ENZYMATIC REDUCTION OF NON-HEME IRON PROTEIN (ADRENODOXIN*) BY REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

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In a previous communication from this laboratory (Suzuki and Kimura, 1965), we had reported the isolation of a non-heme iron protein (adrenodoxin) in a homogeneous state from the adrenal mitochondrial fractions and it had also been established that adrenodoxin is one of components of the steroid 11β-hydroxylase complex, which catalyzes the conversion of desoxycorticosterone to corticosterone. Subsequently, we found that the molecular behaviour of adrenodoxin resembled ferredoxin from Clostridium pasteurianum (Tagawa and Arnon, 1962) or photosynthetic pyridine nucleotide reductase (PPNR) from spinach (Horio and Yamashita, 1962), although the catalytic activity of steroid hydroxylase with adrenodoxin could not be replaced with PPNR.

The present paper deals with the enzymatic reduction of adrenodoxin by NADPH, which is catalyed by a new NADPH:adrenodoxin oxidoreductase.

The preparation of adrenal mitochondrial acetone powder has previously been reported (Suzuki and Kimura, 1965). The 11β-hydroxylase was extracted with 0.15 M KCl from the acetone powder and then fractionated with ammonium sulfate. The fraction precipitating at 20-40 % saturation (Fraction A) and

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* We would propose to give the trivial name, "adrenodoxin", for the non-heme iron protein isolated from adrenal mitochondria.

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at 60-80 % saturation (Fraction B) were collected. The purification procedure for adrenodoxin from Fraction B has already been described. Fraction A (2.6 g protein) was incubated with 5 mg of Naja-naja snake venom for 2 hrs at 25°C in 0.1 M tris buffer (pH 8.8). The digest was subjected to centrifugation at 105,400 x g for 40 minutes. The supernatant fluid thus prepared was again fractionated at 30-60 % saturation of ammonium sulfate. The precipitates after centrifugation were dissolved in 0.01 M phosphate buffer (pH 7.4) and dialyzed against the same buffer. All procedures except the incubation with phospholipase were carried out at near 0°C. Precautions were taken to avoid contamination of extraneous metal ions. The preparation obtained has a typical absorption spectrum of flavoprotein which shows a maximal bleach at 450-60 mμ upon the reduction of dithionite.

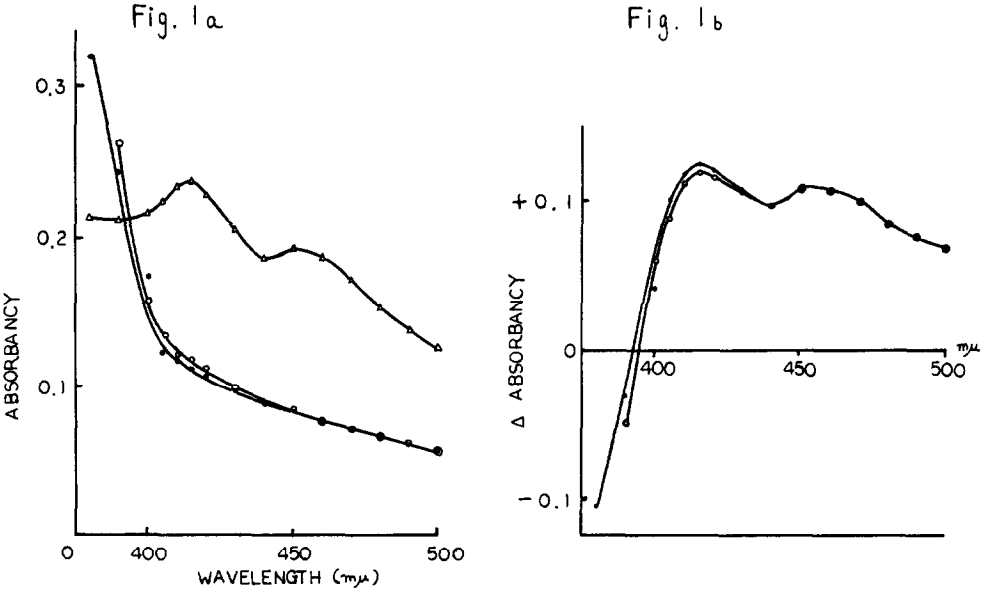

Fig. 1, Spectral Changes of Adrenodoxin by NADPH and NADH
The reaction mixture had: 50 μmoles of K-phosphate buffer (pH 7.0), 26 mμmoles of iron as adrenodoxin, 169 mμmoles of NADPH or 242 mμmoles of NADH if added, 822 μg of the flavoprotein fraction in 1.0 ml as a total volume in an anaerobic cell at 23°C. (Fig. 1a). Difference are shown between the oxidized spectrum and the reduced spectrum (Fig. 1b).
Oxidized form (△——△),NADPH-reduced form (●——●),NADH-reduced form (o——o),Oxidized form minus NADPH-reduced form (●——●), and Oxidized form minus NADH-reduced form (o——o).

Previously, we stated that when the pure oxidized adrenodoxin was incubated with NADPH or NADH either aerobically or anaerobically in the absence of steroid as substrate, the visible spectrum of oxidized adrenodoxin was entirely unchanged. However, the visible color of adrenodoxin could be bleached,when the partially purified flavoprotein fraction was added to the reaction mixture under anaerobic condition. As shown in Fig. 1a, either NADPH or NADH could greatly reduce the absorbancy in the visible range. Difference spectrum between the oxidized adrenodoxin and NADPH- or NADH-reduced form display prominent absorbancy changes at 414 mμ and 450 mμ (Fig. 1b). The complete system without the addition of the flavoprotein fraction did not show any change of absorbancy throughout the visible range. In addition, the rate of the reduction was linear with respect to the reductase concentration below 200 μg of protein per ml.

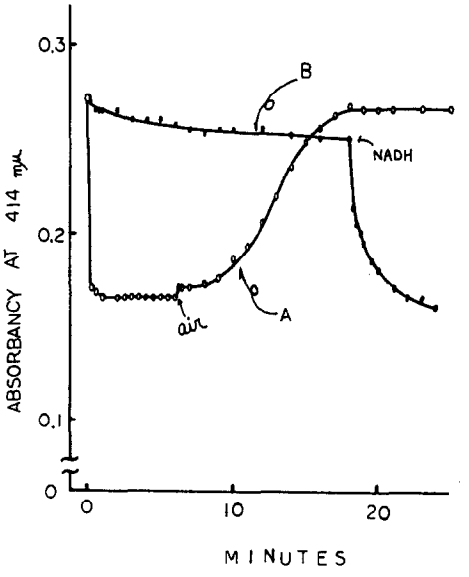

Fig. 2, Rate of Reduction of Adrenodoxin by NADPH and NADH in the Presence of a Flavoprotein Fraction
The reaction mixture had: 50 μmoles of K-phosphate buffer (pH 7.0), 32 mμ-moles of iron as adrenodoxin, 16 mμmoles of NADPH in Curve A or 16 mμmoles of NADH in Curve B, and 1233 μg of the flavoprotein fraction in 1.0 ml as a total volume in an anaerobic cell. Changes of absorbancy at 414 mμ were measured at 23°C by a spectrophotometer. At the indication of arrow, air was introduced in Curve A and 24.2 mμmoles of NADH was added in Curve B.

As indicated in Fig. 2, when the rates of reduction were measured anaerobically at 414 mμ in a Thunberg type cuvette, the rate with 16 mμmoles of NADPH per 32 mμmoles of iron in adrenodoxin was about 400 times faster than that with the same amount of NADH. However, the addition of large excess of NADH bleached adrenodoxin to the same extent as did NADPH. Therefore, it can be concluded that the adrenodoxin reductase is specific for NADPH rather than NADH. The activity of NADPH:adrenodoxin reductase and NADPH:ferricyanide reductase of the crude flavoprotein fraction at 23°C was measured as 0.37 μmoles/min/mg protein and 0.33 μmoles/min/mg protein respectively. It should be mentioned here that an experiment to test whether PPNR can serve as the electron acceptor from NADPH in the assay system, gave a completely negative result.

Significantly, the reduced adrenodoxin was slowly oxidized by simple aeration, until the original absorbancy at 414 mμ was reached. The reoxidized spectrum of adrenodoxin thus obtained coincided with the original spectrum throughout the visible range. Interesting evidence reported by Horio and Yamashita (1963), indicated that PPNR reduced by hydrogen gas in the presence of platinum asbestos could regain partially its original color after aeration. Although adrenodoxin is an auto-oxidizable pigment and one of the components of steroid 11β-hydroxylase, it is still premature to conclude that the slow reoxidation of reduced adrenodoxin is the reaction which activates molecular oxygen in the steroid hydroxylation.

A clear dependence of adrenodoxin-reduction on the presence of the flavoprotein fraction was confirmed as shown in Fig.3. It should be noted here that a marked lag was observed when small amounts of the reductase were used.

In order to know the valency state of iron in adrenodoxin, a preliminary experiment was designed. The extract of non-heme iron with trichloroacetic acid was made from the sample pretreated with 3 moles of mercuric ions per mole of iron in adrenodoxin. Thereafter, with or without

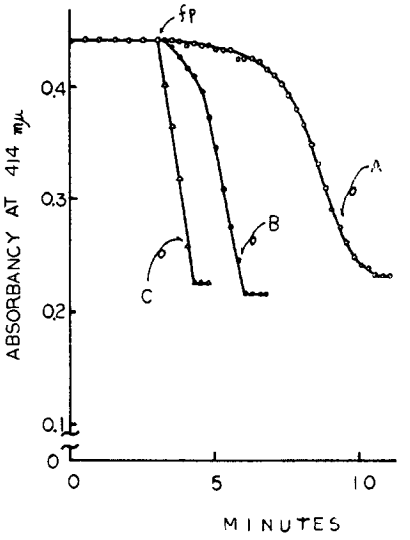

Fig. 3, Dependence of Reduction of Adrenodoxin on Flavoprotein Fraction The reaction mixture had: 50 μmoles of K-phosphate buffer (pH 7.0), 76 mμ-moles of iron as adrenodoxin, 104 mμmoles of NADPH, and 82 μg, 123 μg or 205 μg of the flavoprotein fraction in Curve A,B, and C respectively, in 1.0 ml as a total volume in an anaerobic cell.

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the addition of a reducing agent (ascorbate), color of Fe^{++}-o-phenanthroline chelate was developed. From the result, the majority of iron (more than 80 %) exists as Fe^{+++}. Hence, the visible color of adrenodoxin may be due to Fe^{+++}-protein chelation. Further, upon enzymatic reduction, valency of iron may change with simultaneous decolorization.

Adrenodoxin was titrated with various amounts of NADPH in the presence of the reductase by the measurement of the decrease in absorbancy at 414 mμ in an anaerobic cell. As shown in Fig. 4, the minimum amount of NADPH required to give full enzymatic reduction of adrenodoxin was about half of the amount of iron content in adrenodoxin. From the agreement of theory this shows all iron in adrenodoxin to be functional with respect to reduction. From the difference spectrum between the oxidized form and the NADPH reduced form, the extinction coefficient at 414 mμ was calculated as 3.2×10^3 cm^{-1} mM^{-1}. The value from reduction by dithionite is 4.2×10^3 cm^{-1} mM^{-1}. Hence, 76 % of the chemical full reduction could be performed by the enzymatic reduction.

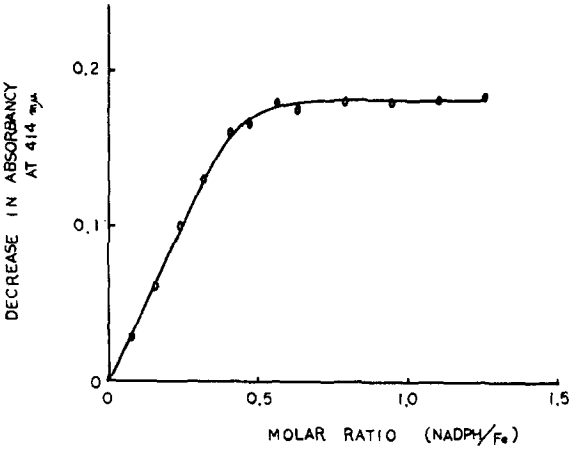

Fig. 4, Titration of Adrenodoxin by NADPH
The reaction mixture had: 50 μmoles of K-phosphate buffer (pH 7.0), 60.8 mμmoles of iron as adrenodoxin, 411 μg of the flavoprotein fraction and various amounts of NADPH as shown in molar ratio (NADPH/Fe), in 1.0 ml as a total volume in an anaerobic cuvette. Decrease of absorbancy at 414 mμ was measured after the full reduction was obtained at 23°C.

According to literature, several enzymes responsible for NADPH-oxidation exists in animal sources. There are NADPH-cytochrome c reductase from liver (Horecker, 1950), NADPH-menadione reductase from liver (Guiditta and Strecker, 1961; Sato et al, 1962), dicoumarol-sensitive diaphorase (Ernster et al. 1962), among others. It may be predicted that one of enzymes known to be responsible for the NADPH-oxidation is the adrenodoxin reductase which is an unique NADPH dehydrogenase with non-heme iron protein as its primary electron acceptor. The most striking similarity of adrenodoxin reductase can be ascribed with ferredoxin-NADP reductase (Shin and Arnon, 1965), although the electron flow is vice versa. Beinert and his colleagues (1961, 1962) have suggested that non-heme iron components of mitochondria, submitochondrial particles and other preparations are reduced upon the addition of appropriate substrates, from the measurements of EPR signal. Since that time, much attention has been payed to non-heme iron components in the electron transfer system. Rieske et al. (1964) isolated a succinylated non-heme iron protein from reduced coenzyme Q-cytochrome c reductase

complex, although the catalytic activity of their iron protein has not yet known. Adrenodoxin demonstrated in this paper is a first indication of iron protein as a natural electron acceptor from NADPH. The relationship of adrenodoxin reduction with steroid 11β-hydroxylation must await further investigations.

Addendum : According to a private letter from Dr.T.Omura in Johnson Research Foundation,he and his colleagues isolated a non-heme iron protein from adrenal cortex and observed the reduction by NADPH in the presence of a flavoprotein fraction. (T.Omura et al. IEG-1, Scientific Memo 346)

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