THE TOPOLOGY OF THE MITOCHONDRIAL 11β-HYDROXYLASE SYSTEM IN BOVINE ADRENAL CORTEX

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Received August 16, 1976

SUMMARY: Binding of deoxycorticosterone to cytochrome P-450 of the 11β-hydroxylase system in adrenal cortex mitochondria was inhibited by the nonpenetrating protein reagent diazobenzenesulfonate in damaged but not in intact mitochondria. The slowly penetrating hydrophilic substrate deoxycorticosterone 21-sulfate showed a slow binding to cytochrome P-450 as compared to the hydrophobic non-esterified steroid. In contrast, the esterified and nonesterified steroids bound equally fast in sonicated, aged or lysolecithin-treated mitochondria. These data imply that the steroid substrates must penetrate the inner mitochondrial membrane to interact with the 11β-hydroxylase system.

INTRODUCTION

Bovine adrenal cortex mitochondria contain a monooxygenase system that catalyzes 11β-hydroxylation of various steroids, e.g., deoxycorticosterone to corticosterone. The system is composed of an NADPH-specific flavoprotein, a nonheme-iron sulfur protein (adrenodoxin) and a cytochrome P-450 (cf. refs. 1 and 2 for reviews).There is evidence that NADPH cannot be supplied to this system from the outside of the intact mitochondria but has to be generated in the mitochondrial matrix, either directly by an NADP-linked dehydrogenase (3), or indirectly from NADH via nicotinamide nucleotide transhydrogenase (4-7). Since the mitochondrial outer membrane but not the inner membrane is permeable to nicotinamide nucleotides (8) the binding site for NADPH of the flavoprotein is most likely localized inside the inner membrane. Although it is generally believed that the 11β-hydroxylase system as a whole is localized in the inner membrane (5,9-12), very little is known about the topology of adrenodoxin and cytochrome P-450. In the present communication the topology of the cytochrome

Table 1

Effect of diazobenzenesulfonate on the extent of binding of deoxycorticosterone to cytochrome P-450 in adrenal cortex mitochondria

Incubation with diazobenzenesulfonate was performed at 30° for 5 minutes after which the reaction was terminated with 10 mM histidine. Protein concentration was 3 mg/ml. Treatment with lysolecithin was carried out prior to the addition of diazobenzenesulfonate, using 2 mM lysolecithin for 10 min at 0°. Sonication, aging and measurements of binding of deoxycorticosterone were carried out as described in Methods. The final addition was 15 μM deoxycorticosterone in 0.5 μl ethanol.

Treatment	Deoxycorticosterone binding (%)	
	− diazobenzene sulfonate	+ diazobenzene sulfonate
none	100	92
sonication	110	< 5
aging	95	< 5
lysolecithin	75	< 5

P-450, in particular its steroid-binding site, was investigated by the use of the nonpenetrating protein reagent diazobenzenesulfonate and the sulfoconjugated steroid deoxycorticosterone 21-sulfate. The results are interpreted to indicate that the steroid-binding site of the cytochrome P-450 component of the 11β-hydroxylase system is localized inside the mitochondrial inner membrane.

MATERIALS AND METHODS

Bovine adrenal cortex mitochondria were prepared as described earlier (13), and stored either at −20° or at 4°, at a protein concentration of 30 mg/ml. At the higher storage temperature, the mitochondria revealed a surprising stability and were intact, i.e., retained intramitochondrial nicotinamide nucleotides for up to 5 days. The impermeability of the mitochondria to nicotinamide nucleotides was abolished by aging in the presence of 50 mM potassium phosphate for 1 h at 30° (13). Sonication of mitochondria in batches of 5 ml was carried out in an ice bath at 7 amp for 2 min at 30 sec intervals (to allow the mixture to cool), using a Branson S 125 sonicator equipped with a micro tip. Spectrofotometric measurements were performed at 30° using an Aminco DW-2 dual wavelength spectrofotometer with 1 ml cuvettes turned 90 degrees from the normal optical pathway. Binding of steroids to cytochrome P-450 was assayed at 420 minus 390 nm in a medium composed of either 14 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-Cl (pH 7.0) and 5 mM KP_i, or 0.1 M KP_i (pH 7.0).

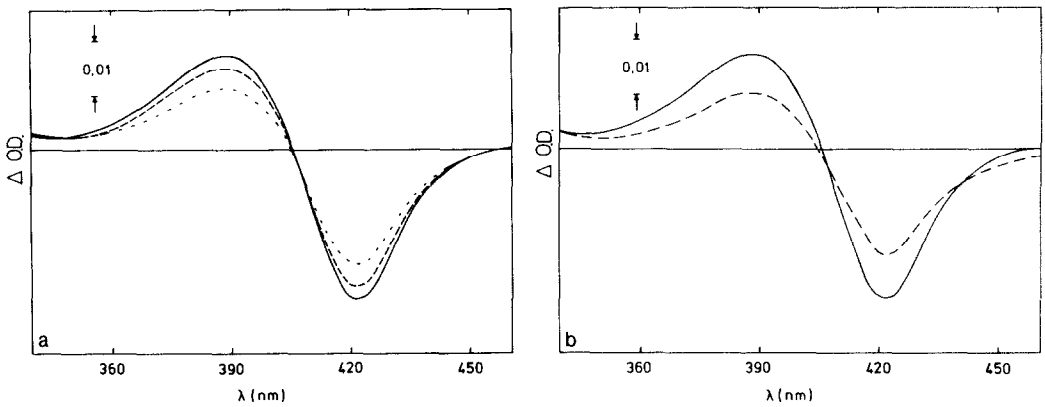

Fig. 1. Difference spectra of mitochondrial cytochrome P-450 produced by deoxycorticosterone 21-sulfate as a function of time (A) and in the absence and in the presence of lysolecithin (B).
(A) dotted, broken and solid line: spectrum was recorded after 0.5, 2 and 5 min, respectively, after the addition of the steroid.
(B) solid line: spectrum was recorded 5 min after the addition of the steroid; dotted line: the spectrum was recorded immediately after the addition of the steroid following incubation with 2 mM lysolecithin for 10 min at 0°. The concentration of protein was 3 mg/ml. Addition was: 0.6 mM deoxycorticosterone 21-sulfate.

Synthesis of diazobenzenesulfonate and treatment of mitochondria with this reagent was performed essentially according to DePierre and Karnovsky (14). All steroids were obtained from Upjohn Co. (Kalamazoo, Mich.). Radioactive and non-radioactive deoxycorticosterone 21-sulfate, cholesterol 3-sulfate and testosterone 17-sulfate were synthesized by the method of Mumma et al. (15), further purified as described by Gustafsson and Ingelman-Sundberg (16) and finally recrystallized from acetone. Lysolecithin was purchased from Sigma Chem. Co. (St. Louis, Mo.).

RESULTS

The diazonium salt of sulfanilic acid is a highly reactive, nonpenetrant protein reagent, that forms covalent bonds with most functional groups in proteins (14,17,18). As shown in Table 1, diazobenzenesulfonate had little or no effect on the binding of deoxycorticosterone to cytochrome P-450 in adrenal cortex mitochondria. However, with sonicated or aged mitochondria a rapid and extensive inhibition of the binding was observed. Low concentrations of the detergent lysolecithin (cf. ref. 19) gave a similar effect. Lysolecithin per se apparently diminished the extent of steroid binding slightly (Table 1, cf. Fig. 1B). These results are interpreted to indicate that cytochrome P-450 is-

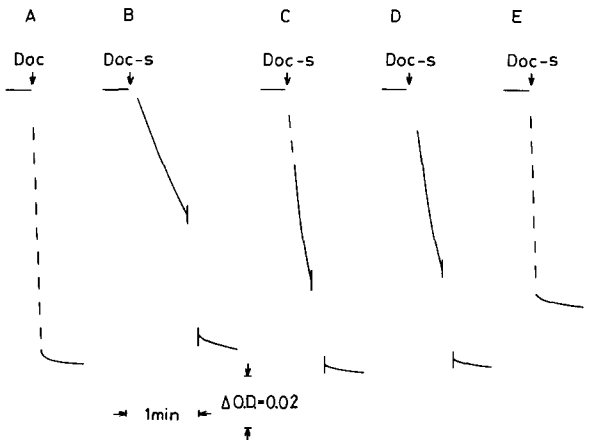

Fig. 2. Binding of deoxycorticosterone (Doc) and deoxycorticosterone 21--sulfate (Doc-s) to mitochondrial cytochrome P-450 as a function of time.
The concentration of protein was 1.2 mg/ml. Additions were: (A) 15 µM deoxycorticosterone; (B) 180 µM deoxycorticosterone 21-sulfate; (C) as in B except that sonicated mitochondria were used; (D) as in B except that aged mitochondria were used; (E) as in B except that mitochondria were pretreated with lysolecithin as described in Fig. 1B. In B, C and D the interval of interruption was 3 min. Binding of steroids to cytochrome P-450 was assayed spectrofotometrically as described in Methods.

not accessible to diazobenzenesulfonate in adrenal cortex mitochondria, unless the inner membrane is disrupted or otherwise made permeable to the protein reagent, suggesting that cytochrome P-450 is localized inside the inner membrane.

To further investigate this possibility the use of nonpermeant steroid derivatives was attempted. A suitable compound was deoxycorticosterone 21-sulfate, which was found to bind to cytochrome P-450 and give rise to a type I absorption spectrum[1] (Fig. 1A), similar to that obtained with deoxycorticosterone, with a peak at about 390 nm and a trough at about 420 nm (cf. ref. 1). However, as may be seen in Fig. 1A, the binding of the conjugated steroid to

[1] Similar spectra were obtained with testosterone 17-sulfate and cholesterol 3-sulfate, in the latter case using acetone-extracted mitochondria. Recently, deoxycorticosterone 21-sulfate was found to be hydroxylated to corticosterone 21-sulfate whereas testosterone 17-sulfate apparently was not hydroxylated. A full account of these results will be published elsewhere (J. Montelius, J.-Å. Gustafsson, J. Rydström and M. Ingelman-Sundberg, manuscript in preparation).

cytochrome P-450 was slow as compared to that of the nonesterified steroid, maximal binding of the sulfate being obtained after about 5 min. This difference in reaction time was abolished in the presence of lysolecithin (Fig. 1B). Although the maximal absorption change was somewhat diminished[2] a large excess of lysolecithin (approx. 10 mM) could be added essentially without further altering the spectrum. Similar results were obtained with testosterone 17-sulfate.

Fig. 2B shows the time course for binding of deoxycorticosterone 21-sulfate to cytochrome P-450 as measured at fixed wavelengths. For a comparison, the rapid binding of deoxycorticosterone is shown in Fig. 2A. The pronounced stimulation of the rate of binding of the steroid sulfate by lysolecithin (Fig. 2E) may be mimicked by sonication (Fig. 2C) or aging (Fig. 2D). The effect of aging or sonication of the mitochondria can be increased substantially by further exposing the mitochondria to these treatments. Also, it is interesting to note that uncouplers of oxidative phosphorylation, e.g., carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, cause a slight increase in the rate of binding of the steroid sulfate to cytochrome P-450 (not shown).

Lysolecithin exerted its effect at relatively low concentrations as compared to those required for solubilization of membranes (*cf.* refs. 19 and 20). Maximal enhancement of the rate of binding of deoxycorticosterone 21-sulfate to cytochrome P-450 was achieved within a concentration range of 0.2 to 0.6 mM (not shown).

DISCUSSION

The distribution and topology of the components of the microsomal cytochrome P-450-linked monooxygenase systems have recently acquired attention as important aspects of the cellular organization of these systems (21). These aspects have attracted less attention in the case of the corresponding mitochondrial systems, e.g., the steroid-metabolizing system of adrenal cortex mito-

[2] The concentrations of deoxycorticosterone used in this communication are saturating. However, with deoxycorticosterone 21-sulfate, as well as with other steroid sulfates, or in the presence of lysolecithin, the affinity of cytochrome P-450 for the substrate is decreased. Decreased absorption changes obtained under these conditions may therefore depend on an incomplete saturation of the cytochrome P-450 (cf. footnote 1).

chondria, in spite of the fact that these organelles seem to be well suited for studies of problems concerning the membrane localization of hydroxylases (cf. however ref. 22).

The data presented in this communication indicate that the steroid-binding site of the cytochrome P-450 involved in 11β-hydroxylation in adrenal cortex mitochondria is localized inside the inner membrane. In view of the inaccessibility of the cytochrome P-450 to diazobenzenesulfonate in intact mitochondria it appears that no part of the protein related to steroid binding is exposed to the outside of the inner membrane. Ready binding of steroid sulfates to cytochrome P-450 was obtained in mitochondria made permeable to these substrates without the use of detergents. These findings suggest that the steroid-binding site may not be buried in the membrane but relatively accessible to the water phase of the matrix.

Steroid-metabolizing mitochondria, in particular those derived from zona fasciculoreticularis in adrenal cortex, show vesicular inner structures, which are not found in liver or heart mitochodnria (23-27). It may be speculated that these structures are separate from the inner membrane proper and are the site of the steroid hydroxylase system. The present results are not incompatible with this hypothesis. Attempts to isolate these vesicles are now in progress.

The intramitochondrial localization of cytochrome P-450 implies that the steroids must cross the mitochondrial inner membrane in order to undergo hydroxylation. Whether this occurs by simple diffusion or through a carrier-mediated transport mechanism remains to be established. A transport of cholesterol to the site of cholesterol side chain cleavage has been proposed (28,22), although direct evidence supporting this theory is not yet available. This problem is presently being investigated.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Natural Science Research Council (K 2162-024), the Swedish Cancer Society (75:124) and the Swedish Medical Research Council (Nr 03X-2819).

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