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KINETIC STUDIES OF RAT OVARIAN 20α-HYDROXYSTEROID DEHYDROGENASE

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Rat ovarian 20α-hydroxysteroid dehydrogenase was purified 230-fold with a 48% recovery through a 3-step process involving hydrophobic, gel filtration and green dye affinity chromatography. The purified enzyme was demonstrated to be a single polypeptide chain of M_r 36000. Initial velocity studies of all four substrates in the forward and reverse reactions indicated a sequential mechanism for the enzyme. Product inhibition and dead-end inhibition studies with substrate analogs were consistent with an ordered bi-bi mechanism in which NADP is the first substrate bound to the enzyme and NADPH, the second product released. Several NADP analogs were demonstrated to function as coenzymes in the reaction catalyzed. The purified enzyme was denatured at moderate temperatures and the binding of NADP protected the enzyme against thermal denaturation.

Introduction

The enzyme 20α-hydroxysteroid dehydrogenase (EC 1.1.1.149), in the presence of reduced pyridine nucleotide coenzyme converts progesterone, a biologically active hormone, to 20α-hydroxypregn-4-en-3-one, a steroid with little biological activity [1,2]. The enzyme has been demonstrated in various animal tissues [1–13], of which only those from reproductive and immunodefence systems have been widely studied and the importance of the enzyme in these systems has been proposed [13–16]. In pregnant rats, the induction of ovarian 20α-hydroxysteroid dehydrogenase and the increase in peripheral 20α-hydroxypregn-4-en-3-one immediately before parturition were coincident with a decrease in the plasma progesterone level [16]. During labor in humans, the placental enzyme was demonstrated to increase, accompanied by a decrease in the ratio of tissue progesterone: 20α-hydroxypregn-4-en-3-one [17]. Rat ovarian and human placental 20α-hydroxysteroid dehydrogenases were thus proposed to maintain normal parturition since these enzymes decrease the level of either peripheral or tissue progesterone [16,18].

The very important function proposed for this dehydrogenase in parturition has recently prompted interest in more extensive studies of the enzyme. In recent years [11,19], 20α-hydroxysteroid dehydrogenase was chromatographically purified from human placenta and rat ovaries. Many studies [20–24] have reported a dual oxidoreductase activity for the human placental enzyme involving both 17β- and 20α-hydroxysteroids. Estimates of kinetic parameters have been reported for the rat ovarian enzyme but only for a 15-fold purified enzyme preparation [25]. Although numerous physiological studies of 20α-hydroxysteroid dehydrogenase have been published, relatively little attention has been paid to specific properties of the enzyme such as kinetic mechanism, thermal stability, molecular weight, subunit

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structure and coenzyme specificity. The present study evaluates these and related properties of an extensively purified preparation of this enzyme.

Experimental procedures

Materials

Progesterone, 20α-hydroxypregn-4-en-3-one, 17β-estradiol and protein molecular weight standards were purchased from Sigma. All dinucleotides were also obtained from Sigma except thionicotinamide adenine dinucleotide phosphate, 3-aminopyridine adenine dinucleotide phosphate, and 3-aminopyridine, $1,N^6$-ethenoadenine dinucleotide phosphate which were prepared by published procedures [26–28]. Other chemicals used were of reagent grade.

Female DUB:(SD) rats (Sprague-Dawley-derived) weighing between 201 and 225 g were obtained from Dominion Lab, Inc., VA.

Methods

Isolation of rat ovarian 20α-hydroxysteroid dehydrogenase. Rats were killed by CO_2 inhalation at random stages of estrous cycle. Ovaries were removed, trimmed of fat, minced, and hand-homogenized in a solution containing, 1 mM EDTA, 12 mM monothioglycerol, and 0.01 M potassium phosphate, pH 8.0. The homogenate was filtered through cheesecloth and centrifuged at $12000 \times g$ for 15 min in a Sorvall RC2-B centrifuge. The supernatant was further centrifuged in a Beckman L2-65B ultracentrifuge at $104000 \times g$ for 75 min and was then filtered through a 0.45 μm filter to remove insoluble materials including fat. The crude cytosolic fraction obtained at this step contained 20α-hydroxysteroid dehydrogenase activity, and was subjected to chromatographic purification. All isolation and purification steps were carried out at 4°C.

Purification of 20α-hydroxysteroid dehydrogenase. Crude cytosolic fraction (30 mg protein) was applied to a phenyl-Sepharose CL-4B hydrophobic column (1.5 × 28 cm) equilibrated with a 0.01 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 12 mM monothioglycerol, and 1 M KCl. The dehydrogenase was eluted from the column by the equilibrating mixture minus KCl. Fractions were pooled, concentrated by ultrafiltration and applied to a Matrex Gel Green dye affinity column (1.0 × 8.5 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, and 12 mM monothioglycerol. Enzyme was eluted by a salt gradient, 0–2 M KCl in the equilibrating buffer. Fractions concentrated by ultrafiltration were applied to a Sephadex G-100 gel filtration column (2.5 × 65 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0, containing 1 mM EDTA, 12 mM monothioglycerol and 1 M KCl. Enzyme eluted at approximately twice the void volume by the same equilibrating buffer. Fractions containing enzyme were pooled and concentrated by binding and elution from a small phenyl-Sepharose column (1.0 × 7.0 cm) equilibrated with a solution containing 1 mM EDTA, 12 mM monothioglycerol, 1 M KCl, and 0.01 M potassium phosphate, pH 8.0. The column was washed with the same buffer minus monothioglycerol. The enzyme was then eluted with a solution containing 0.2 M KCl, 50% propylene glycol, and 0.01 M potassium phosphate, pH 8.0, in one-fifth the volume applied.

From these successive chromatographic steps, 20α-hydroxysteroid dehydrogenase was purified approx. 230-fold with a 48% yield and a specific activity of 3.5 units/mg as shown in Table I. This preparation was used for all studies of enzyme properties.

Assays of 20α-hydroxysteroid dehydrogenase activity. The assay mixtures contained 40 μM 20α-hydroxypregn-4-en-3-one, 50 μM NADP, 3.8% ethanol to keep the steroid solubilized, and 0.01 M Hepes buffer, pH 7.8, and was brought to 37°C prior to the addition of the enzyme. Initial velocities were followed spectrophotometrically at 340 nm for 2–3 min. Specific activity was determined by defining 1 unit of enzyme as the amount capable of reducing 1 μmol NADP per min.

A second fluorometric assay was used to monitor enzyme activity during column purification and for most kinetic studies. The fluorometric assay was 20-times more sensitive than the spectrophotometric assay. The assay mixture was the same as that used spectrophotometrically except that it was performed at 22°C. Fluorescence emission was monitored at 460 nm with the excitation at 340 nm. Enzyme activity was expressed as relative fluorescence increase per min.

TABLE I

PURIFICATION OF RAT OVARIAN 20α-HYDROXYSTEROID DEHYDROGENASE

Fraction	Total protein (mg)	Total act. (munits)	Specific act. (munits/mg)	Yield (%)	Purification (-fold)
1. Cytosol	29.5	450	15.3	100	1
2. Phenyl-Sepharose	3.8	423	106.4	94	7
3. Matrex Gel Green A	0.32	348	1070	77	70
4. Sephadex G-100	0.062	216	3480	48	228

Spectrophotometric measurements were performed on a Beckman Acta MVI spectrophotometer. Fluorescence was measured on a Perkin-Elmer 650-40 spectrofluorometer. Protein concentrations were determined by the microprotein assay of the Coomassie blue method [29] using bovine serum albumin as a standard. Ultrafiltration was carried out using Amicon YM-10 membranes.

Molecular weight determination. Molecular weight of the enzyme was determined under the nondenaturing conditions of a gel filtration Sephadex G-100 column and also under the denaturing conditions of SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed on a 0.1% SDS/10% polyacrylamide gel according to Weber and Osborn [30].

Results

Kinetic mechanism of 20α-hydroxysteroid dehydrogenase

The effects of pH and ionic strength on the kinetic parameters of the purified dehydrogenase were initially studied in order to establish optimal conditions for the subsequent studies of the kinetic mechanism of the enzyme. Initial velocities of NADP reduction were measured as a function of pH between pH 6.0 and 8.5 using three overlapping buffers, 0.01 M potassium phosphate, 0.01 M Hepes and 0.01 M Tris-HCl. At each of the nine pH values studied, initial velocities were measured by varying the concentration of one substrate at a saturating concentration of the second substrate. K_m and V_{max} values for each substrate at each pH were determined from double reciprocal plots. Under the conditions of either varying NADP concentration at saturating 20α-hydroxypregn-4-en-3-one or varying 20α-hydroxypregn-4-en-3-one at saturating NADP, the value of V_{max}/K_m exhibited an optimum pH of 7.8. No specific buffer effects were observed in these studies. When the ionic strength of assay medium at pH 7.8 was increased by addition of KCl from 1 to 20 mM neither the V_{max} nor the K_m values for either substrate were significantly affected. Using these optimal assay conditions, kinetic constants for both substrates in the forward and reverse directions were determined through initial velocity studies varying one substrate at several fixed concentrations of the second substrate. The initial velocities obtained by varying NADPH at five fixed concentrations of progesterone are shown in Fig. 1. The replotting (Fig. 1) of slopes and intercepts [31] was used to determine the values of K_m and V_{max}. In the same

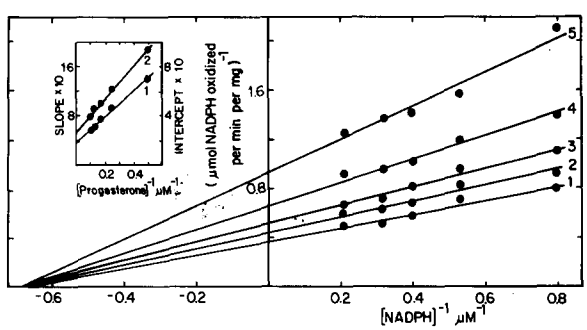


Fig. 1. Effect of NADPH and progesterone concentrations on initial velocities. The assay mixtures contained concentrations of NADPH varying from 1.3 to 4.8 μM at 5 fixed concentrations of progesterone in 0.01 M Hepes buffer, pH 6.8. Reactions were initiated by the addition of 500 ng 20α-hydroxysteroid dehydrogenase and initial velocities were measured spectrophotometrically. The concentrations of progesterone used were as follows: Line 1, 10 μM; line 2, 8 μM; line 3, 6 μM; line 4, 4 μM; line 5, 2 μM. Inset is a replot of slopes (line 1) and intercepts (line 2).

TABLE II

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES FOR SUBSTRATES OF RAT OVARIAN 20α-HYDROXYSTEROID DEHYDROGENASE

The assay mixtures for NADPH oxidation were as stated under Fig. 1. The assay mixtures for NADP reduction contained concentrations of NADP varied from 0.4–4 μM at several fixed concentrations of 20α-hydroxypregn-4-en-3-one from 0.6–3.2 μM. The buffer in the assay system was 0.01 M Hepes, pH 7.8. Reactions were initiated by the addition of 500 ng 20α-hydroxysteroid dehydrogenase for spectrophotometric measurement or 25 ng 20α-hydroxysteroid dehydrogenase for fluorometric measurement.

Substrate	Assay method	K_m (μM)	V_{max} (μmol/min per mg)
Progesterone	Spectrophotometric [a]	5.38	3.85
NADPH	Spectrophotometric [a]	1.38	3.85
20α-Hydroxyprogesterone	Spectrophotometric [b]	1.02	1.99
	Fluorometric [b]	1.62	
NADP	Spectrophotometric [b]	0.88	1.99
	Fluorometric [b]	0.70	

[a] Assays were done at pH 6.8.
[b] Assays were done at pH 7.8.

manner, the kinetic parameters for all four substrates for the forward and reverse reactions were determined and are listed in Table II. The results obtained by varying each of the four substrates gave converging line relationships similar to that depicted in Fig. 1 indicating the presence of a sequential mechanism.

In order to distinguish between an ordered or random sequential process, the following set of product inhibition experiments were performed. Using NADP as the variable substrate, product inhibition by four concentrations of progesterone was evaluated at an unsaturating concentration of the second substrate, 20α-hydroxypregn-4-en-3-one, yielding noncompetitive inhibition and at a saturating concentration of 20α-hydroxypregn-4-en-3-one, yielding uncompetitive inhibition. When inhibition by progesterone was studied with 20α-hydroxypregn-4-en-3-one as the varied substrate, noncompetitive inhibition was observed at both unsaturating and saturating concentrations of NADP. By replotting intercepts, a K_i value of 2.27 μM was determined for progesterone inhibition.

Using NADP as the variable substrate, inhibition by three concentrations of NADPH at an unsaturating concentration of 20α-hydroxypregn-4-en-3-one was competitive with respect to NADP. Competitive inhibition was also observed at a saturating concentration of 20α-hydroxypregn-4-en-3-one. When inhibition by NADPH was studied with 20α-hydroxypregn-4-en-3-one as the varied substrate at unsaturated NADP, noncompetitive inhibition by NADPH was observed. No inhibition by NADPH was obtained under these conditions when a saturating concentration of NADP was employed. From replotting slopes, a K_i value of 0.24 μm was determined for NADPH as a product inhibitor.

In addition to the product inhibition studies, dead-end inhibition by 2′-phosphoadenosine diphosphoribose, a structural analog of NADP and 17β-estradiol, a structural analog of 20α-hydroxypregn-4-en-3-one was evaluated. Inhibition by 2′-phosphoadenosine diphosphoribose was competitive when NADP was used as the variable substrate and noncompetitive when 20α-hydroxypregn-4-en-3-one was used as the variable substrate. Inhibition by 17β-estradiol was uncompetitive when studied with NADP as the variable substrate and competitive when 20α-hydroxypregn-4-en-3-one was the variable substrate. The K_i values determined for 2′-phosphoadenosine diphosphoribose and 17β-estradiol were 0.87 and 3.7 μM, respectively. The results of the studies of product and deadend inhibition indicate an ordered bi-bi mechanism with NADP being the first substrate bound to the enzyme and NADPH the second product released.

Coenzyme specificity

The structural requirements for the functioning of coenzymes were investigated. Rates of reduction of NADP analogs were followed spectrophotometrically or fluorometrically under optimal assay conditions containing 50 μM dinucleotides and 25–500 ng of purified enzyme. Those analogs observed to function as coenzymes were further studied at five concentrations between 1 and 30 μM and the K_m and V_{max} values were determined as described above for the natural substrates. Table III shows the K_m and V_{max} values for these analogs relative to those obtained for NADP. Ana-

TABLE III

MICHAELIS CONSTANTS AND RELATIVE MAXIMUM VELOCITIES FOR NADP ANALOGS

The assay mixtures contained 40 μM 20α-hydroxypregn-4-en-3-one in 0.01 M Hepes, pH 7.8. The concentration of analog was varied between 1 and 30 μM with a minimum of 5 concentrations used. 25 or 500 ng of 20α-hydroxysteroid dehydrogenase were added to initiate reactions depending on the analog used and the sensitivity of assay techniques.

Analogs	K_m (μM)	Relative maximum velocities (%)
Nicotinamide adenine dinucleotide phosphate	0.88	100
Thionicotinamide adenine dinucleotide phosphate	1.86	18
3-Acetylpyridine adenine dinucleotide phosphate	2.08	68
Nicotinamide hypoxanthine dinucleotide phosphate	58.8	23
Nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate	142.9	64

logs investigated which did not function as coenzymes with 500 ng of purified enzyme were NAD, 3-aminopyridine adenine dinucleotide phosphate, and 3-aminopyridine 1,N^6-ethenoadenine dinucleotide phosphate. As a structural analog of NADP, 3-aminopyridine adenine dinucleotide phosphate was studied as an inhibitor of 20α-hydroxysteroid dehydrogenase. Inhibition by this analog was observed to be competitive with respect to NADP, and from the replotting method a K_i value of 3.3 μM was determined.

Enzyme stability and molecular weight determination

The purified enzyme at a concentration of 5 μg/ml in 0.01 M potassium phosphate buffer, pH 8.0, containing 50% propylene glycol, and 0.2 M KCl, was found to be stable at −15°C for at least 2 months with no apparent loss in activity. However, the purified enzyme preparation was denatured at moderate temperatures. When the remaining activity of the enzyme was assayed at intervals during incubation at different temperatures, the rate of thermal denaturation followed first order kinetics as shown in Fig. 2. The enzyme was shown to be stable at 25°C for at least 20 min but had a very short half-life of 7.6 min at 38°C. When NADP was included in the incubation mixture with the enzyme and samples withdrawn at intervals for assay as before, it was observed that NADP protected the enzyme against thermal denaturation (Fig. 3). In contrast, the enzyme was not protected against thermal denaturation by the second substrate, 20α-hydroxypregn-4-en-3-one.

The highly purified enzyme exhibited an apparent M_r of 35 000 when analyzed under nondenaturing conditions of gel filtration. SDS-gel electrophoresis of the denatured enzyme resulted in a protein band of M_r 38 000 indicating a single polypeptide chain with no subunit structure under the experimental conditions employed. Poly-

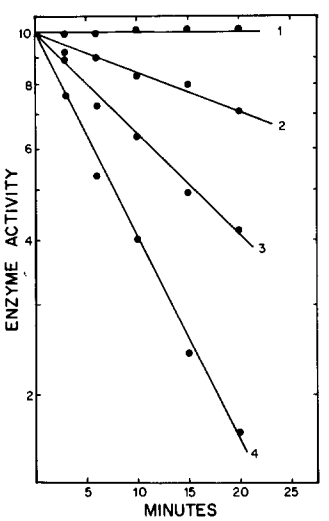


Fig. 2. Thermal denaturation of 20α-hydroxysteroid dehydrogenase at pH 8.0. The incubation mixtures of 0.2 ml contained 15% propylene glycol, 60 mM KCl, 300 ng 20α-hydroxysteroid dehydrogenase, and 7 mM potassium phosphate, pH 8.0. Enzyme samples were withdrawn at timed intervals for fluorometric assay. Line 1, 25°C; line 2, 30°C; line 3, 35°C; line 4, 38°C.

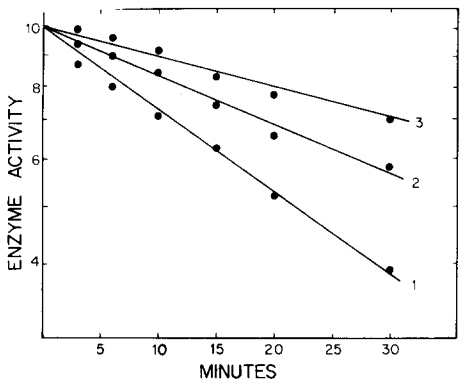


Fig. 3. NADP protection of 20α-hydroxysteroid dehydrogenase denaturation at pH 8.0, 35°C. The incubation mixtures were the same as described under Fig. 2. Line 1, no NADP; line 2, 5 μM NADP; line 3, 10 μM NADP.

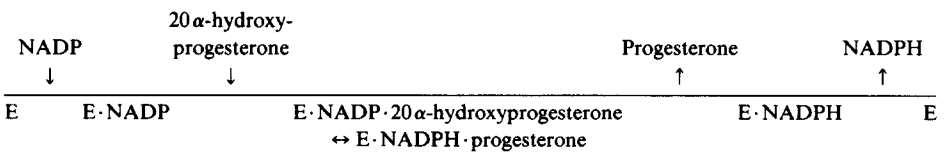
acrylamide gel electrophoresis of the purified enzyme at pH 8.3 showed one protein band corresponding to 20α-hydroxysteroid dehydrogenase as determined by activity staining techniques.

Discussion

Considering the reported importance of 20α-hydroxysteroid dehydrogenase to parturition in the rat [16], it was of interest that many properties of the enzyme including the kinetic mechanism were never evaluated with a highly purified preparation of the enzyme. In the present study, rat ovarian 20α-hydroxysteroid dehydrogenase was isolated from nonpregnant rats and purified to apparent electrophoretic homogeneity through a 3-step procedure involving hydrophobic, gel filtration, and affinity chromatography. The molecular weight of the purified enzyme was determined by both gel filtration and gel electrophoresis methods. The enzyme exhibited a M_r of 36000 under either denaturing or nondenaturing conditions indicating the absence of subunit structure. The 20α-hydroxysteroid dehydrogenase purified from porcine testes was also reported to be a single polypeptide chain of M_r 35000 [6] while the corresponding human placental enzyme was a dimer of M_r 68000 [11,22,33]. The molecular weight of the rat ovarian 20α-hydroxysteroid dehydrogenase was previously estimated [34] to be 75000; however, these studies were performed on a crude preparation of enzyme without using activity staining techniques. The M_r of 36000 determined in the present study for the rat ovarian enzyme is consistent with the subunit molecular weight of most dehydrogenases; however, the rat ovarian enzyme differs in lacking the quaternary structures observed in many other dehydrogenases [35–37].

The reaction catalyzed by rat ovarian 20α-hydroxysteroid dehydrogenase is bireactant in both directions and could be governed by a number of different kinetic mechanisms. Initial velocities of NADP reduction carried out by varying one substrate at several fixed concentrations of the second substrate (Fig. 1) led to converging line relationships in double reciprocal plots with each substrate varied, indicating the reaction followed a sequential mechanism. A similar set of converging line relationships was obtained by varying progesterone or NADPH and monitoring NADPH oxidation. The K_m values for the steroid substrates and the pyridine nucleotide coenzymes (Table II) determined through replotting techniques were considerably lower than ranges reported for these ligands using less pure enzyme preparations [25] and a K_m value for NADPH was not previously reported. In addition, the K_m values for NADP and 20α-hydroxypregn-4-en-3-one were obtained through both spectrophotometric and fluorimetric techniques.

The question of whether the sequential mechanism for the 20α-hydroxysteroid dehydrogenase-catalyzed reaction was of an ordered or random type was investigated through product and dead-end inhibition studies. The uncompetitive inhibition by progesterone when NADP was varied at a saturating concentration of 20α-hydroxypregn-4-en-3-one is characteristic of the ordered bi-bi mechanism. The remaining product inhibition profile is likewise consistent with this mechanism. In the dead-end inhibition studies, the observed uncompetitive inhibition by 17β-estradiol when studied as a function of NADP concentration is also consistent with the ordered bi-bi mechanism since noncompetitive inhibition would have been expected in a random process. Therefore, both types of inhibitor profiles indicated an ordered bi-bi mechanism (Scheme I) with NADP the first substrate binding and NADPH the second product dissociating from the enzyme. Knowledge of the order of addition of substrates and release of



Scheme I. The pattern of ordered bi-bi mechanism of rat ovarian 20α-hydroxysteroid dehydrogenase.

products is of value for future experiments designed to map properties of the substrate binding sites and to prepare selective inhibitors for modifying enzyme activity.

As reported in earlier studies of partially purified enzyme [25], NAD did not serve as a coenzyme for rat ovarian 20α-hydroxysteroid dehydrogenase; however, in the present coenzyme specificity studies, a number of NADP analogs were observed to function as coenzymes (Table III). Since the absorption maxima of the reduced forms of the thionicotinamide and acetylpyridine analogs differ considerably from that of NADPH, utilization of these dinucleotides can be of importance in those cases where 340 nm - absorbing contaminants may interfere with the NADP-dependent assay of the enzyme. The functioning of these analogs also provide the opportunity for determining analog reduction rate ratios for comparative enzyme studies [38,39]. Of the analogs that did not serve as coenzymes for the rat ovarian enzyme, one analog, 3-aminopyridine adenine dinucleotide phosphate, was shown to selectively inhibit the enzyme through coenzyme-competitive inhibition.

The purified rat ovarian enzyme was observed to be very stable when stored in 10 mM potassium phosphate buffer, pH 8.0, containing 0.2 M KCl, and 50% propylene glycol at −15°C. Thermal denaturation was observed at moderate temperatures (Fig. 2) and protection of the enzyme against thermal denaturation was afforded by the binding of NADP (Fig. 3). The protection observed with NADP but not with 20α-hydroxypregn-4-en-3-one is consistent with the initial binary complex of enzyme and NADP occurring in the ordered bi-bi mechanism.

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