Biochimica et Biophysica Acta, 612 (1980) 337—343

BBA 68961

DIHYDROPTERIDINE REDUCTASE AND TETRAHYDROPTERIN IN *CRITHIDIA FASCICULATA* CELLS

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(Received May 5th, 1979)
(Revised manuscript received October 10th, 1979)

Key words: Dihydropteridine reductase; Tetrahydropterin; Pteridine metabolism; (Crithidia fasciculata)

Summary

Dihydropteridine reductase was found in extracts of *Crithidia fasciculata* and was demonstrated by the fact that the enzyme required both quinonoid-dihydropterin and NADH as substrates. 7,8-Dihydropterin and dihydrofolate failed to serve as substrates; tetrahydropterin was formed as the reaction product. The molecular weight of the enzyme was estimated to be about 55 000 by Sephadex G-100 gel filtration. NADH was more effective than NADPH as substrate for the enzyme. Tetrahydropterin (1.35 nmol tetrahydrobiopterin equivalents/g cells) was also detected in *C. fasciculata*.

Introduction

Crithidia fasciculata is a flagellate which requires biopterin as a growth factor in the culture medium and has been used for the biological assay of biopterin (see Ref. 1). Kidder and Nolan [3] have suggested that tetrahydrobiopterin might act as a cofactor of dihydroorotic acid hydroxylase. The role of biopterin and which oxido reduction state(s) of the pterin is important have not yet been unequivocally elucidated. In mammals, it is known that tetrahydrobiopterin acts as an electron donor for several hydroxylases of aromatic amino acids, and is oxidized to quinonoid-dihydrobiopterin during the hydroxylation reaction [4]. Since the quinonoid-dihydro form of pterin is very labile and rapidly converted to 7,8-dihydro isomer in aqueous solution [5], quinonoid-dihydropterin is presumably formed only by oxidation of tetra-

hydropterin in vivo. Therefore, dihydropteridine reductase which catalyzes the reduction of quinonoid-dihydropterin but not 7,8-dihydropterin to tetrahydropterin [5], has a quite characteristic function (that of tetrahydropterin regeneration). We considered that there could be a close relationship between the redox state of pterin and its physiological role. We, therefore, looked for dihydropteridine reductase activity in *C. fasciculata* cells to examine the presence of the tetrahydropterin-regenerating system.

In this paper, we report finding dihydropteridine reductase in *C. fasciculata* and some properties of the enzyme. Tetrahydropterin was also detected.

Materials and Methods

C. fasciculata (A.T.C.C. 11745) was obtained from Dr. K. Kobayashi (Tokyo Metropolitan University). The *Crithidia* cells were cultured in stock culture medium as described by Dewey and Kidder [1] for 6 days at 25°C. Cells were collected by centrifugation and washed twice with 0.85% NaCl.

Dihydropteridine reductase activity of *C. fasciculata* was assayed by the same method as that for the bovine liver enzyme [6]. The standard assay mixture (2 ml) contained 80 μmol Tris-HCl (pH 7.6), 100 nmol ferri-cytochrome *c*, 100 nmol NADH, 2 nmol 5,6,7,8-tetrahydro-6-methylpterin and the enzyme preparation. The reaction was carried out at 25°C. One unit of enzyme activity was defined as the amount that reduced 1 μmol cytochrome *c* per min under the standard condition. The enzyme was partially purified as follows: 2.5 g *C. fasciculata* cells were sonicated twice for 5 min with 3 vols. 40 mM potassium phosphate (pH 6.8). After this sample had been centrifuged at 105 000 × *g* for 1.5 h, the supernatant was applied to a DEAE-cellulose column (2.0 × 15 cm) equilibrated with 40 mM potassium phosphate (pH 6.8). The enzyme was not adsorbed and was eluted with the flow-through fractions. The active fractions (12 ml) were concentrated by use of poly(ethylene glycol) (molecular weight 20 000). The concentrated solution was then applied to a Sephadex G-25 column (2.0 × 15 cm) equilibrated with the same buffer. Protein fractions were collected and concentrated using a Collodion bag.

The molecular weight of the *Crithidia* dihydropteridine reductase was estimated by gel filtration through a Sephadex G-100 column (1.5 × 90 cm) equilibrated with 40 mM potassium phosphate (pH 6.8)/0.1 M KCl. The elution volume was determined relative to those of myoglobin (molecular weight, 18 500), α-chymotrypsin (25 700), bovine liver dihydropteridine reductase (50 000 [6]) and bovine serum albumin (68 000).

The product of the *Crithidia* dihydropteridine reductase was analyzed as follows: the reaction mixture (2 ml) containing 40 μmol Tris-HCl (pH 7.0), approx. 8 nmol quinonoid-dihydro-6-methylpterin, 15 nmol NADH and 1.15 units enzyme was incubated at 25°C for 3 min. The reaction was stopped by the addition of 0.4 ml 36% trichloroacetic acid. After removal of precipitated protein by centrifugation, a 2-ml sample of the supernatant was kept at 25°C for 1.5 h to decompose residual quinonoid-dihydro-6-methylpterin. Most of the quinonoid-dehydropterin, which remained, was converted to inactive substance(s) during this period while tetrahydro-6-methylpterin was stable against this treatment. The solution was then shaken with an equal volume of

diethyl ether to remove the trichloroacetic acid. The aqueous layer was assayed for tetrahydropterin content using authentic tetrahydro-6-methylpterin treated by the same procedure as the standard.

To study the tetrahydropterin content, the cells were treated as for the enzyme preparation and the 105 000 × *g* supernatant was analyzed for tetrahydropterin in the phenyalanine hydroxylation system, as cofactor activity using authentic tetrahydrobiopterin as standard. Tyrosine formation was measured using [4-^{3}H]phenylalanine according to Guroff and Abramowitz [7]. The reaction mixture contained 25 μmol potassium phosphate (pH 6.8), 50 nmol L-phenylalanine, 0.5 mg catalse, 2 μmol dithiothreitol, excess phenylalanine hydroxylase (0.33 mg protein), 0.1 μCi [4-^{3}H]phenylalanine (27 Ci/mmol), and sample in a final volume of 250 μl. After incubation at 25°C for 30 min, the reaction was stopped by the addition of 0.1 ml 2 M HCl, then the pH of the solution was neutralized by 2 M NaOH (0.1 ml). To the neutralized solution, 0.1 ml 1 M sodium acetate (pH 5.5) and 0.1 ml freshly-prepared 2% *N*-iodosuccinimide were added. The mixture was kept for 5 min at 0°C, then 50 μl 30% trichloroacetic acid was added. The mixture (0.7 ml) was applied to a Dowex 50 W-X8 (H^+) column (0.8 × 1.5 cm) and then the column was washed with 1 ml water. A 1-ml sample of the eluate was put into a vial and mixed with 10 ml scintillation fluid (0.27% (w/v) 2,5-diphenyloxazole/33% (v/v) Triton X-100/toluene). The radioactivity was counted using a Packard Model 3380 liquid scintillation counter.

Concentrations of authentic tetrahydro-6-methylpterin and tetrahydrobiopterin were determined by oxidation with 2,6-dichlorophenolindophenol according to Kaufman [8]. The following molecular extinction coefficients were used: NADH and NADPH, 6200 at 340 nm; 2,6-dichlorophenolindophenol, 19500 at 604 nm [6]; cytochrome *c*, 21000 (reduced minus oxidized) at 550 nm [9].

Phenylalanine hydroxylase was prepared from rat liver by the method of Kaufman and Fisher [10] except that the purification was performed up to the second $(NH_4)_2SO_4$ step. Bovine liver dihydropteridine reductase was purified by the method of Hasegawa [6]. Ferri-cytochrome *c*, tetrahydrobiopterin and 7,8-dihydro-6-methylpterin were prepared as described previously [11]. Tetrahydro-6-methylpterin was purchased from Calbiochem (A grade) and quinonoid-dihydro-6-methylpterin was prepared from tetrahydro-6methylpterin by the method of Hasegawa [6] just before use. [4-^{3}H]Phenylalanine (27 Ci/mmol) was from the Radiochemical Centre (Amersham, U.K.), dithiothreitol and 2,6-dichlorophenolindophenol were Wako Pure Chemical Industries (Osaka, Japan), NADH (grade III) and NADPH (type I) were Sigma Chemical Co. (St. Louis, U.S.A.), and catalase was from Tokyo Kasei Kogyo (Tokyo, Japan).

Results and Discussion

The crude extract of *C. fasciculata* cells was analyzed for dihydropteridine reductase activity in the standard assay condition. The enzyme activity (tetrahydropterin-dependent reduction of cytochrome *c*) was observed, while tetrahydropterin-independent reduction of cytochrome *c* (i.e., 'diphorase' activity)

TABLE I

REQUIREMENTS FOR THE ACTIVITY OF THE *CRITHIDIA* DIHYDROPTERIDINE REDUCTASE

The complete assay mixture (2 ml) contained 80 μmol Tris-HCl (pH 7.6), 100 nmol ferri-cytochrome *c*, 100 nmol NADH, 2 nmol tetrahydro-6-methylpterin ($6MPH_4$) and the enzyme preparation.

Assay condition	Cytochrome *c* reduction (nmol/min)
Complete	3.10
—Enzyme	0.30
—Enzyme + Boiled enzyme	0.37
—NADH	0.03
—$6MPH_4$	0.35
—$6MPH_4$ + q-$6MPH_2$ [a]	3.25
—$6MPH_4$ + 7,8-$6MPH_2$ [b]	0.44
—$6MPH_4$ + dihydrofolate [c]	0.30

[a] About 2 nmol quinonoid-dihydro-6-methylpterin.
[b] 2 nmol 7,8-dihydro-6-methylpterin.
[c] 2 nmol dihydrofolate was used instead of tetrahydro-6-methylpterin.

also simultaneously appeared. To confirm the existence of *Crithidia* dihydropteridine reductase, partial purification of the enzyme was performed to remove tetrahydropterin-independent activity. The enzyme preparation did not contain detectable amounts of cofactor activity for phenylalanine hydroxylase or any phenylalanine hydroxylase activity. The hydroxylase activity was also undetectable even in the crude extract (supernatant from the ultracentrifugation). Dihydropteridine reductase of *C. fasciculata* could be stored in 40 mM potassium phosphate (pH 6.8) for at least a week at −80°C. The enzyme activity was unstable under acidic (below pH 6.5) or alkaline conditions (above pH 8.0) and to $(NH_4)_2SO_4$ precipitation.

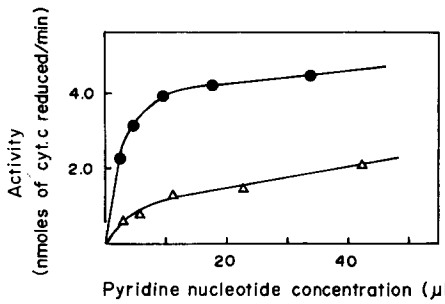
With the preparation thus obtained, the requirement for the dihydropteridine reductase activity was examined. The results are shown in Table I. The *Crithidia* enzyme showed activity in the presence of both tetrahydro-6-methylpterin and NADH in the reaction mixture. The reduction of cytochrome *c* in the absence of the enzyme was due to the spontaneous reduction

TABLE II

CONVERSION OF QUINONOID-DIHYDRO-6-METHYLPTERIN TO THE TETRAHYDRO FORM BY THE *CRITHIDIA* ENZYME

About 8 nmol quinonoid-dihydro-6-methylpterin and 15 nmol NADH in 2 ml 20 mM Tris-HCl (pH 7.0) were incubated at 25°C for 3 min in the presence or absence of the *Crithidia* enzyme (1.15 units). The product was then analyzed for tetrahydropterin content in the phenylalanine hydroxylation system according to Guroff and Abramowitz [7] with the use of authentic tetrahydro-6-methylpterin as standard.

Sample	Radioactivity (cpm)	Tetrahydropterin formed from quinonoid-dihydro-6-methylpterin (nmol)
Product 1 (+enzyme)	4270	6.81
Product 2 (—enzyme)	1140	0.807
Buffer	690	0



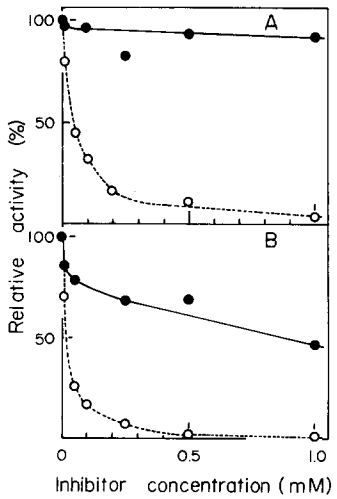


Fig. 1. Effect of reduced pyridine nucleotide concentration on the *Crithidia* dihydropteridine reductase. The assay mixture contained 80 μmol Tris-HCl (pH 7.6), 100 nmol ferri-cytochrome *c*, 2 nmol tetrahydro-6-methylpterin, various amount of NADH (•) or NADPH (△), and the enzyme (4.4 munits) in a final volume of 2 ml.

Fig. 2. Effect of amethopterin and aminopterin on the *Crithidia* dihydropteridine reductase activity. Effect of amethopterin (A) and of aminopterin (B) on the *Crithidia* enzyme were examined and compared with that of these inhibitors on bovine liver dihydropteridine reductase. The *Crithidia* enzyme (2.0 munits, •) or 5.4 munits of the bovine liver enzyme (○) was measured under the standard assay condition in the presence of various concentrations of each inhibitor.

of quinonoid-dihydro-6-methylpterin with NADH, and was usually 0.3—0.4 nmol/min under the condition. The cytochrome *c* reduction in the absence of tetrahydro-6-methylpterin might be due to 'diaphorase' activity slightly contaminated in the preparation. Although dihydropteridine reductase requires quinonoid-dihydropterin as substrate, the quinonoid-dihydro form of the pterin is so labile [5] that the pterin substrate is usually added to assay mixture as the tetrahydro form [6,12,13]. However, we prepared quinonoid-dihydro-6-methylpterin and immediately used it instead of tetrahydro-6-methylpterin to confirm the nature of the pterin substrate of the *Crithidia* enzyme. Quinonoid-dihydro-6-methylpterin could replace tetrahydro-6-methylpterin and almost the same activity was detected as in the case of the tetrahydro form. On the contrary, an equimolar concentration of 7,8-dihydro-6-methylpterin or dihydrofolate was not effective. Even when a higher concentration of these substances (100 μM 7,8-dihydro-6-methylpterin or 79 μM dihydrofolate) was used, activity was not detectable. These results indicated that the *Crithidia* enzyme in the present study is dihydropteridine reductase.

This was further confirmed by product analysis of the *Crithidia* enzyme. The enzyme was incubated with quinonoid-dihydro-6-methylpterin and NADH, then tetrahydro-6-methylpterin formed was measured. As shown in Table II, about 85% of quinonoid-dihydro-6-methylpterin was converted to tetrahydro form. In the control experiment carried out in the absence of the enzyme, spontaneous conversion of quinonoid-dihydro-6-methylpterin to the tetrahydro form by NADH was only about 10%.

The molecular weight of *C. fasciculata* enzyme was calculated to be 55 000 from the gel filtration study. The enzyme was not stable during the gel filtration and only 15% of the activity was recovered.

The *Crithidia* enzyme was suggested to be more specific to NADH than NADPH by the observation that about 50% of the activity was detected when the activity was measured using an equimolar NADPH instead of NADH in the standard assay condition. The effect of concentrations of NADH and NADPH on the enzyme was examined and compared (Fig. 1). With use of tetrahydro-6-methylpterin, the K_m values of the enzyme for NADH and NADPH were estimated to be 2.8 and 8.8 μM, respectively. Apparent maximal velocity of the enzyme was higher with the use of NADH (24.4 nmol cytochrome *c* reduced/min per mg) than NADPH (10.9 nmol cytochrome *c* reduced/min per mg). The *Crithidia* dihydropteridine reductase is similar to the mammalian enzyme with respect to the pyridine nucleotide specificity and the molecular weight [6,12,123].

The effect of aminopterin and amethopterin, which have an inhibitory action on mammalian dihydropteridine reductase [12,13], on the *Crithidia* enzyme was examined (Fig. 2). When 2 munits of the enzyme were used, 50% of the activity was inhibited by 1 mM aminopterin under the standard assay conditions. Amethopterin was less inhibitory than aminopterin, about 10% of the activity being inhibited by 1 mM amethopterin. On the other hand, 50% of the bovine liver dihydropterine reductase activity (5.4 munits) was inhibited by 25 μM aminopterin or 35 μM amethopterin. Though the *Crithidia* dihydropteridine reductase was inhibited by these inhibitors, the sensitivity of the *Crithidia* enzyme was lower than that of the mammalian enzyme. The difference of the sensitivity to inhibitors between mammalian enzyme and flagellate or bacteria enzyme was similarly observed in the case of dihydrofolate reductase as tabulated by Gutteridge et al. [14].

TABLE III

PRESENCE OF TETRAHYDROPTERIN IN THE EXTRACTS OF *CRITHIDIA FASCICULATA*

Tetrahydropterin content in extracts of *C. fasciculata* was analyzed as the cofactor activity in the phenylalanine hydroxylation system by the method of Guroff and Abramowitz [7] with the use of authentic tetrahydrobiopterin as standard. Assay mixture contained 25 μmol potassium phosphate (pH 6.8), 50 nmol L-phenylalanine, 0.5 mg catalase, 2 μmol dithiothreitol, excess phenylalanine hydroxylase (0.33 mg protein), 0.1 μCi [4-^{3}H]phenylalanine (27 Ci/mmol) and sample (165 μl) in a final volume of 250 μl. Incubation was performed at 25°C for 30 min.

Sample	Radioactivity (cpm)	Tetrahydrobiopterin equivalents in assay mixture (nmol)	Tetrahydrobiopterin equivalents in 1 g of cells (nmol)
Extract 1 [a]	1420	0.044	0.800
Extract 2 [b]	2340	0.111	1.35
Medium (before culture)	800	not detected	
Medium (after culture)	830	not detected	
Buffer	830	0	

[a] Extract obtained by sonication with 3 vols. 40 mM potassium phosphate (pH 6.8).
[b] Extract obtained by homogenization with 2 vols. buffer.

Recently, two types of dihydropteridine reductase, ie., NADH-specific and NADPH-specific enzyme, were reported to be present in bovine liver [11]. As we have not separated the *Crithidia* enzyme into NADH-specific and NADPH-specific activities, it is still unclear whether both types of the enzyme exist in *C. fasciculata* as in the case of bovine liver.

The presence of dihydropteridine reductase (tetrahydropterin-regenerating enzyme) in *C. fasciculata* as demonstrated above suggested that biopterin may be reduced to tetrahydro(bio)pterin presumably after incorporation into the cells. We then examined the tetrahydropterin content of *C. fasciculata.*

As shown in Table III, the cofactor activity was detected in both extracts of *C. fasciculata.* A relatively larger amount of tetrahydropterin was recovered in the extract obtained from the cells by homogenization than in that obtained by sonication. The culture medium, either before or after the cell culture, did not contain detectable amount of tetrahydropterin.

The existence of both dihydropteridine reductase and tetrahydropterin in *C. fasciculata,* which requires biopterin as a growth factor, suggests that one of the roles of biopterin is to act, as tetrahydro form, as an electron donor.

Furthermore, since Gutteridge et al. [14] reported that biopterin and 7,8-dihydrobiopterin (each 100 μM) were ineffective as substrate for *Crithidia* dihydrofolate reductase, the presence of tetrahydropterin in the flagellate suggests the interesting possibility that an unconjugated pterin-specific reductase(s) is present in *C. fasciculata.*

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