A FUNCTION OF TETRAHYDROPTERIDINES AS COFACTORS FOR INDOLEAMINE 2,3-DIOXYGENASE

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SUMMARY

Indoleamine 2,3-dioxygenase is known to require both ascorbic acid and methylene blue for maximum activity. Tetrahydrobiopterin and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine were found to act as cofactors of this enzyme. In the reaction with these tetrahydropteridines, methylene blue markedly accelerated the enzymic reaction. However, in contrast to the reaction with ascorbic acid, a substantial stimulation of the enzyme was observed by tetrahydropteridines in the absence of methylene blue, especially in the presence of catalase. It is suggested that tetrahydropteridine may be the natural cofactor of indoleamine 2,3-dioxygenase.

INTRODUCTION

Tryptophan pyrrolase of rabbit small intestine is a heme protein which catalyzes the oxidative ring cleavage of both D- and L-isomers of tryptophan to N-formylkynurenine (1,2). This enzyme also degrades D- and L-5-hydroxytryptophan, tryptamine, and serotonin (3). In view of the broad substrate specificity, this enzyme is better designated as indoleamine 2,3-dioxygenase (4). Recently a similar enzyme purified from rabbit brain has been shown to attack melatonin (4). Indoleamine 2,3-dioxygenases have been shown to require ascorbic acid and methylene blue for maximum activity (2-4). Requirement for this synthetic dye is essential (2), and methylene blue has been considered to be responsible for generation of the $O_2^{\cdot-}$ which participates in the catalytic process of this enzyme *in vitro* (5,6). Tetrahydropteridines are known to function as cofactors in monooxygenase reactions such as hydroxylation of phenylalanine, tyrosine and tryptophan (7). It has recently been shown that $O_2^{\cdot-}$ is generated during the autoxidation of tetrahydropteridines (8,9). It was of interest, therefore, to examine whether tetrahydropteridines could act as cofactors of indoleamine 2,3-dioxygenase. The results of the present study

have indicated that tetrahydropteridines can stimulate this enzymic reaction without the addition of methylene blue.

MATERIALS AND METHODS

Indoleamine 2,3-dioxygenase was purified from rabbit small intestine by the method of Yamamoto and Hayaishi (2). Superoxide dismutase was purified from bovine erythrocytes according to McCord and Fridovich (10). For the determination of activity of this enzyme, the method and units described by the above authors were used. Tetrahydrobiopterin was obtained from Hoffmann-La Roche Ins., and $DMPH_4$[1] from Aldrich Chemical Co. L- and D-tryptophan, DL-kynurenine, xanthine, xanthine oxidase, sodium ascorbate and methylene blue were purchased from Sigma Chemical Co., and catalase from Calbiochem.

Because of interference by tetrahydropteridines in the original spectrophotometric assay of indoleamine 2,3-dioxygenase (2), the product N-formylkynurenine was converted to kynurenine which was determined colorimetrically according to Bratton and Marshall (11). Xanthine oxidase was assayed spectrophotometrically by the oxidation of hypoxanthine (12). Protein was determined by the Lowry method (13).

RESULTS AND DISCUSSION

Function of Tetrahydropteridines as Cofactors - Both tetrahydrobiopterin and $DMPH_4$ were found to stimulate the oxidative ring cleavage of L-tryptophan catalyzed by indoleamine 2,3-dioxygenase in the presence of catalase, and the addition of methylene blue further accelerated this reaction (Table I). Omission of catalase markedly diminished the stimulation caused both by tetrahydrobiopterin alone and by tetrahydrobiopterin plus methylene blue. This catalase effect may be due to the prevention of nonenzymic oxidation of tetrahydrobiopterin by H_2O_2 which is formed during its autoxidation (7), and due to the protection of the enzyme form inactivation by H_2O_2, since catalase also stimulated the enzymic reaction when ascorbic acid and methylene blue were used as cofactors (data not shown). As indicated in Table II, the D-isomer of tryptophan was also degraded by the enzyme using tetrahydrobiopterin or $DMPH_4$ as a cofactor. In this case, too, methylene blue markedly accelerated the reaction, but the degree of stimulation was less than that obtained with ascorbic acid plus methylene blue. The cofactor function of tetrahydro-

[1]Abbreviation used: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

TABLE I. Stimulation of indoleamine 2,3-dioxygenase reaction by tetrahydropteridines using L-tryptophan as substrate

Test Mixture	Kynurenine (nmoles)
Complete (containing methylene blue)	17.8
omit catalase	1.0
$DMPH_4$ instead of tetrahydrobiopterin	12.1
Complete (containing no methylene blue)	5.4
omit catalase	0.5
$DMPH_4$ instead of tetrahydrobiopterin	3.5

The complete incubation mixture (0.2 ml) contained indoleamine 2,3-dioxygenase (10 µg), 150 µM L-tryptophan, 410 µM tetrahydrobiopterin, and catalase (10 µg) in 50 mM postassium phosphate buffer, pH 7.5. Methylene blue was 5 µM when added. $DMPH_4$ was 410 µM when added instead of tetrahydrobiopterin. The reaction was initiated by adding the tetrahydropteridine. After incubation for 10 min at 37°, the reaction was stopped with trichloroacetic acid (3% final concentration) and protein removed by centrifugation. The N-formylkynurenine formed was hydrolyzed by adding HCl to 0.35 N and warming at 37° for 40 min. After appropriate dilution with 3% trichloroacetic acid, kynurenine was assayed colorimetrically (9). The reaction mixture without L-tryptophan was used as a control.

pteridines in the dioxygenase reaction is unique in that the pterin-dependent oxygenases thus far studied are monooxygenases (7).

It is known that xanthine oxidase and hypoxanthine can stimulate the indoleamine 2,3-dioxygenase reaction in place of ascorbic acid plus methylene blue (2) and that certain pteridine derivatives are substrates of xanthine oxidase (14). Although the indoleamine 2,3-dioxygenase preparation used was highly purified, it was not homogeneous. Therefore, if xanthine oxidase was a contaminant of the enzyme preparation, it would have stimulated the oxidative ring cleavage of tryptophan. However, this possibility was ruled out by the following observations;(1) the enzyme preparation did not show appre-

TABLE II. The indoleamine 2,3-dioxygenase reaction with D-tryptophan as substrate

Cofactor	Kynurenine (nmoles) + methylene blue	Kynurenine (nmoles) - methylene blue
Tetrahydrobiopterin	9.5	3.1
$DMPH_4$	5.8	1.8
Ascorbic Acid	41.8	0.5

The conditions were similar to those for the complete systems specified in Table I. D-tryptophan (3 mM) was used instead of L-tryptophan, and the amount of the enzyme used was 12 µg. The concentrations of tetrahydrobiopterin, $DMPH_4$ and ascorbic acid were 410 µM, 410 µM and 4 mM, respectively.

ciable xanthine oxidase activity as assayed spectrophotometrically by the oxidation of hypoxanthine (12), (2) xanthine (220 µM) could not replace a tetrahydropteridine in the indoleamine 2,3-dioxygenase reaction, and (3) xanthine oxidase (18 µg/ml) added to the complete assay mixture did not enhance the enzymic reaction.

Figure 1 shows a comparison of the enzymic activity obtained with tetrahydrobiopterin and with ascorbic acid, which had been used as a cofactor in the original assay (2). In the presence of methylene blue, the degree of stimulation of the oxidative ring cleavage of L-tryptophan by tetrahydrobiopterin was less than that observed with ascorbic acid. However, it should be noted that in the absence of methylene blue, tetrahydrobiopterin gave appreciable stimulation whereas ascorbic acid was essentially inactive. Figure 1 also shows that all the reactions are inhibited at high L-tryptophan concentration, as was originally reported for the reaction with ascorbic acid plus methylene blue as cofactors (2). Similar observations were made with $DMPH_4$ as a cofactor. When D-tryptophan was used as a substrate, substrate inhibition was not observed in the reaction stimulated by tetrahydropteridines. This is

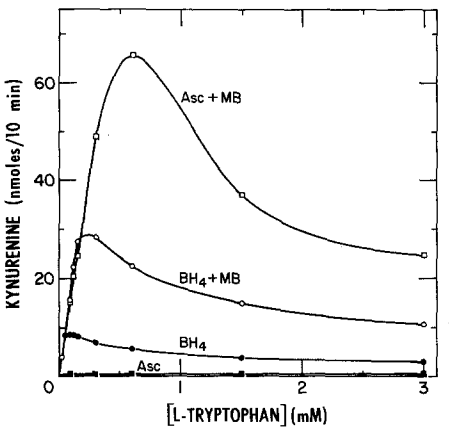

Figure 1 - Comparison of indoleamine 2,3-dioxygenase reactions with tetrahydrobiopterin and ascorbic acid as cofactors. The reaction mixtures (0.2 ml) contained L-tryptophan (concentrations indicated), catalase (10 µg), the enzyme (16 µg), and 410 µM tetrahydrobiopterin in 50 mM potassium phosphate buffer, pH 7.5 in the presence (-o-) and absence (-●-) of 5 µM methylene blue. Kynurenine was assayed colorimetrically after incubating the mixture as specified in Table I. Similar experiments were performed using 5 mM sodium ascorbate in place of tetrahydrobiopterin in the presence (-□-) and absence (-■-) of 5 µM methylene blue.

similar to what was observed with D-tryptophan in the reaction stimulated by ascorbic acid plus methylene blue (2). The maximum activity obtained with D-tryptophan was less than that obtained with L-tryptophan in either the presence or absence of methylene blue.

The maximum activity for the oxidative ring cleavage of L-tryptophan stimulated by tetrahydrobiopterin alone was observed at 200 µM, and about 70 µM tetrahydrobiopterin gave half-maximal activity. The ability of tetrahydrobiopterin to give substantial activity at such a low concentration suggests that it may act as a natural cofactor of indoleamine 2,3-dioxygenase.

Effect of Superoxide Dismutase - Recent studies have shown that a number of oxygenases involve $O_2^{\cdot-}$ in their mechanisms (5,6,15,16) and it has been indicated that indoleamine 2,3-dioxygenase can utilize $O_2^{\cdot-}$ in its enzymic reaction (5,6). Since $O_2^{\cdot-}$ is formed during the autoxidation of tetrahydropteridines (8,9), it was predicted that the indoleamine 2,3-dioxygenase reaction

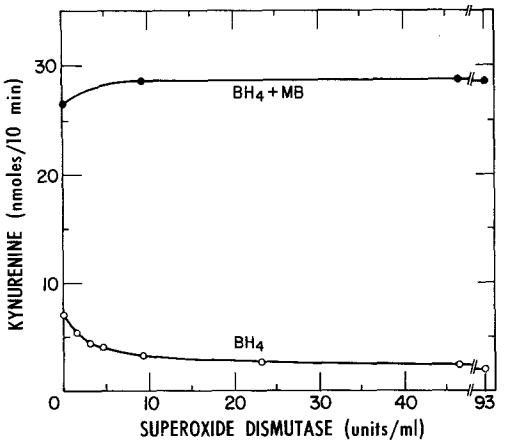

Figure 2 - Effect of superoxide dismutase on the indoleamine 2,3-dioxygenase reaction. The assay mixtures (0.2 ml) contained 150 μM L-tryptophan, 410 μM tetrahydrobiopterin, catalase (10 μg), the enzyme (13 μg) and indicated amounts of superoxide dismutase in 50 mM potassium phosphate buffer, pH 7.5 in the presence (-o-) and absence (-●-) of 5 μM methylene blue. Kynurenine was assayed after incubating the mixtures as specified in Table I.

stimulated by tetrahydropteridine would be inhibited by superoxide dismutase which catalyzes the dismutation of $O_2^{\cdot-}$ (10). As shown in Fig. 2, the reaction stimulated by tetrahydrobiopterin alone was inhibited by superoxide dismutase, maximum inhibition being 70%. Thus, at least 70% of the reaction involves $O_2^{\cdot-}$ ions to which superoxide dismutase is accessible. The amount of superoxide dismutase required to achieve 50% of the maximum inhibition was 3.2 units/ml. The inhibition by superoxide dismutase of the indoleamine 2,3-dioxygenase reaction stimulated by ascorbic acid plus methylene blue was previously reported (6). On the other hand, the reaction stimulated by tetrahydrobiopterin plus methylene blue was slightly activated by superoxide dismutase. This activation is probably due to inhibition of the autoxidation of tetrahydrobiopterin by superoxide dismutase (8,9). The oxygenase reaction stimulated by tetrahydrobiopterin plus methylene blue is not considered to be mediated by $O_2^{\cdot-}$ accessible to the dismutase. In analogy to the mechanism of tryptophan 2,3-dioxygenase (17) the heme of indoleamine 2,3-dioxygenase should be in the ferroheme form when it interacts with tryptophan and O_2. The above findings

suggest that both $O_2^{\cdot-}$ and a tetrahydropteridine serve as electron donor in the reduction of the ferriheme form to ferroheme form.

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