A COMPLEX FORMATION OF THE ADRENAL IRON–SULFUR PROTEIN (ADRENODOXIN) WITH CYTOCHROME *C* AND THE DECOMPOSITION OF THE IRON–SULFUR CENTER

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1. Introduction

The adrenal cortex mitochondrion is unique in the sense that this subcellular organelle has two electron transfer systems [1]: one system is for oxidative phosphorylation and the other for steroid hydroxylation reactions. Both electron transfer systems are believed to be located on the inner membrane. Accordingly, both the adrenal iron–sulfur protein and cytochrome *c* are protein components of the same membrane.

Cytochrome *c* is a basic protein rich in lysyl residues, while the adrenal iron–sulfur protein is an acidic protein rich in aspartyl and glutamyl residues [2]. Thus, the question arises of whether or not they interact with each other by ionic forces. In fact, we have found an intriguing reaction of the iron–sulfur protein with cytochrome *c*, and upon the formation of this complex, the iron–sulfur center subsequently decomposes, resulting in the reduction of cytochrome *c*. Since the electron transfer occurs between proteins with molecular weights of about 10^4, we have decided to elucidate its detailed mechanism.

2. Methods and materials

The bovine adrenal iron–sulfur protein was prepared by the method described previously [3]. The ratio of absorbance at 414 nm to that at 276 nm was 0.86.

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The NADPH-diaphorase (adrenodoxin reductase) was prepared by the method of Chu and Kimura [4]. The ratio of absorbance at 270 nm to that at 450 nm was 9.0. From these values, both protein samples were judged to approximately same molecular weights. Figs. 2C and D illustrate the patterns of the mixtures. It was concluded from these results that the adrenal protein and cytochrome *c* form a complex: in a system containing a mixture of the iron–sulfur protein and cytochrome *c* in a 1:1 ratio, the two peaks corresponding to the complex and uncomplex cytochrome *c* were observed, while in a system containing the mixture with a 3:1 ratio, a single peak corresponding to the complex was detected.

As expected, the force responsible for the formation of the complex was mainly ionic in character, which was evidenced by the rate of decrease of cytochrome *c* reduction with increasing ionic strength of the medium. (fig. 3).

We have previously demonstrated the formation of a complex of the adrenal iron–sulfur protein with its flavoprotein reductase at a 1:1 molar ratio [7]. We have examined whether or not the cytochrome *c* reduction reaction is inhibited by the addition of the reductase. Fig. 4 indicates that in the presence of the reductase, which is tightly bound to the iron–sulfur protein under the conditions used, the rate of reduction largely decreases. This suggests that when the flavoprotein is bound to the iron–sulfur protein, cytochrome *c* is no longer reduced due to the stabilization of the iron–sulfur center. It should be stated here that the addition of NADPH to the reaction mixture containing the iron–sulfur protein, its reductase, and

cytochrome *c* results in a rapid reduction of cytochrome *c*.

3. Discussion

As our results clearly show, the adrenal iron–sulfur protein interacts with cytochrome *c* largely by electrostatic forces. After the formation of a complex between the two proteins, cytochrome *c* is slowly reduced by the oxidized iron–sulfur protein, resulting in the decomposition of the iron–sulfur center (figs. 1–3). It is of interest to point out that this reduction of cytochrome *c* is largely prevented by the addition of the adrenal NADPH-diaphorase (fig. 4). We have previously shown that upon the addition be pure. Cytochrome *c* (horse heart, type III) was obtained from Sigma. In some experiments, the sample was reoxidized by ferricyanide and passed through a Sephadex column. Sephadex gels were purchased from Pharmacia.

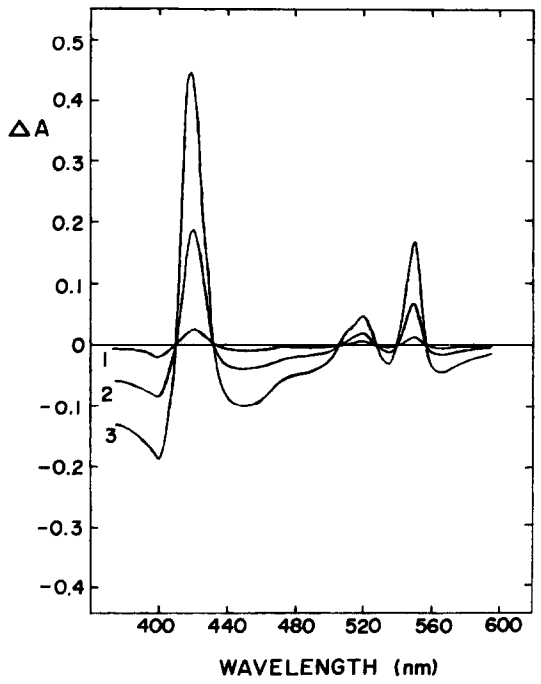

Fig. 1. Reduction of cytochrome *c* by adrenal iron-sulfur protein. Cytochrome *c*, 31.5 nmoles in 1.5 ml of 0.01 M phosphate buffer, pH 7.4, was titrated with adrenal iron–sulfur protein in a tandem cuvet at room temperature. The amounts of the adrenal protein added were, 0.26, 1.58, and 3.65 nmoles for curves 1, 2, and 3, respectively. The spectra were taken after the reaction was completed (60 min after mixing).

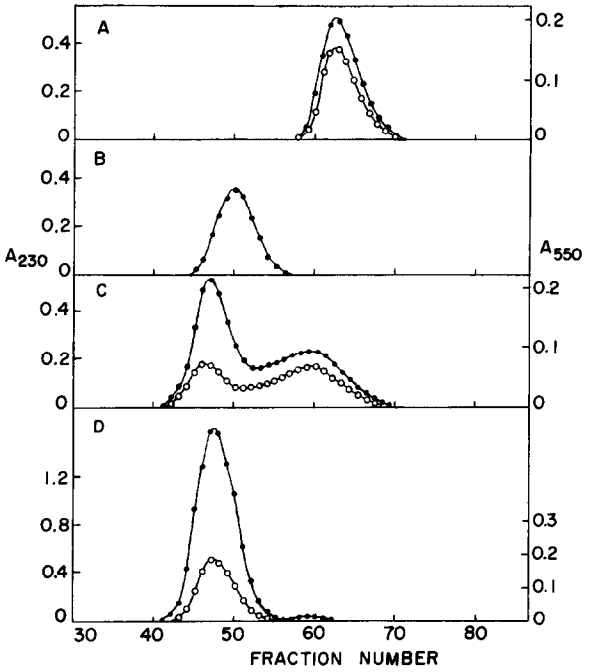

Fig. 2. Sephadex gel filtration of adrenal iron–sulfur protein–cytochrome *c* complex. Protein samples were eluted from a Sephadex G-100 column (1.6 × 62 cm) at a flow rate of 4.0 ml/cm²/hr with 0.01 M phosphate buffer, pH 7.4. Each fraction collected was 1.64 ml in volume. The amounts of proteins used where: A) cytochrome *c* 79 nmoles; B) adrenal iron–sulfur protein 85 nmoles; C) adrenal iron–sulfur protein 85 nmoles + cytochrome *c*, 79 nmoles; D) adrenal iron–sulfur protein 255 mg + cytochrome *c* 79 nmoles. After the absorbance at 230 nm was determined, 40 nmoles of ascorbic acid were added to each fraction and absorbance at 550 nm was then measured. (•), A_{230}; (○), A_{550}.

Optical absorption measurements were carried out by using automatic recording spectrophotometer with a temperature control device.

4. Results

When the oxidized form of adrenal iron–sulfur protein was mixed with ferricytochrome *c*, a slow reduction of cytochrome *c* was observed at room

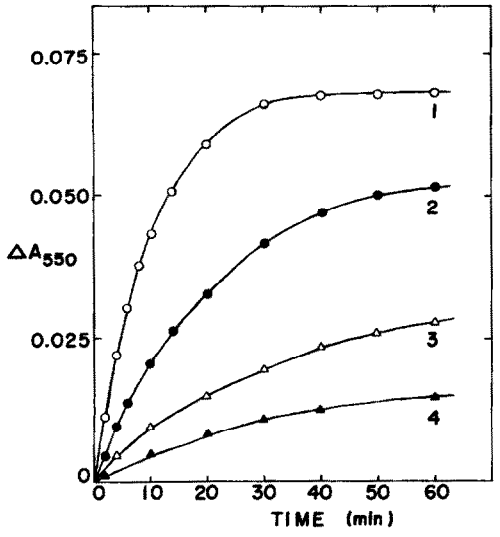

Fig. 3. Effect of KCl concentrations on the time course of the cytochrome *c* reduction. 1.48 nmoles of adrenal iron–sulfur protein were added to a cuvet containing 31.2 nmoles of cytochrome *c* in 3.0 ml of 0.01 M phosphate buffer, pH 7.4. KCl concentrations were: Curve 1, none; Curve 2, 0.05 M; Curve 3, 0.20 M; Curve 4, 0.50 M. The reaction was followed by the changes in absorbance at 550 nm at 30°C.

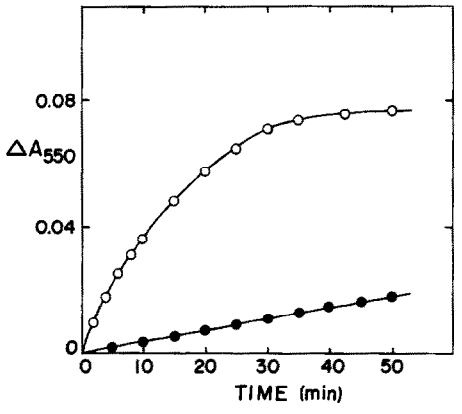

Fig. 4. Effect of NADPH-adrenal diaphorase on the reduction of cytochrome *c*. 1.69 nmoles of adrenal iron–sulfur protein were added to a cuvet containing 29.2 nmoles of cytochrome *c* in 3.0 ml of 0.01 M phosphate buffer, pH 7.4, in the presence (•) and absence (○) of NADPH-adrenal diaphorase (2.47 nmoles). The reaction was followed by the changes in absorbance at 550 nm at 30°C.

temperature under both aerobic and anaerobic conditions. Since both proteins have absorption in the visible range, we utilized tandem cuvettes, where the sample cuvet had a mixture of the two proteins, and the reference cuvet had one protein in one compartment and the other protein in a separate compartment. The difference between the two curvettes are solely due to the mixing of the two proteins. As shown in fig. 1, the solution after the reaction is completed, displays the spectrum of ferrocytochrome *c*. The intensity at 550 nm depends on the amount of cytochrome *c* added. The molar ratio of cytochrome *c* reduced to the iron–sulfur protein added was found to be in the range of 7.0 to 7.5 under aerobic conditions and 8.5 under anaerobic conditions. Since cytochrome *c* accepts one electron per molecule, one molecule of the iron–sulfur protein reduces 7–9 molecules of cytochrome *c*. This reaction is very similar to that of ferricyanide with iron–sulfur proteins (the titer was about 9), as studied by Palmer et al. [5]. Since the adrenal iron–sulfur protein consists of 5 cysteinyl residues, 2 labile sulfur atoms, and 2 ferric ions [6], the value of 9 could be reasonably accepted. Unlike ferricyanide, cytochrome *c* is a much larger molecule. Our effort was extended to investigate how the oxidized adrenal protein reduces cytochrome *c*.

In fig. 2, the complex formation between the adrenal iron–sulfur protein and cytochrome *c* is demonstrated by the use of gel-filtration. Figs. 2A and B represent the elution patterns of cytochrome *c* and the iron–sulfur protein respectively. To note here is the fact that the iron–sulfur protein was always eluted earlier than cytochrome *c* although both proteins have of the iron–sulfur protein, the reductase is markedly stabilized against heat denaturation [7]. Here in the other hand, the iron–sulfur protein was stabilized by the reductase against the attack of cytochrome *c*.

Another interesting feature is the mechanism of reduction following the decomposition of the iron–sulfur center. Since the iron–sulfur protein and cytochrome *c* have mol. wts. of 12 500 and 12 400 respectively, a question arises as to a mechanism of electron transfer from the iron–sulfur center to the heme. The X-ray crystallographic data shows that the heme moiety is largely buried inside the cytochrome *c* molecule [8]. From our ENDOR data, the environment of the iron–sulfur center is hydrophobic, suggesting that the center is not exposed to the protein

surface [9]. At present we do not have any indication of iron atoms, liberated from the iron sulfur protein that may act as electron carriers to the heme. It is most likely that upon the complexing of the iron–sulfur protein with cytochrome *c*, the iron–sulfur protein undergoes a conformational change that lowers the stability of the iron–sulfur center.

The physiological significance of this reaction is not fully understood. This reaction might be involved in the degradation mechanism of iron–sulfur centers. It is our hope that this system would provide a clue for understanding the direct transfer of electrons between electron carrier proteins such as cytochromes, iron–sulfur proteins, and flavoproteins.

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