Cholesterol Side Chain Cleavage in Rat Adrenal Supported by Outer Mitochondrial Membrane NADH-Semidehydroascorbate Reductase*

(Received for publication, July 26, 1984)

Rama D. Natarajan and Boyd W. Harding‡

From the University of Southern California, School of Medicine, Departments of Medicine and Biochemistry, Los Angeles, California 90033

Rat adrenal mitochondria have an active rotenone-insensitive outer mitochondrial membrane NADH-semidehydroascorbate (NADH-SDA) reductase which supports cholesterol side chain cleavage at a rate equal to that supported by malate. Side chain cleavage activity supported by both of these electron donor systems is equally inhibited by cycloheximide. Catalase or butylated hydroxyanisole are required for the NADH-SDA reductase-supported cholesterol side chain cleavage. This requirement can be removed by briefly subjecting the mitochondrial preparations to −20 °C. Ascorbic acid alone or with malate is either inhibitory or has no effect on side chain cleavage activity. These observations demonstrate that outer mitochondrial membrane NADH-SDA reductase in rat adrenal functions to provide cytoplasmic reducing equivalents to intramitochondrial cytochrome P-450om and provides a new explanation for the function of ascorbic acid in corticosteroidogenesis.

Adrenal mitochondrial cytochrome P-450om catalyzes the side chain cleavage (SCC) of cholesterol (1, 2) which is believed to be the rate-limiting step in corticosteroid biosynthesis (3). The rate of cholesterol SCC is stimulated by ACTH (3, 4) and this stimulation is blocked by cycloheximide and other protein synthesis inhibitors (5, 6). Reducing equivalents for oxygen activation by this and other mitochondrial steroid hydroxylases are carried by intramitochondrial NADPH to a steroidogenic electron transport pathway (SETP) consisting of adrenodoxin reductase, a FAD-containing flavoprotein, adrenodoxin, an iron-sulfur oxidation-reduction protein, and cytochrome P-450om (7, 8), all located in the inner mitochondrial membrane (9–11). Intramitochondrial NADPH is believed to be formed primarily by a malic enzyme (12), but also by an energy-linked pyridine nucleotide transhydrogenase (13). In addition a "malate shuttle" has been proposed to function in transferring reducing equivalents from cytosolic NADPH to mitochondrial NADP+ (12).

In the course of examining various electron donors to the SETP, we observed the presence of an active rotenone-insensitive NADH-cytochrome c reductase in rat adrenal mitochondria. This enzyme (cytochrome c reductase, EC 1.6.99.3) was first discovered in the outer mitochondrial membrane of rat liver by Sottocasa et al. (14). Recently Ito et al. demonstrated that this electron transport system was composed of NADH cytochrome b5 reductase together with a b type cytochrome called OM cytochrome b (15, 16) which catalyzed the reduction of semidehydroascorbate (SDA) (17). This SDA reductase (NADH: monodehydroascorbate oxidoreductase, EC 1.6.5.4) has subsequently been identified by Diliberto et al. (18) in a number of tissues including the adrenal. These investigators have provided evidence that in the adrenal medulla this reductase functions to provide reducing equivalents for dopamine β-hydroxylation by maintaining ascorbate in its reduced form which then diffuses between mitochondria and chromaffin granules (19, 20).

It has been suggested that the function of NADH-SDA reductase in the adrenal cortex is to support the free radical scavenging activity of ascorbate (21). In view of the temporal association between ACTH-stimulated corticoid biosynthesis and changes in ascorbate metabolism (22, 23), the function of ascorbate as a cofactor in lysine and proline hydroxylation (24) and its established function as an electron donor for dopamine hydroxylase (19, 25), we considered the possibility that NADH-SDA reductase and ascorbate might participate in an electron transport pathway for the corticosteroiolygonic hydroxylases. This report presents evidence that rat adrenal OMM NADH-SDA reductase transfers reducing equivalents from extramitochondrial NADH to the intramitochondrial SETP which mediates cholesterol SCC.

EXPERIMENTAL PROCEDURES

Animal Treatment—Female Sprague-Dawley rats weighing approximately 200–220 g were each injected intraperitoneally with 0.025 mg of Cortrosyn® (ACTH24) in saline, 10 min before decapitation. The glands were removed after 4 min and transferred to ice-cold 0.25 M sucrose. In some experiments, 10 mg of CHI in ethanol was injected intraperitoneally at the same time as ACTH24.

Preparation of Adrenal Mitochondria—Adrenals were homogenized in ice-cold 0.25 M sucrose, and mitochondria were prepared by differential centrifugation as described previously (26). The washed mitochondrial pellet was resuspended in 0.25 M sucrose to a final concentration of about 4 μM cytochrome P-450 and kept at 4 °C.

Cytochrome P-450 Assay—Total mitochondrial cytochrome P-450 was determined after the procedure of Omura and Sato (27) in a buffer consisting of 96 mM KCl, 13 mM Na2HPO4, 3 mM KH2PO4, and 6 mM MgCl2 using an American Instruments DW-2 spectrophotometer in the split beam mode.

Cholesterol Side Chain Cleavage Activity—Mitochondria equivalent to 0.5 μM cytochrome P-450 were incubated in a volume of 1.6 ml at 30 °C in a buffer consisting of 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine HCl, 10 mM KH2PO4, 5 mM MgCl2, 0.2% bovine serum albumin, 0.2 mM NADP, 6 μM rotenone, and 20 μM 4a-5-epoxy-11β-hydroxy-5α-androstane-3α-carbonitrile. The reaction was initiated by the addition of one or more of the following substrates: malate (7.5 mM), NADH (6.0 mM), sodium ascorbate (7.0

*This work was supported by National Institutes of Health Grant AM27678. A portion of this work was presented at the 74th Annual Meeting of the American Society of Biological Chemists, San Francisco, CA, June 1983 (Natarajan, R. D., and Harding, B. W. (1983) Fed. Proc. 42, 2065 (Abstr. 1797)). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1704 solely to indicate this fact.

‡To whom all correspondence and requests for reprints should be addressed: School of Medicine, University of Southern California, HMR 703, 2025 Zonal Ave., Los Angeles, CA 90033.

1The abbreviations used are: SCC, side chain cleavage; ACTH, adrenocorticotropic hormone; SETP, steroidogenic electron transport pathway; SDA, semidehydroascorbate; OMM, outer mitochondrial membrane; CHI, cycloheximide; BHA, butylated hydroxyanisole; DCPIP, 2,6-dichlorophenolindophenol.
mm), ascorbic acid oxidase (6.0 units), catalase (30 µg/ml), and BHA (100 µM). At various time intervals, 0.2-ml aliquots of the reaction mixture were transferred to 6 ml of ether. The ether extracts were taken to dryness under nitrogen, and the residue was taken up in methanol. Aliquots were assayed for pregnenolone by radioimmuno- 

As described in Table 4, the reductases were assayed according to the procedure of Abraham et al. (25).

Enzyme Assays of Electron Transport—Rates of electron transport at 25°C were determined spectrophotometrically using a DW-2 spectrophotometer. Cytochrome c reduction was monitored at 550 versus 540 nm, essentially after the procedure of Sottocasa et al. (14). The assay mixture consisted of 250 mM sucrose, 15 mM triethanolamine HCl pH 7.6, 1.75 mM KCN, 30 µM cytochrome c, 6.0 µM rotenone, 7.5 µM antimycin A, and mitochondria equivalent to 0.12 µM P-450 in a total volume of 0.85 ml. The reaction was started after a 10-min preincubation by the addition of 0.17 mM NADH.

DCPIP reduction was assayed by monitoring changes in optical density of the dye at 600 nm (29). The assay mixture was the same as that used in the cytochrome c reduction assay except that 0.11 mM DCPIP replaced cytochrome c.

NADH-SDA reductase was assayed according to the procedure of Lto et al. (17) except that the potassium phosphate buffer used in the cytochrome P-450 assay described above was employed.

Materials—[3H]Pregnenolone (10 Ci/mmol) was obtained from New England Nuclear. ACTH1-24 was obtained from Organon Inc. Pregnenolone antiserum was obtained from Radioassay Systems Laboratories. 4a,5-Epoxy-17β-hydroxy-5α-androstane-2α-carbononitrile was a gift from Sterling Winthrop Research Institute. All other materials were obtained from Sigma.

RESULTS AND DISCUSSION

The activities of three rotenone-insensitive reductases studied in intact mitochondria from rats treated with ACTH1-24 and ACTH1-24 plus CHI are shown in Table 1. The rotene-insensitive malate-DCPIP reductase reflects the activity of the peroxisomal SETP, and the rotenone-insensitive NADH-cytochrome c and NADH-SDA reductases reflect the activity of the OMM electron transport system. While the malate-DCPIP assay measures only a small segment of the SETP from NADPH to adrenodoxin reductase (7) this assay was used because we were unable to employ more complete assays of the SETP such as the rotenone-insensitive malate-cytochrome c reductase or malate-cytochrome P-450 reductase (7). In the case of the cytochrome c assay, this electron acceptor has restricted access to the peroxisomal compartment of the SETP in intact mitochondria. In the case of the cytochrome P-450 assay, rat adrenal mitochondria, unlike bovine adrenal cortical mitochondria, contain such large levels of endogenous electron donors that the kinetics of reduction of cytochrome P-450 monitored by the formation of its carbon monoxide adduct cannot be followed. The striking feature of these data is the extremely high electron transport activity of the OMM supported by NADH as compared to that supported by endogenous substrates. One explanation for these results might be that the free radical intermediate, SDA, formed in the ascorbate-supported reactions, dissipates superoxide anion (O2⁻) which mediates the SDA hydroxylation reactions; another might be related to the relatively positive midpoint reduction potential of the SDA/ascorbate couple as compared to that of the steroidogenic electron carriers. In either case it is possible that only under circumstances where the steady state concentration of SDA is maintained at very low levels, either by the activity of NADH-SDA reductase or ascorbic acid oxidase, could ascorbate function as an electron donor in this system.

The possibility that endogenous ascorbate supports the OMM NADH-SDA reductase pathway was tested by preincubating mitochondria with ascorbic acid oxidase for 4 min prior to the addition of NADH. As shown in Fig. 1 this procedure decreased the NADH-supported SCC rate toward that supported by endogenous electron donors alone, suggesting that these isolated washed mitochondria retain some ascorbate which partially supports NADH-SDA reductase.

Fig. 2 demonstrates that cholesterol SCC activity supported by NADH-SDA reductase is equally as sensitive to CHI treatment of the animals as is the malate-supported activity. Mitochondria from ACTH1-24 plus CHI-treated animals support only approximately 38% of the rate of SCC as mitochondria from animals treated only with ACTH1-24, regardless of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Malate DCPIP</th>
<th>NADH-cytochrome c</th>
<th>NADH-SDA reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH1-24</td>
<td>7.14 ± 0.17</td>
<td>7.08 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>ACTH1-24 + CHI</td>
<td>7.08 ± 0.08</td>
<td>260.9 ± 6.4</td>
<td>353.7 ± 14.9</td>
</tr>
</tbody>
</table>

* nmol of DCPIP reduced/nmol of P-450/min.
+ nmol of cytochrome c reduced/nmol of P-450/min.
' nmol of NADH oxidized/nmol of P-450/min.
" S. E. of the mean from 4--9 experiments.

**Fig. 1.** The time course of pregnenolone formation by rat adrenal mitochondria supported by various electron donors. The rats were treated with ACTH1-24 or with ACTH1-24 plus CHI for 10 min before decapitation. Enzyme assays were run as described under "Experimental Procedures," with the addition of the components as indicated: □, malate; ▲, NADH + ascorbate; ×, NADH + ascorbate + ascorbic acid oxidase; □, NADH; V, NADH (preincubated 4 min with ascorbic acid oxidase), none; O, ascorbate.
probably the result of endogenous ascorbate as discussed by standard differential centrifugation procedures. It is possible that this vitamin functions by quenching free radical damage to mitochondrial preparations, providing evidence that peroxisomal preparations results in a marked reduction of the electron donors supporting the activity. Part way into these studies, the NADH-SDA reductase-supported cholesterol SCC activity of these mitochondrial preparations was lost even though malate-supported SCC activity remained intact. After considerable experimental manipulations we discovered that either catalase or BHA could return the NADH-SDA reductase-supported SCC activity. This loss of activity was traced to a faulty temperature regulation of the mitochondrial preparations before decapitation, although these preparations were lost even though malate-supported SCC activity was unchanged. However, such mitochondria treated with NADH plus ascorbate now support maximal cholesterol SCC without any significant effect on the malate- or NADH-supported SCC activities is seen, indicating that the permeability of these cold shocked mitochondria to these electron donors is unchanged. However, such mitochondria treated with NADH plus ascorbate show support maximal cholesterol SCC without the addition of hydrogen peroxide scavengers. The intermediate SCC activity supported by NADH plus catalase alone is probably the result of endogenous ascorbate as discussed above. The fact that malate-supported activity is either unaffected or slightly inhibited by ascorbate appears to rule out the possibility that this vitamin functions by quenching free radicals which adversely affects this hydrolysis.

Boveris et al. (30) have demonstrated that aging of liver peroxisomal preparations results in a marked reduction of hydrogen peroxide (H₂O₂) formation, which in turn could initiate lipid peroxidation of cytochrome P-450₆₇. Hydrogen peroxide is also known to react with ascorbate to produce SDA, thus favorably altering the reduction potential of the SDA/ascorbate couple. However, since “cold shocking” these mitochondrial/peroxisomal preparations has no effect on malate-supported cholesterol SCC and since H₂O₂ scavengers also have no effect on this activity, it is likely that the loss of the NADH-SDA reductase-supported SCC activity in untreated mitochondria is related to an inactivation of the ascorbate-dependent enzyme system. It is known that SDA reductase is unstable and it has been proposed that this is due to the action of peroxides (18, 38).

While reconstitution studies with purified components of the mitochondrial SETP, as the electron donors supporting the activity.

FIG. 2. The effect of cycloheximide on the time course of pregnenolone formation by rat adrenal mitochondria supported by various electron donors. Rats were treated with ACTH₃₄ (solid symbols) or ACTH₁₈₋₃₄ plus cycloheximide (open symbols) 10 min before decapitation, and incubations were performed as described under “Experimental Procedures.” The addition of the components as indicated: ●, malate; ▲, NADH + ascorbate; ○, malate; Δ, NADH + ascorbate; ○, NADH.

The results of some of these observations. Cholesterol SCC is supported optimally in mitochondria prepared at 4 °C only by malate, or by NADH plus ascorbate with either catalase or BHA. If these mitochondria are subjected to -20 °C for 5 min, no significant effect on the malate- or NADH-supported SCC activities is seen, indicating that the permeability of these cold shocked mitochondria to these electron donors is unchanged. However, such mitochondria treated with NADH plus ascorbate now support optimal cholesterol SCC without the addition of hydrogen peroxide scavengers. The intermediate SCC activity supported by NADH plus catalase alone is probably the result of endogenous ascorbate as discussed above. The fact that malate-supported activity is either unaffected or slightly inhibited by ascorbate appears to rule out the possibility that this vitamin functions by quenching free radicals which adversely affects this hydrolysis.

TABLE II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions and treatment</th>
<th>Pregnenolone formed/nmol</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Malate</td>
<td>2.48 ± 0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>NADH</td>
<td>0.65 ± 0.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>NADPH</td>
<td>0.41 ± 0.21</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4</td>
<td>Malate, ascorbate</td>
<td>2.02 ± 0.16</td>
<td>NS*</td>
</tr>
<tr>
<td>5</td>
<td>NADH, ascorbate</td>
<td>0.70 ± 0.18</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6</td>
<td>Malate, catalase</td>
<td>2.33 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>NADH, catalase</td>
<td>1.33 ± 0.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>NADH, ascorbate, catalase</td>
<td>2.35 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>9</td>
<td>NADH, ascorbate, BHA</td>
<td>2.53 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>Malate, -26 °C</td>
<td>2.22 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>NADH, -20 °C</td>
<td>0.73 ± 0.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>12</td>
<td>NADH, ascorbate, -20 °C</td>
<td>2.26 ± 0.15</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Pregnenolone formed by endogenous electron donors has been subtracted from these values.

NS, not significant.

source for H₂O₂ generation. The cytochrome b₅-like flavoprotein reductase of the NADH-SDA reductase pathway of the OMM could result in a one or two electron reduction of oxygen (32, 33). The dismutation of superoxide anion arising from cytochrome P-450 (34) or the reaction of ascorbate with protein bound Fe²⁺ (35) could also generate H₂O₂.

Hydrogen peroxide could inhibit cholesterol SCC in several ways. In the presence of transition metal ions it can form hydroxyl radicals (36) which in turn could initiate lipid peroxidation of cytochrome P-450₆₇. Hydrogen peroxide is also known to react with ascorbate to produce SDA (37), thus unfavorably altering the reduction potential of the SDA/ascorbate couple. However, since “cold shocking” these mitochondrial/peroxisomal preparations has no effect on malate-supported cholesterol SCC and since H₂O₂ scavengers also have no effect on this activity, it is likely that the loss of the NADH-SDA reductase-supported SCC activity in untreated mitochondria is related to an inactivation of the ascorbate-dependent enzyme system. It is known that SDA reductase is unstable and it has been proposed that this is due to the action of peroxides (18, 38).

While reconstitution studies with purified components of the mitochondrial SETP are in progress, no direct evidence for the site of electron donation by the OMM NADH-SDA reductase to the intramitochondrial SETP is yet available. However, because this reductase supports all the known adrenal cortical mitochondrial hydroxylases while being unable to support any microsomal hydroxylase, it is possible that electron transfer to the SETP is dependent upon adrenodoxin as indicated in the following working model for electron transfer.

(i) NADH → Fp → OMM cytochrome b → ascorbate

(ii) NADPH → adrenodoxin reductase → adrenodoxin → cytochrome P-450₆₇

OMM NADH-SDA reductase, which reduces SDA to ascorbate via the flavoprotein, NADH-cytochrome b₅ reductase (Fp), and an OMM b-type cytochrome (OMM cyt b), which is similar to but distinct from cytochrome b₅ (10–17), is shown

R. D. Natarajan and B. W. Harding, unpublished data.
in pathway i. It is not yet clear whether or not OMM cytochrome b reduces SDA directly or via another component. This scheme does not address the question of how ascorbate reaches adrenodoxin of the inner mitochondrial membrane, pathway ii.

These studies, which demonstrate that ascorbic acid acts as an electron carrier transferring cytoplasmic reducing equivalents to a mitochondrial hydroxylase, provide a new explanation for the function of this vitamin in adrenal cortical steroid biosynthesis.

REFERENCES