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# Ascorbate stimulates monooxygenase-dependent steroidogenesis in adrenal zona glomerulosa ☆

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**Abstract**

It is well known that ascorbic acid (Asc) is highly concentrated in the adrenal gland, but its function in the gland is not thoroughly elucidated. We therefore examined the possibility that Asc participates in steroidogenic monooxygenase systems of the adrenal cortex with the aid of the regenerating system including outer mitochondrial membrane cytochrome *b* (OMb). When Asc availability was limited in rat mutants unable to synthesize Asc, the increase in plasma aldosterone concentration under Na-deficiency was suppressed without effect on plasma corticosterone concentration. Aldosterone formation in the isolated mitochondrial fraction of the zona glomerulosa (zG) of the adrenal cortex was stimulated by the addition of Asc and NADH, while corticosterone formation was not. Consistently zG showed a high level of Asc regeneration activity and was rich in OMb among adrenocortical zones. Taken together, the enhanced aldosterone formation that is catalyzed by one of the steroidogenic monooxygenases, P450aldo, may be supported by Asc with its regenerating system.


*Keywords:* Steroidogenesis; Aldosterone formation; Adrenal cortex; Ascorbic acid; Aldosterone synthase cytochrome P450aldo; Outer mitochondrial membrane cytochrome *b*; Semidehydroascorbic acid reductase

The level of ascorbic acid (Asc) in the adrenal gland is very high as compared with other organs [1,2]; the Asc value of almost 20 mM is 10–15-fold greater than tissues with the second highest Asc level such as the liver and the brain. Well known functions of Asc are to protect organs as an antioxidant from damaging lipid peroxides [3] and to participate as cofactors for some enzyme systems including dopamine-β-hydroxylase and prolyl hydroxylase [4,5]. The function of Asc in the adrenal cortex, however, is not thoroughly elucidated, though the involvement of Asc in steroidogenesis in the adrenal cortex has been suggested for a long time.

The steroid synthetic pathway in the adrenal cortex starting from cholesterol is now fully understood. All enzymes involved belong to a family of heme-containing monooxygenases called cytochrome P450, except for 3β-hydroxysteroid dehydrogenase/isomerase. The adrenal cortex consists of three concentric zones, the zona glomerulosa (zG), the zona fasciculata (zF), and the zona reticularis (zR). Each zone secretes specific steroid hormones, mineralocorticoids from zG, glucocorticoids from zF, and in a case of primates, adrenal androgens from zR, which is known as "functional zonation of the adrenal cortex" [6]. We established the molecular basis for this theory, that is, the functional zonation with respect to aldosterone (a strong mineralocorticoid) and corticosterone (a strong glu-

☆ *Abbreviations used:* OMb, outer mitochondrial membrane cytochrome *b*; $b_5$, microsomal membrane cytochrome $b_5$; SDAR, semidehydroascorbate reductase; Asc, ascorbic acid; SDA, semidehydroascorbic acid; ODS rat, osteogenic disorder Shionogi rat; ACTH, adrenocorticotropic hormone; DOC, deoxycorticosterone; COR, corticosterone; $P450_{11\beta}$, steroid 11β-hydroxylase; P450aldo, aldosterone synthase; $P450_{17\ \alpha}$, cytochrome P450 catalyzing the 17α-hydroxylation and C17–20 bond cleavage of pregnenolone and progesterone; zG, zona glomerulosa; zF, zona fasciculata; zR, zona reticularis; zFR, zonae fasciculata-reticularis; SDS, sodium dodecyl sulfate; Na, sodium.

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cocorticoid in a case of rodents) secretion is ascribable to the localization of specific monooxygenases responsible for each corticosteroid biosynthesis; aldosterone synthesizing enzyme, cytochrome P450aldo (P450aldo), in zG and corticosterone synthesizing enzyme, cytochrome $P450_{11\beta}$, in zF as shown in Fig. 1 [7].

It is well known that under conditions of stimulation by adrenocorticotropic hormone (ACTH) a marked depletion of Asc from the adrenal gland occurs followed by a slower fall in cholesterol level, suggesting the participation of Asc in the steroidogenesis [8]. Natarajan and Harding [9] first reported the participation of Asc as an electron donor in cholesterol side-chain cleavage reaction and 11β-, 18-hydroxylation of deoxycorticosterone. On the other hand, Yanagibashi et al. [10] proposed using bovine adrenocortical mitochondrial fraction that Asc provides a source of reducing equivalents especially for the last step of aldosterone synthesis. Redmann et al. [11] then demonstrated employing guinea pig rather a permissive role of Asc in the adaptation of aldosterone secretion in response to sodium (Na)-deficiency. Asc has been reported not to affect microsomal hydroxylases such as 17α- and 21-hydroxylase [9]. Meanwhile, Hornsby et al. [12] reported using bovine adrenocortical cells that Asc functions as an antioxidant especially to protect aldosterone synthesis from lipid peroxides.

The former three groups also suggested that Asc functions in corticosteroidogenesis with the aid of the regeneration system. The system catalyzes NADH-dependent reduction of the Asc free radical, semidehydroascorbic acid (SDA), to Asc and is thought to contain NADH-cytochrome $b_5$ reductase and a *b*-type cytochrome called outer mitochondrial membrane cytochrome *b* (OMb) besides NADH-semidehydroascorbate reductase (SDAR, EC 1.6.5.4). The SDAR activity was first reported by Staudinger et al. [13] in adrenocortical microsomes. OMb, an isoform of cytochrome $b_5$ ($b_5$) of the endoplasmic reticulum [14], was isolated from the outer mitochondrial membrane of rat liver by Ito et al. [15] and demonstrated as one of the components of rotenone-insensitive outer mitochondrial membrane NADH-cytochrome *c* reductase (EC 1.6.99.3). OMb has been found also in microsomal fractions of some tissues [16].

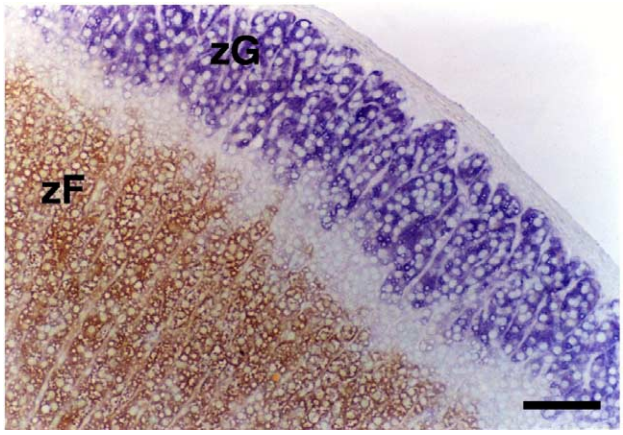


Fig. 1. Immunohistochemcal localization of P450aldo and $P450_{11\beta}$ in the adrenal gland. A fresh frozen section of an adrenal gland from a male SD rat fed on a Na-deficient diet for 20 days was stained with anti-rat P450aldo and anti-rat $P450_{11\beta}$ antibodies simultaneously. Blue-violet and brown color show the presence of P450aldo in zG and $P450_{11\beta}$ in zF, respectively. Nuclei (light blue) were poststained with methyl green. zG and zF denote the zona glomerulosa and the zona fasciculata, respectively. Bar = 50 μm.

Recently, using highly purified OMb without any contamination of $b_5$, we found that OMb but not $b_5$ could regulate rat testicular androgen synthesis by modifying the lyase activity of $P450_{17\alpha}$ [16]. We were also able to obtain very specific antibodies against OMb using the purified OMb. In order to elucidate whether effects of Asc on steroidogenesis are specific for certain steroidogenic reactions in the adrenal cortex and whether OMb is required as one of the components of SDAR, in vivo and in vitro studies were performed using rat mutants unable to synthesize Asc and purified OMb. Our data suggest that Asc with the aid of its regenerating system including OMb participates in aldosterone formation in zG by such a manner as an electron donor when level of the P450-reducing system is low.

## Materials and methods

*Animals.* Sprague-Dawley (SD) rats, Wistar- Kyoto rats, and osteogenic disorder Shionogi (ODS) rats (male, body weight of about 200 g) were purchased from CLEA Japan, Tokyo. The ODS rat is a mutant Wistar-Kyoto rat genetically deficient in Asc-synthesizing enzyme, L-gulono-γ-lactone oxidase [17,18]. Since ODS rats grow normally as long as they are supplied with sufficient Asc, they were fed on standard lab chow (CLEA Rodent diet CE-2) with adequate Asc in drinking water (0.1% Asc) until use. They were divided into four groups fed on Asc-deficient diet (Asc-deficient diet lab chow of CL-2 containing <1 mg% of Asc, CLEA Japan, Tokyo) with drinking water containing 0.1% Asc (group 1. Rats in this group were fed on the standard lab chow), 0.01% Asc (group 2), 0.009% Asc (group 3), and no Asc (group 4). In the other four groups (group 1′-4′), they were kept on Asc- and Na-deficient diet (CLEA Japan) with various Asc-supplements corresponding to those in groups 1–4. Under Na-deficiency, P450aldo is induced enormously [19]. The lab chow of CL-2 was autoclaved at 120 °C for 7 min before use to ensure the absence of Asc. The rats were kept for three weeks before sacrifice under the different conditions as described above. SD rats were treated with metyrapone (Sigma, St. Louis, MO) for 5 days or fed on Na-deficient diet for 10 or 20 days as described before [20]. All animals were treated in accordance with the Institutional Animal Care Guidelines of the School of Medicine, Keio University.

*Purification of OMb and the recombinant rat OMb expressed in Escherichia coli.* OMb was highly purified from rat liver as described before [16]. The recombinant OMb was expressed in *E. coli* and purified as previously described [16]. Since biological activities of the recombinant OMb were indistinguishable from those of the native forms in a case of rat testis [16], the recombinant OMb was mainly used in this study.

*Immunohistochemical localization of cytochrome P450s and OMb.* Localization of cytochrome P450s and OMb on 6-μm sections of fresh frozen adrenal glands was performed using specific antibodies against rat P450aldo, $P450_{11\beta}$, and OMb as previously described [19].

*Measurements of activities of 11β-hydroxylation and aldosterone formation.* The mitochondrial fractions were prepared from the capsular portion (mainly zG) of group 1′ (fed on Na-deficient diet with 0.1% Asc in drinking water) and the decapsular portion (mainly zFR and the medulla) of group 1 (fed on the standard lab chow with 0.1% Asc in drinking water)

according to the conventional procedure [21]. After being confirmed by immunoblot analysis that the mitochondrial fraction of the capsular portion contains mostly P450aldo and of the decapsular portion contains mostly $P450_{11\beta}$, the former and the latter fractions were used as enzyme sources of P450aldo and $P450_{11\beta}$, respectively. The 11β-hydroxylase and aldosterone synthase activities were assayed by essentially the same procedure as previously described [7]. The mitochondrial fractions were employed after disruption by freezing and thawing five times. Asc was added with NADH at the concentration of 20 mM when necessary, because Asc is present in the adrenal gland at around such concentration [1]. The reaction was achieved at 37 °C in a partially reconstituted enzyme system containing each mitochondrial fraction described above and the P450-reducing system which consists of adrenodoxin and adrenodoxin reductase. Deoxycorticosterone (DOC) (Sigma) was used as the substrate in both reaction systems. Adrenodoxin and adrenodoxin reductase were purified from bovine adrenal cortex according to methods previously reported [22,23].

*Immunoblot analysis.* Mitochondrial proteins of zG and of zFR from groups 1, 1′, 3, and 3′ were separated on 12.5% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electrophoretically blotted onto Immobilon-P membranes (Millipore, Bedford, MA) according to standard procedures. The membranes were treated with rabbit anti-rat P450aldo, anti-rat $P450_{11\beta}$, anti-bovine adrenodoxin, or anti-bovine adrenodoxin reductase antibodies overnight at 4 °C. They were then incubated with a secondary antibody of goat anti-rabbit IgG (Fab′) conjugated with horseradish peroxidase (DAKO Japan) for 2 h at room temperature. Bound secondary antibodies were detected by enhanced chemiluminescence (Amersham Biosciences, UK). The chemiluminiscence of the immunoreactive bands of adrenodoxin reductase was quantified using Quantity One (version 4.2.1, Bio-Rad, USA). Antibodies specific for rat P450aldo [24], rat $P450_{11\beta}$ [24], bovine adrenodoxin [25], and bovine adrenodoxin reductase [25] were raised in rabbit as described previously.

*Other measurements and statistics.* Content of Asc in plasma and in tissues was determined by essentially the same method as reported elsewhere [26], except that Asc was detected at 265 nm on high pressure liquid chromatography using COSMOSIL 5C18-AR-II (4.6 × 150 mm, Nacalai Tesque, Japan) instead of an electrochemical detection system. Plasma concentrations of aldosterone, corticosterone (COR) and renin activities were determined using commercially available RIA kits of Yamasa (Japan), Amersham (UK), and Yamasa (Japan), respectively. The renin activities in plasma were expressed as the amount of angiotensin I generated from endogenous angiotensinogen during a 60-min incubation at 37 °C. Rotenone-insensitive NADH-cytochrome *c* reductase and SDAR activities were measured at 30 °C according to the method of Takesue and Omura [27], and Nishino and Ito [28], respectively. The concentration of cytochrome P450 was estimated spectrophotometrically using a molar extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ for the carbon monoxide difference spectrum of the reduced form [29]. Protein concentration was determined by the method of Lowry et al. using BSA (Sigma, St. Louis, MO) as a standard [30]. Statistical significance was analyzed using one-way ANOVA. When the data variances followed normal distribution, differences in mean values among groups were analyzed by unpaired Student's *t* test. A value of $p < 0.05$ was considered statistically significant. Data were expressed as means ± SD ($n = 3$).

## Results and discussion

### *Effect of Asc availability on steroidogenesis in the adrenal cortex in vivo*

In order to examine the effect of Asc availability on steroidogenesis in vivo, we employed rat mutants (ODS rat) unable to synthesize Asc [17,18], since rats in general have the capacity to synthesize Asc. In ODS rats, Asc availability could be controlled as described in Materials and methods. With 0.1% Asc-supplement (group 1), ODS rats grew normally as expected. Asc-supplement of 0.009% in drinking water (group 3) was still found to be good enough to help them grow normally without signs of scurvy that occurred with no Asc-supplement (group 4) [31]. As shown in Fig. 2, under 0.009% Asc-supplement (group 3), the concentrations of Asc in plasma decreased to less than 1/10 (Fig. 2A, left), and those in the adrenal gland (Fig. 2B, left) and the liver (Fig. 2C, left) to about 1/6 as compared to animals receiving 0.1% Asc-supplement (group 1), indicating that we succeeded in lowering the concentration of Asc

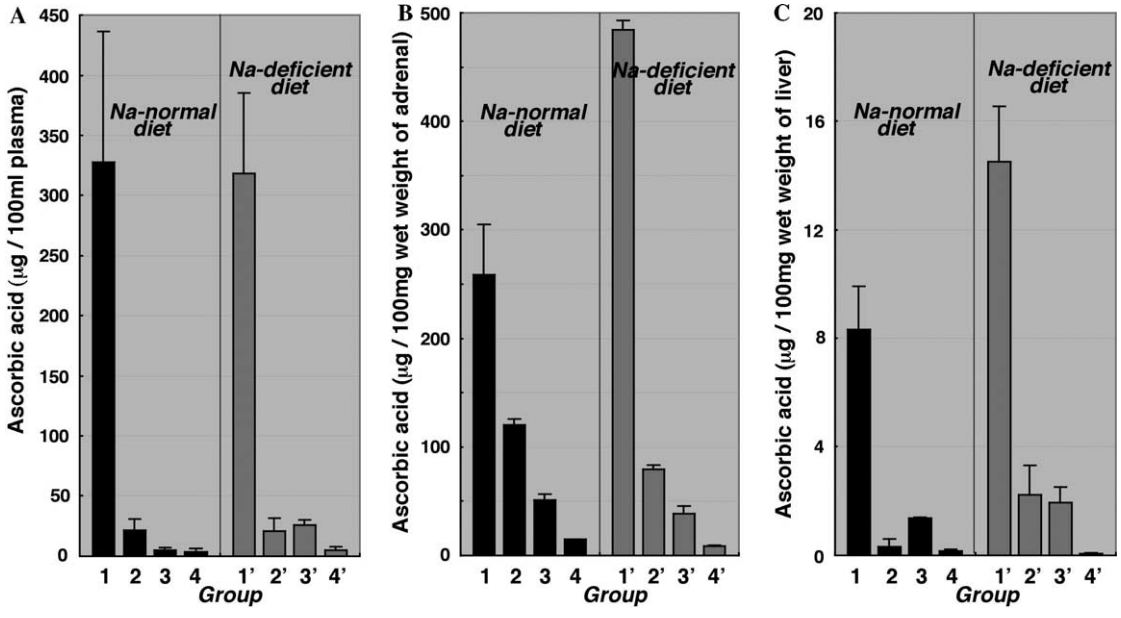


Fig. 2. Concentrations of Asc in plasma (A), in the adrenal gland (B), and in the liver (C) from ODS rats of groups 1–4 (fed on Na-normal diet) and 1′-4′ (fed on Na-deficient diet). The feeding conditions of each group are described in Materials and methods. Values: means ± SD ($n = 3$).

in a body thoroughly without scurvy. Such decrease in the Asc level was also observed in rats under Na-deficiency with various Asc-supplements (group 1′-4′) (Figs. 2A,B,C, right).

When fed on Na-deficient diet with 0.1% Asc-supplement (group 1′), renin activity and aldosterone concentration in plasma increased about 3-fold (Fig. 3A) and about 13-fold (Fig. 3B), respectively, in comparison with those of group 1 (Na-normal diet with 0.1% Asc-supplement). The degree of increment in aldosterone concentration, however, became reduced in parallel with the decrease in Asc-supplement (Fig. 3B, right): with 0.01% Asc-supplement (group 2′), plasma aldosterone concentration decreased to about 80%; with 0.009% Asc-supplement (group 3′) to about 60%; and with no Asc-supplement (group 4′) to about 33% of the value with 0.1% Asc-supplement (group 1′). Such effects of Asc availability on aldosterone levels in plasma were not significantly observed among rats fed on Na-normal diet (Fig. 3B, left). Meanwhile, the concentration of COR in plasma was unaffected by the degree of Asc-supplement and Na-deficiency (groups 1–3 and 1′-3′) (data not shown), suggesting that at least cholesterol side-chain cleavage reaction, steroid 21-hydroxylation, and steroid 11β-hydroxylation were not affected significantly by the limiting amounts of Asc-supplement. In fact, levels of progesterone, DOC, and COR in adrenal glands were not greatly different among groups 1–3. The data also imply that rats with limiting amounts of Asc-supplement (groups 3 and 3′) were not under great stress.

On immunoblot analysis, the terminal enzyme of aldosterone biosynthesis, P450aldo, was found to express almost equally in zG of group 1′ (Na-deficient diet with 0.1% Asc-supplement) and in zG of group 3′ (Na- and Asc-deficient diet with 0.009% Asc-supplement) (Fig. 4A, lanes 1′ and 3′ on the right, P450aldo in zG). Under Na-normal diet, P450aldo was hardly detected in zG (Fig. 4A, lanes 1 and 3 on the right, P450aldo in zG). No significant change in the levels of $P450_{11\beta}$ in zFR (Fig. 4A, lane 1, 1′, 3 and 3′ on the left, $P450_{11\beta}$ in zFR) and of the components of a P450-reducing system, adrenodoxin reductase, and adrenodoxin, in zG and zFR was observed among groups (Figs. 4B and C). These results were also confirmed by immunohistochemical studies (data not shown). Taken together, the data suggest that the decrease in the stimulated level of plasma aldosterone in group 3′ was not derived from the decrease in the level of P450aldo and the reducing system, but rather from the rate of aldosterone formation itself in zG.

*Effect of Asc on steroidogenesis in partially reconstituted systems using mitochondrial fractions and a P450-reducing system*

We therefore directly analyzed the effect of Asc on steroidogenesis in the partially reconstituted system using mitochondrial fractions either from zG (P450:

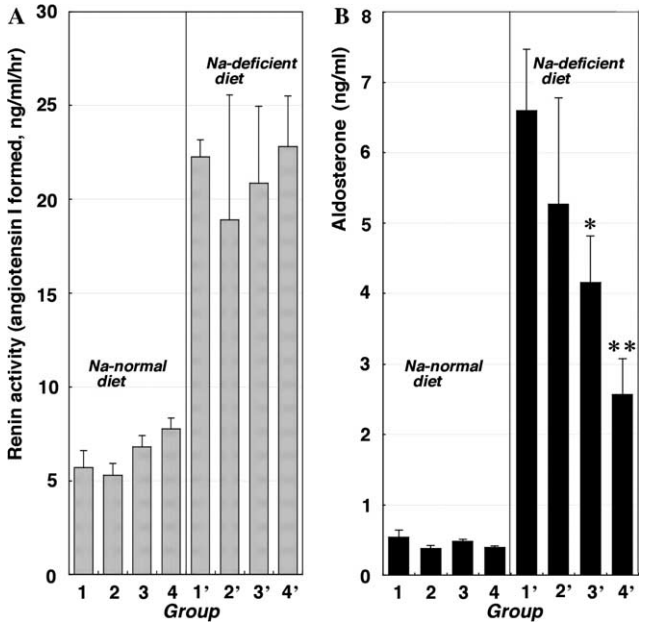


Fig. 3. Renin activity (A) and aldosterone concentration (B) in plasma from ODS rats of groups 1–4 (fed on Na-normal diet) and 1′-4′ (fed on Na-deficient diet). The feeding conditions of each group are described in Materials and methods. Values: means ± SD ($n = 3$). *$p < 0.05$ and **$p < 0.01$ significantly different from the value of group 1′.

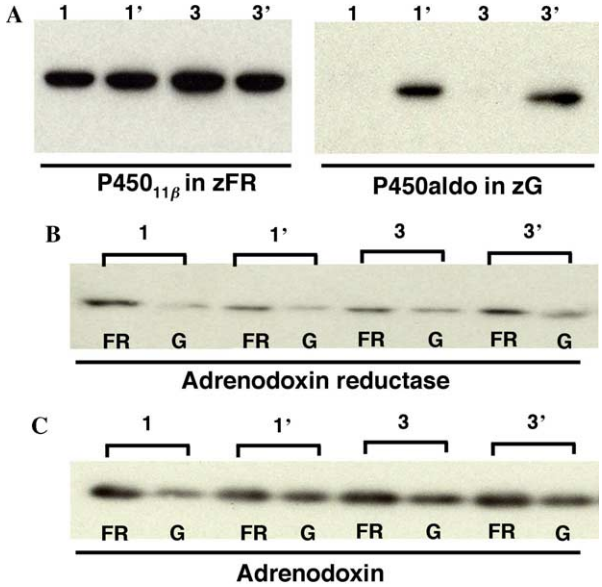


Fig. 4. Immunoblot analysis of amounts of P450 system. Mitochondrial proteins (2.5 μg) of zG or of zFR of groups 1, 1′, 3, and 3′ were separated on 12.5% (wt/vol) SDS–polyacrylamide gel electrophoresis and electrophoretically blotted onto Immobilon-P membranes as described under Materials and methods. The membrane was then treated with rabbit anti-rat P450aldo (A, right), anti-rat $P450_{11\beta}$ (A, left), anti-bovine adrenodoxin reductase (B), or anti-bovine adrenodoxin (C) antibodies. After treated with secondary anti-rabbit IgG (Fab′)-horseradish peroxidase complex, bound secondary antibodies were visualized by enhanced chemiluminescence (Amersham Biosciences, UK). Numbers of 1, 1′, 3, and 3′ correspond to groups 1, 1′, 3, and 3′ of ODS rats described in Materials and methods. G and FR denote the zona glomerulosa and the zonae fasciculata-reticularis, respectively.

$0.77 \pm 0.19$ nmol/mg of mitochondrial protein) of group 1′ as the source of P450aldo or from zFR (P450: $1.20 \pm 0.09$ nmol/mg of mitochondrial protein) of group 1 as the source of $P450_{11\beta}$ as described in Materials and methods. Further the effect was studied as a function of the concentration of purified adrenodoxin reductase added to the reaction medium, since the amount of adrenodoxin reductase in mitochondrial fraction of zG was found to be very low, about one-third of that in zFR as quantified by immunoblot analysis (control rat zG: $0.58 \pm 0.11$, control rat zFR: $1.42 \pm 0.20$; Na-deficient rat zG: $0.27 \pm 0.06$ and Na-deficient rat zFR: $0.87 \pm 0.11$ nmol/mg of mitochondrial protein). The range of concentrations of adrenodoxin reductase (0.06–1.5 μM) was chosen based on our former findings that the initial velocity of 11β-hydroxylation of DOC in the reconstituted system using partially purified P450 from bovine adrenal cortex reached a maximal level at 1 μM adrenodoxin reductase [32]. The concentration of purified adrenodoxin added to the system, on the other hand, was fixed at 14 μM, because the adrenodoxin level in zG was not greatly different from that in zFR (Fig. 4C).

Aldosterone is formed by a single enzyme, P450aldo, from DOC through three successive reactions, 11β-hydroxylation of DOC to COR, 18-hydroxylation of COR to 18-hydroxycorticosterone (18-OH COR), and finally aldehyde formation of 18-OH COR to aldosterone [6,33]. As shown in Fig. 5A, the aldosterone formation from DOC catalyzed by P450aldo in the zG-Mit-containing reaction medium was enhanced about 1.6-fold by the addition of Asc with NADH at 0.3 μM adrenodoxin reductase among tested. The stimulatory effect of Asc decreased with increase in concentration of adrenodoxin reductase in the medium. The addition of anti-OMb antibodies inhibited this increment to about half. The level of 18-OH COR, one of the intermediates, was also changed in parallel to that of aldosterone, although the level of the other intermediate, COR, was not affected significantly. The data suggest that Asc specifically supports the last two steps of the aldosterone formation, which have been reported to be accelerated by an efficient electron supply from the P450-reducing system [10,33]. The COR formation from DOC catalyzed by $P450_{11\beta}$ in the zFR-Mit-containing reaction medium was also unchanged or slightly decreased in the presence of Asc and NADH, irrespective of the concentration of the reductase, 0.3 and 1.5 μM (Fig. 5B). One possible explanation for the effect is that Asc could serve as an auxiliary electron donor especially at the last two steps of the aldosterone synthesizing system in zG where the level of adrenodoxin reductase is low.

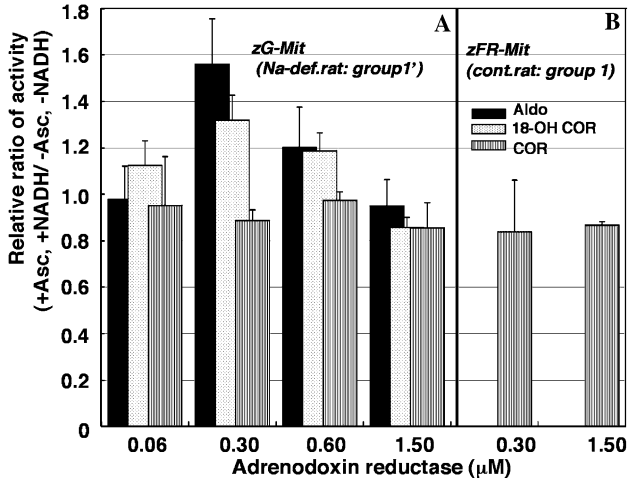


Fig. 5. The effect of Asc on deoxycorticosterone metabolism in a partially reconstituted system as a function of the concentration of adrenodoxin reductase. Mitochondrial fractions (Mit) from zG (A) and zFR (B) of ODS rat of group 1′ (Na-def. rat) and of group 1 (cont. rat), respectively, were incubated with NADPH, purified bovine adrenodoxin, and various amounts of purified bovine adrenodoxin reductase with or without addition of Asc and NADH as described under Materials and methods. Deoxycorticosterone was used as the substrate in both systems. Values on the ordinate express relative ratios between the activities (estimated as the initial velocity) in the presence of Asc with NADH and those in their absence. Aldo, 18-OH COR, and COR denote aldosterone, 18-hydroxycorticosterone, and corticosterone, respectively. Values: means ± SD ($n = 3$).

*Possible participation of OMb in the steroidogenesis*

Accumulated data have shown that the primary oxidation product of Asc coupled to an Asc-dependent enzyme system such as dopamine-β-hydroxylase system is SDA and most of tissues have the regenerating system of Asc from SDA [5]. As described above, the system is thought to consist of NADH-cytochrome $b_5$ reductase, OMb, and SDAR. The SDAR activity has been found to be a part of the activity of rotenone-insensitive NADH-cytochrome *c* reductase [28]. Recently, we have shown that SDAR activity in rat adrenal cortex is very high, which is comparable to those in the liver and the kidney [31].

Employing highly purified OMb and the antibody, the participation of OMb in the activity of rotenone-insensitive NADH-cytochrome *c* reductase in the liver and the adrenal cortex of normal SD rats was examined. As shown in Fig. 6, OMb stimulated the activity about 2-fold in both mitochondrial and microsomal fractions of the liver and about 1.4-fold in both mitochondrial and microsomal fractions of zG and zFR of the adrenal cortex. Anti-OMb antibody decreased the activity to about 70-80% of that in the reaction medium without added OMb (data not shown). The effect of OMb on SDAR activity was examined in mitochondrial fractions of zG and zFR of the adrenal cortex. OMb slightly increased the activity: about 1.2-fold in zG and 1.3-fold in zFR (data not shown). The addition of anti-OMb antibody resulted in the decrease to 60–70% of the activity without added OMb. The data thus demonstrate for the first time that OMb directly participates in a rotenone-insensitive NADH-cytochrome *c* reductase and SDAR systems carrying electrons from NADH to cytochrome *c* and SDA, respectively. Further studies on this matter are required using both purified reductases.

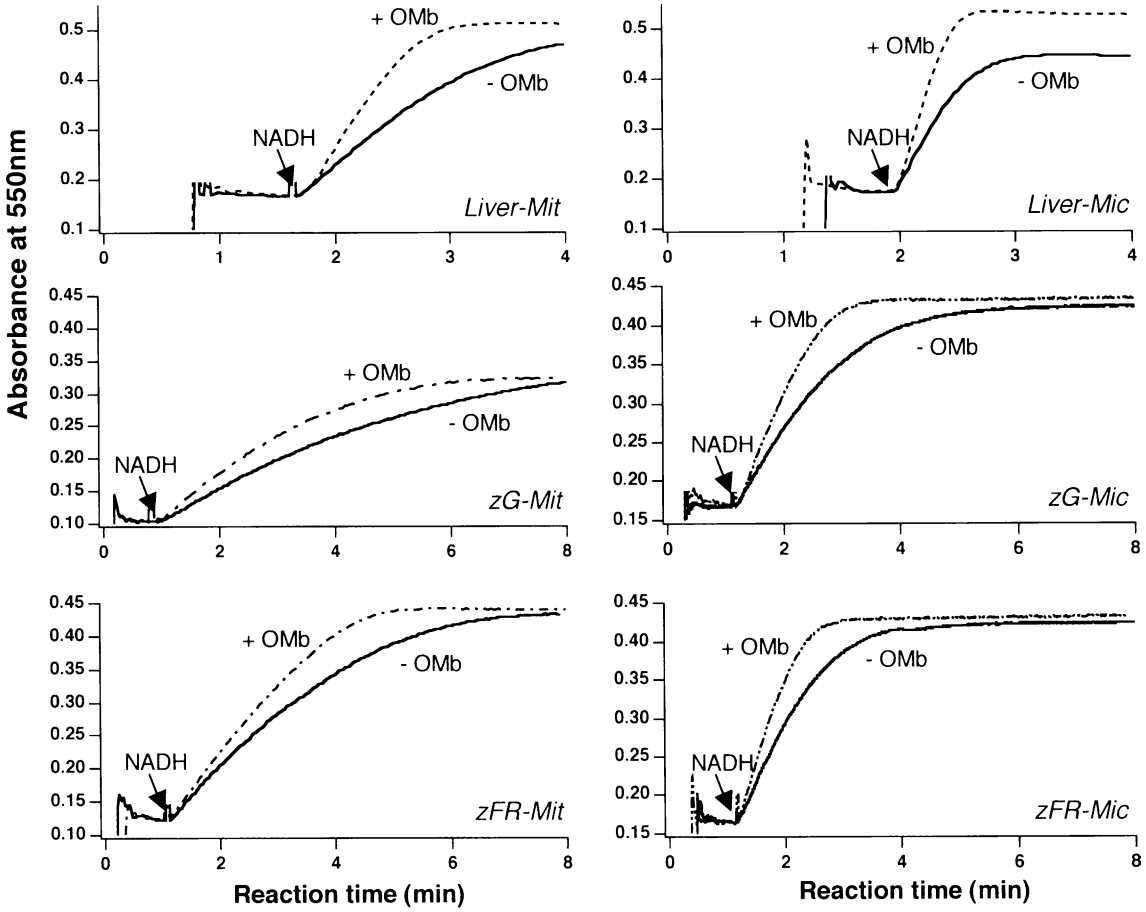


Fig. 6. The effect of OMb on rotenone-insensitive NADH-cytochrome *c* reductase activity. Rotenone-insensitive NADH-cytochrome *c* reductase activity was measured in the absence (-OMb, solid lines) or the presence of purified OMb at the concentration of 1.6 nM (+OMb, broken lines). The reaction mixture in the absence of OMb contained 1.0 ml of 0.1 mM NADH, 0.3 mg/ml of mitochondrial or microsomal fractions from the liver, zG or zFR of SD rats, 20 μM cytochrome *c*, 1 mM KCN, 1 μM rotenone, and 100 mM sodium phosphate buffer, pH 7.4, in a total volume of 100 ml. The reaction was started by the addition of NADH (indicated by an arrow) with magnetic stirring and the reduction of cytochrome *c* was followed at 550 nm at 30 °C. Mit and Mic denote the mitochondrial and microsomal fractions, respectively. zG and zFR denote the zona glomerulosa and the zonae fasciculata-reticularis, respectively.

We then attempted to localize OMb in the adrenal gland. As shown in Fig. 7A, OMb was immunohistochemically detected in the cortical cells but not in the medullary cells. In the cortex, it is of interest to notice that OMb is more abundant in zG cells than in cells of other zones (Figs.7A and B). On the other hand, cytochrome $b_5$ was rich rather in zFR cells when compared with that in zG cells [31]. The expression level of OMb in zG increased on angiotensin II stimuli elicited by feeding on Na-deficient diet under which the expression level of P450aldo is enhanced (Fig. 7C), and decreased on ACTH stimuli elicited by administration of metyrapone under which the expression level of P450aldo is suppressed (Fig. 7D) [34], suggesting that the expression of OMb is closely connected with the function of zG cells. Meanwhile, amounts of Asc in the capsular portion (mostly zG) and the decapsular portion (mostly zFR and medulla) of control and Na-deficient rat adrenals (Wistar-Kyoto rat, male 7w) were not different with each other (control rat zG: 241.45 + 26.5, control rat zFR: 229.9 + 18.1, Na-deficient rat zG: 295.4 + 46.0, and Na-deficient rat zFR: 291.7 + 60.6 μg/100 mg wet weight of tissue). The data imply that Asc functions in various ways in the adrenal gland that remain to be investigated.

It is no doubt that Asc functions as an antioxidant in the adrenal gland [12,35,36]. Present study further shows that Asc may serve as an auxiliary electron donor to the aldosterone formation system in zG where the level of adrenodoxin reductase is considerably low as compared with that in zFR. In addition, the effect of Asc appears greatly in the stimulated aldosterone formation under Na-deficiency, where more reducing equivalents are required. The standard redox potential ($E_0'$) of the Asc/SDA couple has been reported to be +0.34V, which was obtained by non-enzymic reaction of Asc/SDA with $b_5$ ($Fe^{2+}$)/$b_5$ ($Fe^{3+}$) [37]. In this context, high SDAR activity with the aid of OMb in zG may be required to maintain SDA at very low level. Asc level in the adrenal gland is about 20 mM as mentioned above; thus, the redox potential of Asc/SDA couple in vivo should be greatly less than $E_0'$ of Asc/SDA couple [38]. Lower redox potential of Asc/SDA couple is favorable

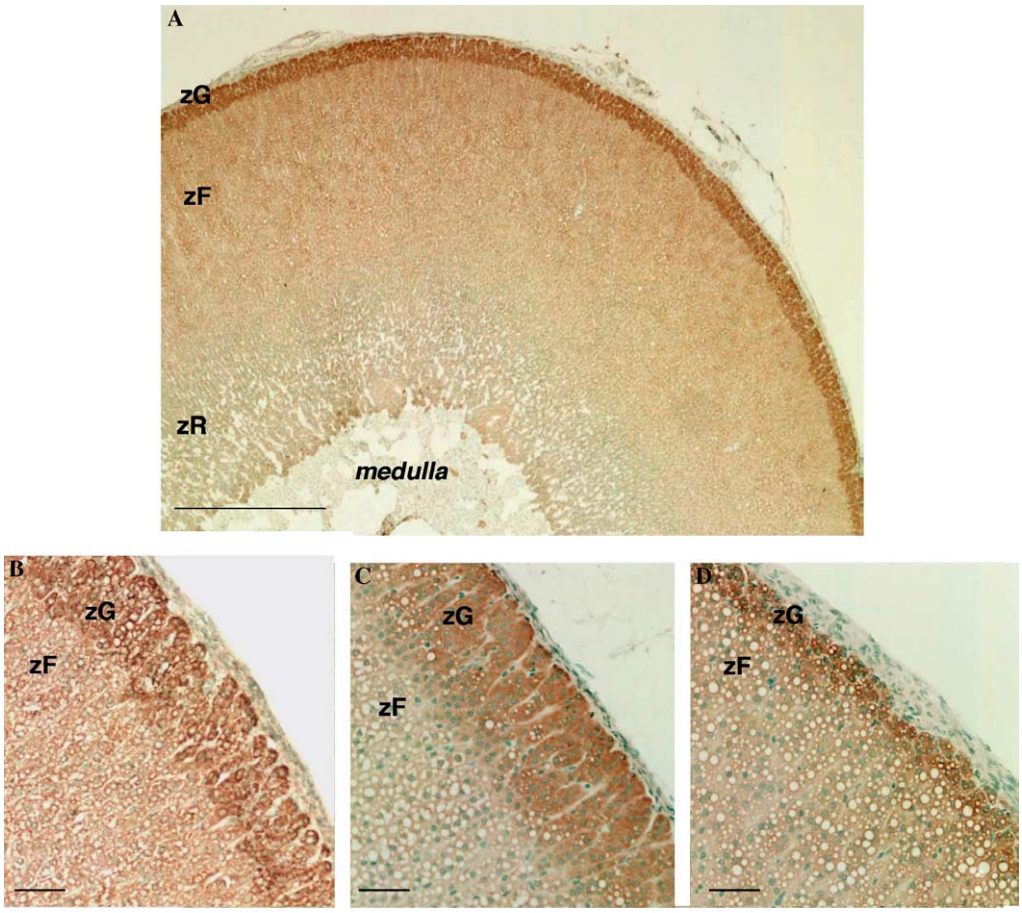


Fig. 7. Immunohistochemical localization of OMb in the adrenal gland under various physiological conditions. Fresh frozen sections of adrenal glands from SD rats fed on a normal diet (A and B), fed on a Na-deficient diet for 10 days (C) or treated with metyrapone for 5 days were stained with anti-rat OMb antibodies as described in Materials and methods. Brown color shows the presence of OMb. Nuclei (light green) were poststained with methyl green. zG, zF, and zR denote the zona glomerulosa, the zona fasciculata, and the zona reticularis, respectively. Bars = 500 μm in (A) and 50 μm in (B–D).

for transferring reducing equivalents of Asc to P450-relating system [39].

Further studies are needed to elucidate whether or not Asc provides reducing equivalents directly or via another factor and to which site those equivalents are transferred during aldosterone formation.

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