Biochimica et Biophysica Acta, 1100 (1992) 155–159

BBABIO 43597

Spectrophotometric detection of the interaction between cytochrome *c* and heparin

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(Received 2 October 1991)

Key words: Cytochrome *c*; Cytochrome *c* oxidase; Heparin

Heparin inhibits transport of electrons from reduced cytochrome *c* to cytochrome *c* oxidase. The effect is due to the interaction of heparin with cytochrome *c*. It has been observed that binding of heparin to the reduced or oxidized cytochrome *c* changes the spectrum of cytochrome *c* at the Soret region. Affinity chromatography of heparin in cytochrome *c* immobilized to thiol-Sepharose shows that commercial heparin is eluted in the low-affinity and high-affinity fractions. Both participate in the interaction with cytochrome *c*. Polylysine induces decay of the cytochrome *c*-heparin complex.

Introduction

Cytochrome *c* is a basic protein which participates in a transfer of electrons in the respiratory chain. It forms tight complexes with its biological redox partners through electrostatic interactions [1–5]. It was suggested that this process optimizes the orientation between the heme of cytochrome *c* and the heme of the redox partner. Moreover, binding of redox partner to cytochrome *c* induces a rearrangement of the protein matrix near the heme [6, 7] and thereby decreases the height of the barrier for electron transport. Nevertheless, despite intensive investigation, the precise mechanism of the formation of these complexes is still unclear.

It is evident that the electron absorption spectrum of the cytochrome *c* heme is dependent on the type of ligand and polarity of the medium [8–10]. Binding of redox partners to cytochrome *c* alters its optical absorption spectrum, mainly in the Soret region [11,12]. This fact enables spectrophotometric indication of the formation of complexes and conformational changes which occur near the heme of cytochrome *c*. Since similar changes are characteristic for the heme-containing biological redox partners of cytochrome *c* [12] the interpretation of results is very complicated. For this reason, the extensive effort has been developed for the application of polyanions (not containing a heme) in the studies of conformational changes of the cytochrome *c*. It has been observed that polytungstates alter structure of cytochrome *c* similarly to biological redox partners [13,14]. A disadvantage of polytungstates is their low stability in alkaline solution [13].

Heparin is one of the strongest polyanions that occurs naturally in organisms [15]. Unlike the proteins and nucleic acids, the flexibility of heparin molecule that is induced by the flexibility of glycosidic bonds is very high [16]. The high content of negative charges on heparin causes an electrostatic repulsion between the parts of the chain, so the chain of the free molecule is hydrophilic and unfolded [16]. Heparin creates very tight complexes with basic proteins through electrostatic interactions [15]. Binding of heparin to these proteins induces conformational rearrangement and alters their activity [15,17]. Petersen and Cox observed an expressive inhibition of reduction of cytochrome *c* with ascorbic acid due to heparin [18]. They suggested that formation of the cytochrome *c*-heparin complex is related to the replacement of a positive charge on the free cytochrome *c* by negative charges. Since heparin, like polytungstates, does not absorb light in the visible region, it can serve as a useful model system for characterization of complexes of basic heme proteins with polyanions. We have studied the formation of the complex of cytochrome *c* with heparin and its effect on the electron absorption spectrum of cytochrome *c*

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Materials and Methods

Cytochrome *c* III, cytochrome *c* *Saccharomyces cerevisiae* VIII and 2-mercaptoethanol were obtained from Sigma. Tris-HCl and polylysine were from Serva and heparin from Sigma and Spofa, Prague, Czechoslovakia. No differences were observed in the effect of these two types of heparin on cytochrome *c*. All other chemicals were purchased from Lachema, Brno, Czechoslovakia. Cytochrome *c* oxidase from bovine heart was isolated according to the method of Yonetani [19] and stored in liquid nitrogen.

The concentration of cytochrome *c* was determined from the extinction coefficient red-ox$_{550}$ $\epsilon = 21\,000$ M cm^{-1}. The concentration change of heparin for affinity chromatography was indicated from the changes of eluent absorbance at 250–300 nm. Affinity chromatography of heparin was performed on thiol-Sepharose 4B (Pharmacia) with linked *S. cerevisiae* cytochrome *c* through disulfide bonds. The length of a column was 10 cm and diameter 2.5 cm. Thiol-Sepharose 4B-cytochrome *c* was prepared as described in Ref. 20 at a ratio of 15 mg of cytochrome *c* per 2.5 g of activated thiol-Sepharose 4B.

Reduced cytochrome *c* as a substrate for the measurement of the activity of cytochrome *c* oxidase was prepared by adding a small amount of sodium dithionite which was removed from the solution using a column G-25 (Pharmacia). The activity of cytochrome *c* oxidase was determined from the decrease of absorption spectrum of reduced cytochrome *c* in reaction mixture at 550 nm.

The effect of heparin on the optical spectrum of oxidized and reduced cytochrome *c* was measured in 1 cm cuvette, for 0.12 mM concentration of cytochrome *c* in 0.1 cm cuvette. The reduced cytochrome *c* for spectroscopic measurements was prepared by 20 min incubation of oxidized cytochrome *c* with 3 mM 2-mercaptoethanol in a buffer solution at pH 7.4. For the measurement of the spectral changes of the oxidized cytochrome *c*, the cytochrome *c* was kept in oxidized state by 22 μM potassium ferricyanide in a buffer of pH 7.4. In the reference cuvette, cytochrome *c* was omitted. Potassium ferricyanide did not create a complex with heparin and prevented partial reduction of cytochrome *c* caused by higher concentrations of heparin. All the spectrophotometric measurements were performed by spectrophotometer Shimadzu UV-3000.

The other experimental conditions are described in the legends to the figures.

Results and Discussion

In Fig. 1, the effect of heparin on the rate of oxidation of cytochrome *c* (Fe^{2+}) by cytochrome *c* oxidase is illustrated. Heparin inhibits the rate of oxidation of cytochrome *c* and 50% inhibition is observed for concentrations of 10 units/ml. The transport of electrons from cytochrome *c* to cytochrome *c* oxidase is realized by formation of the cytochrome *c*-cytochrome *c* oxidase complex and is inhibited by higher ionic strength of the medium [21]. The inhibitive effect of heparin, negatively charged in the neutral water solution, can be explained by an increase in the ionic strength of the medium. On the other hand, if we consider that each chain of heparin contains a high number of negative charges participating in the interaction with multiply positively charged polymers, the effect of heparin can be different.

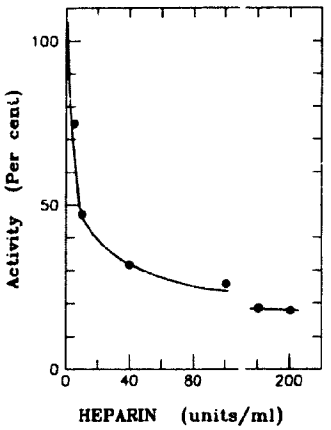

Fig. 1. Inhibition of cytochrome *c* oxidase activity induced by heparin. Cytochrome *c* oxidase activity without heparin was 30 μM cytochrome *c* min^{-1} at 25°C. The assay was carried in 50 mM Hepes (pH 7.4) with 0.5% Tween 80 and 0.12 μM cytochrome *c* oxidase and 2 μM ferrocytochrome *c*.

The positively charged lysine amino groups on cytochrome *c* participate in the formation of complexes between cytochrome *c* and its natural redox partners or polytungstates. The number and arrangement of negative groups on polytungstates effect the conformation of the cytochrome *c* (near a heme) in the cytochrome *c*-polyanion, as has been observed in Refs. 13 and 14. Fig. 2. shows that addition of heparin in excess of the nonionic reduction agent 2-mercaptoethanol leads to the appearance of absorption maximum in the difference spectrum of reduced cytochrome *c* at 417 nm. The dependence of the changes of the absorption maximum at 417 nm on the concentration of heparin is illustrated in Fig. 3. The maximum change is attained at 4 units of heparin/ml. It is very difficult to determine the exact value of the binding constant and binding ratio of the cytochrome *c*, since commercial heparin consists of different polymeric molecules with various number of carboxyl and sulfate groups. A further complication in determination of the binding ratio of cytochrome *c* and heparin is that very

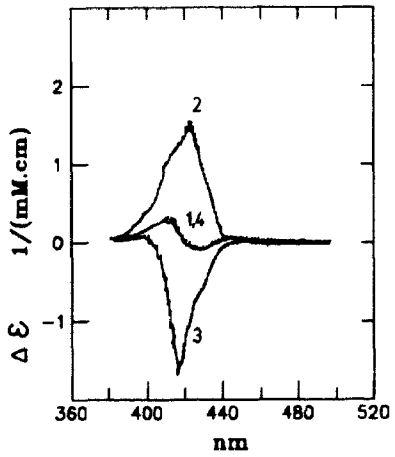

Fig. 2. Spectral change on binding of heparin to reduced cytochrome *c*. Absorption difference spectrum of reduced cytochrome *c* was measured in 50 mM Tris-HCl 2 mM mercaptoethanol (pH 7.4); concentration of cytochrome *c* was 5.2 μM. Baseline (curve 1), absorption difference spectrum obtained by adding to reduced cytochrome *c* 4 units/ml heparin (curve 2), 25 units/ml heparin (curve 3) and 25 units/ml heparin + 0.1 M KCl (curve 4).

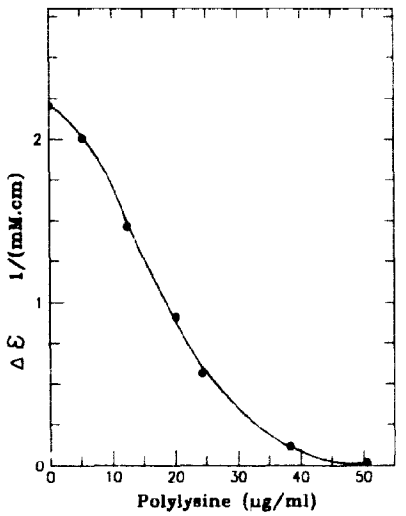

Fig. 4. Effect of polylysine on the change of the absorption coefficient at 417 nm of the complex of reduced cytochrome *c* with heparin in 50 mM Tris-HCl (pH 7.4)/2 mM 2-mercaptoethanol; cytochrome *c* concentration 7.5 μM, heparin 4 units/ml.

high concentrations of heparin induce a decrease in the absorption spectrum with a minimum at 414 nm (Fig. 2).

Fig. 4 demostrates the participation of the negative charged groups on heparin in the formation of the cytochrome *c*-heparin complex. Addition of polylysine to the complex of cytochrome *c* with heparin induces a decrease in the absorption maximum at 417 nm. Half maximum is attained at 17 μg of polylysine/ml. The spectrum of cytochrome *c* in the presence of 50 μg polylysine and 4 units heparin/ml is identical to that of the free cytochrome *c* (not shown). Electrostatic nature of the interaction between cytochrome *c* and heparin is shown in Fig. 2 (curve 4) as the spectral alteration of the cytochrome *c* in the presence of heparin is not observed if 0.1 M KCl is used.

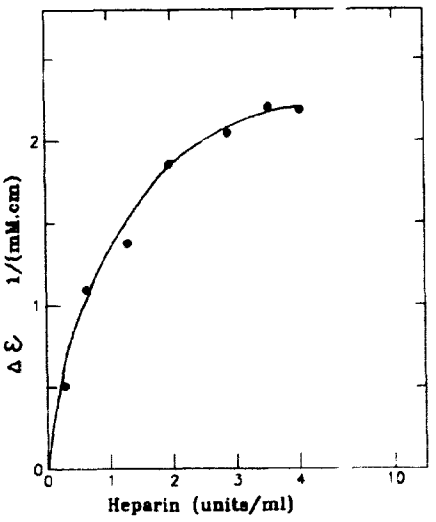

Fig. 3. Effect of heparin on the change of reduced cytochrome *c* absorption coefficient at 417 nm in 50 mM Tris-HCl (pH 7.4)/2 mM 2-mercaptoethanol; cytochrome *c* concentration 7.5 μM.

Spectrophotometric measurements indicate sensitivity of cytochrome *c* in oxidized state to the addition of heparin, too. Mauk et al. [11] and Michel et al. [12] observed that cytochrome b_5 and cytochrome *c* oxidase affect the height and red-shift of the Soret band of the difference spectrum of oxidized cytochrome *c*. Similar heparin-induced changes in the difference spectrum of cytochrome *c* are demonstrated in Fig. 5. Heparin at a concentration of 80 units/ml gave rise to two absorption bands, at 412 nm and smaller one at 362 nm (Fig. 5, curve 2). Conservation of the absorption band at 695 nm (not shown) referred to the fact that spectral changes of cytochrome *c* in the Soret region are not caused by interruption of the bond between iron in heme of cytochrome *c* and methionine-80. Higher concentrations of heparin, similarly as for reduced cytochrome *c*, induce a decrease of the absorption spectrum with a minimum at 408 nm. Fig. 6 shows the dependence of the changes of the absorption maximum of oxidized cytochrome *c* at 414 nm on the concentration of heparin. At lower concentration of cytochrome *c*, when the maximum absorption difference is achieved, a slow decrease after addition of higher concentrations of heparin has been observed. At higher concentra-

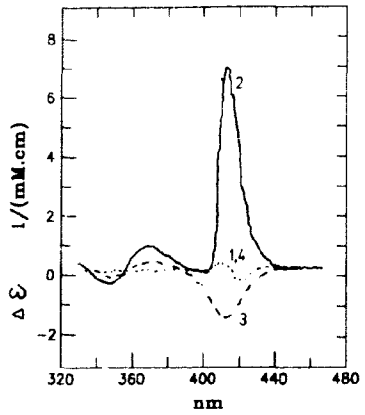

Fig. 5. Spectral change on binding of heparin to oxidized cytochrome *c*. Absorption difference spectrum of oxidized cytochrome *c* was measured in 50 mM Tris-HCl/0.2 mM potassium ferricyanide (pH 7.4); cytochrome *c* concentration 120 μM. Baseline (curve 1), absorption difference spectrum obtained by adding to oxidized cytochrome *c* 80 units/ml heparin (curve 2), 200 units/ml heparin (curve 3) and 200 units/ml heparin + 0.5 M KCl (curve 4). Concentration of oxidized cytochrome *c* in the last two cases was 5.2 μM.

tions of cytochrome *c* (0.12 mM), heparin induces a gradual incerase in absorbance at 414 nm up to the concentration level of 80 units/ml. The molar extinction coefficient at maximum absorption difference is similar to that for the complex of cytochrome *c*-cytochrome *c* oxidase ($\epsilon \simeq 8$ mM cm^{-1}) [12] or cytochrome *c*-cytochrome b_5 ($\epsilon \simeq 3$ mM cm^{-1}) [11].

As in the case of reduced cytochrome *c*, the formation of an oxidized cytochrome *c*-heparin complex is affected by ionic strength. It is clear that in 0.5 M KCl, formation of the complex is inhibited (Fig. 5, curve 4). The dependence of inhibition of the complex formation on the ionic strength is illustrated in Fig. 7. There is no specificity in the effects of different ions. The effectiveness of ions increases with the number of negative groups in the molecule of the ion. Similarly to the reduced cytochrome *c*, polylysine eliminated heparin from the complex but less efficiently (Fig. 8).

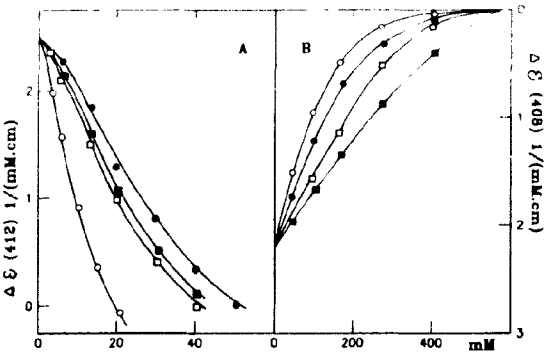

Fig. 7. Effect of ionic strength on the changes of cytochrome *c* absorption coefficient induced by heparin in 50 mM Tris-HCl (pH 7.4); cytochrome *c* concentration 5.2 μM, heparin concentration 2 units/ml at 412 nm (A) and 100 units/ml at 408 nm (B); ○, potassium; □, Na_2SO_4; ■, KCl; ●, potassium phosphate.

Commercial heparin is a mixture of polysaccharides which differ in chain length, number and position of sulfate, carboxyl and of hydroxyl groups. So, it is probable that individual fractions of heparin interacted with cytochrome *c* differently. Fig. 9 shows the affinity chromatography of heparin in yeast cytochrome *c* immobilized to thiol-Sepharose 4B. The elution of the

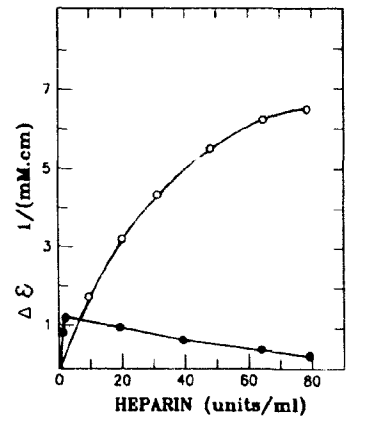

Fig. 6. Effect of heparin on the change of cytochrome *c* absorption coefficient at 412 nm in 50 mM Tris-HCl (pH 7.4); 0.2 mM potassium ferricyanide, cytochrome *c* concentration 0.12 mM (○) and 5.2 μM (●).

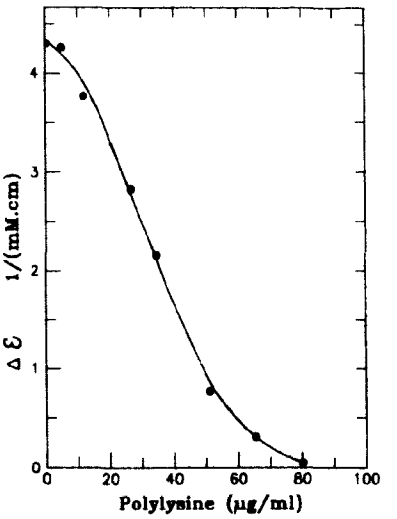

Fig. 8. Effect of polylysine on the change of the absorption coefficient at 412 nm of the complex between oxidized cytochrome *c* and heparin in 50 mM Tris-HCl (pH 7.4), 50 μM potassium ferricyanide; cytochrome *c* concentration 7.5 μM, heparin 4 units/ml.

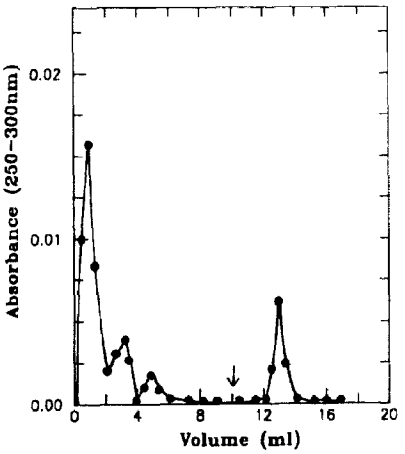

Fig. 9. Affinity chromatography of heparin in cytochrome *c* immobilized to thiol-Sepharose 4B. 3 mg of heparin per 200 μl of 50 mM Tris (pH 7.4)) was applied to a column and eluted with 50 mM Tris-HCl (pH 7.4). Arrow indicates addition of 1 M KCl.

column with 50 mM Tris-HCl (pH 7.4), when heparin has been applied, caused a partial separation of the low-affinity fraction of heparin binding to cytochrome *c*. Addition of 1 M KCl induces a release of the high-affinity fraction of heparin. This follows from the fact that electrostatic interactions between cytochrome *c* and heparin are applied.

We suppose that the observed spectral changes of cytochrome *c* in the Soret region are caused by surface contact of cytochrome *c* with heparin which lead to the rearrangement of the heme environment as in the case of biological redox partners. Conservation of the absorption band at 695 nm for oxidized cytochrome *c*-heparin complex indicated that alteration can be induced by a change in polarity in the microenvironment around the heme, as was suggested by Michel et al. [8] for cytochrome *c* and cytochrome *c* oxidase complexes.

Also, the heparin-induced spectral changes in cytochrome *c* at the Soret region, when the absorption band at 695 nm is conserved, may be explained by the transfer of the charge from protein surface to the heme through aromatic amino acids.

The effect of ions on the formation of the cytochrome *c*-heparin complex shows that electrostatic interactions are applied in this process. The elimination of heparin from the complex with cytochrome *c* by polylysine suggests that the groups which can participate in the interaction are positively charged amino groups on the cytochrome *c*.

Acknowledgements

We thank Dr. A. Musatov for providing cytochrome *c* oxidase and Prof. V. Dadák for help in the preparation of this paper.

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