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SEPIAPTERIN REDUCTASE EXHIBITS A NADPH-DEPENDENT DICARBONYL REDUCTASE ACTIVITY

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SUMMARY: We have found a new ability of sepiapterin reductase, which has been known to show a strict substrate specificity for the 6-lactyl sidechain of sepiapterin to produce 6-dihydroxypropyl sidechain of dihydrobiopterin in the biosynthesis of tetrahydrobiopterin, to reduce many dicarbonyl compounds with NADPH as effectively utilized substrates. By analysis of diacetyl reduction by purified sepiapterin reductase, it was observed that both of the carbonyl groups of the compound are finally sequentially reduced by the enzyme with NADPH to hydroxyl groups. And we expect that this enzyme may reduce "Compound X", which is an intermediate of tetrahydrobiopterin synthesis and would be a dicarbonyl derivative of pteridine (Tanaka et. al., 1980), to dihydrobiopterin via sepiapterin.

Tetrahydrobiopterin (6(R)L-erythro-1',2'-dihydroxypropyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine; BH_4) is an important component for controling the concentration of catecholamines or indoleamines in the brain and other nerve tissues (1). During the last 10 years, the pathway of BH_4 biosynthesis from GTP is now believed to have been apparently established by many workers as shown in Fig. 1. Recently, however, some new pathways of BH_4 synthesis from GTP have been demonstrated (2,3). And accurate experiments are immediately required for determination of the substrate specificity and other properties of the enzymes which are involved in BH_4 biosynthesis. In the metabolic pathway of BH_4, sepiapterin was indicated to be an intermediate (4,5); and sepiapterin reductase (EC 1,1.1.153; SPR), which was purified to homogeneity from rat whole brain (6), has been considered to play the role of the reversible catalysis of sepiapterin (2-amino-4-hydroxy-6-lactyl-7,8-dihydropteridine) with NADPH to dihydrobiopterin (2-amino-4-hydroxy-6-1',2'-dihydroxypropyl-7,8-dihydropteridine)(7,8,9,10). This enzyme

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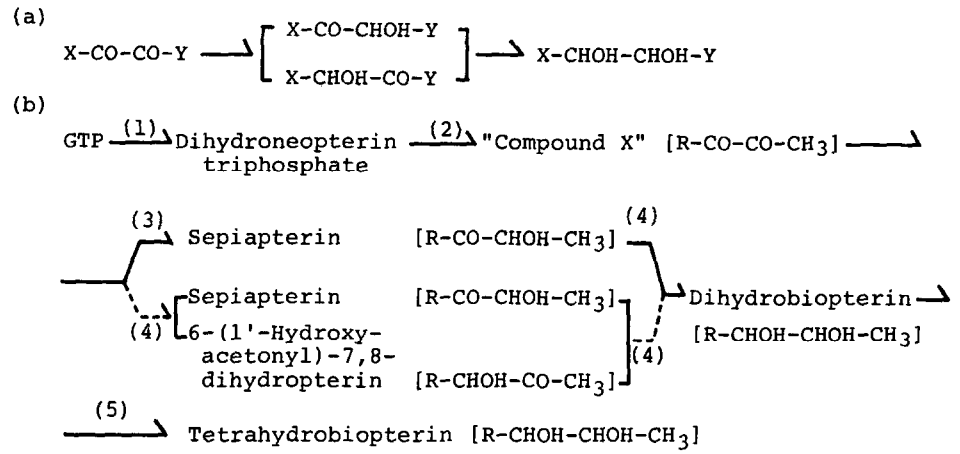

Fig. 1. (a)The reduction of a dicarbonyl compound with NADPH by sepiapterin reductase, and (b)Biosynthetic pathway of tetrahydrobiopterin with additional pathway of postulated reaction by sepiapterin reductase (----).

(1) GTP cyclohydrolase, (2) Fraction A2 of Tanaka et.al.
(3) Fraction A1 of Tanaka et. al.
(4) Sepiapterin reductase, (5) Dihydrofolate reductase.
R: Pteridine ring.

has been reported to show a strict substrate specificity for the 6-lactyl group of sepiapterin and to scarcely catalyze the conversion of analogues of sepiapterin such as deoxysepiapterin (2-amino-4-hydroxy-6-propyonyl-7,8-dihydropteridine) and xanthopterin B_2 (2,4-dihydroxy-6-lactyl-7,8-dihydropteridine)(8,9).

We have now discovered a new property of SPR, that is, catalysis of the reduction of many dicarbonyl compounds in the presence of NADPH as effectively as in the case of sepiapterin. And we expect that "Compound X", designated as an intermediate of BH_4 and demonstrated as a dicarbonyl compound of a pteridine derivative (2-amino-4-hydroxy-6-1',2'-dioxopropyl-7,8-dihydropteridine) by Tanaka et. al.(5), may be reduced by SPR in the presence of NADPH to produce successively sepiapterin and dihydrobiopterin in the biosynthetic pathway of BH_4 (Fig.1).

MATERIALS AND METHODS

Materials: NADPH and NADH were the products of Sigma. G6P and G6P-dehydrogenase were obtained from P.L.Biochemicals and Boehringer, respectively. Diacetyl and acetoin were from Wako Chemical Co., and 2,3-butanediol, from Tokyokasei Kogyo Co.. Sepiapterin was prepared from adult se mutant of Drosophila melanogaster (11).

Preparation and assay of SPR: Purified SPR (homogeneous by disc electrophoresis) was prepared from rat erythrocytes according to the method by Sueoka and Katoh (11). SPR activity with a carbonyl compound (50 μM) as substrate was determined as described by Katoh(8) except at pH 6.8 and 340 nm measurement (100 mM potassium phosphate buffer, 100 μM NADPH) in the presence of 40 mU SPR (Shimazu Photometer 210A). The reaction, with SPR or NADPH omitted, was performed, for each compound, as controls. One unit of enzyme is defined as the amount which catalyzes the reduction of 1 μmol of sepiapterin at pH 6.4 and 25 °C.

Assay of diacetyl, acetoin, and NADPH: Commercial acetoin was further purified just before use by the method of Westerfeld (12). Gas chromatography of acetoin was performed using a Shimazu Gas Chromatopraph (GC 7A) with a flame-ionization detector with the stainless column (Porapak type Q, 80-100 mesh (Waters), 3 mm x 2 m) with a column temperature of 200 °C and an injection temperature of 240 °C. N_2 gas (30 ml/min) was used as the carrier gas.

Photometric determination of diacetyl and acetoin were performed by the method of Westerfeld (12). One ml of sample was added with 0.2 ml of 0.5% creatine solution and 0.2 ml of 5 % α-naphthol solution. After incubation at room temperature for 10 min and 60 min for diacetyl and acetoin, respectively, the absorbance at 540 nm was measured. One ml of distilled water was used as a reference. By this method acetoin could not be determined in the presence of a large amount of diacetyl, and acetoin was assayed at the time when no further decrease in absorbance at 340 nm was observed (13) in the SPR system. The amount of acetoin in the SPR reaction mixture was obtained by subtracting the amount measured after the 10 min incubation from the amount found after the 60 min incubation. 2,3-Butanediol is not detected by this method.

The amount of NADPH oxidized during the incubation in the SPR system was determined by using the G6P-dehydrogenase system (14). To the mixture of 0.2 μmol $G6PN_a$, 40 μmols $MgCl_2$, and 1U G6P-dehydrogenase in 200 μmols potassium phosphate buffer, pH 7.5, an aliquot (1 ml) of the reaction mixture was added to give a final vol of 2.0 ml. The increase in the absorbance at 340 nm was measured to determine the amount of $NADP^+$ formed in the SPR reaction mixture.

RESULTS AND DISCUSSION

When a number of carbonyl compounds were incubated with purified SPR from rat erythrocytes and NADPH at neutral pH, a decrease in absorbance at 340 nm was observed. In a series of carbonyl compounds examined including quinones, diketones, monoketones, and aldehydes, 9,10-phenanthrenequinone and some dicarbonyl compounds listed in Table I showed the greatest decrease in absorbance. As SPR is known to reduce the carbonyl group (-CO-) on the sidechain of sepiapterin with NADPH to produce the (-CHOH-) group, the carbonyl group(s) of these compounds would be expected to be reduced in the same way. Only diacetyl (CH_3-CO-CO-CH_3) of the dicarbonyl compounds listed in Table I was available

Table I
Michaelis constants and maximal velocities of sepiapterin reductase against various dicarbonyl compounds

Substrates	K_m (μM)	V_{max} (μmol/ min/mg)	(V_{max}/K_m)	Coenzyme requirement NADH/NADPH(%)
Sepiapterin[a]	14.3	24.1	1.69	23.3
Phenylpropanedione (-CO-CO-CH_3)	21.8	35.1	1.61	23.3
Benzil (-CO-CO- )	20.0	2.9	0.15	35.7
Diacetyl (CH_3-CO-CO-CH_3)	1000	45.1	0.045	10.3

The apparent values of the Michaelis constant and maximal velocity were estimated by double reciprocal plots of Lineweaver-Burk with NADPH. All reactions were performed in the presence of 100 mM potassium phosphate buffer, 100 μM NADPH and 5 mU sepiapterin reductase at pH 6.4 and 25°C. Coenzyme requirement was examined with 50 μM substrate and 100 μM coenzyme. [a](11).

for the analysis of this enzyme reaction since authentic compounds of its postulated reducing products by SPR and NADPH, acetoin (CH_3-CO-CHOH-CH_3) and 2,3-butanediol (CH_3-CHOH-CHOH-CH_3), could be obtained commercially. 5 mM diacetyl was incubated with 2.48 mM NADPH and 80 mU of the purified SPR at pH 6.8 in 100 mM potassium phosphate buffer, at 37 °C. When no further decrease in absorbance at 340 nm was observed (after about a 30 min incubation), the reaction mixture was mixed with 1 vol of chloroform. An aliquot of the chloroform layer of the mixture was used for gas chromatography. Authentic acetoin was detected at 9.8 min in the system described in "Materials and Methods". After the incubation of diacetyl with SPR, a new peak at 9.8 min, corresponding to acetoin, was observed. At various times during the incubation, the amounts of diacetyl, acetoin, and NADPH oxidized in the reaction mixture were determined by photometric methods described in the "Materials and Methods" (Fig. 2). The amounts of the decrease and increase in the absorbance at 340 nm of the reaction mixture and in the G6P-dehydrogenase system, respectively, were quite identical. Almost all of diacetyl in the reaction mixture disappeared at the equilibrium time of the reaction. The results of Fig. 2 indicate that the dicarbonyl compound, diacetyl, was reduced by SPR to monocarbonyl compound, acetoin, with NADPH. At the equilibrium time of the reaction, the ratio

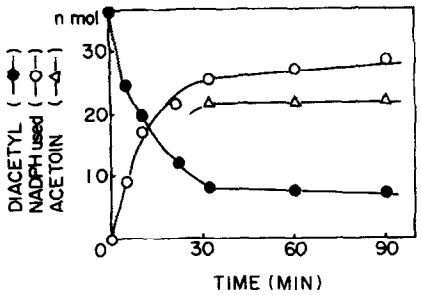

Fig. 2. NADPH-dependent formation of acetoin from diacetyl by sepiapterin reductase reaction.
The reaction mixture containing 100 mM potassium phosphate buffer, pH 6.8, 36 μM diacetyl, 100 μM NADPH and 500 mU of sepiapterin reductase was incubated at 25 °C. Diacetyl and acetoin were determined by the method of Westerfeld(12), and the amount of NADPH oxidized was measured per 1 ml of reaction mixture at various times by the G6P-dehydrogenase system(14) as described in "Materials and Methods".

between the decrease in diacetyl and NADPH oxidized was about 1 (1 mol:0.95 mol), and the ratio between the decrease in diacetyl and the increase in acetoin concentration was also about 1 (1 mol:0.75 mol). In the case of phenylpropanedione (50 μM) as substrate of SPR (5 mU), 1:1 ratio between the amounts of decreased substrate and the increased NADPH oxidized was also observed during the incubation. These results showed that the two carbonyl groups of the dicarbonyl compound were not reduced at the same time. One carbonyl group of a molecule was reduced to a hydroxyl group in diacetyl reaction.

When 50 μM acetoin was added to the usual SPR system as described in "Materials and Methods", no significant decrease in absorbance at 340 nm was detected. However, when a large amount of acetoin (5 mM) was added to the system with 91.5 mU SPR, a slight but significant decrease in the absorbance was observed (3.0 μM NADPH oxidized per 30 min). As it was unsuccessful to find a suitable method for analyzing a small amount of 2,3-butanediol for this experiment, the formation of this compound was undetectable in the reaction mixture after the incubation. NADPH production, on the other hand, was observed in the presence of 50 mM 2,3-butanediol and 0.5 mM $NADP^+$ in the reverse reaction of SPR with 1000mU SPR at pH 7.5 (4.8μM $NADP^+$ reduced per 30 min at 37°C). By measuring the activity of NADPH oxidizing in the G6P-dehydrogenase system, the K_m value of 30 mM was

obtained for acetoin at pH 6.4, of which value is 30 times larger than that for diacetyl(Table I). These results indicate that the monocarbonyl compound, acetoin, can be reduced by SPR, and that when the dicarbonyl compound is reduced by SPR, the rate of the first reaction against one of the two carbonyl groups is far more rapid than the second reaction against the remaining group. Though both carbonyl groups are finally reduced by the SPR reaction one after another, the order of the reduction of the two groups and the configuration of the two hydroxyl groups formed by the reaction are yet unknown (Fig. 1(a)).

Table I shows the kinetic constants, K_m and V_{max}, of active dicarbonyl compounds by measuring the amount of NADPH oxidized with a relatively small amount of SPR(5mU)for 5min. Since the product of 9,10-phenanthrenquinone by the SPR reaction was reoxidized under aerobic conditions, kinetic constants for it could not be determined. Phenylpropanedione and benzil showed similar values for Michaelis constants, and phenylpropanedione and diacetyl showed the same order of maximal velocities as those of the natural substrate, sepiapterin. Thus, based on the apparent pseudo-minute order rate constant (V_{max}/K_m) phenylpropanedione is quite similar to sepiapterin as an effectively utilized substrate.

All three of these compounds required NADPH as coenzyme rather than NADH, as observed in the case of sepiapterin (Table I).

SPR purified to homogeneity from rat whole brain (6) was also observed to show a NADPH-oxidizing activity in the presence of diacetyl. It was peculiar that some monoketotestosterones showed a slight activity in the SPR system. Various properties of this new activity of SPR will be described in detail elsewhere.

The physiological role of this non-specific NADPH-dependent carbonyl reductase activity of SPR is not clear, but this activity quite resembles those of carbonyl reductases from human brain reported by B. Wermuth (15), in which the role of the enzyme is thought to involve decomposition of metabolic intermediates or exogenous drugs (aldehydes and ketones) in the body.

At any rate, in this report, we have found a new ability of SPR to reduce, utilizing NADPH, many carbonyl

compounds (especially some dicarbonyl compounds) other than sepiapterin. And when a dicarbonyl compound is catalyzed by SPR, a stepwise reduction of the two carbonyl groups is indicated. This activity of SPR against many non-pteridine derivatives was unexpected because this enzyme shows a strict specificity for the 6-lactyl group and the 2-amino and 4-hydroxy substituted derivatives of pteridines (3,8,9). These findings, however, suggest that SPR may also reduce a pteridine derivative with a dicarbonyl group on its sidechain, if it occurs. Recently Tanaka et. al. (5) designated in the dihydrobiopterin biosynthetic pathway, an intermediate as "Compound X" which is derived from dihydroneopterin triphosphate by catalysis involving Fraction A2. And they demonstrated that "Compound X" is a pteridine with a dicarbonyl group on the 6-sidechain and that it would be further catalyzed by Fraction A1 to sepiapterin in the presence of NADPH (5) (Fig. 1(b)). According to the results of the experiments in this report, we postulate that SPR may reduce "Compound X" to finally produce dihydrobiopterin via sepiapterin or possibly 6-(1'-hydroxyacetonyl)-7,8-dihydropterin (Fig. 1(b)). It is quite necessary to test the specificity of SPR for "Compound X" and to compare the properties of SPR with those of Fraction A1 of Tanaka et. al. (5), which has the activity of reducing "Compound X" to sepiapterin with NADPH in the biosynthetic pathway of BH_4.

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