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Conformational stability of ferricytochrome *c* near the heme in its complex with heparin in alkaline pH

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Abstract

The stability of the methionine 80 sulfur–heme iron bond of ferricytochrome *c* (cyt *c*) in its complex with heparin has been studied by absorption spectroscopy in the alkaline pH region at temperatures of 20–80°C and low ionic strength. According to spectral data, the midtransition temperature ($T_{1/2}$) of the cleavage of the sulfur–iron bond was 57.5 ± 0.5 and 52.5 ± 0.5°C for cytochrome *c* and cytochrome *c*–heparin complex, respectively, at neutral pH. The increasing in pH caused an expressive fall of cyt *c* transition temperature while the $T_{1/2}$ for cyt *c* in its complex with heparin was constant and from pH > 7.7, this value was higher than that for the free cyt *c*. Addition of heparin to cyt *c* evoked a moderate increase of 695 nm band intensity at neutral or slightly alkaline pH. It was shown that heparin stabilises the conformation of cyt *c* in state III (the Met 80–heme iron bond is presented) in alkaline pH region and physiological temperature range.

Keywords: Heparin; Ferricytochrome *c*; Met 80–heme iron bond; Alkaline transition

1. Introduction

Cytochrome *c*-mediated electron transfer in the mitochondrial respiratory chain is accompanied by the creation of the complexes with proteins acting as redox partners (Hildebrandt, Vanhecke, Buse, Soulimane & Mauk, 1993; Mauk, Mauk, Weber & Matthew, 1986; Osheroff, Brautigan & Margoliash, 1980). Negatively charged groups of the redox partner interact via electrostatic interactions with positively charged groups at the surface of cyt *c*. The formation of the cyt *c*–redox partner complexes is accompanied by a conformational rearrangement of both protein molecules in the vicinity of the hemes (Weber, Michel & Bosshard, 1987). However, the properties of cyt *c* in such complexes is difficult to be examined owing to overlapping signals from both redox partners in spectrometric measurements. So, it seems to reasonable to use suitable model system allowing to mimic the negatively charged binding domains of the physiological redox partners. Therefore, a lot of effort was devoted to the understanding of the interaction of cyt *c* with various negatively charged surfaces, such as electrode, lipid bilayers and inorganic polyanions (Choi & Swanson, 1995; Chottard, Michelon, Hervé & Hervé, 1987; Collinson & Bowden, 1992).

The interactions of proteins with polysaccharide polyanions are relatively intensively studied from various points of view (Gurov, Larichev, Krylov & Tolstoguzov, 1978; Jackson, Bush & Cardin, 1991; Spillman & Lindhal, 1994; Tolstoguzov, 1991; Tolstoguzov, Grinberg & Gurov, 1985; Van Deerlin & Tollefsen, 1992). There are several evidences that binding occurs between specific domain of clustered basic amino acid of the protein and sulphate and carboxylate groups of polysaccharide heparin (Jackson et al., 1991). Cyt *c* formed specific complexes with its redox partners through the positively charged lysine-rich front surface of cyt *c* (Lys 72, 73, 79, 86) and negatively charged binding site of redox partners. It was found that binding site of cytochrome *bc*1 complex for cyt *c* contains eight consecutive glutamic acid on NH_2-terminal of subunit 8 (Iwata et al., 1998). It indicates that negatively charged field of the redox partner domain leads to the formation of the defined cyt *c* conformation given by the intrinsic properties of this protein. Thus, the polysaccharides and other polyanions present an appropriate model for studying the interactions of cyt *c* with its physiological partners. This approach was supported by various experimental data (Antalík, Bona & Bágel'ová, 1992a; Antalík, Bona, Gažová

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& Kuchár, 1992b; Bágel'ová, Antalík & Tomori, 1997; Chottard et al., 1987; Hildebrandt, 1990; Sedlák, 1997).

The polyanions create very tight complexes with basic proteins by means of electrostatic interactions and induce their conformational rearrangements. We focused our attention on "linear" polyanions, such as heparin, polyglutamate and polygalactouronate, which are stable over a wide range of pH values. The spectral changes of cyt *c* occurring after addition of these polyanions are similar to those of addition of cytochrome *c* oxidase or cytochrome b_5 (Antalík et al., 1992a,b). The heparin as well as cytochrome *c* oxidase retained the Met 80 sulfur–heme iron bond of ferricytochrome *c* at neutral pH, physiological temperature. Besides, heparin decreased the denaturation temperature of cyt *c* (measured by differential scanning calorimetry) (Bágel'ová, Antalík & Bona, 1994) as natural redox partners of cyt *c* (Kresheck & Erman, 1988; Yu, Steidl & Yu, 1983; Yu, Gwak & Yu, 1985).

The alkaline transition of cyt *c* from so-called neutral state (state III, Met80–heme iron bond is presented) to the alkaline state (state IV, Met80–Fe bond is disrupted) with an apparent pK_a of about 9.3 (at room temperature) includes a structural rearrangement of the heme pocket leading to a change of the coordination state. In the alkaline cyt *c* form, the methionine is replaced by an another strong field ligand, probably by the Lys72 or Lys79 (Dopner, Hildebrandt, Rosell & Mauk, 1998; Ferrer, Guillemette, Bogumil, Inglis, Smith & Mauk, 1993; Rosell, Ferrer & Mauk, 1998). Formation of cyt *c*–cytochrome *c* oxidase complex is accompanied by a significant increase of the pK_a constant of alkaline transition of cyt *c* and the similar effect was detected for "linear" polyanions (Antalík et al., 1992a). The saturated concentration of heparin increases the apparent pK_a of cyt *c* by 1.5 units to 10.45 (Antalík et al., 1992a).

It was shown (Atanasov & Mitova, 1971) that the conformational changes of myoglobin near the heme induced by increasing pH (fixed temperature) and those temperature-induced (fixed pH) are the same in the alkaline pH region. The reality that final characteristics of the protein seem independent of the sequence of pH or temperature variation is described in several papers (Privalov, 1979, 1997). The cyt *c* conformational changes near the heme during alkaline transition are followed by the loss of 695 nm band. This band also disappears by a moderate increase in temperature (Schejter & George, 1964) at temperature considerably lower than that at which protein denatures thermally, as it was determined calorimetrically at neutral pH (Bágel'ová et al., 1997). The study of stability of the Met 80–heme iron bond of the cyt *c* in its complex with physiological redox partners against temperature-induced changes in alkaline pH region was not performed owing to irreversibility and aggregation of such systems. Reversibility is, however, observed for cyt *c*–heparin complex.

In this paper, we present study of the stability of the Met 80–heme iron bond of the free cyt *c* and cyt *c*–heparin complex against temperature-induced changes in the alkaline pH region.

2. Material and methods

Horse heart cytochrome *c*, type VI, and heparin were obtained from Sigma and were used without further purification. All other chemicals were purchased from Fluka. The concentration of cyt *c* was determined spectrophotometrically (Butt & Keilin, 1962). All measurements were performed in 2 mM phosphate buffer. Prior to use, cyt *c* was converted to the fully oxidised form by adding of 2 μM $K_3Fe(CN)_6$. The pH of the solutions was changed by adding of concentrated NaOH and determined with a Sensorex glass microelectrode. The pH of the samples was checked and readjusted after all additions.

Temperature dependence of the absorption spectra of cyt *c* and cyt *c* complex with heparin were measured in a SHIMADZU UV-3000 spectrophotometer with a temperature-controlled cuvette holder. Temperature in the sample was monitored by means of a copper–constantan thermocouple. The heating rate was 1°C/min. The difference optical spectra of cyt *c*and cyt *c*–heparin complex induced by temperature were measured between 20 and 80°C; the spectrum at 20°C was taken as reference. From these spectra, the dependence of absorption differences ($\Delta A_{345-395}$ and $\Delta A_{675-695}$) on temperature were determined. The other experimental conditions are described in the legends to the figures.

3. Results and discussion

Ferricytochrome *c* is known to form at least five stable conformational states. The pH-induced transitions between these conformations have been extensively studied by various spectroscopic techniques such as magnetic resonance methods (Gupta & Koenig, 1971; Morishima, Ogawa, Yonezawa & Iizuka, 1977), Raman spectroscopy (Uno, Nishimura & Tsuboi, 1984), infrared spectroscopy (Tonge, Moore & Wharton, 1989), and absorption spectroscopy (Gadsby, Peterson, Foote, Greenwood & Thompson, 1987; Theorel & Åkesson, 1941). Experimental results obtained by these methods show that cyt *c* alkaline transition occurs with an apparent pK_a of about 8.9–9.3 depending on ionic strength. The cyt *c* alkaline isomer possesses a six-coordinate, low-spin heme iron retaining the native ligand of His18 while the sulphur atom of Met80 is replaced by an another strong-field ligand (Dopner et al., 1998; Ferrer et al., 1993). Interest in alkaline form of the cyt *c* has intensified as the result of evidence suggesting that cyt *c* may undergo related conformational changes upon binding to other electron-transfer proteins (Weber et al., 1987).

The characterisation of cyt *c* transition from the neutral state (state III) to alkaline state (state IV) is experimentally very simple by using absorption spectroscopy. This alkaline

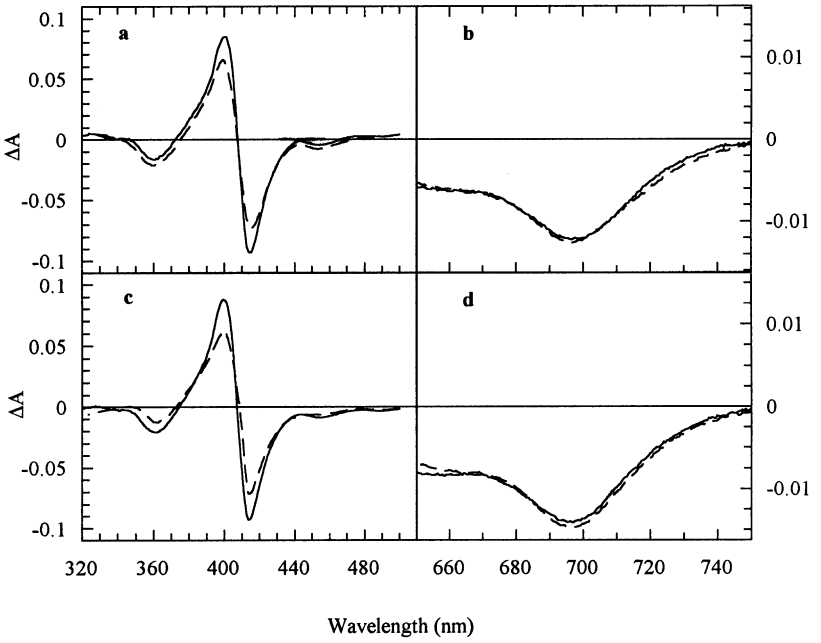

Fig. 1. Absorption difference spectra of cyt *c* (a) and (b) and cyt *c* with heparin (c) and (d); solid line—spectra obtained after change of pH from 7.0 to 10.3 (a) and (b), respectively, from 7.0 to 11.2 (c) and (d) at 20°C; dashed line—spectra obtained after temperature change from 20°C to 80°C (a) and (b), respectively, from 20 to 70°C (c) and (d) at pH 7.0. The concentration of cyt *c* was 5–7 μM (a) and (c), and 30 μM (b) and (d), heparin concentration was 1 mg/ml, 2 mM phosphate solution.

transition causes a shift in most of the major absorption bands of the cyt *c* and the elimination of the absorption at 695 nm although the protein remains in the low-spin state. Temperature-induced cyt *c* conformational change is also accompanied by the loss, upon heating, of the 695 nm band. Fig. 1a and b shows the difference spectra of cyt *c* (pH 10.3 versus pH 7.0 at 20°C, and $T = 80$°C versus $T = 20$°C at pH 7.0) at Soret region (Fig. 1a), and in the 750–650 nm region (Fig. 1b). We can see that the difference spectra of cyt *c* as a result of the pH change are very similar to those of the temperature-induced ones. The variations between these differences probably result from the temperature effect on the electron level energy of the heme as from some differences in cyt *c* conformations at alkaline physiological temperature form and neutral high-temperature form. Fig. 1c and d shows absorption difference spectra of cyt *c*–heparin complex after pH, and temperature-induced transitions. We can see that the increasing temperature changes are nearly identical to those obtained by increasing pH from 7.0 to 11.2.

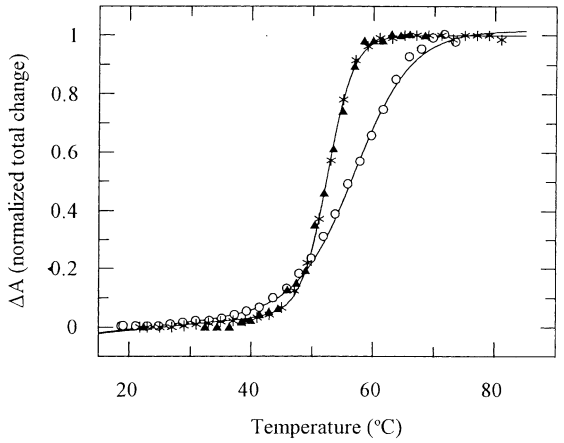

Fig. 2. The temperature dependence of absorbance changes of cyt *c* and cyt *c*–heparin complex at pH 7.0. The data are normalised on the maximum of absorption difference $\Delta A_{345-395}$ (○—cyt *c*; *—cyt *c* + heparin; cyt *c* concentration 5–7 μM), resp. $\Delta A_{675-695}$ (▲—cyt *c* + heparin, cyt *c* concentration 30 μM). All experiments were performed in 2 mM phosphate solution, concentration of heparin was 1 mg/ml.

In spite of the fact that final states are nearly identical, some differences have been observed when midpoint of pH and temperature transitions are characterised. Fig. 2 shows the temperature dependence of the absorbance changes for cyt *c* and cyt *c*–heparin complex at pH 7.0. The change in cyt *c* absorption slightly starts at about 20°C and strongly increases from 50 up to 70°C for $\Delta A_{345-395}$ as well as for $\Delta A_{675-695}$ (data not shown). The midpoint of the thermal transition of cyt *c* was found at 57.5 ± 0.5°C. The absorption change of cyt *c*–heparin complex begins at a higher temperature and the transition is more narrow in comparison with cyt *c* dependence. The midpoint of the thermal transition of cyt *c*–heparin complex is observed at 52.5 ± 0.5°C. Analysis of the spectral data for cyt *c* and cyt *c*–heparin complex gives values of the change of the van't Hoff enthalpy $\Delta H_{vH} = 198 \pm 22$ and 395 ± 29 kJ/mol, respectively. This indicates that cyt *c*–heparin complex thermal transition is more cooperative. The same results were obtained analysing the absorption change $\Delta A_{675-695}$ for cyt *c* as well as cyt *c*–heparin complex.

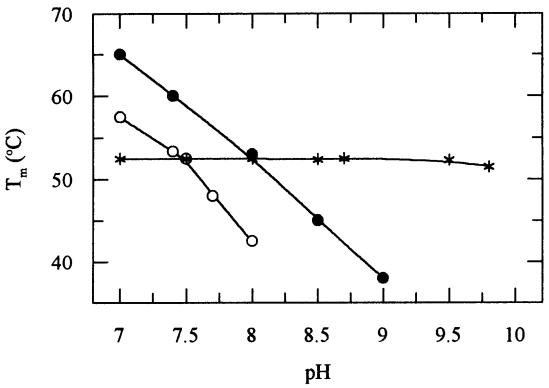

Fig. 3. Dependence of the temperature transition on pH for cyt *c* (○), cyt *c* + 0.5 M NaCl (●), and cyt *c* + heparin (*). All experiments were performed in 2 mM phosphate solution, cyt *c* concentration was 5–7 μM, 1 mg/ml of heparin.

Schejter, Luntz, Koshy and Margoliash (1992) observed a correlation between the value of pK_a for alkaline transition and transition temperature for cyt *c* mutants. The higher apparent pK_a value corresponds to a higher transition temperature. In our previous papers, we have shown that addition of polyglutamate increases both the apparent pK_a of alkaline transition of cyt *c*, and the transition temperature of cyt *c* (Antalík et al., 1992a; Bágel'ová et al., 1994). However, our results for heparin are contrary. We showed previously (Antalík et al., 1992a) that addition of heparin increased the apparent pK_a of alkaline transition from 9.3 to 10.45 and, as it is demonstrated in Fig. 2, the temperature of spectral transition of cyt *c* in complex with heparin is lower than that for free cyt *c* at pH 7.0. We propose that this paradox may be a consequence of higher pK value of cyt *c*–heparin complex characterising the transition from high-spin (state II) to low-spin state (neutral state III). It has been found that presence of heparin increases the pK value of the cyt *c* acid transition from 2.5 to 4.7 at room temperature (Sedlák, 1997).

The obtained results prompted us to investigate the dependence of transition temperature for alkaline pH region. Fig. 3 shows the effect of pH on the midpoint of the thermal transition of cyt *c* near the heme at various conditions (low ionic strength, high ionic strength and its complex with heparin at low ionic strength). The transition temperature of the free cyt *c* decreases from 57.5 to 42.5°C by increasing pH from 7.0 to 8.0. In pH range from 7.0 to 9.0, a similar transition temperature decline (from 64.9 to 38.0°C) is observed for cyt *c* in 0.5 M NaCl. The transition temperatures, and ΔH_{vH} values of cyt *c* in the complex with heparin are constant in the pH region from 7.0 to 9.5 and are equal to 52.0 ± 0.5°C, and 395 ± 29 kJ/mol, respectively. As follows from our measurements, the transition temperature of cyt *c*–heparin complex at pH 7.0 is lower than the transition temperature for cyt *c* at low ionic strength. Whereas increase in pH causes expressive fall of the transition temperature of cyt *c*, the transition temperature of cyt *c*–heparin complex is nearly constant and from pH ~ 7.7 is significantly higher than that for cyt *c* at low ionic strength at the same pH.

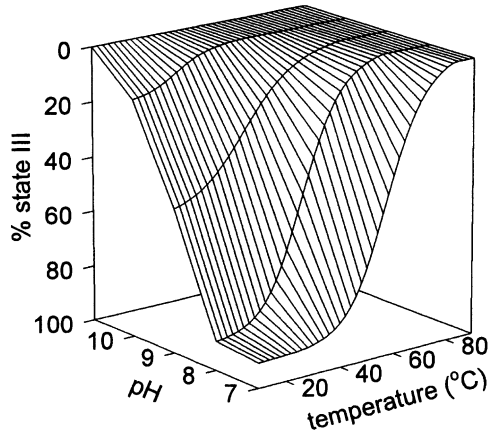

Fig. 4. The pH and temperature dependencies of the % amount of cyt *c* at state III, low ionic strength.

Interaction of cytochrome *c* oxidase with cyt *c* results in a change of the cyt *c* spectrum. As a consequence of this interaction, maxima appear near 410, 526 and 562 nm while a band at 695 nm is observed (Mauk, Reid & Mauk, 1982; Michel & Bosshard, 1984; Michel, Proudfoot, Wallace & Bosshard, 1989). In our previous paper (Antalík et al., 1992b), we demonstrated that addition of polyanion heparin affects the cyt *c* absorption spectrum in the Soret region similarly, and 695 nm band is preserved alike as in the case of the complex formation of cyt *c* with cytochrome *c* oxidase or various hydrophilic polyanions (Antalík et al., 1992a). Moreover, intensity of this band is moderately greater than that for free cyt *c*, at pH 7.0 and room temperature. The moderate increase of absorbance from 0.0125 to 0.014 in difference spectrum is observed for complex cyt *c*–heparin (Fig. 1d) in comparison with cyt *c* (Fig. 1b) in the 695 nm region. It indicates that at neutral pH, low ionic strength and room temperature, there is a small population of cyt *c* molecules (~10%) with disruption of the Met80–Fe bond. In other words, addition of heparin forms more homogenous population of cyt *c* (practically all cyt *c* molecules are in state III). This could be extended for pH region up to 9.5 and physiological temperature range. The state IV is not easily to characterise, however, the state III (or presence of Met80–heme iron bond) is unambiguously characterised by the existence of the 695 nm band. The pH and temperature dependencies of the state III population for cyt *c* and its complex with heparin are shown in Figs. 4 and 5. The percentage of the state III complexed and free cyt *c* was calculated from the 695 nm band height, where the values for cyt *c*–heparin complex was taken as a 100% at pH 7.0, 10°C. Fig. 4 confirms that an increase in pH leads to a sharp changes in fractional concentration of cyt *c* in state III for pH 9–11. Similarly, increase in temperature results in decreasing of cyt *c* population in the state III. The value of transition temperature falls to lower temperatures as the

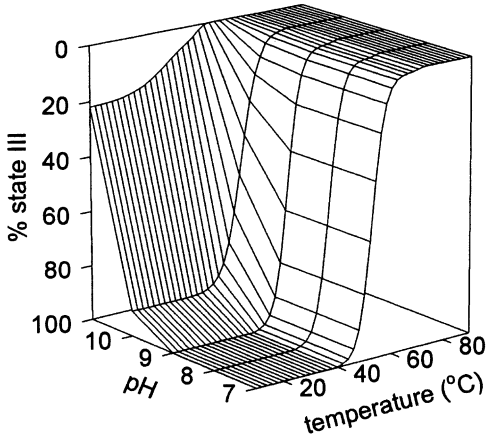

Fig. 5. The pH and temperature dependencies of the % amount of the complex cyt *c*–heparin at state III, low ionic strength.

pH is increasing. As it was mentioned, heparin stabilises the cyt *c* population in state III at physiological temperatures. Although the transition temperature of cyt *c*–heparin complex is lower than that for free cyt *c* at neutral pH, it is higher at more alkaline pH (pH > 7.5) and it does not depend on pH for pH range from 7.0 to 9.5. For cyt *c*–heparin complex, there is a wider range of pH and temperature where cyt *c* is in state III (Fig. 5).

These findings point to the substantial importance of the negatively charged groups in induction of a stabilising effect on cyt *c* structure near the heme. Based upon these and our previous results (Antalík et al., 1992a,b; Bágel'ová et al., 1994), we believe that the cyt *c*–heparin complex can provide useful model for the characterisation of the properties of cyt *c* in its complexes with redox partners. So, the system cyt *c*–heparin may have biological implication in understanding the origin of the conformational changes of this protein induced by interaction with its biological redox partners.

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