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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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We have estimated the concentrations of cytochromes *P*-450$_{scc}$ and *P*-450$_{11\beta}$ 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$_{scc}$ and *P*-450$_{11\beta}$, in equal concentrations) and 1:2.5:3 in the corpus luteum (which has only *P*-450$_{scc}$). 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$_{scc}$) in three consecutive cycles of monooxygenation (hydroxylation) each of which consumes two electrons, a H^+, and O_2 [1–6]. The electrons are transferred from NADPH to cytochrome *P*-450$_{scc}$ by means of an electron transport chain that includes a flavoprotein (adrenodoxin reductase), and a ferredoxin (adrenodoxin) [1–5]:

NADPH → adrenodoxin reductase → adrenodoxin → cytochrome *P*-450.

In the adrenal cortex there is an additional mitochondrial *P*-450 that utilizes the same electron transport chain. This *P*-450$_{11\beta}$ 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$_{scc}$ and *P*-450$_{11\beta}$ 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$_{scc}$, *P*-450$_{11\beta}$, 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$_{scc}$ and cytochrome *P*-450$_{11\beta}$ 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

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Abbreviation. PBS, phosphate-buffered saline.

Enzymes. Adrenodoxin reductase (EC 1.18.1.2); cytochrome *P*-450$_{scc}$ (EC 1.14.15.6); cytochrome *P*-450$_{11\beta}$ (EC 1.14.15.4).

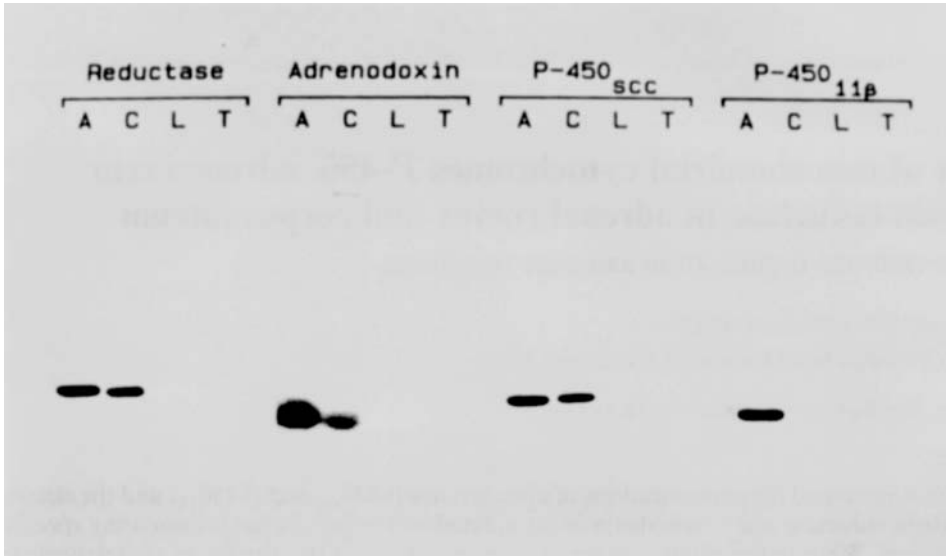

Fig. 1. *The specificity of the antisera developed against adrenodoxin reductase, adrenodoxin, cytochrome* P-450_{scc} *and cytochrome* P-$450_{11\beta}$ *isolated from the bovine adrenal cortex.* Autoradiograms of Western blots of total tissue protein (20–80 μg) from adrenal cortex (A), corpus luteum (C), liver (L) and testis (T). The blots were reacted with the antisera developed against the enzyme listed on top of the figure

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 ^{125}I-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*-450_{scc} antisera with liver protein, and that of *P*-$450_{11\beta}$ 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*-450_{scc} reacted well with the corpus luteum enzymes (Fig. 1). Southern blot analyses, using a cloned *P*-450_{scc} cDNA, suggest that there may be only one gene for *P*-450_{scc} 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*-450_{scc} 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

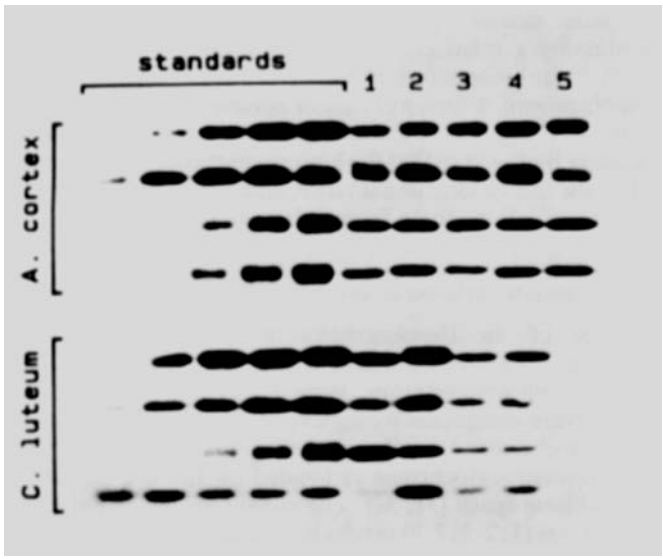

Fig. 2. *Quantitation of adrenodoxin reductase, adrenodoxin, cytochrome* P-450_{scc} *and cytochrome* P-$450_{11\beta}$ *in bovine adrenal cortex and corpora lutea and comparison with cytochrome* c *oxidase subunit levels.* Each row shows a cut of the autoradiogram of the Western blot of one gel reacted with antisera developed against: (1) adrenodoxin reductase (first row from the top), (2) adrenodoxin (second row), (3) cytochrome *P*-450_{scc} (third row), (4) cytochrome *P*-$450_{11\beta}$ (fourth row only for adrenal cortex) and (5) cytochrome *c* oxidase (fourth row only for corpus luteum). The first five lanes marked as 'standards' contained increasing concentrations of the respective purified enzyme (0.5–5 pmol) with the exception of the oxidase results, which show five adrenal and five corpus luteum samples side by side for comparison as they were run on the same gel together. The lanes marked 1–4 included total protein from four different cows, but each adrenal and corpus luteum sample bearing the same number was from the same cow. Lane 5 for adrenal was total protein from a bull adrenal cortex. Lane 5 for corpus luteum was total protein from a pool of ovaries isolated from five cows. The lanes marked 1–5 contained 10 μg (rows 2–4 for adrenal), 20 μg (row 1 for adrenal, and rows 2–3 for corpus luteum), 40 μg (row 1 for corpus luteum), or 80 μg protein (last row, oxidase), with the exception of lane 5 on the right (ovary), which contained 50% more protein in each row

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 ($N = 5$)

Tissue	Reductase	Adrenodoxin	*P*-450_{scc}	*P*-$450_{11\beta}$
	pmol/mg protein			
Adrenal cortex				
Mean ± SD	109 ± 12	306 ± 57	391 ± 18	403 ± 27
Range	90 – 128	235 – 365	370 – 415	370 – 445
Corpus luteum				
Mean ± SD	69 ± 35	163 ± 49	250 ± 148	–
Range	34 – 128	110 – 240	100 – 480	–
Ovary	< 5	< 5	< 5	–

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_{scc} and *P*-$450_{11\beta}$ are relatively constant in different animals and show no significant sex differences, in corpora lutea they vary considerably (Fig. 2). As compared to ovary their concentrations increase at least fiftyfold in corpora lutea. The concentrations of *P*-450_{scc} in corpora lutea may reach levels similar to the adrenal cortex, but the total concentration of mitochondrial cytochromes *P*-450 is highest in the adrenal cortex, which contains *P*-450_{scc} and *P*-$450_{11\beta}$ in nearly equal concentrations (Table 1).

The average relative concentrations of adrenodoxin reductase, adrenodoxin and *P*-450 are 1:3:8 in the adrenal cortex and 1:2.5:3 in the corpus luteum (Fig. 3, Table 1). Previously the molar ratio of adrenodoxin reductase to adrenodoxin in bovine adrenal cortex was reported as about 1:8 to 1:20 [10]. One possible explanation for this significant difference may be differential recovery of enzymes during the tissue fractionation procedures employed in the earlier studies (see above). Previously, using spectroscopic methods, adrenodoxin and total *P*-450 were shown to be present in equal molar amounts in rat adrenal cortex mitochondria [22, 23]. Our results indicate that in the bovine adrenal cortex and corpus luteum adrenodoxin is present at lower concentrations.

The Western blots with cytochrome *c* oxidase antibody revealed results that were similar to the steroidogenic enzymes in terms of their relative concentrations (Fig. 2) (the correlation coefficient between cytochrome oxidase subunit autoradiogram band intensity and reductase concentration was 0.72 for adrenal cortex and corpus luteum together, and 0.62 for corpus luteum alone). We could not measure the molar concentrations of these subunits. But differences in autoradiogram band intensity apparently reflected differences in the absolute levels of these subunits as half of the total amount of tissue protein gave nearly half-peak intensity on the autoradiograms. Thus, while the levels of the subunits of this enzyme in the adrenal cortex are very similar in different animals, they show significant differences between corpora lutea and ovary in a manner that parallels the concentrations of the steroidogenic electron-transfer proteins. But, since we could not measure the low levels present in the ovary relative to corpus luteum, at present the relative degree of induction of these two enzymes remains unclear.

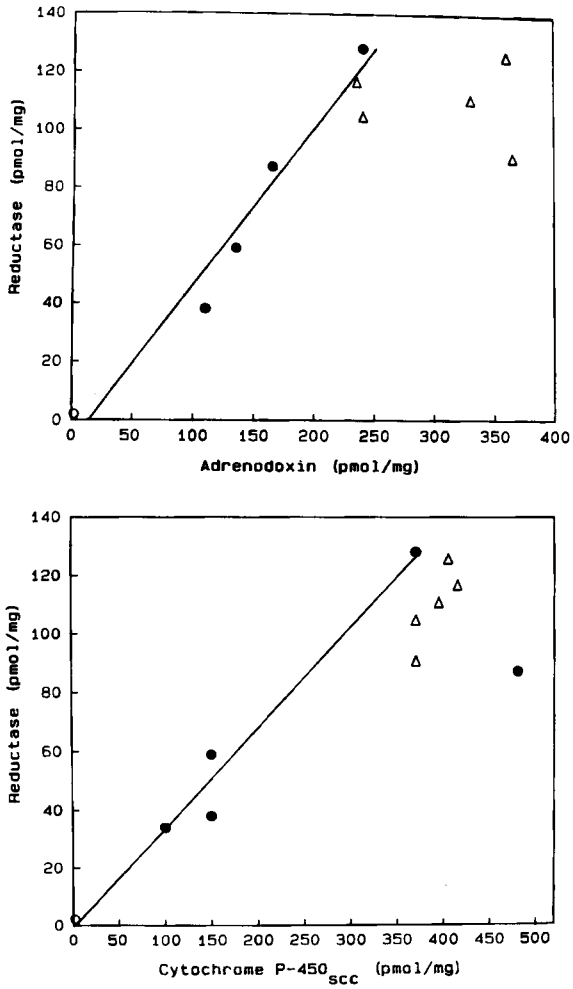

Fig. 3. *Correlation of the levels of adrenodoxin reductase with adrenodoxin and cytochrome* P-*450$_{scc}$ in corpora lutea.* (●) Corpus luteum, (○) ovary, (△) adrenal cortex results. The line of linear regression was fitted only to corpus luteum and ovary samples. The correlation coefficients (r) were 0.98 for adrenodoxin reductase versus adrenodoxin, and 0.87 or 0.99 (with or without the most deviating point) for adrenodoxin reductase versus cytochrome *P*-450$_{scc}$

DISCUSSION

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 III) 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 $P\text{-}450_{scc}$ and 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 [37–40]. 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.

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