The Participation of a Second Molecule of Adrenodoxin in Cytochrome P-450-catalyzed 11β Hydroxylation*

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David W. Seybert,‡ J. David Lambeth,‡§ and Henry Kamin∥

From the ‡Department of Biochemistry and the §Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

SUMMARY

We have utilized 11β-hydroxylase activity and visible absorption spectrophotometry to detect possible complex formation among adrenodoxin reductase, adrenodoxin, and cytochrome P-45011β. At low ionic strength, a 1:1 complex between adrenodoxin reductase and adrenodoxin occurs but does not support maximal rates of 11β hydroxylation; at least 1 additional molecule of adrenodoxin in excess of the 1:1 complex is required for full hydroxylase activity. Spectrophotometric titration of a mixture of adrenodoxin reductase and cytochrome P-45011β with adrenodoxin indicates sequential formation of 1:1 complexes between adrenodoxin reductase and adrenodoxin and then between a second adrenodoxin and cytochrome P-45011β; the adrenodoxin-cytochrome P-45011β complex is only detectable when the concentration of adrenodoxin exceeds that of adrenodoxin reductase.

The steroid hydroxylation system of adrenocortical mitochondria is composed of three types of protein: an FAD-containing flavoprotein (adrenodoxin reductase), a ferredoxin-type Fe₂S₄ protein (adrenodoxin), and a hemoprotein (cytochrome P-450) (1-3). There are at least two reaction specific cytochromes P-450, one catalyzing the side chain cleavage of cholesterol, and another catalyzing the 11β hydroxylation of deoxycorticosterone (4, 5). AR and ADX appear to be common to both P-450's. Reducing equivalents are transferred from NADPH through AR and ADX to cytochrome P-450, where they are utilized in the hydroxylation of specific steroid substrates. In the presence of NADPH and adrenodoxin reductase, the 1-electron reductions of both cytochrome P-450 and cytochrome c (a nonphysiologic electron acceptor for this system) show an absolute dependence on the presence of adrenodoxin (6-8). Furthermore, Baron et al. (9) have shown that inhibition by antibodies to ADX of cytochrome c and cytochrome P-450 reduction in sonicated mitochondria occur in parallel, suggesting similar mechanisms of reduction of both hemoproteins. Lambeth and Kamin (10) have described the mechanism of electron transport through AR and ADX using cytochrome c as an electron acceptor, and they have shown that the catalytically active species in cytochrome c reduction is a 1:1 complex between AR and ADX; much less is known of the roles of the components in the hydroxylations catalyzed by cytochrome P-450.

Earlier work (11, 12) suggested that very high ratios of ADX/P-450 were required for optimal rates of hydroxylation; ratios reported were of the order of 10:1 to 50:1. In 1975, however, Mitani et al. (13) demonstrated that an "absolute concentration" of ADX rather than a specific molar ratio of ADX/P-450 was required for maximal hydroxylation rates. Subsequently, a 1:1 complex between ADX and cytochrome P-450 was demonstrated by Katgiri et al. (14).

We have purified cytochrome P-45011β, have sought to ascertain whether there is a ratio of components among AR, ADX, and P-450 which is optimal in 11β-steroid hydroxylations, and have examined possible complex formation among the protein components. Since the apparent Kₐ for ADX increases sharply with increasing ionic strength, we utilized low ionic strength and relatively high enzyme concentrations in our efforts to detect catalytically effective complexes among AR, ADX, and P-450. Additional experiments utilizing different spectrophotometric titration support the conclusions derived from catalytic data.

At the meeting of the American Society of Biological Chemists in Atlanta, Georgia, June 4-8, 1978, N. Orme-Johnson and Light presented data (not included in their abstract (15)) showing that the addition of ADX to P-450 produced perturbations in both the visible absorption and EPR spectra of the P-450c heme moiety. Titration with ADX indicated a 1:1 complex between the two proteins. In the presence of P-450 and AR, however, optical perturbations were only seen when the ratio [ADX]/[AR] was greater than 1. At the same meetings, our group presented analogous experiments (also unreported in our abstract (16)) on P-450; we utilized catalytic activity to detect possible complex formation among AR, ADX, and P-450. Our results demonstrated that the 1:1 complex of AR and ADX is not sufficient to permit P-450-catalyzed hydroxylation, but that ADX in excess of AR is required. Thus, both the Madison and Durham Laboratories independently showed data, using different approaches and different adrenocortical cytochromes P-450, which indicated participation of more than 1 ADX molecule in the structural or functional relationship between AR, ADX, and cytochrome P-450. At that time, both laboratories suggested that a 1:2:1 complex of AR, ADX, and P-450 might account for their findings.

MATERIALS AND METHODS

Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, cholic acid, deoxycorticosterone, and Tween 20 were purchased from Sigma; hexyl agarose was a product of Miles Biochemicals; and NADPH was obtained from P-L Biochemicals. 2',5'-ADP Sepharose and DEAE-cellulose were purchased from Pharmacia and Whatman, respectively. Cholic acid was purified by conversion to the sodium salt followed by

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precipitation from hot 92% acetone.

Bovine adrenal glands were purchased from Martins Wholesale Meats, Inc., Goldsboro, N.C. Adrenodoxin reductase and adrenodoxin were purified using a combination of DEAE-chromatography and affinity chromatography on 2',5'-ADP Sepharose. P-45011p was purified by cholate extraction of sonicated mitochondrial pellets followed by ammonium sulfate fractionation and chromatography on hexyl agarose. The concentrations of AR and ADX were estimated using millimolar extinction coefficients of 10.9 mM⁻¹ cm⁻¹ at 460 nm (17) and 11 mM⁻¹ cm⁻¹ at 414 nm (18), respectively. The concentration of P-45011p was determined using a millimolar absorption coefficient of 91 mM⁻¹ cm⁻¹ for A450 minus A490 of the reduced CO complex (19). The specific content of the P-450 lip was between 8 and 11 nmol/mg.

Fig. 1. 11β-Hydroxylase activity as a function of ADX concentration at two [AR]/[P-450] ratios. Additions of ADX were made to a series of tubes containing a final concentration of P-45011p of 0.7 \( \mu \)M and a final concentration of AR of either 0.5 \( \mu \)M (closed symbols) or 2 \( \mu \)M (open symbols). The arrows indicate an [ADX]/[AR] ratio of 1. The assay buffer contained 5 mM KP, pH 7.0, 3 mM glucose 6-phosphate, 0.3% Tween 20, 0.5 mM deoxycorticosterone, and 1.7 units/ml of glucose-6-phosphate dehydrogenase. 11β-Hydroxylase activity was measured at 30°C in a 200-µl volume. Reactions were initiated by the addition of NADPH at a final concentration of 0.1 mM. Fifty-microliter aliquots were removed at 0, 2, and 4 min, quenched with dichloromethane, and assayed for corticosterone. \( \bullet \), [AR]/[P-450] = 0.7; \( \circ \), [AR]/[P-450] = 2.8.

RESULTS AND DISCUSSION

To examine the amount of ADX required for optimal hydroxylation in the reconstituted 11β-hydroxylase system, we conducted experiments in which increasing amounts of ADX were added to a mixture of AR and P-45011p at two different molar ratios of [AR]/P-45011p. The results are presented in Fig. 1. There is a lag in the rate of hydroxylation with increasing amounts of ADX until a molar ratio of ADX/AR of 1 (see arrows, Fig. 1) is attained, followed by a rapid increase in activity with increasing ADX until a plateau is reached. The extent of the lag phase is dependent only upon the concentration of AR and is independent of the P-45011p concentration; a 4-fold increase in the AR concentration as in Fig. 1 leads to a 4-fold increase in the length of the lag phase. Extrapolation of the second phase of the titration indicates that between one and two additional ADX's are required per P-45011p for maximal activity.

The dissociation constant for the AR-ADX 1:1 complex is of the order of 5 × 10⁻⁹ M under these conditions, whereas the \( K_a \) for the ADX-P-45011p 1:1 complex is reported to be approximately 10⁻⁷ M (14). Assuming a 1:1 ADX-P-45011p complex with a similar \( K_a \), we interpret our results to mean that upon addition of ADX to a mixture of AR and P-45011p, the ADX preferentially associates with AR until the concentration of ADX exceeds that of AR, after which increases in ADX concentration result in markedly increased hydroxylation rates. Apparently, the 1:1 complex of AR-ADX is not sufficient to support maximal P-45011p-catalyzed hydroxylation; an excess of ADX over AR is necessary.

We have conducted converse experiments, titrating AR into a mixture of P-45011p and excess ADX and monitoring 11β hydroxylation (data not shown). We observe a linear increase in hydroxylation with increasing AR concentration at low ratios of AR/P-45011p, followed by a relatively sharp break and subsequent plateau. The molar ratio of AR/P-45011p at the endpoint, however, is variable between 0.5 and 1. Studies are presently underway to ascertain whether the observed ratios reflect a macromolecular complex among all enzyme components.

P-45011p displays a spectral perturbation upon binding ADX, and this property has permitted the description of a 1:1 complex between the two proteins (14,15). We have observed that the addition of ADX to P-45011p also results in spectral perturbations. We have performed a spectrophotometric titration by the addition of ADX to an equimolar mixture of P-45011p and AR, to detect possible complex formation among AR, ADX and P-45011p. Fig. 2 shows the results of such a titration. It is apparent from the data that two independent spectrophotometrically detectable processes occur. The first process (observed at 375 nm minus 340 nm) exhibits a linear increase in \( \Delta \lambda \) with the amount of ADX added until a molar ratio of ADX/P-45011p (or ADX/AR) of 1 is attained, above which there is no further absorbance change. The second process (observed at 365 nm minus 410 nm) shows a lag; there is no absorbance change observed until a molar ratio of ADX/P-45011p (or ADX/AR) of 1 is reached, above which \( \Delta \lambda \) increases with increasing ADX until a molar ratio of 2, after which there are only small absorbance changes.

To identify the physical events which produce the two spectrophotometrically detectable processes, we have determined the spectrum associated with each process by subtracting the difference spectra at four points during the titration, as explained in the legend to Fig. 3. The difference spectrum for the first process was obtained by subtracting the spectrum at 0.33 eq of ADX added from that at 0.95 eq added. The resulting spectrum is shown as Curve A-B in Fig. 3. The spectrum markedly resembles the difference spectrum obtained by Chu and Kimura (5) upon mixing AR and ADX; wavelength maxima and extinction coefficients are virtually identical. Thus, the difference spectrum of the first phase of the ADX titration results from the association of ADX with AR.

The spectrum for the second phase of the ADX titration
was obtained by subtracting the difference spectrum at 1.11 eq of ADX added from that at 2.06 eq added. The resulting spectrum labeled as Curve C-D in Fig. 3 is similar to the difference spectrum obtained by mixing ADX with P-45011p in the absence of AR (data not shown). Thus, the second spectrophotometric process in Fig. 2 appears to arise from the association of ADX with P-45011p.

The spectrophotometric titration thus corroborates our interpretation of the activity titrations. Upon the addition of ADX to a solution of AR and P-45011p, the first ADX binds preferentially to AR, and ADX binding to P-45011p is only observed when [ADX] > [AR]. This latter condition, i.e. [ADX] > [AR], is also necessary for hydroxylation. This does not preclude the possibility that the 1:1 AR-ADX complex formed during the first phase of the titration physically associates with P-45011p, thus forming an AR-ADX-P-45011p ternary complex. However, at this time, we have no spectrophotometric evidence that such a complex exists.

At present, we feel there are several possible explanations for these results. The first possibility is that the active species in steroid hydroxylation is a 1:2:1 complex among AR, ADX, and P-45011p, respectively. There are, however, two points which cause us to hesitate in proposing this explanation. First, we see no perturbation in the spectrum of P-45011p in the presence of equimolar AR and ADX which might indicate a 1:1:1 ternary complex. As discussed earlier, however, this fact alone does not preclude the existence of such a species. The second point which argues against a 1:2:1 complex, however, is the variable ratio of AR/P-45011p at the endpoint of the activity titration with AR. If a quaternary complex among the three proteins were the catalytically active species, a constant molar ratio of AR/P-45011p of 1 should be obtained at the endpoint.

A second possibility consistent with our results is the formation of two binary complexes, one between AR and ADX and one between ADX and P-45011p. During catalysis, the ADX-P-45011p complex could serve as a substrate in accepting reducing equivalent(s) from AR-ADX. This possibility is supported by the correlation between the spectrophotometric and activity titrations with ADX. The two curves (Figs. 1 and 2) are not superimposable, however, since the spectrophotometric titration shows a sharp endpoint whereas the activity titration shows a more gradual saturation; this may suggest that a 1:1 complex between AJX and P-45011p is not a strict requirement for optimal hydroxylation.

Although the two binary complex model is an attractive one, we must also entertain a third possibility, i.e. that reduced ADX is acting as an electron “shuttle” between AR and P-450. The $K_d$ for the AR-ADX complex is $5 \times 10^{-7}$ M, whereas the $K_d$ for an ADX-P-450 complex (at least for P-45011p) is on the order of $10^{-5}$ M (14). We also know that the AR-AIDX complex is less tightly associated when ADX is reduced than when it is oxidized (8). In the presence of free oxidized ADX, reduced ADX could be displaced by oxidized ADX, thereby allowing reduced ADX to associate with and reduce P-450 or an AIDX-P-450 complex. This postulate is especially attractive in view of the report that the molar ratio of ADX to AR is 7.6 in bovine adrenal cortex mitochondria (22).

A specific role in electron transport has been previously proposed for iron-sulfur proteins in cytochrome P-450 hydroxylase systems. Schleyer et al. (12, 23) have demonstrated an absolute specificity for ADX in 11β hydroxylation. Reduced ADX mixed with reduced substrate-bound P-45011p plus oxygen supports the 11β hydroxylation of deoxycorticosterone; reduced electron mediators of similar or lower oxidation-reduction potential are not effective. Small differences in the ADX-P-45011p difference spectrum are observed and appear to depend on the content of cytochrome P-420, the fraction of high spin versus low spin P-45011p, and the age of the preparation. The general features of the spectra are the same, however, and peaks and troughs correlate to within ±5 nm between the spectra.
duction potentials do not substitute for ADX. It should be noted that Tyson et al. have proposed (24) an “effector” role for putidaredoxin in the camphor hydroxylase system from *Pseudomonas putida*. We suggest that the requirement for the “extra” ADX may reflect a specific requirement for provision of the “second electron” for P-450-mediated hydroxylation. This “extra” ADX is not needed for cytochrome c reduction (10), which requires only 1 electron.

In summary, we have demonstrated that at low ionic strength, the 1:1 complex of AR-ADX is not sufficient to support maximal rates of hydroxylation in the reconstituted 11β-hydroxylase system, but that ADX in excess of AR is required. We have also measured spectrophotometric perturbations in a mixture of AR, ADX, and P-450_{11β} which indicate complex formation between AR and ADX and between ADX and P-450_{11β}, but which also show that the ADX-P-450_{11β} complex is only detectable when [ADX] > [AR]. Experiments are currently in progress to ascertain the significance of these observations in the catalytic mechanism of steroid hydroxylation in bovine adrenal cortex mitochondria.

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REFERENCES