) with bands at 1371 (ν_4), 1483 (ν_3, 6-coordinated high spin), 1491 (ν_3, 5-coordinated high spin), 1574 (ν_2, probably due to the overlapping contribution of both high spin species), 1616 (ν_{10}, 6-coordinated high spin), and 1628 cm^{-1} (ν_{10}, 5-coordinated high spin). All these spectroscopic features indicate that the hexa-

coordinated low spin form, present at pH > 3.5, undergoes a conversion to high spin forms at lower pH with one or both histidines being replaced by weak field ligands, such as H_2O. Similar absorption and resonance Raman spectroscopic behavior has been observed in horse heart cytochrome *c* at pH 2.0 in the absence of NaCl (11–13).

The absorption spectroscopic variation has been followed at low ionic strength between pH 3.5 and 1.5 at 406 and 622 nm, giving a closely similar pattern that can be described by a two proton-linked concerted transition with an apparent pK_a of 2.4 ± 0.2 (Fig. 3). Such a behavior indicates the occurrence of a drastic change in the coordination of the heme's iron, which could result either from the cooperative protonation of both His ϵ_2 nitrogens or from the protonation of one His ligand after it has been destabilized by a protonation that disrupts the hydrogen bonding network. The cooperative character of the spectroscopic transition suggests that protonation of one histidine in the oxidized form raises the pK_a for the protonation of a second group, thus facilitating the uptake of two protons in a cooperative fashion.

The pH-dependent conformational change described above in cytochrome *c″* from *M. methylotrophus* elicited interest in the relationship between the coordination change of the heme iron and reduction properties. At neutral pH, the kinetics of reduction by sodium dithionite is bimolecular and depends on the square root of the reducing agent concentration (Fig. 4*A*). This observation suggests that the reductant is SO_2^-, and the observed concentration dependence for the reduction kinetics at pH 7.0 (Fig. 4*A*) gives a second order rate constant k = 4.2 (±0.4) × 10^7 M^{-1} s^{-1}. In the absence of a detailed description of the linkage between protons and sodium dithionite equilibria (5, 6), the pH dependence of the pseudo-first order rate constant for reduction of oxidized cytochrome *c″*, which has to be referred to the proton-linked equilibria of the oxidized molecule, is reported at a fixed concentration of sodium dithionite, namely 0.2 mM (Fig. 4*B*). The pH dependence of k_{obs} is referrable to a proton-linked variation of the intrinsic reduction constant k only (see Equation 1), because (i) a relevant pH dependence of the dissociation constant K (see Equation 1) is not likely over that pH range and (ii) sodium dithionite concentration should not vary significantly at low pH because its exposure to very low pH is limited to a very short time. Therefore, it appears very likely that the pH dependence of k_{obs} mostly refers to the proton-linked effect on the second order reduction rate constant k (see Equation 1) and thus to the protein ionization.

The kinetics of reduction by sodium dithionite displays a bell-shaped pH dependence with two protonation events. The first proton-linked transition is characterized by a 6-fold rate enhancement and displays a pK_{a1} = 5.5 ± 0.3. It is followed by a second proton-linked transition, which displays a less than 2-fold rate decrease and a pK_{a2} = 2.5 ± 0.3 (Fig. 4*B*).

The pK_{a1} value is similar to that obtained in ferricytochrome *c″* from *M. methylotrophus* from observation of the pH dependence of ^{1}H NMR chemical shifts (4). Such a protonation does not induce significant spectral changes, and it can probably be attributed to the protonation of a heme propionate(s) (14), which have already been suggested to be responsible for the "redox Bohr effect" in several cytochromes (15–18). Therefore, the behavior reported in Fig. 4*B* seems to indicate that (i) the negatively charged heme propionic acid decreases the rate of reduction by the negatively charged $S_2O_4^{2-}$ (or SO_2^-), possibly by electrostatic repulsion, and that (ii) protonation of the heme propionates (or at least of one of them) facilitates the approach of the reducing agent, enhancing the reduction rate.

The determination of pK_{a2} indeed is of particular interest,

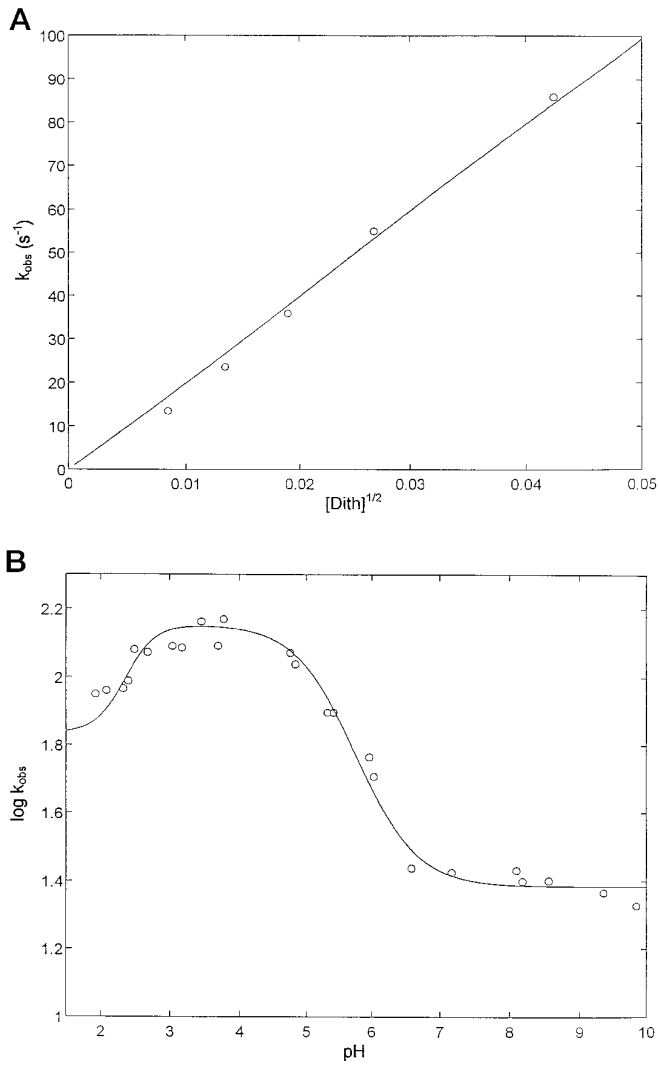

FIG. 4. *A*, dependence of the reduction rate constant of ferricytochrome *c″* from *M. methylotrophus* on the square root of sodium dithionite concentration at pH 7.0 in 0.3 M phosphate at 20 °C. λ = 406 nm. For further details, see text. *B*, pH dependence of the reduction rate constant of ferricytochrome *c″* from *M. methylotrophus* at 20 °C in 0.2 mM sodium dithionite. λ = 406 nm. The *continuous line* is the least squares fit of data points according to the following equation.

$$k_{obs} = k_0/P + k_1 \cdot K_1[H^+]/P + k_2 \cdot K_1K_2[H^+]^3/P \qquad \text{(Eq. 3)}$$

where k_{obs} is the observed rate constant at 0.2 mM sodium dithionite at different pH values and k_0, k_1, and k_2 are the observed reduction rate constants in 0.2 mM sodium dithionite before the first pH-linked transition, after the first pH-linked transition, and after the second pH-linked transition, respectively. K_1 and K_2 are the proton binding affinity of ferricytochrome *c″* from *M. methylotrophus* for the first and the second pH-linked transition, and $P = 1 + K_1[H^+] + K_1K_2[H^+]^3$ is the proton binding polynomial to ferricytochrome *c″*, accounting for the two-proton concerted transition of pK_{a2}. The following parameters have been employed to obtain the continuous curve: k_0 = 24.0 ± 2.3 s^{-1}, k_1 = 133.3 ± 5.4 s^{-1}, k_2 = 84.9 ± 4.7 s^{-1}, $K_1 = 3 \times 10^5$ (±0.4 × 10^5) M^{-1}, $\sqrt{K_2}$ = 3.3 × 10^2 (±0.2 × 10^2) M^{-1}. For further details, see text.

because it is the first time, to our knowledge, that kinetics of reduction has been carried out over this low pH range on a stable cytochrome. The value of pK_{a2} (2.5 ± 0.3, see above) from reduction kinetics is closely similar to that obtained on the basis of the electronic absorption spectroscopic transition (of about 2.4 ± 0.2, see Fig. 3), suggesting that the two events are correlated. This being the case, it indicates that the bis-histidine axial coordination, which is present in the oxidized form at pH > 3.5 (Figs. 1*A* and 2, and see Ref. 1), is lost at pH < 3.5 and

that this event is accompanied by a clear-cut decrease of the reduction rate constant, which is compatible as well with a proton-linked concerted transition (Fig. 4*B*), as described for the spectroscopic transition (Fig. 3).

In conclusion, the extension of the investigation on the kinetics for the reduction process of cytochrome *c″* from *M. methylotrophus* to an unusually large pH range provides evidence that the reduction of the oxidized cytochrome is regulated by (i) ionization of the heme propionates and by (ii) the axial coordination, such that a "low spin to high spin" transition brings about a decreased reduction rate.

The reversible detachment of one of the axial histidines upon reduction is a remarkable feature of this protein (2). This study further indicates that at low pH at least one histidine is detached also in the oxidized form, and yet the process is reversible. These data reveal that the structure of the heme pocket in this protein is extraordinarily resilient and that elucidating its structural basis therefore becomes a matter of particular interest for the design of heme enzymes.

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REFERENCES

1. Costa, H. S., Santos, H., and Turner, D. L. (1993) *Eur. J. Biochem.* **215,** 817–824
2. Santos, H., and Turner, D. L. (1988) *Biochim. Biophys. Acta* **954,** 276–286
3. Safo, M. K., Gupta, G. P., Walker, F. A., and Scheidt, W. R. (1991) *J. Am. Chem. Soc.* **113,** 5497–5510
4. Costa, H. S., Santos, H., Turner, D. L., and Xavier, A. V. (1992) *Eur. J. Biochem.* **208,** 427–433
5. Lambeth, D. O., and Palmer, G. (1973) *J. Biol. Chem.* **248,** 6095–6103
6. Mayhew, S. J. (1978) *Eur. J. Biochem.* **85,** 535–547
7. Choi, S., Spiro, T. G., Langry, K. C., Smith, K. M., Budd, D. L., and LaMar, G. N. (1982) *J. Am. Chem. Soc.* **104,** 4345–4351
8. Marmorino, J. L., and Pielak, G. J. (1995) *Biochemistry* **34,** 3140–3143
9. Goto, Y., Hagihara, Y., Hamada, D., Hoshino, M., and Nishii, I. (1993) *Biochemistry* **32,** 11878–11885
10. Hagihara, Y., Tan, Y., and Goto, Y. (1994) *J. Mol. Biol.* **237,** 336–348
11. Jeng, M.-F., and Englander, S. W. (1991) *J. Mol. Biol.* **221,** 1045–1061
12. Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry* **29,** 3480–3488
13. Jordan, T., Eads, J. C., and Spiro, T. G. (1995) *Protein Sci.* **4,** 716–728
14. Moore, G. R., Pettigrew, G. W., Pitt, R. C., and Williams, R. J. P. (1980) *Biochim. Biophys. Acta* **590,** 261–271
15. Barakat, R., and Strekas, T. C. (1982) *Biochim. Biophys. Acta* **679,** 393–399
16. Jackson, J. T., La Mar, G. N., and Bartsch, R. G. (1983) *J. Biol. Chem.* **258,** 1799–1805
17. Leitch, F. A., Moore, G. R., and Pettigrew, G. W. (1984) *Biochemistry* **23,** 1831–1838
18. Lommen, A., Ratsma, A., Bijlsma, N., Canters, G. W., van Wielink, J., Frank, J., and van Beeumen, J. (1990) *Eur. J. Biochem.* **192,** 653–661

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