AN IRON PROTEIN AS A COMPONENT OF STEROID 11β-HYDROXYLASE COMPLEX

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Among numerous reports concerned with 11β-hydroxylation of steroids is that of Tomkins et al. (1958), who found the enzyme obtained from adrenal mitochondria to consist of at least three components and an unidentified cofactor which could be extracted from adrenal or liver. Later, Sweat et al. (1962) also reported observations on the multiple nature of the 11β-hydroxylase. Hence, it has been established that the steroid 11β-hydroxylase is an enzyme complex which can be resolved into two or three separate enzyme proteins.

The present communication deals with the isolation of an iron protein in a homogeneous state from adrenal mitochondria. This iron protein is one of the components of the enzyme complex which catalyzes 11β-hydroxylation of desoxycorticosterone. Some properties of this protein are described.

Pig adrenal glands were obtained from a local slaughter house and brought immediately on ice to the laboratory. The cortical material from about 70 glands was homogenized for two minutes at low speed of the Waring blendor in 10 volumes of ice cold 0.25 M sucrose containing 0.05 M nicotinamide and adjusted at pH 7.4. All subsequent procedures were carried out at a temperature of 0° C. Throughout the purification, precautions were taken to minimize contamination by extraneous metal ions. The homogenate was then subjected to centrifugation to obtain the mitochondrial fraction. The nuclei and cell debris were sedimented at

700 x g for 10 minutes. The supernatant fluid was again centrifuged at 15,000 x g for 20 minutes. The precipitate thus obtained was washed with the same sucrose solution by centrifugation. An acetone powder was made by desiccation with chilled acetone at -20°C. The 11β-hydroxylase was extracted with 0.15 M KCl and then fractionated with ammonium sulfate. The fractions precipitating at 20-40% saturation (Fraction A) and at 60-80% saturation (Fraction B) were collected. Purification of Fraction A is still under investigation in this laboratory, Fraction B was extensively purified as follows: it was placed on a DEAE-cellulose column previously equilibrated with 0.01 M phosphate buffer (pH 7.4). After washing with a solution of 0.01 M phosphate buffer (pH 7.4) containing 0.20 M KCl, the active component was eluted with 0.01 M phosphate buffer (pH 7.4) containing 0.30 M KCl. The brown eluate was again purified by repeating the DEAE-cellulose column chromatography. The eluate thus obtained was further fractionated with ammonium sulfate precipitation, and the precipitate obtained between 60-80% saturation was dissolved in 0.01 M phosphate buffer (pH 7.4) and dialyzed thoroughly against the same buffer.

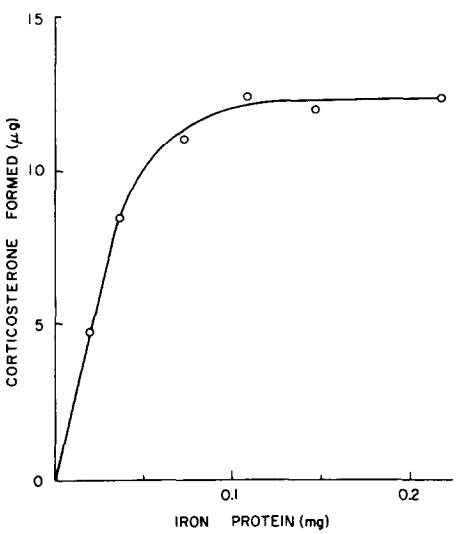

Fig.1, Requirement of the Iron Protein for 11β-Hydroxylation.

The reaction mixture had: 1 μmole of desoxycorticosterone in propylene glycol, 60 μmoles of Tris buffer (pH 7.4), 10 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 1 μmole of mercaptoethanol, 0.38 μmoles of NADPH, 2.4 mg protein of Fraction A, and amounts of purified iron protein as indicated in the figure. After shaking for 30 minutes at 37°C in air phase, corticosterone formed was extracted with dichloromethane and the quantity was estimated by the fluorometric method of Mattingly (1962), with minor modifications.

The preparation purified from Fraction B (which will later be identified as an iron protein) maintained a single homogeneous boundary during sedimentation in the centrifuge, and the sedimentation constant ($S_{20,w}$) was calculated as 1.7 S, assuming a partial specific volume of 0.74. When the redox potential was estimated potentiometrically, using dithionite as a reducing agent, E_o' observed at pH 7.4 was + 0.15 volts.

A reaction mixture containing either Fraction A or the iron protein purified from Fraction B did not catalyze the 11β-hydroxylation, but a combination of both fractions was active. Fig.1 shows that the iron protein is absolutely required for the activity in the presence of a constant amount of Fraction A. Apparently, the result indicates resolution of the iron protein from the 11β-hydroxylase complex.

Absorption spectra of the iron protein are given in Fig. 2. The spectrum of the oxidized enzyme showed absorption maxima at 414 mμ and 450 mμ, and a broad absorption maximum at 320 mμ. Treatment with

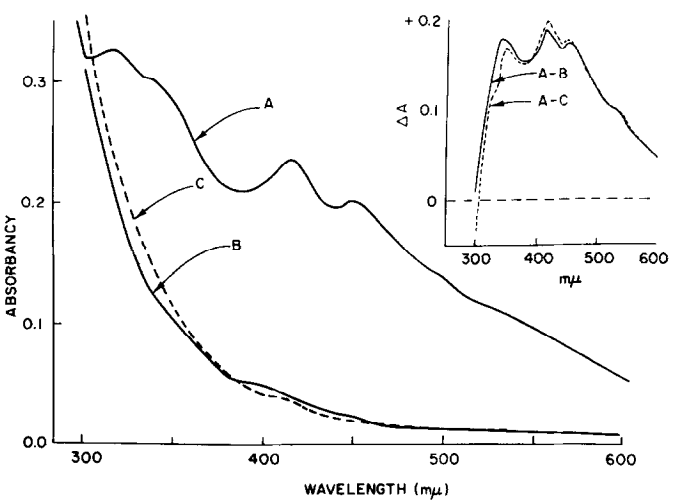

Fig.2, Absorption Spectra of the Purified Iron Protein from Adrenal Mitochondria. Each measurement was performed on a solution of the iron protein containing 0.4 mg/ml of 0.01 M phosphate buffer (pH 7.4) and 45 mμmoles of iron/mg protein. A, in the oxidized form; B, with the addition of 100 mμmoles of $HgCl_2$; C, with the addition of 100 mμmoles of $CuSO_4$.

dithionite, mercuric, cupric, silver ion or p-chloromercuriphenyl sulfonate greatly reduced the absorbancy throughout the visible range, but a considerable increase of ultraviolet absorbancy was observed. As shown in the insert in Fig. 2, difference spectra (oxidized minus mercuric or cupric treated) displayed prominent absorbancy changes at 414 mμ, 450 mμ and 340-50 mμ. Moreover, there was a linear relationship between the absorbancy change at 414 mμ and the molarity of mercuric or silver ion required. It can be computed that the addition of three moles of mercuric ion per mole of iron in the protein are required to eliminate the absorbancy at 414 mμ. Univalent silver ion was found to be exactly half as effective as mercuric ion in this regard. Treatment with ascorbate or borohydride did not produce any significant change of the visible spectrum. When the oxidized enzyme was incubated with NADPH aerobically or anaerobically in the absence of substrate, the absorbancy at 414 mμ of the oxidized enzyme was not effected and the absorbancy at 340 mμ due to NADPH added was not decreased. This observation is not consistent with the result obtained by Tomkins (1958) who reported that the spectrum of the partially purified enzyme extracted with 0.5% digitonin showed an absorption maximum at 413 mμ, which could be depressed by the addition of NADPH.

Total and nonheme iron was determined with o-phenanthroline, as reported by Massey (1957). The total iron exists as nonheme iron and the iron to protein ratio (mμmoles of iron per mg of protein in biuret basis) was estimated as high as 45. The value for the iron content indicates a minimal molecular weight of the protein of about 22,000. Based on the sedimentation constant of 1.7 S, a tentative molecular weight was also be calculated as 22,000, if one can assume 6.8×10^{-7} as $D_{20,w}$. It is reasonable to estimate that there is one mole of iron per molecule of the protein.

In addition, the protein contains "labile sulfur", which is

liberated as hydrogen sulfide upon acidification. After denaturation of the protein, which caused concomitant disappearence of the visible color, the odor of hydrogen sulfide could readily be detected. Therefore, hydrogen sulfide was determined by the method of Fogo and Popowsky (1949) with some modifications. The behaviour of "labile sulfur" in the iron protein will be published later.

It would be of interest to compare the adrenal iron protein with photosynthetic pyridine nucleotide reductase from spinach (Horio and Yamashita, 1962), ferredoxin from Clostridium pasteurianum (Tagawa and Arnon, 1962) and an iron protein (succinylated form) from reduced coenzyme Q - cytochrome c reductase complex in beef heart mitochondria, as recently reported by Rieske et al.(1964) and Coleman et al.(1964). All of these iron proteins have approximately same molecular weight of about 20,000 and have a typical absorption maximum at near 420 mμ, which is possibly due to the iron-protein chelation. All have "labile sulfur". Enzymatic functions of above mentioned iron proteins are quite different: an experiment to test whether photosynthetic pyridine nucleotide reductase from spinach can substitute the adrenal iron protein for the activity of 11β-hydroxylation or not, gave a negative result. Further details of this study will be published elsewhere.

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