Studies on cytochrome *c*–heparin interactions by differential scanning calorimetry

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The effects of heparin on the thermotropic properties of ferricytochrome *c* have been studied using high-sensitivity differential scanning calorimetry. Saturating concentrations of heparin at low ionic strength induced an important shift of the transition temperature T_m from 84.1 °C to 59.8 °C. This was accompanied

by unusually large cooperativity of thermal denaturation of this complex, indicating strong intermolecular interactions between protein molecules. The destabilization of cytochrome *c* when mixed with heparin was not observed at high ionic strength, under which conditions complex was not formed.

INTRODUCTION

Cytochrome *c* is a strongly cationic protein. In the ferricytochrome form, at neutral pH, it carries a net positive charge of approx. +9. The charge distribution is likely to play a crucial role in electrostatic interactions that govern binding of cytochrome *c* to its physiological redox partners [1–4]. There is evidence to indicate that cytochrome *c* binds to cytochrome *c* oxidase in a cleft of the interface between the monomers of the dimeric enzyme [5]. The heparin polyanion is one of the most acidic molecules found in nature. Heparin creates very tight complexes with basic proteins by means of electrostatic interactions and induces conformational rearrangement [6–8]. It has been shown [9] that spectral changes of cytochrome *c* occurring after addition of heparin are similar to those of addition of cytochrome *c* oxidase or cytochrome *b₅*. Formation of cytochrome *c*–cytochrome *c* oxidase complex, just like formation of cytochrome *c*–heparin complex, is followed by a significant increase of the pK_a constant of alkaline transition [10]. Therefore heparin seems to be a good model for studying the interactions of cytochrome *c* with cytochrome *c* oxidase.

In this study we have used differential scanning calorimetry to assess the effect of heparin binding on the thermodynamic stability of ferricytochrome *c*.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type III, Sigma Chemical Co.) was used without any further purification. Heparin (187 units/mg) was obtained from Sigma. All other chemicals used were of analytical grade. Before use, cytochrome *c* was converted into its fully oxidized form by adding 10 μ l of 11 mM $K_3Fe(CN)_6$ to a 1 ml sample of protein (102–111 μ M). All measurements were performed in 2 mM Tris/maleate buffer, pH 7.0. The concentration of cytochrome *c* was determined spectrophotometrically using an absorbance coefficient of ϵ_{550}^{red-ox} 21 mM⁻¹·cm⁻¹.

The differential scanning calorimetric measurements were made on a high-sensitivity DASM-4 microcalorimeter at a heating rate of 1 °C/min. The protein concentration was 100–110 μ M. All the heating curves were corrected using an instrument baseline obtained by heating the buffer. The heparin has no thermal transition.

RESULTS

Figure 1 shows the differential-scanning-calorimetry thermograms of ferricytochrome *c* in the presence of increasing concentrations of heparin in 2 mM Tris/maleate buffer, pH 7.0. The endotherm of cytochrome *c* is characterized by a single peak at 84.1 °C, calorimetric enthalpy (ΔH_{cal}) of 410 kJ/mol and van't Hoff enthalpy (ΔH_{vH}) of 397 kJ/mol. Under these conditions, the thermal denaturation of cytochrome *c* was 50 % reversible. The ratio of ΔH_{cal} to ΔH_{vH} was close to 1, indicating that the thermotropic event may be estimated using a simple two-state model.

In the presence of heparin (7 units/ml) the cytochrome *c* denaturation peak was decreased and its maximum, T_{m1} , was shifted from 84.1 °C to 82.0 °C. Moreover, a new small but sharp peak at a T_{m2} of 60 °C appeared. The higher concentration of heparin (25 units/ml of 100 μ M cytochrome *c* or higher) gave only one thermal transition. No important shift in the transition temperature of the complex occurred up to a heparin concentration of 50 units/ml (T_{m2} = 59.7 °C). The shift of T_{m2} was more significant after adding 75 and 100 units/ml cytochrome *c* (60.9 and 61.4 °C respectively). The thermal denaturation of cytochrome *c*–heparin complex was irreversible when 7–50 units of heparin were added. In the presence of higher concentrations of heparin the reversibility of thermal denaturation of the complex was enhanced, achieving 63 % at 100 units of heparin. For concentrations of heparin ranging between 25 and 100 units values of ΔH_{cal} and ΔH_{vH} for the cytochrome *c*–heparin complex were calculated (Table 1). Strictly speaking the equation $\Delta H_{vH} = 4RT_m^2 C_{pmax}/Q_i$ (where R is the gas constant, C_{pmax} is the height of the heat capacity curve for $T = T_m$ and Q_i is the peak area in energy units) can be applied only to a reversible process. Because the observed heat effect, T_m and the shape of melting curve, do not significantly depend on the heating rate (0.25–2 °C/min), we used this equation for calculation of ΔH_{vH} [11]. In contrast with ΔH_{cal} , the ΔH_{vH} value of the complex is influenced by changes to the heparin concentration. At concentrations of 25–50 units/ml the ratio of $\Delta H_{vH}/\Delta H_{cal}$ is higher than 2, which may result from interactions of two cytochrome *c* molecules bonded to the heparin chain. At concentrations of 75–100 units/ml that ratio is close to 1, as is the case for free cytochrome *c*.

Figure 2 illustrates the differential-scanning-calorimetry

Figure 1 Differential-scanning-calorimetry scans of ferricytochrome *c* in 2 mM Tris/maleate buffer, pH 7.0, with increasing concentrations of heparin

The protein concentration was 105 μM . The number of units of heparin per ml of cytochrome *c* is indicated at the beginning of curves.

Table 1 Thermodynamic parameters characterizing the thermal denaturation of the cytochrome *c*-heparin complex

The concentration of cytochrome *c* was 105 μM and the buffer was 2 mM Tris/maleate, pH 7.0. The values are the arithmetical means of three measurements.

Heparin (units/ml)	T_{m2} (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$
25	59.8	340	850	2.50
50	59.7	355	720	2.02
75	60.9	362	429	1.18
100	61.4	354	432	1.22

Figure 2 Differential-scanning-calorimetry scans of ferricytochrome *c*-heparin in 2 mM Tris/maleate buffer, pH 7.0, with increasing concentrations of NaCl

The protein concentration was 100 μM and heparin concentration 50 units/ml. The NaCl concentrations (mM) are indicated at the beginning of curves.

Table 2 Thermodynamic parameters characterizing the thermal denaturation of ferricytochrome *c*-heparin complex in the presence of NaCl

The concentration of cytochrome *c* was 100–105 μM and the concentration of heparin was 50 units/ml of cytochrome *c*. Experiments were performed in 2 mM Tris/maleate buffer, pH 7.0. The values are the arithmetical means of three measurements.

NaCl (mM)	T_{m2} (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$
0	59.7	355	720	2.02
50	63.8	350	728	2.08
100	65.9	342	801	2.34
200	71.8	360	675	1.87
400	78.6	377	465	1.23
600	79.6	372	387	1.04
1000	79.8	375	398	1.06

Figure 3 Temperature denaturation of ferricytochrome *c* (○) and ferricytochrome *c*-heparin complex (△) as a function of NaCl concentration

thermograms for the cytochrome *c*-heparin complex in the presence of increasing concentrations of NaCl. A relatively sharp transition (thermodenaturation peak) can be seen over the range 0–200 mM NaCl and a broader one at higher concentrations of NaCl. The results are summarized in Table 2. There is no significant change in ΔH_{cal} for denaturation of cytochrome *c*-heparin complex over the range of NaCl concentrations. ΔH_{vH} is decreased with increasing ionic strength and the ratio of $\Delta H_{vH}/\Delta H_{cal}$ approaches 1 at NaCl concentrations of 400 mM or higher. Figure 3 shows the denaturation temperatures of cytochrome *c* and cytochrome *c*-heparin complex as a function of NaCl concentration. The temperature transition of the complex increases with increasing NaCl concentrations. On the other hand, the effect of NaCl concentration on the thermodenaturation of cytochrome *c* is relatively small, as suggested by the reduction of T_{m1} from 84.1 °C to 79.8 °C at 0 and 1 M NaCl

Table 3 Thermodynamic parameters characterizing the thermal denaturation of ferricytochrome *c* in the presence of NaCl

The concentration of cytochrome *c* was 100–105 μ M. Experiments were performed in 2 mM Tris/maleate buffer, pH 7.0. The values are the arithmetical means of three measurements.

NaCl (mM)	T_{m1} (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_{cal}$
0	84.1	410	397	1.03
50	83.0	388	362	1.07
100	82.0	402	385	1.04
200	80.9	386	377	1.02
400	80.1	384	404	0.95
600	79.8	392	398	0.98
1000	79.8	398	404	0.98

respectively. As shown in Table 3, ΔH_{cal} and ΔH_{vH} values for cytochrome *c* do not depend on NaCl concentration.

At 0.6 and 1 M NaCl the thermodynamic parameters characterizing the thermal denaturation of cytochrome *c* and its complex with heparin are practically equal. This implies that high ionic strength prevents the formation of the cytochrome *c*–heparin complex.

DISCUSSION

The denaturation profile of ferricytochrome *c* in 2 mM Tris/maleate, pH 7.0 (Figure 1) is generally similar to the differential-scanning-calorimetric scan of ferricytochrome *c* at neutral pH reported by Long and Hawkriged [12], Ismond et al. [13] and Yu et al. [14]. On the other hand, it has been found previously [15,16] that the main transition of ferricytochrome *c* at neutral pH is preceded by a weaker endotherm. According to Santucci et al. [16], the unfolding process is a two-step transition with separate processes occurring at different parts of the molecules. As shown by our experiments, the thermal transition of ferricytochrome *c* at neutral pH is a simple two-state process, with the ratio $\Delta H_{cal}/\Delta H_{vH}$ close to 1 at all ionic strengths used (Table 3). The level of reversibility of its thermal denaturation varies from 50% to 77% and strongly depends on the temperature at which the protein is heated.

Binding of ferricytochrome *c* to polyanion heparin (containing negatively charged carboxyl and sulphate groups) at low ionic strength results in an overall protein destabilization, as indicated by a large decrease in the denaturation temperature, from 84.1 °C to 60 °C. As seen in Figure 3, increasing the concentration of a simple Cl[−] anion resulted in only small reductions of the T_m of cytochrome *c* from 84.1 °C at 0 mM NaCl to 79.8 °C at 1 M NaCl. Heparin-induced destabilization of the cytochrome *c* molecule may be caused therefore by the parallel effect of several

negative groups on the chain of the polyanion with several positive charges on cytochrome *c*. Such a process can be assumed also in formation of the complexes of cytochrome *c* with its physiological redox partners. A similar decrease of the denaturation temperature of ferricytochrome *c* has been observed after interaction with several proteins. Formation of a cytochrome *c*–cytochrome *c* oxidase complex destabilized cytochrome *c* but not cytochrome *c* oxidase in terms of thermodenaturation [17], i.e. T_m of cytochrome *c* decreased from 84 °C to 62 °C. Very similar observations were made by Yu et al. [14] on the interaction of cytochrome *c* and cytochrome *c*₁. The thermodenaturation temperature of cytochrome *c* falls from 84 °C to 67 °C. The presence of cytochrome *c* peroxidase also decreases the stability of cytochrome *c*, and T_m for cytochrome *c* is decreased from above 80 °C to 60 °C even at higher ionic strengths [18]. It is very difficult to determine the exact value of binding ratio of cytochrome *c* and heparin, as commercial heparin consists of different polymeric molecules with various numbers of carboxyl and sulphate groups. For ferricytochrome *c* associated with heparin (50 units/ml of 100 μ M cytochrome *c*) ΔH_{vH} significantly exceeds ΔH_{cal} at low ionic strength. The unusually high cooperativity of thermal denaturation of the cytochrome *c*–heparin complex suggests strong intermolecular interactions, probably between neighbouring molecules of cytochrome *c* bonded to heparin.

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