Stoichiometry of mitochondrial cytochromes P-450, adrenodoxin and adrenodoxin reductase in adrenal cortex and corpus luteum

Implications for membrane organization and gene regulation

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(Received December 10, 1985/February 3, 1986) — EJB 85 1339

We have estimated the concentrations of cytochromes P-450 and P-450, and the electron-transfer proteins adrenodoxin reductase and adrenodoxin in the adrenal cortex and corpus luteum using specific antibodies against these enzymes. While in the adrenal cortex the concentrations of these enzymes are relatively constant in different animals and show no significant sex differences, in corpora lutea they vary considerably and can increase at least up to fiftyfold over the levels found in the ovary. The average relative concentrations of adrenodoxin reductase, adrenodoxin and P-450 are 1:3:8 in the adrenal cortex (which has two cytochromes P-450, P-450 and P-450, in equal concentrations) and 1:2.5:3 in the corpus luteum (which has only P-450). We further present evidence that the levels of cytochrome c oxidase also show a degree of correlation with the levels of the mitochondrial steroidogenic enzymes.

In all steroidogenic tissues the first and rate-limiting step in the biosynthesis of steroid hormones is the conversion of cholesterol to pregnenolone [1-4]. This reaction is catalyzed by a mitochondrial cytochrome P-450 (P-450,ad) in three consecutive cycles of monoxygenation (hydroxylation) each of which consumes two electrons, a H+ and O2 [1-6]. The electrons are transferred from NADPH to cytochrome P-450,ad by means of an electron transport chain that includes a flavoprotein (adrenodoxin reductase), and a ferredoxin (adrenodoxin) [1-5]:

\[ \text{NADPH} \rightarrow \text{adrenodoxin reductase} \rightarrow \text{adrenodoxin} \rightarrow \text{cytochrome P-450}. \]

In the adrenal cortex there is an additional mitochondrial P-450 that utilizes the same electron transport chain. This P-450,ad catalyzes both 11β and 18-hydroxylation of steroids which are specific for the biosynthesis of glucocorticoids and mineralocorticoids [6-8].

In addition to steroidogenic tissues, electron transfer proteins homologous or identical to adrenodoxin and adrenodoxin reductase have been detected in a number of other tissues [9-16]. In the liver these proteins support specific mitochondrial cytochromes P-450 that catalyze hydroxylation reactions in the pathways of biosynthesis of bile acids and vitamin D [11-13]; and in the kidney they support another step in the activation of vitamin D [14, 15].

The enzymes of the mitochondrial P-450 systems are located on the matrix side of the inner mitochondrial membrane [17-19]. Cytochromes P-450 and P-450, are integral membrane proteins [1-4]. Adrenodoxin reductase may be partly embedded in the membrane [17]. Adrenodoxin is a small (12 kDa) peripheral membrane protein, which appears to bind to P-450 and reductase by ionic but not hydrophobic forces [1-4, 20-21] and it may also associate with head groups of membrane lipids by ionic interactions. Some previous studies have estimated the concentrations of one or two of these enzymes in the adrenal cortex [10, 22, 23] but the spectroscopic methods used could not distinguish between the different cytochromes P-450 present in this tissue, and the relative amounts of reductase and cytochromes P-450 still remain unknown. In this study we have determined the amounts of the mitochondrial cytochromes P-450, P-450, adrenodoxin and adrenodoxin reductase in adrenal cortex and corpus luteum using specific antibodies against these enzymes.

MATERIALS AND METHODS

Adrenodoxin, adrenodoxin reductase, cytochrome P-450, cytochrome P-450, were purified from bovine adrenal cortex to the same purity levels as in our previous reports [5, 20, 21]. The concentrations of the enzymes were measured by their absorption spectra as previously described [20, 21]. The tissues were obtained from bulls and cows within 30 min after slaughter. The adrenal cortex was separated from the medulla and the capsule. The corpus luteum was easily removed from the ovary and its capsule. After this preparation the tissues were stored at -20°C. The tissue homogenates were prepared by blending for 2 min in 50 mM Tris, pH 7.4, and 1 mM EDTA at 15 g tissue/100 ml, and kept frozen at -20°C. This yielded a homogenate of 20-30 mg protein/ml as determined by biuret assay using bovine serum albumin as standard [24].

To generate antibodies 150-200 µg purified enzymes were injected into rabbits in Freund's complete adjuvant. Booster shots were administered after two to four weeks. Blood was collected from rabbits ten days the last injection. Serum was separated from clotted blood cells and stored at -20°C. The antisera developed against bovine heart cytochrome c oxidase

Abbreviation. PBS, phosphate-buffered saline.

Enzymes. Adrenodoxin reductase (EC 1.18.1.2); cytochrome P-450 (EC 1.14.15.6); cytochrome P-450 (EC 1.14.15.4).
were donated generously by Dr Gera Eytan from this department.

The samples of purified proteins and homogenized tissue fractions were dissolved in gel sample buffer (final concentrations: 60 mM Tris, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercaptoethanol), heated to 100°C for 2 min and electrophoresed on 8.5% or 15% (for adrenodoxin only) polyacrylamide gels with sodium dodecyl sulfate. The proteins were transferred to nitrocellulose paper (0.2 μm) [25] and the paper was treated according to the following protocol: (a) incubation in 1% bovine serum albumin in phosphate-buffered saline (PBS: 10 mM potassium phosphate, pH 7.4, 150 mM NaCl) at 37°C for 1 h; (b) addition of antiserum at a 1:100 dilution and incubation for 1.5 h at room temperature; (c) four washes in PBS for 30 min; (d) incubation with 5 μCi 125I-protein A (8.9 μCi/μg, from New England Nuclear) in 0.25% gelatin in PBS for 1.5 h; (e) four washes in PBS for 40 min; (f) two washes with 10 mM potassium phosphate, pH 7.4, 1 M NaCl, 0.4% N-dodecylsarcosine (Sigma) for 1.2 h; (g) final wash in PBS; (h) autoradiography at −70°C.

On every gel, in addition to tissue homogenates, four or five different concentrations of purified enzyme were run to establish a standard curve. The relative intensities of the bands observed on autoradiograms were measured using a densitometer. These results were then used to estimate the amounts of the enzymes in tissue homogenates on the basis of standard curves using least-squares regression analysis.

RESULTS

Specificity of the antibodies

In the Western blots of all enzyme preparations except adrenodoxin one major band reacted with the antisera and that comigrated with the purified enzyme used as the antigen (Fig. 1). The best evidence for the specificity of the antisera was the lack of any cross-reactivity with proteins from tissues that do not have the enzymes. Thus, lack of cross-reactivity of P-450sc antisera with liver protein, and that of P-45011β antisera with corpus luteum and testis (no bands were seen on the autoradiograms even at long exposure intervals) further validated the specificity of these antisera. Adrenodoxin and adrenodoxin reductase antisera showed very faint bands of cross-reactivity with protein from liver and testis but we did not attempt to quantify these. Both of these tissues contain mitochondrial monooxygenase systems that include these two electron-transfer proteins at very low concentrations as compared to adrenal cortex [9–13]. The cytochrome c oxidase antibody recognizes two subunits of the enzyme but one more strongly than the other.

Quantitation of the enzymes in tissues

In order to quantitate the mitochondrial enzymes we undertook Western blot analysis, as this method was previously shown to be a sensitive, and reliable assay for the quantitation of microsomal cytochromes P-450 [26] and many other proteins. The sensitivity of our different antisera preparations varied, but the ranges wherein the amount of the antibody bound to protein on nitrocellulose blots increased linearly were within 0.5–5 pmol for purified enzymes and 5–40 μg for total tissue protein. Almost every quantitative result reported here was based on at least two independent determinations. The methods used yielded reproducible results as the average difference of the replicates from their means was 8.6% over the entire experiment and at most 11% for each gel run.

As previously observed [27, 28] the antibody against adrenal cortex adrenodoxin and cytochrome P-450sc reacted well with the corpus luteum enzymes (Fig. 1). Southern blot analyses, using a cloned P-450sc cDNA, suggest that there may be only one gene for P-450sc in the bovine genome [29]. Thus, the enzymes in both tissues may be the product of the same gene. This would further validate the use of the antibody against adrenal cytochrome P-450sc to quantify this enzyme in the corpus luteum. At present, the number of genes that code for adrenodoxin and adrenodoxin reductase is not known. Thus, in our calculations here we assume that the
enzymes in both tissues share the same antigenic determinants. This assumption is supported by the observation that quantitation of the corpus luteum and adrenal cortex enzymes yielded similar results with two different antisera preparations.

In the experiments shown in Figs 1 and 2 the total protein from the different tissues was obtained by homogenization of the whole tissue without separation of the mitochondria. In initial experiments the tissues were homogenized in a buffer with 0.25 M sucrose to prevent lysis of the mitochondria, and the nuclear pellet and postnuclear fractions were separated by differential centrifugation. In order to determine whether there was a differential recovery of the enzymes in the different subcellular fractions the quantity of the enzymes present in both of the fractions was determined by Western blot analysis. These experiments indicated that the recovery of the different enzymes in the postnuclear supernatant were not similar. Therefore, to avoid biased estimations of the amounts of enzymes, total tissue homogenates were prepared as described above. Since this is the simplest procedure for tissue preparation, it is probably easier to replicate these conditions than those that require subcellular fractionation.

Comparison of enzyme concentrations in steroidogenic tissues

While in the adrenal cortex the concentrations of adrenodoxin reductase, adrenodoxin, and cytochromes P-450

| Tissue          | Reductase (pmol/mg protein) | Adreno- doxin (pmol/mg protein) | P-450

<table>
<thead>
<tr>
<th>Mean ± SD</th>
<th>Range</th>
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<tr>
<td>Adrenal cortex</td>
<td>109 ± 12</td>
<td>90−128</td>
<td>306 ± 57</td>
<td>235−365</td>
<td>391 ± 18</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>69 ± 35</td>
<td>34−128</td>
<td>163 ± 49</td>
<td>110−240</td>
<td>250 ± 148</td>
</tr>
<tr>
<td>Ovary</td>
<td>&lt;5</td>
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Table 1. The concentrations of the mitochondrial cytochrome P-450 system enzymes in the adrenal cortex, corpus luteum and ovary. The results are presented as pmol/mg total tissue protein (N = 5)
Membrane organization of the mitochondrial cytochrome P-450 systems

The observed ratio of components of the mitochondrial cytochrome P-450 systems precludes the structural organization of these enzymes in linear arrays of electron-transport chains. The in vitro kinetic behavior of these enzymes also indicates that a 1:1:1 ternary complex of adrenodoxin reductase, adrenodoxin and P-450 does not form under equilibrium or steady-state turnover conditions [4, 5, 20, 21, 30]. During catalytic turnover adrenodoxin appears to act as a mobile electron carrier: its oxidized form binds to reductase, accepts one electron from it and dissociates, and then binds to P-450 and unloads its electron [4, 5, 20, 21, 30].

Oxidized adrenodoxin molecules, however, can also bind to P-450 in competition with reduced adrenodoxin and thus inhibit the catalytic activity of the P-450 [5, 20, 21]. Consequently the efficient functioning of this electron-transfer system requires that most of the adrenodoxin molecules are maintained in reduced form and unbound to reductase. These biological design specification are apparently met by maintaining a relatively low concentration of reductase to minimize the competition of reductase with P-450 for binding to adrenodoxin, a turnover rate of adrenodoxin reduction by reductase that is much faster than the rate of adrenodoxin oxidation by P-450 so that the low concentrations of reductase suffice for the system, and a redox equilibria that favors dissociation of adrenodoxin from reductase after reduction [4].

Similarities and differences among electron-transport systems

Some of the characteristics of mitochondrial P-450 systems noted above are similar to those of the mitochondrial oxidative phosphorylation system, which includes four multienzyme complexes (complexes I - IV) embedded in the inner mitochondrial membrane, and a small peripheral membrane protein (cytochrome c) located on the side of the intermembrane space [31, 32]. The stoichiometry of these five components (1:2:3:7:9) precludes a model of rigid structural organization of enzymes on the membrane [34]. Electron transfers between complexes I, II and III is mediated by ubiquinone, and between complexes III and IV by cytochrome c. A functional 1:1:1 complex of complex III, cytochrome c, and cytochrome c oxidase apparently cannot form as the same domain on cytochrome c is involved in binding both to its reductase (complex II) and oxidase (complex IV) [31]. Thus, like adrenodoxin, cytochrome c functions as an electron shuttle between its reductase and oxidase, and not as an 'electron bridge' between the two enzymes [31, 32].

It is significant that both the mitochondrial P-450 and respiratory systems include small peripheral membrane proteins that act as electron carriers between enzymes that are embedded in the inner mitochondrial membrane. A major reason for this may be that the diffusion of proteins may be faster on the surface than in the plane of the inner mitochondrial membrane, which has a particularly high protein content. At present we have no information on the mobility of adrenodoxin, P-450 and reductase in the membrane, but indeed the diffusion rate of cytochrome c is at least tenfold faster than those of the membrane-embedded complexes [32].

In contrast to the mitochondrial P-450 systems the microsomal cytochrome P-450 systems include two integral membrane proteins: an NADPH:cytochrome P-450 oxidoreductase and several different forms of P-450, all of which are dependent on the same reductase. The molar ratios of these two enzymes in liver microsomes vary from 1:20 to about 1:100 [26]. The reductase transfers an electron to P-450 directly after the formation of a 1:1 complex of the two enzymes [33, 34]. A model that postulates the organization of the enzymes in clusters can not fully explain the reduction of all P-450 molecules [35, 36]. Thus, while electron transfer between the microsomal reductase and P-450 is accomplished by direct interaction between the two enzymes after their diffusion in the plane of the membrane, the mitochondrial systems utilize small peripheral membrane proteins as electron carriers between integral membrane proteins.

The regulation of the levels of cytochrome P-450 system enzymes

The present results show that while in the adrenal cortex the levels of the five enzymes is quite constant across animals, in the corpus luteum they can vary at least severalfold and...
can rise at least 50-fold over the levels in the ovary. In the luteal phase of the reproductive cycle or during pregnancy as the corpus luteum develops from the granulosa cells of the ovary, the blood levels of progesterone dramatically increase. Several recent studies have shown that the levels of P450, adrenodoxin and their mRNAs can be increased by luteotrophic hormones in bovine and porcine granulosa cells in culture indicating that the increased steroidogenic capacity of the corpus luteum is a result of the induction of these enzymes at the level of the transcription of their genes. The present measurement of the absolute levels of these enzymes in tissue confirm that indeed these two enzymes are very highly induced in the corpus luteum as compared to the ovary and provide evidence that adrenodoxin reductase and cytochrome c oxidase are also similarly induced (Fig. 2). The correlation of the concentrations of the enzymes in the corpus luteum and ovary (Fig. 3) further indicates that the ebb and flow of the levels of these enzymes during the reproductive cycles do not result in major changes in the relative molar ratios of these enzymes.

Most interestingly the increase in the levels of cytochrome oxidase subunits in correlation with the levels of the steroidogenic enzymes would indicate some degree of coordinate regulation of the mitochondrial oxidative phosphorylation chain enzymes with steroidogenic enzymes. This is understandable as increased steroidogenic capacity of the cell would most likely increase the energy requirement in the cell as well.

This research was supported by a grant from the US National Institutes of Health and a Technion V. P. R. Fund-Henri Gutwirth Award. We are grateful to Ms. Irena Reiter for assistance in the purification of the enzymes, and to Dr Gera Eytan (Dept of Biology, Technion) for generously providing us with anti-cytochrome c oxidase antisera. We are also grateful to Tsfonit HaEmek Technion Co. for their generous supply of bovine tissues used for enzyme purifications and subsequent studies.

REFERENCES