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

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Massimo Coletta‡§, Helena Costa†, Giampiero De Sanctis‡, Francesca Neri**,
Giulietta Smulevich**, David L. Turner‡‡, and Helena Santos††

From the ‡Department of Experimental Medicine and Biochemical Sciences, University of Roma Tor Vergata, Via di Tor
Vergata 135, I-00133 Roma and the Department of Biochemical Sciences “Alessandro Rossi” Panelli, * University of Roma
“La Sapienza,” I-00185 Roma, Italy, the ††Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa,
P-2780 Oeiras, Portugal, the ‡Department of Molecular, Cellular, and Animal Biology, University of Camerino, I-62032
Camerino (MC), Italy, the **Department of Chemistry, University of Firenze, I-52100 Firenze, Italy, and the
‡‡Department of Chemistry, The University, Southampton, United Kingdom

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 pKa = 5.5 and 2.4, and a spectroscopic
transition with a pKa ≈ 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
transition 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 pKa 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 pKa ≥ 8.1 for the ε2-NH in the reduced form and a pKa < 2 in the oxidized form (1, 4).
However, this very low pKa 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 H2O, 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 m). 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

\[ k_{obs} = k \cdot \sqrt{[SO_2^-]/K} \]  

(Eq. 1)

where \( k \) is the intrinsic reduction rate constant and \( K = 7.1 \times 10^4 \text{ M}^{-1} \) (5) is the equilibrium constant for the association of sodium dithionite
**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. 1A), 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 (\( \nu_1 \)) , 1504 (\( \nu_2 \)) , 1587 (\( \nu_3 \)) , and 1639 cm\(^{-1} \) (\( \nu_4 \)).

Upon pH lowering, the absorption spectrum remains unchanged until pH 3.5. Further acidification brings about a transition from pH 3.5 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. 1B), 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. 1C, spectrum 1). This difference, which mainly consists in the absence of a negative Cotton effect in cytochrome c^"^ from *M.

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

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\(^2\)/mol 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.
methylophilus, 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. 1B) and by an increase of the random coil arrangement (from the increase of ellipticity below 200 nm, see spectrum 2 in Fig. 1B). 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 th in the Soret region, and by a red-shift in the near-UV region (Fig. 1C, 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. 1B) or transferred at pH 1.4 into a high ionic strength medium (1 M NaCl, see spectrum 4 in Fig. 1B). 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. 1B), or it undergoes a transition toward a "state A" (9) upon increasing the ionic strength (see spectrum 4 in Fig. 1B). 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 repulsion 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 in 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 (see spectrum 4 in Fig. 1A). 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_A), 1483 (ν_B, 6-coordinated high spin), 1491 (ν_C, 5-coordinated high spin), 1574 (ν_D, probably due to the overlapping contribution of both high spin species), 1616 (ν_E, 6-coordinated high spin), and 1628 cm⁻¹ (ν_O, 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 H2O. 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 ε2 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. 4A). This observation suggests that the reductant is SO_2^-_2, and the observed concentration dependence for the reduction kinetics at pH 7.0 (Fig. 4A) 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. 4B). The pH dependence of k_obs is referable 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_0sS 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. 4B).

The pK_a1 value is similar to that obtained in ferricytochrome c' from M. methylotrophus from observation of the pH dependence of 1H 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. 4B seems to indicate that (i) the negatively charged heme propionic acid decreases the rate of reduction by the negatively charged S_O_S^-_2 (or SO_2^-_2), possibly by electrostatic repulsion, and (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, 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. 1A 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. 4B), 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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