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A DIRECT ASSAY FOR LIVER PHENYLALANINE HYDROXYLASE

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SUMMARY

1. An assay for liver phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) based on the non-enzymatic regeneration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine from 2-amino-4-hydroxy-6,7-dimethyldihydropteridine (quinonoid form) by dithiothreitol is described.

Phenylalanine + O_2 + 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine → tyrosine + H_2O + 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (quinonoid form)

2-Amino-4-hydroxy-6,7-dimethyldihydropteridine (quinonoid form) + dithiothreitol → 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine + oxidized dithiothreitol

Dithiothreitol not only regenerates the tetrahydropteridine from dihydropteridine but it also effectively destroys peroxide formed during the aerobic oxidation of the tetrahydropteridine.

2. The apparent K_m values for each of the substrates were: 1.12 mM phenylalanine; 0.31 atm O_2, 67 μM 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine.

INTRODUCTION

Much progress in elucidating the mechanism of the enzymatic hydroxylation of phenylalanine to tyrosine has been made since MITOMA[1] showed this process to require two enzymes and DPN^+. Subsequently, the decisive work of KAUFMAN[2–4] not only identified the enzymes as dihydropteridine reductase and phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) but also demonstrated the role of unconjugated pteridines in the conversion of phenylalanine to tyrosine. These studies of KAUFMAN[2–4] defined for the first time a metabolic role for unconjugated pteridines which previously were known as naturally occurring growth factors for protozoa[5–7]. Recently, GUROFF *et al.*[8] have presented strongly suggestive evidence that several enzymatic hydroxylations including that of

Abbreviations: Pt, 2-amino-4-hydroxy-6,7-dimethylpteridine; 7,8-H_2Pt, 2-amino-4-hydroxy-6,7-dimethyldihydropteridine; H_4Pt, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; DCIP, 2,6-dichlorophenolindophenol.

phenylalanine occur by mechanisms involving enzyme-bound OH^+ as the direct hydroxylating agent. Recently STORM AND KAUFMAN[9] have suggested that oxygen is reduced to peroxide prior to hydroxylation and that all three substrates are bound to the enzyme before release of any of the products.

Nevertheless, the study of the enzymatic hydroxylation of phenylalanine has been hampered by the lack of an assay which measures the hydroxylase unambiguously in the absence of dihydropteridine reductase and a TPNH-generating system. This paper describes such an assay, based on the observation of KAUFMAN[10] that certain reductants can rapidly reduce 2-amino-4-hydroxy-6,7-dimethyldihydropteridine (7,8-H_2Pt) formed during the reaction to 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (H_4Pt), and some of the properties of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Phenylalanine hydroxylase was prepared from rat liver through the second $(NH_4)_2SO_4$ step of the procedure of KAUFMAN[11]. None of the preparations used destroyed tyrosine. H_4Pt, prepared by catalytic hydrogenation[12] from recrystallized[13] 2-amino-4-hydroxy-6,7-dimethylpteridine (Pt) (Aldrich), was divided into separate portions which were stored in separate tubes at $-15°$ (ref. 14). The reduction was always quantitative as determined by titration with 2,6-dichlorophenolindophenol (DCIP)[15] standardized with ascorbic acid. The reduction product chromatographed* as a single component on silica gel G (Brinkmann) developed by 0.05 M potassium phosphate (pH 6.8) under 100% nitrogen. The chromatographed product was visualized fluorometrically and by decolorizing DCIP. 7,8-H_2Pt, prepared from H_4Pt by aerobic oxidation in 0.1 M potassium phosphate (pH 6.8) had the same spectra in acid and alkali as a similar preparation described by KAUFMAN[15]. Phenylalanine, tyrosine, DPNH, cysteine, and GSH were obtained from Sigma. Dithiothreitol, dithioerythritol, and 2-mercaptoethylamine were products of Calbiochem. D-Araboascorbic acid was purchased from Matheson, Coleman and Bell. Protein was measured by the biuret test with bovine serum albumin (Sigma) as standard[16].

Assay for phenylalanine hydroxylase

The standard system (1.0 ml) contained (in μmoles): Tris–HCl (pH 7.2), 100; freshly prepared dithiothreitol, 10; H_4Pt, 0.4; L-phenylalanine, 2; and enzyme. Acidic or basic solutions were neutralized with KOH or HCl before they were added to the system. The complete system except H_4Pt was incubated for 5 min at 25°. H_4Pt was then added and, unless otherwise indicated, the system was incubated for 20 min at 25° with shaking in air. The reaction was stopped by the addition of 2 ml 12% trichloroacetic acid. Tyrosine in 2-ml aliquots of the deproteinized solution was estimated colorimetrically[17] or fluorometrically[18]. Occasionally the reaction was initiated with phenylalanine instead of H_4Pt.

Processing of kinetic data

Double reciprocal plots of substrate concentrations against initial velocities

* T. A. LLOYD, personal communication.

(formation of tyrosine during a 20-min period) were made. Any point obviously in error was discarded. The kinetic constants were then calculated from the remaining data by a digital computer with the program of CLELAND[19].

RESULTS

Effect of varying enzyme concentration and time on the reaction

The data in Fig. 1a show that the rate of formation of tyrosine is directly proportional to the amount of enzyme added over a 40-fold range in protein concentration. The formation of tyrosine is also linear with time for 30 min at a single enzyme level (Fig. 1b). However, if the reaction is begun by the addition of enzyme rather than H_4Pt, the rate of formation of tyrosine is still linear with time but occasionally the extrapolated line intersects the ordinate above the origin. These results are likely due to inhibition of the enzyme by sulfhydryl compounds[11].

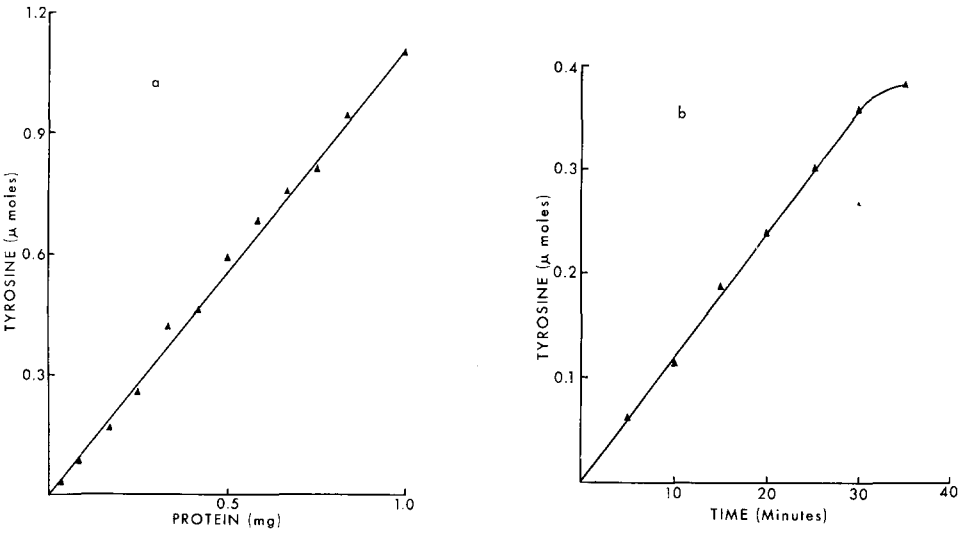

Fig. 1. The influence of enzyme concentration and time on the reaction. a. The indicated concentrations of protein were incubated for 20 min in the system described under EXPERIMENTAL PROCEDURE. b. 0.12 mg of protein was incubated for the indicated times.

Specificity of reducing agent

The ability of various reductants to replace dithiothreitol in the incubation system is shown in Table I. All sulfhydryl compounds examined and DPNH stimulated the reaction; ascorbate was without effect and ferrous sulfate was inhibitory. Dithioerythritol, dithiothreitol, and GSH produced the greatest stimulations. However, GSH as well as the other monothiols interfered with colorimetric estimation of tyrosine[17]. The amount of color produced by these compounds in zero-time controls in the nitrosonaphthol test varied somewhat from one experiment to another. Typical results are given in Table I. On the other hand, the two dithiols tested not only were most effective in stimulating the reaction, but they consistently did not interfere with the determination of tyrosine.

TABLE I

SPECIFICITY FOR REDUCING AGENT

The system used was that described under EXPERIMENTAL PROCEDURE with 0.34 mg of the second $(NH_4)_2SO_4$ fraction and with the indicated replacements for dithiothreitol.

Addition	*Amount (μmoles)*	*Tyrosine* (nmoles)*	
		At zero time	*After 20-min incubation*
None		8	64
L-Ascorbate	10	22	58
D-Araboascorbate	10	9	68
GSH	10	192	660
Cysteine	10	132	475
Mercaptoethylamine	10	27	205
Mercaptoethanol	10	55	143
Thioglycolate	10	16	272
Dithioerythritol	5	0	672
Dithiothreitol	5	5	662
$FeSO_4$	2	6	21
DPNH	4	30	151

* The apparent amount of tyrosine measured colorimetrically.

The effect of varying pH on the reaction

The rate of the reaction (Fig. 2) increases as the pH is raised from 6.0 to 7.2 and falls as the pH is increased beyond 7.2. The observation that the velocities of the reactions in Tris and phosphate buffers at pH 7.2 are the same indicates that the chemical reduction of the quinonoid form of 7,8-H_2Pt formed during the reaction is much faster than the phosphate-catalyzed isomerization of the quinonoid form of

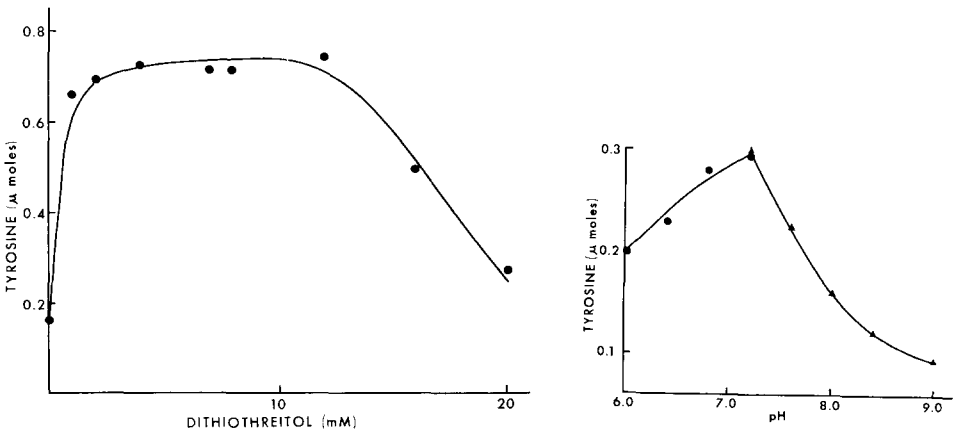

Fig. 2. Effect of varying pH on the activity of phenylalanine hydroxylase. The system used was that described under EXPERIMENTAL PROCEDURE with 0.17 mg of protein and with the buffers indicated. ●, in 0.1 M potassium phosphate; ▲, in 0.1 M Tris–HCl.

Fig. 3. The effect of various concentrations of dithiothreitol on the reaction. The system used is that described under EXPERIMENTAL PROCEDURE except that the concentration of dithiothreitol was varied as indicated; 0.33 mg of protein was used.

7,8-H_2Pt to 7,8-H_2Pt (ref. 20). Dithiothreitol does not reduce 7,8-H_2Pt to H_4Pt (see below).

The influence of O_2 tension on the reaction

The velocity of the reaction increases with increasing O_2 tensions from 0.042 to 1.00 atm. Double reciprocal plots were linear over the range tested; the apparent K_m for O_2 is 0.31 $\pm$ 0.02 atm* and the $v_{max} = 1.50 \pm 0.04$ μmoles tyrosine per 20 min per 0.15 mg protein. It was necessary to initiate the reaction in this experiment with phenylalanine rather than H_4Pt, since 1 atm O_2 destroyed H_4Pt without dithiothreitol in the side arm of the Warburg vessel during the equilibration period.

The effect of various concentrations of phenylalanine on the reaction

Double reciprocal plots of the effect of varying the concentration of phenylalanine from 0.1 to 8.0 mM are linear; the apparent K_m of phenylalanine is 1.12 $\pm$ 0.05 mM and the $v_{max} = 209 \pm 5$ nmoles tyrosine per 20 min per 46 μg protein.

The influence of varying the concentration of dithiothreitol on the reaction

The rate of the reaction increases with increasing concentrations of dithiothreitol to about 2 mM (Fig. 3). The velocity of the reaction does not change as the concentration of dithiothreitol is raised from 2 to 12 mM, but concentrations above 12 mM are distinctly inhibitory.

The influence of the concentration of H_4Pt on the reaction at three concentrations of dithiothreitol

In determining the apparent K_m of H_4Pt, it is essential to eliminate the possibility that the rate of the chemical reduction of 7,8-H_2Pt by dithiothreitol influences the observed constant. If the enzymatic reaction is rate-limiting at a given concentration of dithiothreitol, the apparent K_m should not be affected by the faster rate of reduction of 7,8-H_2Pt to H_4Pt at a higher concentration of dithiothreitol. Analysis of the computor-fitted kinetic constants for the data in Fig. 4 by the "Students" t test reveals no significant difference between the effects of different concentrations of H_4Pt at 2.5, 5, and 10 mM dithiothreitol. Thus, changing the concentration of dithiothreitol in this range has no noticeable effect on either v_{max} or the apparent K_m (67 $\pm$ 4 μM) for H_4Pt. Therefore, the rate of regeneration of H_4Pt from 7,8-H_2Pt is faster at these three concentrations of dithiothreitol than the enzymatic hydroxylation and the observed apparent K_m for H_4Pt is independent of the rate of the nonenzymatic regeneration of H_4Pt.

The effect of 7,8-H_2Pt on the reaction

Since the quinonoid form of 7,8-H_2Pt produced during the reaction readily isomerizes to 7,8-H_2Pt (refs. 20, 21), dithiothreitol could reduce either or both of these isomers. Unfortunately, the quinonoid form is too unstable for quantitative experiments. However, the data in Table II shows that dithiothreitol does not reduce 7,8-H_2Pt to H_4Pt, since the addition of dithiothreitol and 7,8-H_2Pt to a system which is active in the presence of H_4Pt and dithiothreitol does not lead to the formation of tyrosine. A double reciprocal plot of [H_4Pt] *vs.* the velocity of the reaction at three

* 1 atm is 627.51 mm Hg in Denver.

TABLE II

THE FAILURE OF DITHIOTHREITOL TO REDUCE 7,8-H_2Pt

The system used is that described under EXPERIMENTAL PROCEDURE with 0.30 mg protein, 250 μmoles potassium phosphate (pH 6.8), and the indicated pteridines.

Pteridines added (μmoles)		*Tyrosine formed (nmoles)*
7,8-H_2Pt	*H_4Pt*	
—	0.2	310
1.0	—	1
1.0	0.2	267
2.0	—	1
2.0	0.2	219

concentrations of 7,8-H_2Pt is shown in Fig. 5. The secondary replot of the slopes *vs.* the inhibitor concentration is linear showing 7,8-H_2Pt to be a linear competitive inhibitor (K_i = 0.35 mM) with respect to H_4Pt.

Effect of catalase on the rate of the reaction at different concentrations of H_4Pt in the absence of dithiothreitol

The time course of the reaction in the absence of dithiothreitol at three different concentrations of H_4Pt was followed in an effort to learn why relatively little tyrosine is formed in the absence of a H_4Pt-regenerating system (Table I). The data in Fig. 6a show that under these conditions the rate of tyrosine formation progressively falls and that there is an inverse relationship between tyrosine formation and the initial

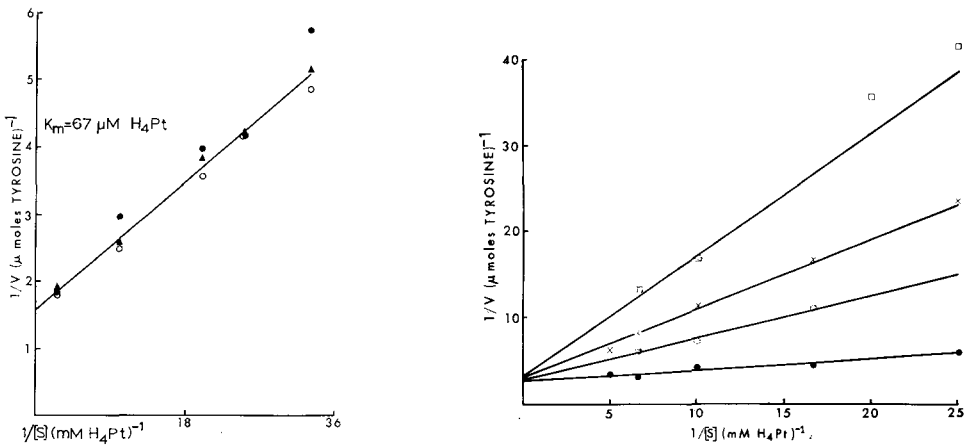

Fig. 4. Double reciprocal plots of the effect of various concentrations of H_4Pt on the reaction at three concentrations of dithiothreitol. The system used is that described under EXPERIMENTAL PROCEDURE except that the concentrations of H_4Pt and dithiothreitol were as indicated. ●, 2.5 mM dithiothreitol; ▲, 5 mM dithiothreitol; ○, 10 mM dithiothreitol.

Fig. 5. Lineweaver–Burk plots for H_4Pt at three concentrations of 7,8-H_2Pt. The system used is described under EXPERIMENTAL PROCEDURE with 0.17 mg protein in the absence of 7,8-H_2Pt (●), at 1 mM 7,8-H_2Pt (○), at 2 mM 7,8-H_2Pt (×), at 4 mM 7,8-H_2Pt (□). The vertical intercepts (in μmoles tyrosine^{-1}) for the computer-derived lines are in order of increasing concentrations of 7,8-H_2Pt: 2.54 ± 0.15, 2.72 ± 0.45, 2.94 ± 0.38, 3.14 ± 1.48. The calculated K_i for 7,8-H_2Pt = 0.35 ± 0.03 mM.

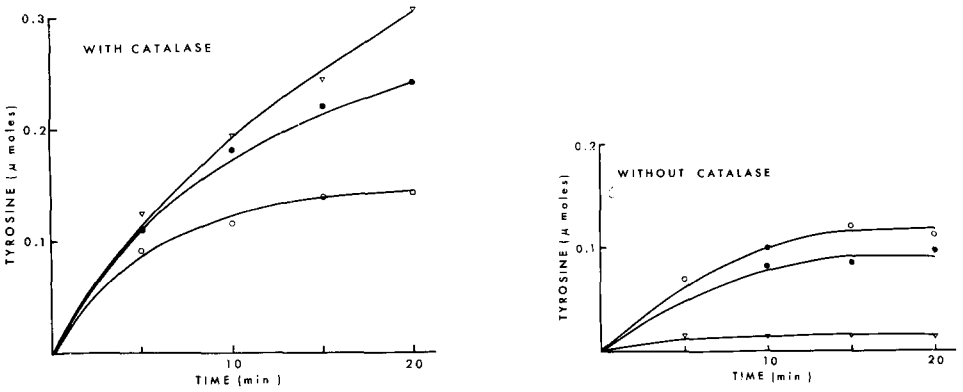

Fig. 6. The effect of catalase on the reaction at various concentrations of H_4Pt in the absence of dithiothreitol. The system used was that described in the text except that dithiothreitol was omitted, the indicated amounts of H_4Pt were added, and the incubation was run for the indicated times. 0.27 mg protein was added. ○—○, 0.2 mM H_4Pt; ●—●, 0.4 mM H_4Pt; △—△, 1.0 mM H_4Pt. Catalase, 0.2 mg, was added where indicated.

concentration of H_4Pt. The decreases in velocities cannot be attributed entirely to depletion of H_4Pt since increasing the concentration of H_4Pt actually inhibits tyrosine formation. Depression of tyrosine formation must be due in part to a compound produced from H_4Pt which is removed by dithiothreitol, since Fig. 3 shows that in the presence of dithiothreitol, concentrations of less than 2 mM H_4Pt are not inhibitory. Since it is known that H_4Pt is aerobically oxidized to 7,8-H_2Pt and H_2O_2 (ref. 20), the time course of the reaction was followed at various concentrations of H_4Pt in the presence of catalase. The rate of formation of tyrosine is faster when catalase is added than when it is omitted (Fig. 6). Of greater significance is the observation that the rate of the reaction with catalase increases as the concentration of H_4Pt is raised. Thus peroxide formed from H_4Pt in the absence of catalase is causing the inverse relationship between the concentration of H_4Pt and reaction rate. The fall in rate of tyrosine formation with time in the presence of catalase appears to be due primarily to depletion of H_4Pt. KAUFMAN[2] previously described the protection of H_4Pt by catalase.

DISCUSSION

This paper describes an assay system for phenylalanine hydroxylase based on the chemical rather than the enzymatic regeneration of H_4Pt from the quinonoid form of 7,8-H_2Pt. Dithiothreitol and dithioerythritol were the most effective reducing agents in stimulating the reaction as well as the only sulfhydryl compounds which did not interfere with the colorimetric determination of tyrosine. The chemical reduction of 7,8-H_2Pt by three different concentrations of dithiothreitol does not appear to be rate-limiting even at the lowest level of H_4Pt, since the apparent K_m's for H_4Pt at these three concentrations of dithiothreitol are not significantly different. However, the rates of the enzymatic reaction measured in this system are those of a slightly inhibited enzyme, since 10 mM dithiothreitol depresses the activity of the hydroxylase in the system of KAUFMAN[11] about 10% (C. BUBLITZ, unpublished observation). Nevertheless,

these compounds are distinctly less inhibitory than 2,3-dimercaptopropanol[11]. Dithiothreitol also appears to protect the enzyme from peroxide formed during the aerobic oxidation of H_4Pt, since the velocity of the reaction in the absence of dithiothreitol is inversely related to the concentration of H_4Pt. However, there is a direct relation between the reaction rate and the concentration of H_4Pt if catalase is added. KAUFMAN[2] has found that catalase protects H_4Pt from aerobic oxidation.

Michaelis–Menten kinetics are followed by varying the concentrations of all three substrates at low concentrations. When assayed under the present conditions, none of the substrates inhibited the reaction.

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