Kinetic Studies on Bovine Cytochrome $P450_{11\beta}$ Catalyzing Successive Reactions from Deoxycorticosterone to Aldosterone†

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ABSTRACT: The reactions for the synthesis of aldosterone from deoxycorticosterone were investigated kinetically in the membrane-reconstituted system with bovine cytochrome $P450_{11\beta}$ at 37 °C. Reaction rapid-quenching experiments for the metabolism of deoxycorticosterone by $P450_{11\beta}$ showed that aldosterone was produced via corticosterone, not via 18-hydroxydeoxycorticosterone. The kinetic analysis revealed that aldosterone was formed successively from fractions of intermediate metabolites which did not dissociate from $P450_{11\beta}$. The rate of each reaction step in the successive reactions was estimated from the combination of results of the rapid-quenching experiments and the metabolism of deoxycorticosterone in the presence of an excess amount of substrate, in which the dissociation of final product, aldosterone, from the enzyme was the slowest step in the synthesis from deoxycorticosterone. Under steady-state reaction conditions, the interaction of $P450_{11\beta}$ with $P450_{scc}$ stimulates the production of corticosterone from deoxycorticosterone by about 10-fold but inhibits further reactions from corticosterone. The rapid-quenching experiments showed, however, that the rate constant for the 11β-hydroxylation of deoxycorticosterone for corticosterone production in the presence of $P450_{scc}$ was almost the same as that without $P450_{scc}$. The interaction of $P450_{11\beta}$ with $P450_{scc}$ in the reaction system for deoxycorticosterone metabolism was found to slow the rate of the subsequent 18-hydroxylation of the produced corticosterone and to accelerate the dissociation of the corticosterone from $P450_{11\beta}$, which stimulated the corticosterone production and inhibited the further reaction for aldosterone synthesis in the steady-state reaction.

Aldosterone is an essential steroid hormone for mammals, which regulates sodium and potassium ion excretion in the kidneys. Aldosterone is produced in the adrenal cortex from cholesterol by the catalysis of cytochrome P450s and 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase (*1*, *2*). The terminal reaction for aldosterone synthesis is catalyzed by $P450_{11\beta}$[1] in bovine adrenal cortex, in which only zona glomerulosa out of three zones can produce aldosterone. No specific localization of $P450_{11\beta}$ was, however, found in the cortex (*3*−*5*). Recently, we found in the membrane-reconstituted system that the interaction between $P450_{scc}$ and $P450_{11\beta}$, both purified from bovine adrenals, has a strong stimulating effect on 11β-hydroxylase activity of $P450_{11\beta}$ but suppresses the production of aldosterone (*6*, *7*). The specific secretion of aldosterone from bovine adrenal zona glomerulosa can be attributed to the strong interaction of these P450s in zona fasciculata-reticularis and to the weak interaction in the zona glomerulosa (*1*).

The mechanism regulating $P450_{11\beta}$ reaction with $P450_{scc}$ has been studied in the steady state using the membrane-reconstituted system (*6*, *7*). $P450_{11\beta}$ interacting with $P450_{scc}$ has a 40 times larger K_d value for corticosterone than that without $P450_{scc}$, which might explain no production of aldosterone in the reaction system containing $P450_{11\beta}$ and $P450_{scc}$. A similar inhibitory effect on $P450_{11\beta}$ catalyzing aldosterone production can be observed in the presence of detergents without $P450_{scc}$ (*7*, *8*). It was deduced that bovine $P450_{11\beta}$ has 2 forms, one for corticosterone production (form C) and the other for aldosterone production (form A) (*6*). The former might be induced by the interaction with $P450_{scc}$ or by the addition of detergents.

The production of ^{3}H-aldosterone from ^{3}H-deoxycorticosterone was decreased only slightly by the presence of the nonradioactive intermediate molecular species expected in the synthetic pathway from deoxycorticosterone to aldosterone, 18-hydroxydeoxycorticosterone, corticosterone, or 18-hydroxycorticosterone, suggesting that aldosterone is produced via fractions of the intermediates which do not dissociate from the enzyme (*9*). There are two possible pathways (pathway 1 and 2 in Figure 1) for aldosterone synthesis from deoxycorticosterone, since it has been reported that aldosterone can be produced from deoxycorticosterone, corticosterone, 18-hydroxydeoxycorticosterone, or 18-hydroxycorticosterone (*10*, *11*). In pathway 1, deoxycorticosterone is converted sequentially into corticosterone, then to 18-hydroxycorticosterone, and finally to aldosterone. In

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[1] Abbreviations: $P450_{11\beta}$, cytochrome P450XIB1; $P450_{scc}$, cytochrome P450XIA1; $P450_{17\alpha,lyase}$, P450XVIIA1; $P450_{arom}$, cytochrome P450XIXA1; $P450_{C21}$, P450XXIA1; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DOC, 11-deoxycorticosterone; COR, corticosterone; 18OH-COR, 18-hydroxycorticosterone; 18OH-DOC, 18-hydroxydeoxycorticosterone; ALDO, aldosterone.

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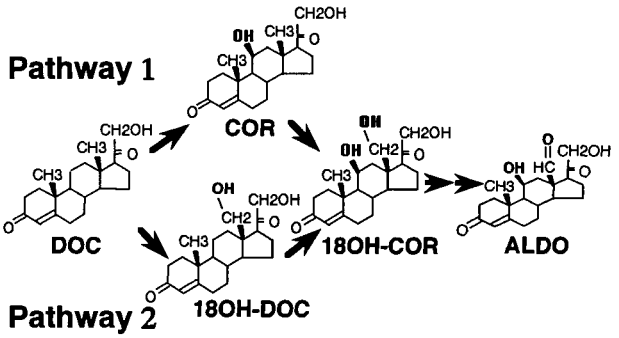

FIGURE 1: Two possible pathways for aldosterone synthesis from deoxycorticosterone catalyzed by $P450_{11\beta}$. DOC, COR, 18OH-COR, 18OH-DOC, and ALDO represent deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone, and aldosterone, respectively.

pathway 2, deoxycorticosterone is first 18-hydroxylated to 18-hydroxydeoxycorticosterone, which is then metabolized to 18-hydroxycorticosterone and finally to aldosterone. We cannot distinguish which is the real pathway for aldosterone synthesis from the results of the reactions in the steady state.

The steroidogenic cytochromes P450, $P450_{scc}$, $P450_{17\alpha,lyase}$, and $P450_{arom}$ catalyze multistep reactions in which certain fractions of the intermediate metabolites do not leave the enzymes, which are referred to as successive reactions (*12*−*20*). Under steady-state reaction conditions, it is difficult to detect the intermediate metabolites bound in the enzyme, because very few bound intermediates are formed during the reaction. The kinetics of the successive reactions from progesterone to androstenedione catalyzed by guinea pig $P450_{17\alpha,lyase}$ were analyzed in detail using a reaction rapid-quenching device (*17*). In the early stage of the reaction, the decrease of progesterone coincided with the increase of 17α-hydroxyprogesterone, and the subsequent decay of 17α-hydroxyprogesterone led to the formation of androstenedione, which clearly demonstrated that the intermediate metabolite is 17α-hydroxyprogesterone in the successive reactions from progesterone to androstenedione.

In this study, reaction rapid-quenching experiments were carried out for the $P450_{11\beta}$-catalyzing reactions from deoxycorticosterone to aldosterone to clarify the reaction pathway. Detailed kinetic analysis was performed for the reaction in the presence of $P450_{scc}$ to reveal how the interaction of $P450_{11\beta}$ with $P450_{scc}$ inhibits the successive reactions of $P450_{11\beta}$ from deoxycorticosterone to aldosterone.

MATERIALS AND METHODS

Materials. [1,2-^{3}H]-Progesterone and [1,2-^{3}H]-corticosterone (^{3}H-COR) were obtained from Amersham International (Amersham, UK). 11-[1,2-^{3}H]-Deoxycorticosterone (^{3}H-DOC) was prepared from [1,2-^{3}H]-progesterone in this laboratory by the reaction catalyzed by liposomal $P450_{C21}$ and purified by a normal phase HPLC system (*21*, *22*). 11-[4-^{14}C]-Deoxycorticosterone (^{14}C-DOC) and [1-^{14}C]-L-α-dipalmitoylphosphatidylcholine were from DuPont-NEN (Boston, MA). Phosphatidylcholine (egg yolk, Type II-E), cardiolipin (bovine heart), 11-deoxycorticosterone (DOC), corticosterone (COR), 18-hydroxycorticosterone (18OH-COR), 18-hydroxydeoxycorticosterone (18OH-DOC), aldosterone (ALDO), sodium cholate, and alumina Cγ were purchased from Sigma (St. Louis, MO). NADPH and dithiothreitol (DTT) were from Boehringer-Yamanouchi (Tokyo). Phosphatidylethanolamine (egg yolk) was from Lipid Product (Surrey, UK). 1-*O*-*n*-Octyl-β-D-glucopyranoside (octylglucoside) was from Nacalai Tesque (Kyoto). All other chemicals were of the highest grade commercially available. ω-Aminooctyl sepharose 4B was prepared in this laboratory according to the method of Cuatrecasas (*23*).

Preparation of Enzymes. $P450_{11\beta}$ was purified as described previously with some modification (*9*, *24*). ω-Aminooctyl sepharose 4B columns were used instead of aniline sepharose columns. At the second ω-aminooctyl sepharose 4B chromatography, $P450_{11\beta}$ was eluted with 50 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM EDTA, 0.1 mM DTT, 50 mM DOC, 0.7% (w/v) octylglucoside, 0.05% (w/v) of phospholipid mixture, 20% (v/v) glycerol, and 0.7% (w/v) sodium cholate. The phospholipid mixture consists of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin at a weight ratio of 2:2:1 which simulates the phospholipid composition of adrenal mitochondria (*9*). The elution of $P450_{11\beta}$ with phospholipids improved the yield of $P450_{11\beta}$ in the incorporation into liposomal membranes. $P450_{scc}$, adrenodoxine, and NADPH−adrenodoxine reductase were purified from bovine adrenocortical mitochondria according to methods previously reported (*24*−*26*). The concentration of cytochrome P450s determined from the dithionite-reduced CO difference spectra using the difference absorption coefficient $\Delta\epsilon$ (450−490 nm) = 91 mM^{-1} cm^{-1} (*27*). The concentrations of adrenodoxine and NADPH−adrenodoxine reductase were determined from their optical absorption spectra (*25*, *26*). All these enzymes show only one band in sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Preparation of Proteoliposomes. $P450_{11\beta}$ was incorporated into liposomal membranes according to a method previously described with some modifications to improve the yield and to shorten the preparation time (*7*). The phospholipid mixture in chloroform solution was evaporated under vacuum, and 10 μL of 20% (w/v) sodium cholate solution was added per milligram of the phospholipids. After the phospholipid solution became clear with the aid of sonication (Heat-system W-225, Farmingdale, NY), it was diluted to 10 mg of lipids/mL with 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM DTT, 0.03 mM 2,6-di-*tert*-butyl-*p*-crezol (BHT), 0.1 mM EDTA, 20% (v/v) glycerol, 10 μM DOC, 500 mM NaCl, and about 10 000 cpm of ^{14}C-L-α-dipalmitoylphosphatidylcholine (^{14}C-DPPC) for the estimation of the lipid recovery during the proteoliposome preparation. $P450_{11\beta}$ from the second ω-aminooctyl sepharose 4B column was mixed with the phospholipids at a ratio of 1 nmol of $P450_{11\beta}$/2000 nmol of lipids and dialyzed as described previously (*7*). The dialysis period was shortened to 23 h from 43 h. The precipitates formed during the dialysis were removed by centrifugation at 26500*g* for 45 min at 4 °C. The proteoliposomes in the supernatant were concentrated by centrifugation at 420000*g* at 4 °C for 2 h, and the recovered liposome solution was applied to a combination of TSK-PWH (7.5 mm × 7.5 cm, Tosoh) and phenyl-5PW guard columns (Tosoh) to remove the DOC. With these procedures, about 25% of the $P450_{11\beta}$ and 50% of the lipids were recovered and the ratio of P450 to the lipids became about 1:2000−3000 (mol/mol). The concentrations of $P450_{11\beta}$ and DOC in the preparation were about

5−10 μM and 0.1−0.2 μM, respectively. The prepared proteoliposomes were frozen in liquid N_2 until use.

Reaction Rapid-Quenching Experiments. A reaction rapid-quenching device (OJOH 3) was constructed in our laboratory. The detail of the instrument has been described elsewhere (*28*). One-hundred microliters of reaction solution contained 50 pmol of P450$_{11\beta}$ in the liposome membranes, 250 pmol of NADPH−adrenodoxine reductase, 2.5 nmol of adrenodoxine, and 500000 cpm ^{3}H-DOC (5 pmol) without nonradioactive DOC in 50 mM potassium phosphate buffer, pH 7.2 and 0.1 mM EDTA. The reaction solution was preincubated at 37 °C for 2 min, and the reaction was initiated with rapid addition of 100 nmol of NADPH from a micropipet. At 200 ms after the initiation, nonradioactive DOC was added to the solution as a chaser to prevent the rebinding of dissociated steroids to P450$_{11\beta}$ during the reaction. Changes in the timing of the addition of the chaser after the initiation in the range from 0.1 to 0.7 s did not affect the reaction kinetics. The reaction was terminated by the rapid addition of 50 μL of 1M HCl in 20% (v/v) glycerol. The steroid metabolites were extracted with 1 mL of chloroform containing 10 000 cpm ^{14}C-progesterone for the estimation of the recovery of steroids in the whole assay procedure. The extracted steroids were separated with an HPLC system consisting of a PU980 HPLC pump (JASCO Co., Tokyo), a UV-8000 UV detector (Tosoh, Tokyo), a AS-48 autosampler (Tosoh, Tokyo), and a Gilson 202 fraction collector with a reverse-phase column (RP-18, Kanto Chemicals, Tokyo) using a concentration gradient elution program from 67:33 (v/v) of water/acetonitrile to 100% acetonitrile. The radioactivities of the separated metabolites were measured with a liquid scintillation counter (Beckman LC-6500, Beckman Instruments Inc., Filerton, CA). In some experiments, ^{3}H-corticosterone (2 000 000 cpm, 20 pmol) was used as the substrate.

Assay of P450$_{11\beta}$ Activity in the Presence of an Excess Amount of Substrate. The steroid metabolizing activity of P450$_{11\beta}$ was measured at 37 °C under aerobic conditions with 100 μL of 50 mM potassium phosphate buffer (pH 7.2) containing 50 pmol of P450$_{11\beta}$ in the liposome membranes, 250 pmol of NADPH−adrenodoxine reductase, 2.5 nmol of adrenodoxine, 50 nmol of DOC with 500 000 cpm ^{3}H-DOC, and 0.1 mM EDTA. The reaction was started by the addition of 100 nmol of NADPH and terminated by violent stirring with 1 mL of chloroform. The steroid metabolites were separated with HPLC and the radioactivities were measured as described above.

Other Methods. Optical absorption spectra were measured with a Beckman DU-7. Centrifugation and ultracentrifugation were performed with a Sorval RC-5 (Dupont) and a TL-101 tabletop ultracentrifuge (Beckman).

RESULTS

Rapid-Quenching Experiments for DOC Metabolism. Rapid-quenching experiments were performed to identify the intermediate metabolites in the reactions from DOC to aldosterone catalyzed by P450$_{11\beta}$. Five picomoles of ^{3}H-DOC (500 000 cpm) was preincubated with 50 pmol of liposomal P450$_{11\beta}$ in the presence of sufficient amounts of adrenodoxine and adrenodoxine reductase, where most of the ^{3}H-DOC can be considered to bind to P450$_{11\beta}$ on the basis of the calculation using a K_d value of 0.28 μM which was determined at 37 °C in the present study using the substrate-induced difference spectra (*7*). The reaction was initiated by the rapid addition of NADPH to the reaction mixture and quenched at the appropriate times by the addition of quencher. The quenched reaction solutions were subjected to the reverse-phase HPLC to separate the metabolites (Figure 2). A peak for corticosterone (COR) was detected at the reaction time of 0.5 s (Figure 2b), followed by the sequential appearances of 18OH-corticosterone (18OH-COR) and aldosterone (ALDO) (Figure 2c,d). A small peak of 18OH-DOC was detected at 30 s (Figure 2d). The amounts of steroid metabolites separated by HPLC were estimated from the radioactivities and are plotted in Figure 3. A rapid decay of DOC was observed in two seconds accompanying a sharp increase in corticosterone. After 2 s, the amount of corticosterone decreased while that of 18OH-corticosterone increased. The increase of aldosterone occurred slowly and leveled off after 15 s. The amount of 18OH-DOC was the smallest of all the metabolites. This figure clearly shows that aldosterone is produced by pathway 1, from DOC sequentially to corticosterone, 18OH-corticosterone and finally to aldosterone. The lines in Figure 3 are theoretical curves drawn using the rate constants in Figure 6a obtained by fitting the observed data to the equations derived for the reaction scheme shown at the upper part of Figure 3. The theoretical equations (a1−a5) are listed in the Appendix. The good fittings show the plausibility of the reaction scheme.

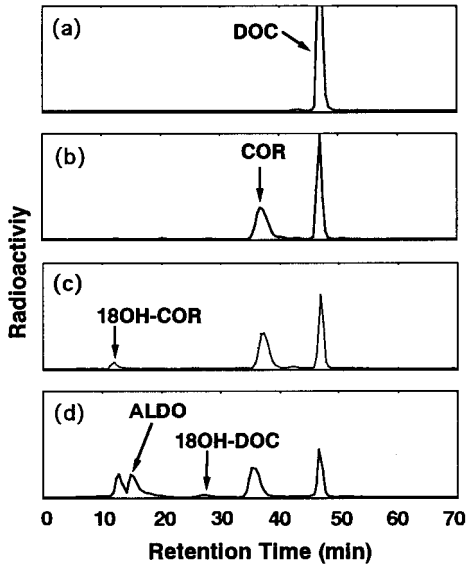

FIGURE 2: The separation with HPLC of the metabolites trapped in the rapid quenching experiments for deoxycorticosterone metabolism at 37 °C by liposomal P450$_{11\beta}$. a, b, c, and d show the separation of metabolites in the reaction solution quenched at 0, 0.5, 1, and 30 s, respectively, after the initiation. Details are in Materials and Methods.

Rapid-Quenching Experiments for Corticosterone Metabolism. The metabolism of corticosterone by P450$_{11\beta}$ was measured under the same condition as in Figure 3 except for the substrate. The decay curve of corticosterone in Figure 4 is apparently similar to that of corticosterone in Figure 3 except for the initial 2 s, and the concomitant increases of 18OH-COR and aldosterone with the decrease of corticosterone differ little between the two figures. These results

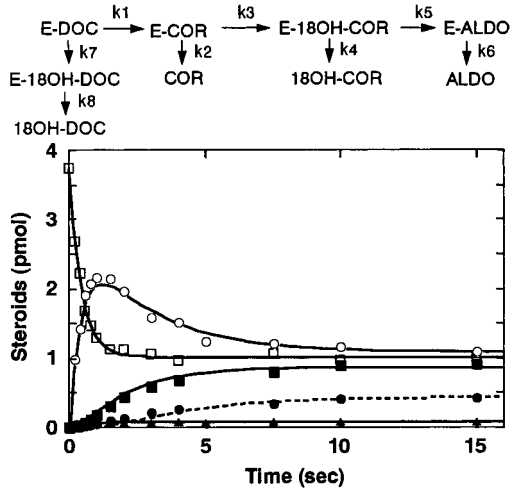

FIGURE 3: The time course of deoxycorticosterone metabolism in the rapid quenching experiments with liposomal $P450_{11\beta}$. The open squares, open circles, closed squares, closed circles, and closed triangles represent the amounts of deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, aldosterone, and 18-hydroxydeoxycorticosterone, respectively. The lines were drawn using the equations a1−a5 in the Appendix with the rate constants in Figure 6a. The reactions were carried out at 37 °C. Details are in Materials and Methods.

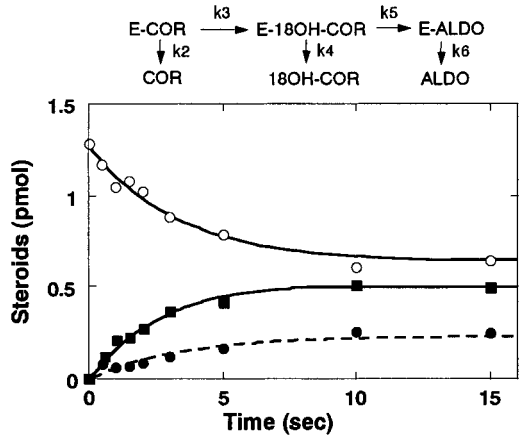

FIGURE 4: The time course of corticosterone metabolism in the rapid-quenching experiments with liposomal $P450_{11\beta}$. The open circles, closed squares, and closed circles represent amounts of corticosterone, 18-hydroxycorticosterone, and aldosterone. The lines were drawn using the equations b1−b3 in the Appendix with the rate constants in Figure 6b. The reaction conditions were the same as in Figure 3 except for the substrate.

suggest that almost the same reactions occur for corticosterone as for corticosterone produced from DOC. The curves in Figure 4 were drawn using the best-fitting rate constants in Figure 6b which were similar to the corresponding rate constants in Figure 6a. The theoretical equations (b1−b3) are listed in the Appendix.

Reactions in the Presence of an Excess Amount of the Substrate. In the steady state, $P450_{11\beta}$ converts DOC into corticosterone, 18OH-DOC, 18OH-corticosterone, and aldosterone (*6*, *7*, *9*). In the presence of $P450_{scc}$, the productions of 18OH-corticosterone and aldosterone from deoxycorticosterone were almost completely inhibited. Table 1 shows the activities of $P450_{11\beta}$ for DOC and corticosterone metabolisms in the presence of an excess amount of the substrate. The presence of $P450_{scc}$ stimulated the corticosterone production from DOC about 10-fold but completely inhibited further reactions. It is worth noting that the 18OH-DOC formation was also stimulated, about 5-fold. The activity of the corticosterone formation without $P450_{scc}$ was about four times less than that observed in a previous study (*7*) in which the reaction solution contained 20% (v/v) glycerol. Glycerol is not appropriate for rapid-quenching experiments, because rapid mixing of the solutions is essential for the experiments. Thus, we did not use glycerol in the present study. We confirmed the stimulation of activities by the presence of 20% (v/v) glycerol. Of interest is how glycerol activates the activities of $P450_{11\beta}$. It might perturb the dissociation of substrates or intermediate metabolites from the enzyme. We conducted experiments to elucidate the effect of the viscosity of the reaction solution on the $P450_{11\beta}$ successive reactions but obtained no clear results yet.

Table 1: The Activities of $P450_{11\beta}$ in the Metabolisms of Deoxycorticosterone and Corticosterone in the Presence of an Excess Amount of the Substrate[a]

		activity (mol min^{-1} (mol of $P450_{11\beta}$)$^{-1}$)			
substrate	additives	COR	18OH-COR	ALDO	18OH-DOC
DOC	no	4.0 ± 0.2	1.8 ± 0.07	1.0 ± 0.06	0.6 ± 0.02
COR	no	nd	2.2 ± 0.6	0.9 ± 0.3	nd
DOC	$P450_{scc}$[b]	44 ± 2	<0.01	<0.01	2.8 ± 0.4

[a] The reaction was performed at 37 °C. The values are the average of at least three experimental results. ND means not determined. DOC, COR, 18OH-COR, ALDO, and 18OH-DOC represent deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, aldosterone and 18-hydroxydeoxycorticosterone, respectively. The details are in Materials and Methods. [b] The reaction solution contained $P450_{scc}$ at concentration 5 times greater than that of liposomal $P450_{11\beta}$.

The Effect of $P450_{scc}$ in the DOC Metabolism. To investigate how the interaction between $P450_{11\beta}$ and $P450_{scc}$ affects $P450_{11\beta}$ activities, rapid-quenching experiments were performed under the same conditions as for Figure 3 but with the concentration of $P450_{scc}$ 5 times greater than that of $P450_{11\beta}$. Figure 5 shows the results of rapid quenching experiments of DOC metabolism in the presence of $P450_{scc}$. The decay of DOC was accompanied by an increase in the amount of corticosterone and a slight increase in 18OH-DOC. None of the other metabolites were detectable under these conditions. To our surprise the decay curve for DOC differed little from that without $P450_{scc}$ except for the amount of the decrease. The rate constant for 11β-hydroxylation for corticosterone production (k_1) in Figure 6c is about the same as that in Figure 6a. The curves in the figure were drawn using rate constants obtained by the curve fittings to the observed data using the equations (c1−c3) in the Appendix.

DISCUSSION

Kim et al. pointed out the possibility of the $P450_{11\beta}$-mediated DOC conversion to 18OH-corticosterone through corticosterone rather than through 18OH-DOC, based on the higher activity for corticosterone production from DOC than that for 18OH-DOC production in the steady state (*10*). Vinson et al. preferred, however, pathway 2 in Figure 1 for the production of aldosterone in $P450_{11\beta}$-catalyzing DOC metabolism. They found a steroid binding protein specific to 18OH-DOC in the zona fasciculata-reticularis in the rat

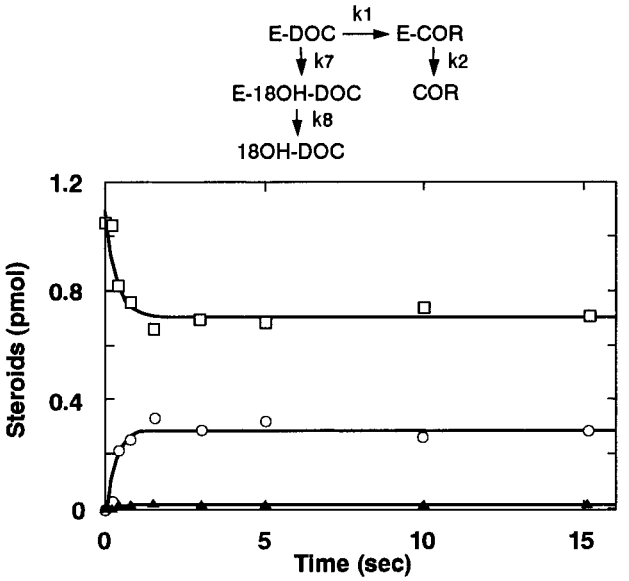

FIGURE 5: The time course of deoxycorticosterone metabolism in the rapid-quenching experiments with liposomal P450$_{11\beta}$ in the presence of P450$_{scc}$. The open squares, open circles, and closed triangles represent the amounts of deoxycorticosterone, corticosterone, and 18-hydroxydeoxycorticosterone, respectively. The lines were drawn using the equations c1−c3 in the Appendix with the rate constants in Figure 6c. The reaction conditions were the same as in Figure 3 except for the presence of P450$_{scc}$ at a concentration 5 times greater than that of liposomal P450$_{11\beta}$.

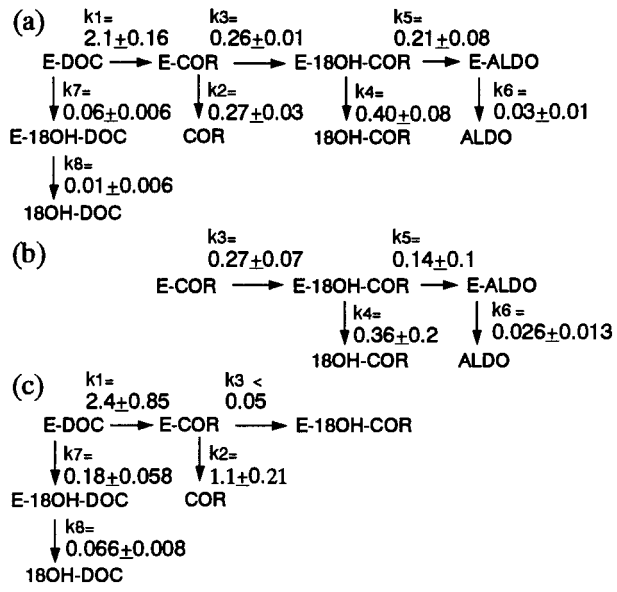

FIGURE 6: The rate constants (s^{-1}) in (a) deoxycorticosterone metabolism, and (b) corticosterone metabolism, catalyzed by liposomal P450$_{11\beta}$ without P450scc and (c) those of deoxycorticosterone metabolism by liposomal P450$_{11\beta}$ in the presence of P450scc. The rate constants in a, b, and c were obtained by fitting the observed data of rapid quenching experiments at 37 °C to the theoretical equations a1−a5, b1−b3, and c1−c3 in the Appendix, respectively. The dissociation rate constants of the products were calculated using the production rates of metabolites in the presence of excess amounts of substrates. The values are the average of at least three experimental results. The details are in the Discussion and Appendix.

adrenal and speculated that the binding of 18OH-DOC to that protein prevents aldosterone formation in that zone (*29*). As proved in a previous paper, aldosterone is produced successively from fractions of the intermediate metabolites which did not dissociate from P450$_{11\beta}$ (*9*). The observed metabolites in the steady state are those released from the enzyme, whose amounts are not necessarily proportional to the amounts of bound intermediates during the successive aldosterone formation. The low activity for 18OH-DOC production from DOC in the steady state could be indicative of a fast conversion of 18OH-DOC to 18OH-corticosterone. We used rapid-quenching methods to determine the synthetic pathway for the successive reactions for aldosterone formation from DOC. 18OH-Corticosterone could be detected after the appearance of corticosterone but almost no peak was detected at the position of 18OH-DOC at the early stage of the reaction (Figure 2b,c), which clearly shows that the 18OH-corticosterone was formed through corticosterone from DOC. The reaction scheme for the aldosterone formation was assumed to be that in Figure 7a, where aldosterone was produced from fractions of corticosterone and 18OH-corticosterone which did not dissociate from the enzyme and the released metabolites were not metabolized further. The rationality of the scheme is guaranteed by the good fittings of the theoretical curves to the observed data.

The rate equations for successive aldosterone formation from DOC in Figure 7a can be solved as equations a1−a5 in the Appendix, and the rate constants in Figure 6a were obtained by curve fittings to the observed data using the equations. The value for $k_1 + k_7$ was assumed to be equal to k_1 in the curve fittings for DOC, COR, 18OH-corticosterone, and aldosterone using equations a1, a2, a3, and a4, respectively, because the amount of 18OH-DOC was small, almost in the error range of the amounts of the other metabolites. The rate constant k_7 was estimated from the curve fitting to the data of 18OH-DOC using eq a5 and the obtained k_1 value by the above treatments. The rate constants for the reaction from corticosterone in Figure 6b and those from DOC with P450$_{scc}$ in Figure 6c were obtained by almost the same procedures as above using equations b1−b3 and c1−c3, respectively. The obtained rate constants for corticosterone metabolism differed little from the corresponding rate constants in DOC metabolism without P450$_{scc}$. It must be noted that the final amounts of each metabolite in the rapid quenching experiments can be expressed by the time-independent terms in the equations.

The rate of metabolite production in the steady state can be solved using the King−Altoman method as in equations d1−d4 in the Appendix for DOC metabolism without P450$_{scc}$, e1−e2 for corticosterone metabolism, and f1−f2 for the DOC metabolism with P450$_{scc}$ (*17*, *30*). The ratio of final amounts of corticosterone ($Ak_1k_2/(XY)$ in eq a2) to that of 18OH-corticosterone + aldosterone ($Ak_1k_3/(XY)$ from a3 + a4) in the rapid-quenching experiments for the DOC metabolism without P450$_{scc}$ is equal to k_2/k_3, which must be equal to the ratio of the rate of formation of corticosterone (eq d1) to the total rate of formation of 18OH-corticosterone + aldosterone (equations d2 + d3) in the presence of an excess amount of substrate DOC without P450$_{scc}$. The k_2/k_3 value of 1.04 obtained by the rapid-quenching experiments was similar to the observed ratio 1.4 for corticosterone formation to 18OH-corticosterone + aldosterone in the presence of an excess amount of substrate.

The values k_6 and k_8 cannot be estimated from the rapid-quenching experiments, because the observed amounts are the summations of the released and the bound metabolites.

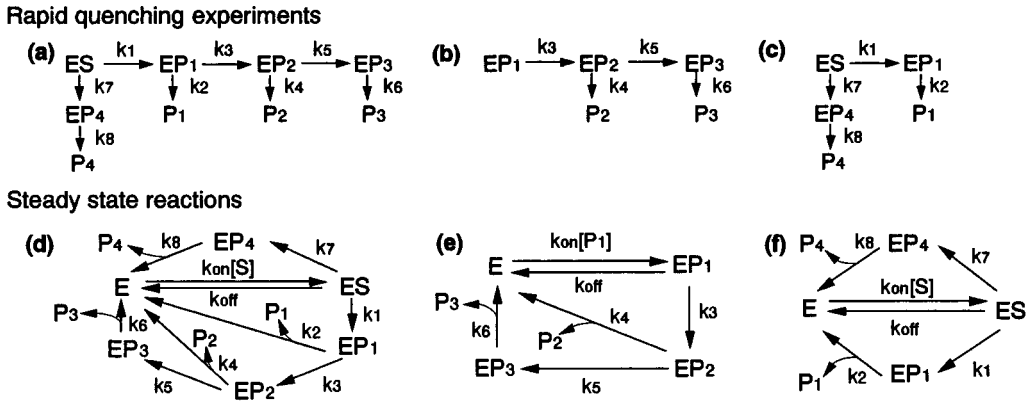

FIGURE 7: The reaction schemes for $P450_{11\beta}$ catalyzing reactions. Reaction schemes in a, b, and c are for the rapid-quenching experiments of deoxycorticosterone and corticosterone metabolisms by liposomal $P450_{11\beta}$ without $P450_{scc}$, and of deoxycorticosterone metabolism by liposomal $P450_{11\beta}$ in the presence of $P450_{scc}$, respectively. Those in d, e, and f are for the reactions of liposomal $P450_{11\beta}$ in the presence of an excess amount of substrate—deoxycorticosterone, corticosterone, and deoxycorticosterone, respectively—in which the reaction of f is in the presence of P450scc. S, P_1, P_2, P_3, and P_4 represent deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, aldosterone, and 18-hydroxydeoxycorticosterone, respectively. E, ES, and EP_i represent $P450_{11\beta}$, $P450_{11\beta}$-deoxycorticosterone, and $P450_{11\beta}$-other metabolite complexes, respectively. k_i represents the rate constant of each step. The equations in the Appendix are derived on the basis of these reaction schemes.

We previously reported a method for the estimating the rate of the product dissociation using the rate constants obtained from the rapid-quenching experiments and the data from the reactions in the presence of an excess amount of substrate (*17*). The rate of aldosterone formation in DOC metabolism without $P450_{scc}$ in the presence of an excess amount of substrate can be written as eq d3 which contains k_6k_8. We decided to simplify the reaction scheme which neglects the pathway for very little 18OH-DOC formation in the calculation of the value for k_6. k_8 was calculated with eq d4 using the k_6 value obtained in the above treatment. Almost the same treatments were carried out for the estimation of k_6 for corticosterone metabolism using eq e1 and of k_2 and k_8 for DOC metabolism with $P450_{scc}$ using equations f1 and f2. The obtained dissociation constants are shown in Figure 6. The values of k_6 both for the DOC metabolism without $P450_{scc}$ and for corticosterone metabolism are the smallest rate constants in the successive aldosterone formation, suggesting that the rate-limiting step for aldosterone formation in both cases is the release of the final product. In the successive reaction of guinea pig $P450_{17\alpha,lyase}$-mediated progesterone metabolism, the rate-limiting step was also the dissociation of the final androstenedione (*17*). The dissociation of the final product was not always the slowest step in cytochrome P450-mediated steroidogenesis. In bovine $P450_{17\alpha,lyase}$-catalyzing progesterone metabolism, the formation of 17α-hydroxyprogesterone is 5 times slower than its dissociation from the enzyme (*30*).

In a previous paper (*7*), we had shown in the steady-state metabolism of DOC by $P450_{11\beta}$ that the interaction of $P450_{11\beta}$ with $P450_{scc}$ increased the K_d value of the $P450_{11\beta}$-corticosterone complex, and we concluded that the large increase in the dissociation tendency of corticosterone from the enzyme prevented the further metabolism of corticosterone in the successive reaction to aldosterone. The ratio of the amounts of observed corticosterone to the total amount of further metabolites in the quenching experiments is determined by k_2/k_3 (see Appendix), and in the presence of $P450_{scc}$, the ratio must be larger than 20 (detectable limit), meaning that k_3 must be less than 0.05 s^{-1}. This clearly showed that the inhibition is due not only to an approximate 4-fold increase in the dissociation rate, k_2, of corticosterone from $P450_{11\beta}$ but also to the decrease in the rate constant of k_3.

It is clear that $P450_{11\beta}$ catalyzes mainly 11β-hydroxylation and 18-hydroxylation reactions. This means that steroids in the active site of $P450_{11\beta}$ can have two configurations with respect to the activated oxygen atom bound to the iron atom at the heme. Corticosterone which has just formed from DOC must have the configuration of the 11β position close to the iron, and in order to undergo further 18-hydroxylation, the configuration of the corticosterone must change to direct the 18 position to the iron. In the rapid-quenching reaction with corticosterone as the substrate (Figure 4), the corticosterone might bind to the enzyme with a favorable configuration for 18-hydroxylation. The k_3 values for DOC metabolism and that for corticosterone were about the same, which means the change in the configuration of the produced corticosterone from that for 11β-hydroxylation to that for 18-hydroxylation must be fast, compared with other rate constants of the reaction. In our previous paper, we suggested that the interaction with $P450_{scc}$ induces some structural change in $P450_{11\beta}$ into C form which accelerates 11β-hydroxylation of DOC but prevents aldosterone formation from DOC (*6*). $P450_{11\beta}$ without $P450_{scc}$ can produce aldosterone, which has a different structure, A form. In this context, it is reasonable to conclude that the A form is favored for having the 18 position of corticosterone close to the iron and the C form for the 11β position of DOC close to the iron.

In conclusion, aldosterone is produced by liposomal $P450_{11\beta}$ from DOC in the scheme, deoxycorticosterone → corticosterone → 18OH-corticosterone → aldosterone. Aldosterone is produced from fractions of the intermediate metabolites which do not dissociate from the $P450_{11\beta}$ in the successive reactions. The rate-limiting step of aldosterone formation from DOC in the presence of an excess amount of substrate is the dissociation of final aldosterone from $P450_{11\beta}$. The interaction of $P450_{scc}$ with $P450_{11\beta}$ accelerates the dissociation of corticosterone from the enzyme and decreases the reaction rate of the 18-hydroxylation of

corticosterone, which prevents successive reactions for aldosterone formation.

APPENDIX

The abbreviations used in the equations are the same as those in Figure 1.

(1) Kinetics in Rapid-Quenching Experiments.

(a) DOC Metabolism without $P450_{scc}$.

$$[\mathrm{DOC}] = [\mathrm{ES}] = A\exp(-Xt) \quad \text{(a1)}$$

$$[\mathrm{COR}] = [\mathrm{EP_1}] + [\mathrm{P_1}] = (Ak_1(X - k_2)/(X(-X + Y)))\exp(-Xt) - (Ak_1k_3/(Y(-X + Y)))\exp(-Yt) + Ak_1k_2/(XY) \quad \text{(a2)}$$

$$[\mathrm{18OH\text{-}COR}] = [\mathrm{EP_2}] + [\mathrm{P_2}] = (Ak_1k_3(X - k_4)/(X(-X + Y)(-X + Z)))(\exp(-Xt) + k_4/(X - k_4)) - (Ak_1k_3(Y - k_4)/(Y(-X + Y)(-Y + Z)))(\exp(-Yt) + k_4/(Y - k_4)) + (Ak_1k_3k_5/(Z(-X + Z)(-Y + Z)))(\exp(-Zt) + k_4/k_5) \quad \text{(a3)}$$

$$[\mathrm{ALDO}] = [\mathrm{EP_3}] + [\mathrm{P_3}] = -(Ak_1k_3k_5/(X(-X + Y)(-X + Z)))(\exp(-Xt) - 1) + (Ak_1k_3k_5/(Y(-X + Y)(-Y + Z)))(\exp(-Yt) - 1) - (Ak_1k_3k_5/(Z(-X + Z)(-Y + Z)))(\exp(-Zt) - 1) \quad \text{(a4)}$$

$$[\mathrm{18OH\text{-}DOC}] = [\mathrm{EP_4}] + [\mathrm{P_4}] = -(Ak_7/X)\exp(-Xt) + Ak_7/X \quad \text{(a5)}$$

where $X = k_1 + k_7$, $Y = k_2 + k_3$, $Z = k_4 + k_5$ and A represents the amount of metabolized deoxycorticosterone.

(b) COR Metabolism.

$$[\mathrm{COR}] = [\mathrm{EP_1}] = A\exp(-k_3t) \quad \text{(b1)}$$

$$[\mathrm{18OH\text{-}COR}] = [\mathrm{EP_2}] + [\mathrm{P_2}] = (A(k_3 - k_4)/(-k_3 + k_4 + k_5))\exp(-k_3t) - (Ak_3k_5/((-k_3 + k_4 + k_5)(k_4 + k_5)))\exp(-(k_4 + k_5)t) + Ak_4/(k_4 + k_5) \quad \text{(b2)}$$

$$[\mathrm{ALDO}] = [\mathrm{EP_3}] + [\mathrm{P_3}] = -(Ak_5/(-k_3 + k_4 + k_5))\exp(-k_3t) + Ak_3k_5/((-k_3 + k_4 + k_5)(k_4 + k_5))\exp(-(k_4 + k_5)t) + Ak_5/(k_4 + k_5) \quad \text{(b3)}$$

A represents the amount of metabolized corticosterone.

(c) DOC metabolism with $P450_{scc}$.

$$[\mathrm{DOC}] = [\mathrm{ES}] = A\exp(-Xt) \quad \text{(c1)}$$

$$[\mathrm{COR}] = [\mathrm{EP_1}] + [\mathrm{P_1}] = -(Ak_1/X)\exp(-Xt) + Ak_1/X \quad \text{(c2)}$$

$$[\mathrm{18OH\text{-}DOC}] = [\mathrm{EP_4}] + [\mathrm{P_4}] = -(Ak_7/X)\exp(-Xt) + Ak_7/X \quad \text{(c3)}$$

where $X = k_1 + k_7$ and A represents the amount of metabolized deoxycorticosterone.

(2) Kinetics in the Presence of an Excess Amount of Substrate. $[\mathrm{E_o}]$ represents the total concentration of $P450_{11\beta}$ which is the summation of $[\mathrm{E}] + [\mathrm{ES}] + \Sigma[\mathrm{EP_i}]$.

(d) DOC Metabolism without $P450_{scc}$.

$$V_{\mathrm{COR}} = \mathrm{d}[\mathrm{P_1}]/\mathrm{d}t = k_2[\mathrm{EP_1}] = k_{\mathrm{on}}k_1k_2(k_4 + k_5)k_6k_8[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(d1)}$$

$$V_{\mathrm{18OH-COR}} = \mathrm{d}[\mathrm{P_2}]/\mathrm{d}t = k_4[\mathrm{EP_2}] = k_{\mathrm{on}}k_1k_3k_4k_6k_8[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(d2)}$$

$$V_{\mathrm{ALDO}} = \mathrm{d}[\mathrm{P_3}]/\mathrm{d}t = k_6[\mathrm{EP_3}] = k_{\mathrm{on}}k_1k_3k_5k_6k_8[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(d3)}$$

$$V_{\mathrm{18OH-DOC}} = \mathrm{d}[\mathrm{P_4}]/\mathrm{d}t = k_8[\mathrm{EP_4}] = k_{\mathrm{on}}k_6k_7k_8(k_2 + k_3)(k_4 + k_5)[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(d4)}$$

$$\Sigma = (k_{\mathrm{off}} + k_1 + k_7)(k_2 + k_3)(k_4 + k_5)k_6k_8 + k_{\mathrm{on}}[\mathrm{S}](k_2 + k_3)(k_4 + k_5)k_6k_8 + k_{\mathrm{on}}[\mathrm{S}]k_1(k_4 + k_5)k_6k_8 + k_{\mathrm{on}}[\mathrm{S}]k_1k_3k_6k_8 + k_{\mathrm{on}}[\mathrm{S}]k_1k_3k_5k_8 + k_{\mathrm{on}}[\mathrm{S}](k_2 + k_3)(k_4 + k_5)k_6k_7$$

In the case where there is an excess amount of substrate in the reaction mixture, $((k_{\mathrm{off}} + k_1 + k_7)(k_2 + k_3)(k_4 + k_5)k_6k_8)/[\mathrm{S}]$ can be assumed to be 0. V_{COR}, $V_{\mathrm{18OH-COR}}$, V_{ALDO}, and $V_{\mathrm{18OH-DOC}}$ are the activities of $P450_{11\beta}$ for the production of corticosterone, 18-hydroxycorticosterone, aldosterone, and 18-hydroxydeoxycorticosterone, respectively, in the presence of an excess amount of deoxycorticosterone.

(e) COR Metabolism.

$$V_{\mathrm{18OH-COR}} = \mathrm{d}[\mathrm{P_2}]/\mathrm{d}t = k_4[\mathrm{EP_2}] = k_{\mathrm{on}}k_3k_4k_6[\mathrm{S}][\mathrm{E_o}]/\Sigma = k_3k_4k_6[\mathrm{E_o}]/((k_3 + k_4 + k_5)k_6 + k_3k_5) \quad \text{(e1)}$$

$$V_{\mathrm{ALDO}} = \mathrm{d}[\mathrm{P_3}]/\mathrm{d}t = k_6[\mathrm{EP_3}] = k_{\mathrm{on}}k_3k_5k_6[\mathrm{S}][\mathrm{E_o}]/\Sigma = k_3k_5k_6[\mathrm{E_o}]/((k_3 + k_4 + k_5)k_6 + k_3k_5) \quad \text{(e2)}$$

$$\Sigma = (k_{\mathrm{off}} + k_3)(k_4 + k_5)k_6 + k_{\mathrm{on}}[\mathrm{S}](k_4 + k_5)k_6 + k_{\mathrm{on}}[\mathrm{S}]k_3k_6 + k_{\mathrm{on}}[\mathrm{S}]k_3k_5$$

In the case where there is an excess amount of substrate in the reaction mixture, $((k_{\mathrm{off}} + k_3)(k_4 + k_5)k_6)/[\mathrm{S}]$ can be assumed to be 0. $V_{\mathrm{18OH-COR}}$ and V_{ALDO} are the activities of $P450_{11\beta}$ for the production of 18-hydroxycorticosterone and aldosterone, respectively, in the presence of an excess amount of corticosterone.

(f) DOC Metabolism with $P450_{scc}$.

$$V_{\mathrm{COR}} = \mathrm{d}[\mathrm{P_1}]/\mathrm{d}t = k_2[\mathrm{EP_1}] = k_{\mathrm{on}}k_1k_2k_8[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(f1)}$$

$$V_{\mathrm{18OH-DOC}} = \mathrm{d}[\mathrm{P_4}]/\mathrm{d}t = k_8[\mathrm{EP_4}] = k_{\mathrm{on}}k_2k_7k_8[\mathrm{S}][\mathrm{E_o}]/\Sigma \quad \text{(f2)}$$

$$\Sigma = (k_{\mathrm{off}} + k_1 + k_7)k_2k_8 + k_{\mathrm{on}}[\mathrm{S}]k_2k_8 + k_{\mathrm{on}}[\mathrm{S}]k_1k_8 + k_{\mathrm{on}}[\mathrm{S}]k_2k_7$$

In the case where there is an excess amount of substrate in the reaction mixture, $((k_{\mathrm{off}} + k_1 + k_7)k_2k_8)/[\mathrm{S}]$ can be assumed to be 0. V_{COR} and $V_{\mathrm{18OH-DOC}}$ are the activities of $P450_{11\beta}$ for the production of corticosterone and 18-hydroxydeoxycorticosterone, respectively, in the presence of an excess amount of deoxycorticosterone.

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