Effect of the Hinge Protein on the Heme Iron Site of Cytochrome c_1†

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Received November 23, 1988; Revised Manuscript Received December 27, 1988

ABSTRACT: X-ray absorption spectroscopic (XAS) studies on cytochrome c_1 from beef heart mitochondria were conducted to identify the effect of the hinge protein [Kim, C. H., & King, T. E. (1983) *J. Biol. Chem. 258*, 13543–13551] on the structure of the heme site in cytochrome c_1. A comparison of XAS data of highly purified "one-band" and "two-band" cytochrome c_1 [Kim, C. H., & King, T. E. (1987) *Biochemistry 26*, 1955–1961] demonstrates that the hinge protein exerts a rather pronounced effect on the heme environment of the cytochrome c_1: a conformational change occurs within a radius of approximately 5 Å from the heme iron in cytochrome c_1 when the hinge protein is bound to cytochrome c_1. This result may be correlated with the previous observations that the structure and reactivity of cytochrome c_1 are affected by the hinge protein [Kim, C. H., & King, T. E. (1987) *Biochemistry 26*, 1955–1961; Kim, C. H., Balny, C., & King, T. E. (1987) *J. Biol. Chem. 262*, 8103–8108].

Cytochrome c_1 is an essential carrier in electron transfer from the cytochrome b–c_1 complex to cytochrome c in the respiratory chain of mitochondria. Although structural properties of cytochrome c_1, including the amino acid sequence (Wakabayashi et al., 1982a; King, 1983; Kim & King, 1987), have been extensively studied, the molecular mechanism of electron transfer between cytochrome c_1 and c is still not clearly understood because of a lack of detailed structural information.

Recently we have reported the role of the hinge protein (Kim & King, 1981, 1983; Wakabayashi et al., 1982b; Kim, 1987), which stabilizes the cytochrome c_1–c complex (Chiang et al., 1976; Kim & King, 1981), in electron-transfer reaction between cytochrome c_1 and cytochrome c (Kim et al., 1987a,b). The effect of the hinge protein on some properties of cytochrome c_1 has been also reported (Kim & King, 1987). In those reports, we proposed that the hinge protein may function as a regulator in the electron-transfer reaction between cytochromes c_1 and c by stabilizing cytochrome c_1 in a specific conformation.

In order to identify the effect of the hinge protein on the structure of cytochrome c_1, we performed X-ray absorption spectroscopic (XAS) studies of cytochrome c_1 without the hinge protein ("one-band" c_1) and of cytochrome c_1 with the hinge protein ("two-band" c_1). X-ray absorption spectroscopy is particularly suited to detect small structural differences such as subtle changes in heme stereochemistry (Shulman et al., 1978), as has been successfully demonstrated for cytochrome c (Labhardt & Yuen, 1979; Korszun et al., 1982). In this paper we present the first X-ray spectroscopic evidence for conformational changes on the heme iron site of cytochrome c_1 when the hinge protein is bound to cytochrome c_1.

† This work was supported by grants from the American Heart Association (861254) and the National Institutes of Health (RR01633, GM16767, and GM39612).

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Experimental Procedures

Enzyme Preparations. One-band and two-band cytochromes c_1 were prepared by the method previously reported (Kim & King, 1987). Cytochromes c_1 and c were oxidized with an excess amount of solid potassium ferricyanide and passed through a Sephadex G-25 column equilibrated with 50 mM phosphate buffer, pH 7.4. Cytochromes c_1 (both two-band c_1 and one-band c_1) were then concentrated in an Amicon ultrafiltration cell with a PM-10 Diaflo ultrafiltration membrane or an Amicon Macrosolute concentrator (Minicon B15). All samples were stored at −80 °C prior to use.

X-ray Absorption Studies. X-ray absorption experiments were performed at room temperature at the National Biostructures PRT beam line X9-A of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. Cytochrome solutions were placed in a 1-mL Mylar window sample cell, and fluorescence XAS spectra were measured in the front-face fluorescence mode with a Stern–Heald-type ionization chamber with a MnO_2 filter and Soller slits for suppression of elastically scattered background radiation. Silicon-111 monochromator crystals were used, and the monochromator was detuned to minimize third harmonic contamination. Each sample was oriented such that the front face of the sample cell was at 45° with respect to both the direction of the X-ray beam and detector. Spectra were accumulated in 5-eV steps with 1.0-s collection time per step from 6.9 to 7.9 keV except for 2-eV steps between 6.96 and 7.19 keV.

X-ray absorption data were collected first for the oxidized cytochromes: one-band cytochrome c_1, 1–2 mM; two-band cytochrome c_1, 1.5–2.5 mM; horse heart cytochrome c, 2–4 mM, in 50 mM phosphate buffer, pH 7.4. After each EXAFS experiment a small amount for sample was withdrawn for optical spectral analysis to verify if radiation damage or photoreduction occurred in the sample during X-ray exposure. The rest of the sample was then reduced by adding an excess amount of solid sodium dithionite, and EXAFS experiments on the reduced cytochromes were performed.

Results and Discussion

Figure 1 shows the background-corrected fluorescence EXAFS spectra of both oxidized and reduced forms of horse

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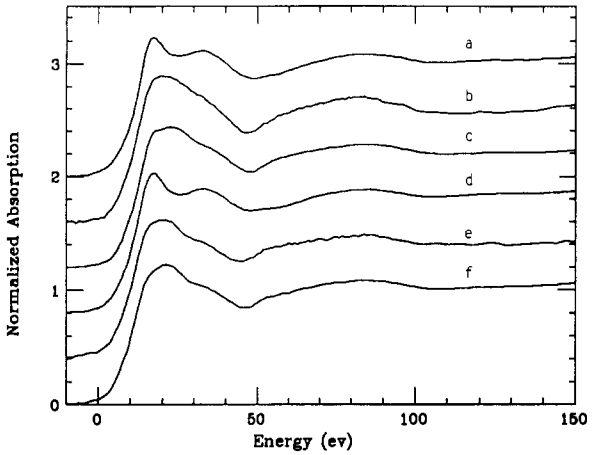

FIGURE 1: X-ray K-edge fluorescence excitation spectra of cytochromes c_1 and c: (a–c) oxidized forms of two-band c_1 (cytochrome c_1 with the hinge protein), one-band c_1 (cytochrome c_1 without the hinge protein), and cytochrome c, respectively; (d–f) reduced forms of two-band c_1, one-band c_1, and cytochrome c, respectively. The preedge linear background has been subtracted, and the data are normalized to unit edge step. The spectra are offset for clarity. The energy zero is 7100 eV.

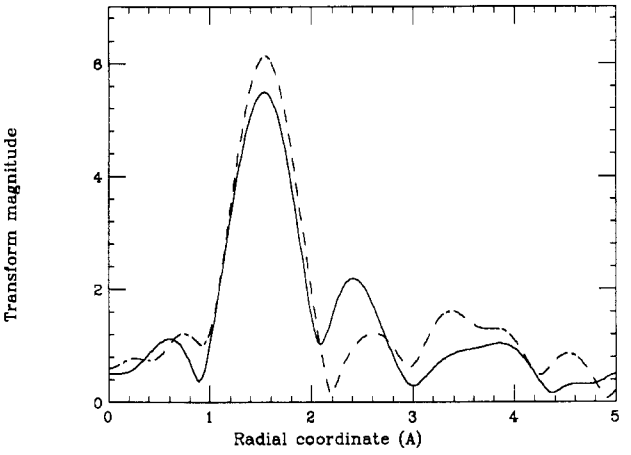

FIGURE 2: Fourier transforms of two-band cytochrome c_1 (solid line) and cytochrome c (dashed line) EXAFS data. Both cytochromes are oxidized forms. The background subtracted data were k^3 weighted, and the transform range is k = 3.0–8.8 Å^{-1}. The magnitude of transformed spectra is in normalized arbitrary units.

heart cytochrome c, one-band cytochrome c_1, and two-band cytochrome c_1. The spectra of oxidized and reduced horse heart cytochrome c agree well with those previously reported by Korszun et al. (1982). The spectrum of one-band cytochrome c_1 is quite similar to that of the corresponding redox state of cytochrome c, although some small differences are evident. In contrast, the spectra of two-band cytochrome c_1 are strikingly different from those of cytochrome c and one-band c_1. Specifically, double peaks immediately above the edge were observed in the spectrum of two-band c_1. However, this double peak structure cannot be a transient experimental artifact, because two separate experiments using two different enzyme preparations gave the same result. Furthermore, the data acquisition interval for the two-band c_1 was interleaved with those for cytochrome c and one-band c_1, and the spectra of cytochrome c and one-band c_1 have singly peaked principal maxima, although some fine structure is evident. Radiation damage is not a likely explanation, because no progressive changes in the spectra with time were observed, and the optical spectra appeared to be normal after X-ray irradiation. It is possible that the observed effect is due to the reaction of two-band cytochrome c_1 with X-ray photoproducts, but this seems to be unlikely, since the heme iron site in two-band cytochrome c_1 is less exposed than that in the one-band cytochrome c_1 (Kim & King, 1987). One would expect that one-band cytochrome c_1 would be more easily altered than two-band cytochrome c_1.

X-ray absorption spectra are sensitive to structural changes only within a radius of approximately 5 Å from the photoabsorbing iron atom because of the relatively short photoelectron mean free path (Stern, 1974) and the intrinsic lifetime broadening of the core hole. Therefore, it can be presumed that substantial conformational changes occur in the neighborhood of the iron atom when the hinge protein is bound to cytochrome c_1. The structural significance of this observation is not yet clear in detail, but possible explanations are (i) replacement of a first-shell ligand with another type; (ii) other strereochemical changes in the first coordination sphere, with retention of ligands; and (iii) stereochemical changes beyond first coordination spheres, i.e., changes in heme conformation.

The EXAFS spectra and corresponding Fourier transforms of cytochrome c and one-band cytochrome c_1 can be superimposed except for somewhat higher noise in the one-band cytochrome c_1 spectrum (see Figure 1). Figure 2 shows the Fourier transforms of the EXAFS data of oxidized two-band cytochrome c_1 and cytochrome c. The Fourier transform spectrum of two-band cytochrome c_1 is compared with that of cytochrome c, because the quality of the Fourier transform of cytochrome c is better than that of one-band cytochrome c_1. The background subtracted EXAFS data were weighted by k^3, and the transform range was k = 3.0–8.8 Å^{-1}. The first-shell Fourier transform peaks of two-band cytochrome c_1 and cytochrome c are very similar in modulus (Figure 2) and phase (not shown). In contrast, the second-shell transform peak of two-band cytochrome c_1 is significantly different from that of cytochrome c, being aboout 90° out of phase on average, and the amplitude of the corresponding peak of two-band cytochrome c_1 is slightly larger than that of cytochrome c. This shift in the average EXAFS phase over the data range may be caused by interferences between the atoms composing the second shell. The Fourier transforms of the data suggest that the first-shell environment of two-band cytochrome c_1 is similar to that of cytochrome c, but there are significant structural differences beyond the first shell. Preliminary EXAFS analysis (Kim et al., 1987c) indicates that the average shell distances, coordination numbers, and Debye–Waller factors are only slightly different between two-band c_1 and one-band c_1. From these results, it is conceivable that either the second or third coordination shell environments of the heme in cytochrome c_1 may change when the hinge protein is bound to cytochrome c_1.

For all of the cytochromes, the positions of the edge (as judged by the maximum of the first derivative) are shifted slightly to lower energy upon reduction. Edge shifts are 1.8 ± 0.3 eV for cytochrome c, 0.8 ± 0.3 eV for one-band c_1, and 0.7 ± 0.4 eV for two-band c_1. These observed edge shifts are comparable to the 1.5-eV shift reported by Korszun et al. (1982). The smaller shifts for cytochrome c_1 are probably not caused by X-ray-induced reduction of the nominally oxidized samples, because the extent of reduction after EXAFS experiments was only 5–10% for both one-band and two-band cytochrome c_1.

Since the discovery of the hinge protein and its obligatory role for the formation of cytochrome c_1–c complex (Kim & King, 1981, 1983; Wakabayashi et al., 1982b; Kim, 1987), the function of the hinge protein in the mitochondria has remained to be investigated. Studies of structural and func-

tional properties of cytochrome c_1 in the presence and absence of the hinge protein (Kim & King, 1987; Kim et al., 1984, 1987a) showed that the hinge protein may play a role for stabilizing the structure of cytochrome c_1. It appears that when the hinge protein is bound to the cytochrome c_1, the structure of cytochrome c_1 is stabilized and somewhat protected from photoreduction, autoxidation, and other reactions (Kim & King, 1987). Mukai and Matsubara (1987) also demonstrated that the stable binding of the hinge protein to cytochrome c_1 is necessary to protect cytochrome c_1 from autoxidation.

Furthermore, we have recently found that the hinge protein seemed to play a certain role in electron-transfer reaction between cytochrome c_1 and c, and the effect of the hinge protein is most probably mediated through a change of the conformation of cytochrome c_1 (Kim et al., 1987a,b).

Although it is not clear at present if any explanation for structure–function relationships of cytochrome c_1 with or without the hinge protein can be made from the current XAS results of cytochrome c_1, we are inclined to think that the observed effect of the hinge protein on the heme site of cytochrome c_1 may be correlated with the observed function of the hinge protein in electron-transfer reaction between cytochrome c_1 and c (Kim et al., 1987a,b). More detailed XAS studies will be conducted to substantiate our current hypothesis and, further, to characterize the structural changes induced in cytochrome c_1 when the hinge protein is bound to cytochrome c_1.

Acknowledgments

We acknowledge the valuable technical assistance of S. Chace and M. Seaman. We are grateful to Dr. J. C. Salerno for his valuable suggestions and discussion.

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