CONTINUOUS FLOW-RESONANCE RAMAN SPECTROSCOPY OF AN INTERMEDIATE REDOX STATE OF CYTOCHROME *c*

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ABSTRACT An intermediate redox state of cytochrome *c* at alkaline pH, generated upon rapid reduction by sodium dithionite, has been observed by resonance Raman (RR) spectroscopy in combination with the continuous flow technique. The RR spectrum of the intermediate state is reported for excitation both in the (α, β) and the Soret optical absorption band. The spectra of the intermediate state are more like those of the stable reduced form than those of the stable oxidized form. For excitation at 514.5 nm, the most prominent indication of an intermediate state is the wave-number shift of one RR band from 1,562 cm^{-1} in the stable oxidized state through 1,535 cm^{-1} in the intermediate state to 1,544 cm^{-1} in the stable reduced state. For excitation at 413.1 nm, a band, present at 1,542 cm^{-1} in the stable reduced state but not present in the stable oxidized state, is absent in the intermediate state. We interpret the intermediate species as the state where the heme iron is reduced but the protein remains in the conformation of the oxidized state, with methionine-80 displaced as sixth ligand to the heme iron, before relaxing to the conformation of the stable reduced state, with methionine-80 returned as sixth ligand.

INTRODUCTION

The existence and lifetime of intermediate states of enzymes are of interest in elucidating enzyme mechanisms in general and biological energy conversions in particular (1, 2). The study of protein conformational transitions by observation of the spectral properties of the prosthetic group of an enzyme is based on the interaction between this group and the polypeptide chain.

Cytochrome *c* acts as an electron transfer enzyme in the respiratory chain of mitochondria (3). Its prosthetic group is a heme group, which is covalently attached to the polypeptide chain through thioether linkages of two cysteine residues to the porphyrin and through the bonds between the iron and the residues acting as fifth and sixth ligands to the iron. The heme group is responsible for two optical absorption bands, the (α, β)-band in the visible, and the Soret band in the near ultraviolet, spectral region. Mainly through the direct interactions described, conformational transitions of the polypeptide chain influence the spectral properties of the heme group, e.g., the optical absorption, circular dichroism, and resonance Raman scattering.

Cytochrome *c* undergoes a large conformational transition upon change of the oxidation state of the heme iron at alkaline pH (4–13). The residue that serves as sixth ligand to the iron at neutral pH, i.e., methionine-80, is displaced in the oxidized state at alkaline pH. The residue that replaces methionine-80 is not established, though lysine-72 and lysine-79 are favored candidates. Upon reduction of the heme, methionine-80 returns as sixth ligand to the heme iron. The kinetics of the reduction of cytochrome *c* at alkaline pH has been studied by various methods for the two cases of chemical reduction and reduction by hydrated electrons. The stopped-flow technique for rapid chemical reduction has been combined with detection of optical absorption (4–7), circular dichroism (12, 13), and, using rapid freezing, electron spin resonance (6). The reduction by hydrated electrons has been accomplished by the pulse-radiolysis technique and detected by optical absorption spectroscopy (8–11). In both cases of reduction, several phases of the reduction have been distinguished. The primary phase, which is fast, corresponds to the second-

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order reaction between oxidized cytochrome *c* and reductant. The primary step is followed by first-order intramolecular processes, interpreted as conformational transitions.

Resonance Raman (RR) spectroscopy has been applied rather successfully to the study of heme proteins (14–17). Their RR spectra have proved to be characteristic of oxidation state, spin state, and iron-ligand interactions; thus they provide a very sensitive spectroscopic tool for electronic and structural studies of this class of molecules. A few years ago, this technique was applied for the first time to the study of short-lived intermediates (18, 19). Time-resolved RR spectroscopy has since been used to study biological processes that involve heme proteins and are initiated by laser pulses (20–25) or, in the case of cytochrome *c* reduction, by pulse radiolysis (26).

In the present work, the chemical reduction of cytochrome *c* by sodium dithionite in alkaline aqueous solution has been studied by RR spectroscopy in a continuous mixed-flow experiment. The combination of these two methods has been used previously to investigate redox processes (27–31), but is here for the first time applied to the study of electron transfer enzymes.

EXPERIMENTAL METHODS

Cytochrome *c* from horse heart (Sigma Chemical Company, St. Louis, MO, type VI), $Na_2B_4O_7$ (E. Merck, Darmstadt, Germany), and NaCl (Merck) were analytical reagents. $Na_2S_2O_4$ (Fisons Scientific Apparatus, Loughborough, Leics., England) was 85% assay, and the water was doubly distilled before use.

RR spectra were obtained with a spectrometer consisting of a Spex model 1403 (Spex Industries, Inc., Metuchen, NJ) double monochromator fitted with a UVISIR sample compartment and controlled by the Spex Compudrive and Scamp minicomputer (Spex Industries). One exit port of the 1403 was equipped with a slit assembly and a cooled phototube, RCA type C31034-A02 (RCA Electro-Optics and Devices, RCA Solid State Div., Lancaster, PA). Using 1,800 gr/mm gratings, this system provided normal single-channel spectra. At the other exit port a cooled vidicon detector, the PAR (EG&G Princeton Applied Research Corp., Princeton, NJ) model 1205, was mounted to obtain multichannel RR spectra and this was connected with a PAR 1215 controller and PAR 1216 system processor. This OMA-2 system (EG&G) was used in conjunction with 150 gr/mm ruled gratings fitted in the Spex 1403, to provide a spectral range of ~1,400 cm^{-1} at the detector.

Excitation was provided by Spectra Physics model 170 Kr^+ (Spectra-Physics Inc., Mountain View, CA) and Coherent Radiation model CR4 Ar^+ lasers (Coherent Inc., Palo Alto, CA). N_2-bubbled aqueous solutions of 2×10^{-4} M cytochrome *c* and 5×10^{-2} M $Na_2S_2O_4$, buffered to pH 10.5 by addition of NaOH to 2.5×10^{-2} M $Na_2B_4O_7$ in 0.3 M NaCl, were flowed together through a four-way mixer. The cytochrome *c* solution contained ~5% of the stable reduced form as estimated from separate optical absorption measurements. A 1-mm i.d. thin-walled capillary tube of fused silica was used as the flow cell, the Raman laser beam being focused transversely through this tube 18 mm downstream from the point of mixing, corresponding to a time delay of 55 ms. A motor-driven twin-syringe system was used to push the solutions through the mixer and flow cell assembly at a constant rate for the duration of an experiment. Repetitive scan accumulations were used to build up high signal strength on the vidicon detector and thus achieve approximately linear response as well as good signal-to-noise ratios. Typically 2,000 scans at 45 ms/scan were accumulated, although the sample was exposed to laser excitation for only ~70 s of this 90-s experiment time. The ~70-s period of flow was marked at each end by a microswitched voltage step which was used to operate a small flag in the laser beam. At a flow rate of ~7.5 cm^3/min for each reactant, the volume of cytochrome *c* solution used in each experiment was ~8.75 cm^3.

Using the OMA-2 detector, the wave-number calibration of the system was found to be sensitive to the optical alignment within the sample compartment. Special care was therefore taken to calibrate each set of multichannel data with an indene spectrum obtained under identical conditions. Both multichannel and conventional single-channel spectra were obtained from the stable oxidized and reduced forms of cytochrome *c* held in a standard spinning cell as well as in the capillary flow cell, thus providing a further check on the wave-number calibration which is believed to be good to ±2 cm^{-1} throughout.

RESULTS

RR spectra of cytochrome *c* excited at a wavelength of 514.5 nm in resonance with the β-optical absorption band

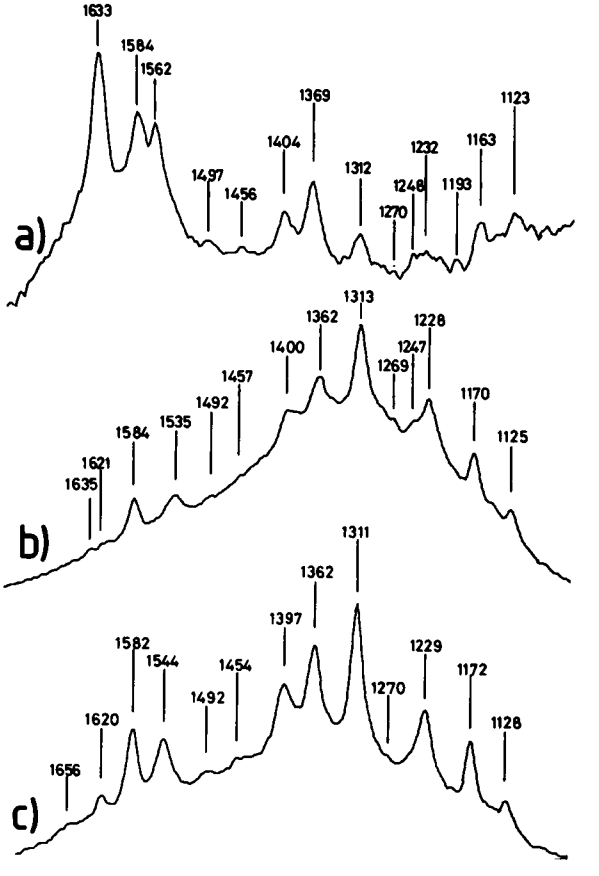

FIGURE 1 Resonance Raman spectra of cytochrome *c* at pH = 10.5, excited by a continuous-wave Ar^+-laser at 514.5 nm generating ~300 mW on the sample in a continuous flow capillary, and detected by a vidicon OMA. (*a*) stable oxidized state observed from a stationary solution of 0.1 mM oxidized cytochrome *c*, which contains ~5% of the stable reduced form. (*b*) intermediate state observed from a flowing reaction mixture ~55 ms after mixing, in the volume-ratio 1:1, of solutions of 0.2 mM oxidized cytochrome *c* as in (*a*), and 50 mM $Na_2S_2O_4$. (*c*) stable reduced state observed from a stationary solution resulting from mixing as in (*b*). Stationary samples (*a* and *c*) were exposed to the laser for ~7 s, flowing solution (*b*) exposed throughout the flow-time of ~70 s. All solutions prepared in a buffer of 0.25 mM $Na_2B_4O_7$, pH = 10.5 by addition of NaOH, and 0.3 M NaCl and deoxygenated by bubbling with N_2.

are shown in Fig. 1. Fig. 2 shows the corresponding spectra, obtained under identical conditions as in Fig. 1, but with 413.1 nm excitation in resonance with the Soret band. The upper spectra (*a*) in Figs. 1 and 2 are those obtained from the stable oxidized species before mixing, while the lower ones (*c*) derive from the stable reduced form several minutes after reduction by sodium dithionite. Finally, the spectra (*b*) in the middle were obtained from a reaction mixture approximately 55 ms after mixing.

From the figures it can be seen that the spectra (*b*) of the reaction mixtures are very similar, though not identical, to the spectra (*c*) of the stable reduced form, but are quite different from the spectra (*a*) of the stable oxidized form.

Excitation at 514.5 nm

In Fig. 1 the most prominent difference between the RR spectra of the reaction mixture (*b*) and the stable reduced form (*c*) is the wave-number shift of a band which, in the stable reduced state, appears at 1,544 cm^{-1}. In the stable oxidized state the corresponding band is located at 1,562 cm^{-1} and shifts to 1,535 cm^{-1} in the intermediate spectrum.

The band that is present at 1,247 cm^{-1} in the intermediate spectrum is of lower intensity in the spectrum of the stable reduced state. There is a band at this wave-number in the spectrum of the stable oxidized state, but a comparison of the band intensities (e.g., at 1,633 cm^{-1}) in spectra *a* and *b* of Fig. 1 shows that the oxidized form is not present in a sufficient amount to account for this band in the spectrum of the reaction mixture.

Other differences between spectra *b* and *c* are the wave-number shifts of the bands located at 1,454; 1,397; and 1,128 cm^{-1} in the spectrum of the stable reduced state. The shifts of these bands between the intermediate spectrum and that of the stable reduced state are small, ~3 cm^{-1}, but outside the experimental error. Furthermore, the band at 1,620 cm^{-1}, characteristic of the stable reduced form and absent from spectrum *a*, is only weakly evident in spectrum *b*.

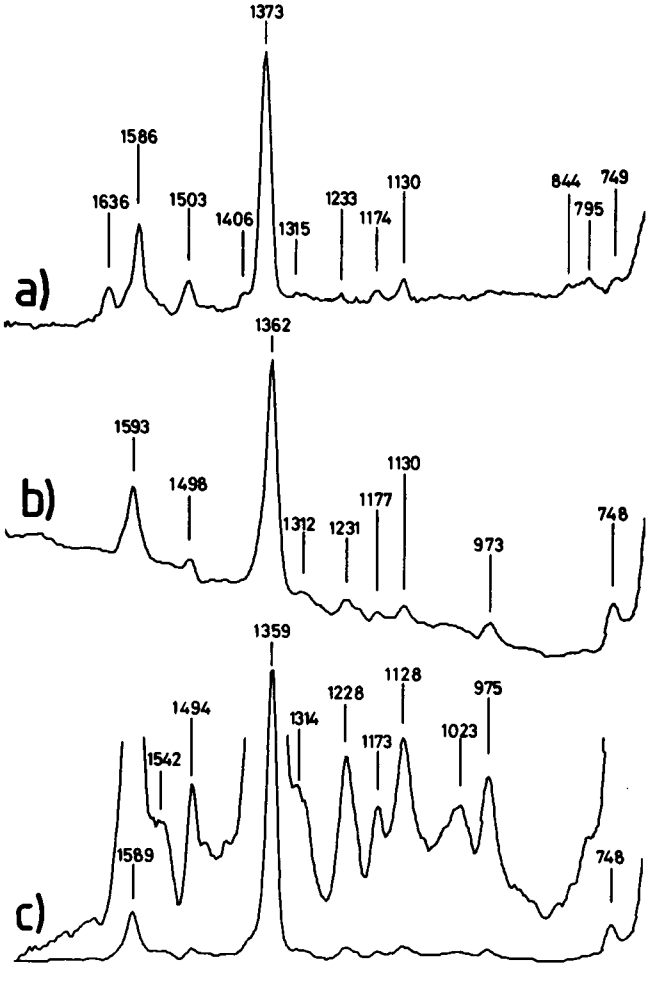

FIGURE 2 As in Fig. 1, but resonance Raman spectra excited by a continuous-wave Kr^+-laser at 413.1 nm generating ~120 mW on the sample.

Excitation at 413.1 nm

In Fig. 2, the band that is present at 1,542 cm^{-1} in the spectrum of the stable reduced state is not found in the reaction-mixture spectrum.

Apart from this difference other bands undergo small (3 or 4 cm^{-1}), though significant, wave-number shifts between the spectra *b* and *c*, viz. the bands at 1,589; 1,494; 1,359; 1,228; and 1,173 cm^{-1} given by the stable reduced state.

DISCUSSION

The two excitation wavelengths used provide complementary information to each other on the normal modes of cytochrome *c* (32, 33). In both cases, it is Raman scattering from in-plane modes that is resonance enhanced, because the optical absorption bands involved in the resonance originate from porphyrin $\pi \rightarrow \pi^*$ electronic transitions polarized in the porphyrin plane. Resonance of the exciting light with the α-band, or its vibronic side band, the β-band, enhances scattering from modes that are not totally symmetrical. The scattering mechanism in this case is of B-term type (34), i.e., involves a vibronic coupling between two excited states, viz. the excited states of the α-band and Soret-band transitions. The selection rules for vibronic coupling imply that the modes with increased scattering activity are of symmetries A_{2g}, B_{1g}, and B_{2g} in the point group D_{4h}, although B_{2g} modes have not been observed in practice. A_{2g} modes produce inversely polarized scattering, whereas B_{1g} modes generate depolarized scattering. The *A*-term type of scattering mechanism (34), with only one excited state involved, is operative during excitation in resonance with the Soret band. In that case, polarized scattering from totally symmetric A_{1g} modes is generated.

From the significant differences between spectra *b* on the one hand and spectra *a* and *c* on the other, we conclude that a distinct intermediate species different from the stable oxidized and reduced forms must be present in the reaction mixture. It has previously been noted that certain vibrational bands can be used as markers of oxidation and spin state (33). In particular, an intense polarized band at 1,374 or 1,362 cm^{-1} (excitation at Soret-band) and a depolarized one at 1,636 or 1,620 cm^{-1} (excitation at [α, β]-band) are characteristic of the oxidized and reduced form, respectively. By inspection of spectra *b* it can be seen that the intermediate spectra arise from a reduced species, the amount of oxidized form present being negligible. As to the relative concentrations of intermediate reduced and stable reduced species, we estimate from the relative intensities in spectra 1 *b* and *c* of the depolarized bands, which are located at 1,544 and 1,620 cm^{-1} in the spectrum of the stable reduced form, that the intermediate spectra *b* consist of approximately 80% intermediate and 20% stable reduced form.

The three bands with wave numbers 1,620, 1,582 cm^{-1} (excitation at [α, β]-band) and 1,494 cm^{-1} (excitation at Soret band) in the spectra of the stable reduced form serve as markers of the spin state since they show significant wave-number shifts between compounds containing high- and low-spin iron (33). The absence of such shifts for these bands between the spectra from the intermediate and the stable reduced state indicates that the intermediate state contains low-spin iron as already has been established for the stable reduced state (35).

Most published RR spectra of the stable forms of cytochrome *c*, excited at the (α, β)-band (32, 33, 36) or at the Soret band (33, 37), are obtained at neutral pH. The influence of pH on the RR spectra of cytochrome *c* has previously been shown, using excitation at 514.5 nm, to be significant for the oxidized form only (38).

For a characterization of those normal modes that we observe to function as markers of the intermediate state, we refer to a previous normal coordinate analysis (39). In the wave-number region studied, the RR spectrum of cytochrome *c* is dominated by porphyrin ring modes with contributions from stretching of C—C and C—N bonds and bending of C—H bonds. Let C_a and C_b denote the innermost and outermost C atoms of the pyrrole rings, respectively, and C_m the C atoms in between different pyrrole rings.

Excitation at 514.5 nm. The normal mode assigned to the band at 1,544 cm^{-1} in the stable reduced state consists mainly of C_b—C_b bond stretching. This contributes 57% of its character. The next largest component of this mode is methine bridge C_a—C_m stretching, accounting for 16%. The C_b—C_b bonds are the most susceptible to influence by conformational transitions of the protein, because C_b atoms are involved in the covalent linkages between the protein and the porphyrin, viz. the thioether linkages from two cysteines. The wave-number shift to 1,535 cm^{-1} corresponds to a decrease of the bond order in the intermediate state as compared with the stable reduced state. The symmetry assignment of this mode is B_{1g}, and its Raman scattering is depolarized. The band at 1,247 cm^{-1}, observed in the spectrum of the intermediate state has not been assigned by the normal coordinate analysis.

Excitation at 413.1 nm. There is also no literature assignment for the 1,542 cm^{-1} band which was given by the stable reduced state but not by the intermediate state. The selection rules for the RR scattering associated with Soret band excitation predict the mode to be of A_{1g} symmetry, and thus the band to be polarized. However, as depolarized bands at positions identical to those given by 514.5-nm excitation have been reported at 750; 1,230; 1,397; and 1,550 cm^{-1} (37), the possibility that the band at 1,542 cm^{-1} generated by 413.1-nm excitation is due to the same mode as that at 1,544 cm^{-1} for 514.5-nm excitation discussed above cannot be ruled out completely. The assignment of the 1,589 cm^{-1} band given by the stable reduced state, viz. 54% C_b—C_b stretching and 16% C_a—C_m stretching, is very similar to that of the 1,544 cm^{-1} band given by the stable reduced state and discussed above. One might, therefore, have expected a comparable difference between the intermediate and the stable reduced state with regard to the band at 1,589 cm^{-1} to that which is observed for the band at 1,544 cm^{-1}.

As described in the Introduction, it is well established that at alkaline pH cytochrome *c* undergoes a large conformational transition when the oxidation state is changed. The residue that serves as sixth ligand to the heme iron at neutral pH (i.e., methionine-80) is displaced in the oxidized state at alkaline pH. We interpret the intermediate state observed in the present experiments as the state where the heme iron is reduced but the protein remains in a conformation corresponding to that of the oxidized state before it has relaxed to its new equilibrium conformation. The fact that we observe the intermediate state most distinctly from the initial, stable oxidized, and final, stable reduced, states as late as 55 ms after mixing of the reactants might be due to a possible stabilizing influence on the intermediate state by the high ionic strength.

This interpretation of the form of the intermediate state is supported by observations on the di-carboxymethylated form of cytochrome *c* at alkaline pH. In this modified form of cytochrome *c*, where methionine-65 and methionine-80 are carboxymethylated, methionine-80 is displaced as sixth ligand to the heme iron at alkaline pH in both oxidation states (40). The type of intermediate state that is generated when the native form of cytochrome *c* is reduced at alkaline pH, as discussed above, thus is stabilized in this modified form of cytochrome *c*. A similarity between the

molecular nature of the intermediate state that we observe and of the stable reduced state of dicarboxymethylated cytochrome *c* at alkaline pH is indicated by the RR band at 1,535 cm^{-1} that is present in the spectra of both (41, 42) and has been proposed above to be a marker of our intermediate state.

Another indication of the molecular nature of our intermediate state is provided by the model compound ferrous protoporphyrin bis-imidazole, which shows RR bands at 1,534 and 1,583 cm^{-1} (43), again similar to our intermediate state. It has also been observed that the RR band at 1,544 cm^{-1} in the stable reduced state is the most sensitive to replacement of the sixth ligand to the heme iron (38).

Our interpretation of the intermediate state also is consistent with the very recent observations by time-resolved RR spectroscopy of pulse-radiolytically reduced cytochrome *c* (26). In that investigation, the exciting dye-laser was tuned to resonance with the α-optical absorption band. The same RR band as observed here using excitation at 514.5 nm, viz. the 1,535 cm^{-1} band given by the intermediate state, was then similarly distinguished to serve as a marker of an intermediate state generated upon reduction. From studies of the influence on the intermediate state of varying proportions of the neutral and alkaline forms of cytochrome *c*, it was concluded that the intermediate state was specific to the alkaline form. It follows from the present work that the molecular nature of the intermediate state is similar whether it is generated by rapid chemical reduction by sodium dithionite or by pulse-radiolytic reduction by hydrated electrons.

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