INHIBITION OF PHENYLALANINE HYDROXYLASE BY p-CHLOROPHENYLALANINE; DEPENDENCE ON COFACTOR STRUCTURE

J. E. Ayling and G. D. Helfand
Department of Biological Chemistry, School of Medicine,
University of California, Los Angeles, California 90024

Received September 16, 1974

The reported discrepancy between the in vitro and in vivo properties of p-chlorophenylalanine as an inhibitor of phenylalanine hydroxylase (E.C.1.14.3.1) was investigated. It was demonstrated that the lack of inhibition, in vitro, was not due to (1) non-physiological pH or temperature of the in vitro assay system, (2) inhibition by m-chlorotyrosine, a product of the enzymatic hydroxylation of p-chlorophenylalanine, or (3) a slow irreversible reaction of p-chlorophenylalanine with enzyme. However, when the inhibitory properties of p-chlorophenylalanine were determined using the natural cofactor, tetrahydrobiopterin, instead of the pseudocofactor 6,7-dimethyltetrahydropterin, which had been utilized in the reported in vitro studies, it was shown that p-chlorophenylalanine is a potent inhibitor of the enzymatic hydroxylation of phenylalanine. The apparent K_i is 0.03mM with tetrahydobiopterin as cofactor, compared to 1.5mM with 6,7-dimethyltetrahydropterin. The dependence of the inhibitory properties of an aromatic amino acid analog on the structure of the cofactor may be a general phenomenon with all tetrahydrobiopterin dependent aromatic amino acid hydroxylases.

p-Chlorophenylalanine has been used extensively as an in vivo inhibitor of phenylalanine hydroxylase for the induction of symptoms of phenylketonuria in experimental animals (e.g. 1,2). Yet it has been reported that, in vitro, p-chlorophenylalanine inhibits phenylalanine hydroxylase poorly, if at all, at concentrations which are effective in vivo (1,3). Although various hypotheses have been proposed to account for the lack of agreement between in vitro and in vivo data (4,5,6), little attention has been given to differences in the environment of the enzyme when assayed in vitro compared to its physiological environment. For example, differences in pH, temperature, or the use of pseudosubstrates or pseudocofactors could sufficiently affect an enzyme that its response to an inhibitor may be changed.

In this report we demonstrate that the discrepancy between the in vivo and in vitro inhibition by p-chlorophenylalanine can, at least in part, be attributed to pseudocofactor used in published in vitro assay systems. These results may be of relevance in the interpretation of inhibitory data with all tetrahydrobiopterin dependent hydroxylases.

MATERIALS AND METHODS

6,7-Dimethyltetrahydropterin, DL-p-chlorophenylalanine and tyrosine were purchased from Calbiochem; L-phenylalanine, dithiothreitol and catalase from Sigma; DL-m-chlorotyrosine from K and K; and protamine sulfate from Elanco division of Eli Lilly. Tetrahydrobiopterin was generously donated by Dr. K. J. M. Andrews, Roche Products Ltd., Hertfordshire, England. Since D-phenylalanine has no sub-

Copyright © 1974 by Academic Press, Inc.
All rights of reproduction in any form reserved.

strate or inhibitory activity with phenylalanine hydroxylase, it was assumed that D-chlorophenylalanine, also, would not react with enzyme. The concentration of p-chlorophenylalanine in kinetic experiments was, therefore, taken as half the total concentration of DL-p-chlorophenylalanine.

Phenylalanine hydroxylase was partially purified from rat livers. Livers were homogenized in 3 volumes of 0.2M Tris-HCl-10mM mercaptoethanol, pH 7.5. The homogenate was centrifuged at 20,000 g for 15 minutes, and the supernatant was treated with 0.2 volumes of 2% protamine sulfate in 0.1M Tris-HCl, pH 7.5. The protamine sulfate supernatant was fractionated with ammonium sulfate. Phenylalanine hydroxylase precipitates between 35 and 45% saturation with ammon sulfate. The precipitate containing the enzyme was dissolved in 0.1M Tris-HCl, pH 7.5 and frozen in liquid N_2, in aliquots.

Enzyme activity was monitored spectrophotometrically as the phenylalanine dependent rate of dihydropteridine formation, at 340nm for dihydrobiopterin, and at 330nm for 6,7-dimethyldihydropterin (7). Rates were measured in a double beam spectrophotometer in which the reference cell contained all the components of the reaction cell, except phenylalanine. The kinetics of inhibition by p-chlorophenylalanine, with 6,7-dimethyltetrahydropterin as cofactor, were also determined from the amount of tyrosine formed after a fixed incubation time in the presence of 5mM dithiothreitol to regenerate cofactor (8). Tyrosine was quantitated as its nitrosonaphthol derivative, either spectrophotometrically (9), or fluorometrically (10). Tyrosine standards, containing all components of the reaction mixture, were run with each experiment. Catalase, 0.25 mg (4,000 units), was included in all reactions to diminish the non-enzymatic rate of oxidation of the cofactor. For either method of assay, enzyme was equilibrated with buffer, phenylalanine and catalase for 5 minutes at the reaction temperature before pteridine cofactor was added to initiate the reaction. The total volume was 1 ml and, unless otherwise stated, the temperature was 27°. All kinetic assays were performed at atmospheric O_2, in 0.1M Tris-HCl, pH 7.4, with 4-12 mU rat liver phenylalanine hydroxylase. 1mU is an amount of enzyme which produces 1 nmole of product per min. at pH 7.4 and 27° in the presence of 1mM phenylalanine and 0.2mM 6,7-dimethyltetrahydropterin. Kinetic data were analyzed by a graphics computer program (11).

RESULTS

Inhibition as a function of p-chlorophenylalanine concentration. In the presence of 6,7-dimethyltetrahydropterin, hydroxylation is only slightly inhibited by 0.5 mM p-chlorophenylalanine (Fig. 1). This is in agreement with the published findings of others (1,3). However, with the natural cofactor, tetrahydrobiopterin, the reaction is >90% inhibited by 0.5mM p-chlorophenylalanine (Fig. 1A). In both cases Michaelis-Menten kinetics are observed, since

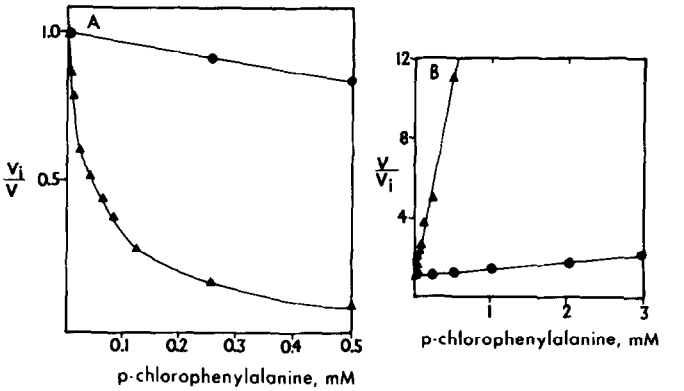

Figure 1. Inhibition by p-chlorophenylalanine in the presence of two different cofactors. (●) 6,7-Dimethyltetrahydropterin as cofactor at a concentration of 0.2mM; phenylalanine concentration is 1mM. (▲) Tetrahydrobiopterin as cofactor, at a concentration of 0.05mM; phenylalanine concentration is 0.3mM. The concentrations of cofactor and phenylalanine have been chosen to give comparable conditions with either cofactor, since the K_m of 6,7-dimethyltetrahydropterin is 3-4 times higher than the K_m of tetrahydrobiopterin (12), and the K_m of phenylalanine with 6,7-dimethyltetrahydropterin as cofactor is 3-4 times higher than with tetrahydrobiopterin. (A) The ratio of v_i, the inhibited rate, to v, the rate in the absence of inhibitor, is plotted as a function of inhibitor (p-chlorophenylalanine) concentration. (B) The reciprocals of the rates in (A) are plotted as a function of inhibitor concentration (Dixon plot (13)).

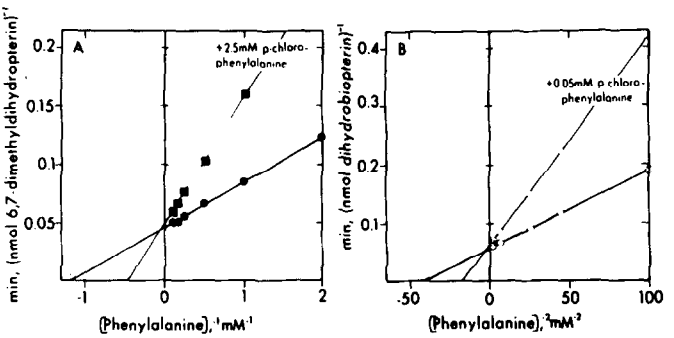

Figure 2. Inhibition by p-chlorophenylalanine with respect to phenylalanine. (○) Control curve; (□) + p-chlorophenylalanine. See legend to Fig. 1. (A) The cofactor is 6,7-dimethyltetrahydropterin, 0.2mM, and the inhibitor concentration is 2.5mM. Reciprocal velocity is plotted against reciprocal phenylalanine concentration. (B) The cofactor is tetrahydrobiopterin, 0.05mM, and the inhibitor concentration is 0.05mM. The reciprocal of the velocity is plotted against the reciprocal of the square of the phenylalanine concentration, since, in the presence of tetrahydrobiopterin, the K_m curve for phenylalanine is sigmoidal, with a Hill coefficient of 2 (14).

---

a plot of control rate/inhibited rate vs, the inhibitor concentration (Dixon plot) gives a straight line (Fig. 1B). Under the conditions of the assay, 50% inhibition is reached at 0.045 mM p-chlorophenylalanine when tetrahydrobiopterin is cofactor, but 2.5 mM p-chlorophenylalanine is required to give 50% inhibition with 6,7-dimethyltetrahydropterin.

Type of inhibition and K_i. The type of inhibition with respect to phenylalanine is close to competitive, regardless of which cofactor is used (Fig. 2A, 2B). When the inhibition constants are determined under comparable conditions, the apparent K_i is 1.5mM with 6,7-dimethyltetrahydropterin as cofactor, and 0.03mM with tetrahydrobiopterin.

When phenylalanine concentration is held constant and cofactor concentration is varied, extrapolation to infinite cofactor concentration does not remove the inhibition. The observed mixed inhibition means that p-chlorophenylalanine, in addition to inhibiting the interconversion of ES complexes to form products, appears to increase the Km of the cofactor. This occurs with either cofactor (Fig. 3A,B).

Inhibition by product. p-Chlorophenylalanine is slowly hydroxylated by phenylalanine hydroxylase. The major product is m-chlorotyrosine (15). Since in vivo inhibition could result from a metabolite of p-chlorophenylalanine, the effect of m-chlorotyrosine on the enzyme was determined. There appeared to be no inhibition of phenylalanine hydroxylase by concentrations of m-chlorotyrosine up to 1mM, with 6,7-dimethyltetrahydropterin as cofactor, as already noted (1), and only slight inhibition with tetrahydrobiopterin as cofactor.

Effect of temperature on inhibition. The preceding kinetic experiments were all performed at 27°. To ascertain whether or not the degree of inhibition and the cofactor dependence of the inhibition would be the same at physiological temperatures, the inhibition as a function of p-chlorophenylalanine concentration was compared at 27° and 37°. The percentage inhibition, and the cofactor dependence of the inhibition, was very similar at the two temperatures.

Reversibility of inhibition. The above interpretation of the kinetics of

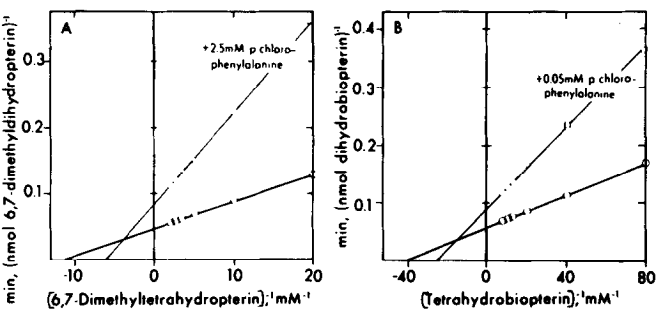

Figure 3. Inhibition by p-chlorophenylalanine with respect to cofactor. (○) control curve; (□) + p-chlorophenylalanine. See legend to Fig. 1. (A) The phenylalanine concentration is 1mM, and the inhibitor concentration is 2.5mM. Reciprocal velocity is plotted against reciprocal 6,7-dimethyltetrahydropterin concentration. (B) The phenylalanine concentration is 0.3mM, and the inhibitor concentration is 0.05mM. Reciprocal velocity is plotted against reciprocal tetrahydrobiopterin concentration.

inhibition by p-chlorophenylalanine was based on the assumption that the inhibition of phenylalanine hydroxylase was reversible. However, the in vivo effectiveness of p-chlorophenylalanine could also be due to irreversible inhibition of the enzyme. In order to demonstrate whether or not this was the case, a concentrated solution of enzyme was preincubated with 1mM p-chlorophenylalanine for 48 hours at 0. The preincubated enzyme, 0.02 ml, was assayed with 0.2 μmole 6,7-dimethyltetrahydropterin and 1 μmole phenylalanine in a total volume of 1 ml, so that p-chlorophenylalanine was diluted out to 0.02mM. Activity was measured directly from the rate of cofactor utilization. There was no inhibition or loss of enzyme activity other than that which occurred in control enzyme. Since it was possible that irreversible inhibition of enzyme by p-chlorophenylalanine might only occur in the presence of cofactor, enzyme was preincubated with 1mM p-chlorophenylalanine, 0.05mM tetrahydrobiopterin and 20mM dithiothreitol for 96 hours at 0°, or for 9 hours at 25°. Activity was measured by the tyrosine assay, in the same reaction mixture as above. Under these conditions, also, it was not possible to demonstrate any irreversible inhibition of phenylalanine hydroxylase. Therefore, if p-chlorophenylalanine can irreversibly inactivate the enzyme, it must be an extremely slow reaction.

Similar experiments were conducted with 0.2mM 6,7-dimethyltetrahydropterin instead of tetrahydrobiopterin in the preincubation mixture, and also with 1mM m-chlorotyrosine in place of p-chlorophenylalanine. In all cases, no irreversible inhibition of enzyme could be detected.

DISCUSSION

Previously reported studies of the in vitro inhibition of phenylalanine hydroxylase by p-chlorophenylalanine utilized the commercially available pseudocofactor 6,7-dimethyltetrahydropterin (1,3). Under these conditions, high concentrations of p-chlorophenylalanine are required to produce significant inhibition of the enzymatic hydroxylation of phenylalanine. The results reported here show that when the natural cofactor, tetrahydrobiopterin, is used, the reaction of phenylalanine hydroxylase is highly sensitive to inhibition by p-chlorophenylalanine. Thus when the in vivo conditions are more closely simulated in vitro, p-chlorophenylalanine is a potent inhibitor of this enzyme. The apparent K_i is 0.03mM using a concentration of tetrahydrobiopterin close to that found in rat liver (16). At lower tetrahydrobiopterin concentrations, the inhibition would be even greater. The increased inhibition with decrease in cofactor concentration has been incorrectly interpreted to mean that p-chlorophenylalanine inhibits competitively with respect to cofactor (17, p71). However, increase in inhibition with decrease in cofactor (or substrate) concentration also occurs with mixed inhibition. As shown here, inhibition is mixed with respect to cofactor, and competitive with respect to substrate.

It has been observed that the active phenylalanine hydroxylase content of the liver is decreased to about 10% of control level 1-2 days after injection of rats with a single dose of p-chlorophenylalanine (316-360 mg/Kg, i.p.) (4,18,19), but after prolonged treatment, the levels of phenylalanine hydroxylase are 25-45% of control animals (20,21), and may eventually return to normal (22). However, even a quarter of normal activity would probably be sufficient for normal metabolism, considering that phenylalanine hydroxylase activity in rat liver is 3-4 times higher than that in human liver (23), and that humans heterozygous for phenylketonuria are able to maintain normal serum phenylalanine levels. The in vivo effectiveness of p-chlorophenylalanine, in producing symptoms of phenylketonuria in experimental animals, may thus be accounted for by the results given here, that p-chlorophenylalanine is a potent reversible inhibitor of phenylalanine hydroxylase in the presence of the natural cofactor (Fig. 1, 2B, 3B).

It appears that the dependence of the inhibitory potency of an amino acid analog on the structure of the cofactor is not a unique property of p-chlorophenylalanine. Preliminary experiments show that p-fluorophenylalanine, also, is about a 50 times more potent inhibitor with tetrahydrobiopterin as cofactor than with 6,7-dimethyltetrahydropterin (14).

The observation that the potency of an inhibitor can be controlled to such a large degree by a small change in the structure of the cofactor is not only of theoretical interest in the mapping of the active site of the enzyme, but also has significant practical value in drug design. In addition to the production of inhibitors of phenylalanine hydroxylase, analogs of aromatic amino acids have been extensively investigated in the development of inhibitors of tyrosine hydroxylase and tryptophan hydroxylase with the objective of producing clinically useful drugs for the regulation of nor-epinephrine and serotonin biosynthesis, respectively (3,17). In virtually all of this work the pseudo-cofactor, 6,7-dimethyltetrahydropterin, has been used in the in vitro assays. The results reported here suggest that a meaningful in vitro evaluation of a potential drug may require the use of the natural cofactor in the in vitro assay system.

ACKNOWLEDGMENTS. We would like to thank Dr. K. Andrews for supplying us with the tetrahydrobiopterin. This work was supported by USPHS Grants CA-15659 and HD-06576. Computing assistance was obtained from the Health Sciences Computing Facility, University of California at Los Angeles, sponsored by National Institutes of Health Special Research Resources Grant RR-3.

REFERENCES

1. Lipton, M.A., Gordon, R., Guroff, G., and Udenfriend, S. (1967) Science 156, 248-250.
2. Andersen, A.E., Rowe, V., and Guroff, G. (1974) Proc. Nat. Acad. Sci. 71, 21-25.

3. Counsell, R.E., Desai, P., Smith, T.D., and Chan, P.S. (1970) J. Med. Chem. 13, 1040-1042.
4. Guroff, G. (1969) Arch. Biochem. Biophys. 134, 610-611.
5. Gal, E.M., Roggeveen, A.E., and Millard, S.A. (1970) J. Neurochem. 17, 1221 1235.
6. Gal, E.M., and Millard, S.A. (1971) Biochim. Biophys. Acta. 227, 32-41.
7. Ayling, J.E., Pirson, R.A., Pirson, W.D., and Boehm, G.R. (1973) Anal. Biochem. 51, 80-90.
8. Bublitz, C. (1969) Biochim. Biophys. Acta. 191, 245-256.
9. Udenfriend, S., and Cooper, J.R. (1952) J. Biol. Chem., 196, 227-233.
10. Waalkes, T.P., and Udenfriend, S. (1957) J. Lab. Clin. Med. 50, 733-736.
11. Ayling, J.E. and Zarky, M. (1974) in press.
12. Ayling, J.E., Boehm, G.R., Textor, S.C., and Pirson, R.A. (1973) Biochem. 12, 2045-2051.
13. Dixon, M. (1953) Biochem. J. 55, 170-171.
14. Ayling, J.E., and Helfand, G.D. (1974) unpublished results.
15. Guroff, G., Kondo, K., and Daly, J. (1966) Biochem. Biophys. Res. Comm. 25, 622-628.
16. Guroff, G., Rhoads, C.A., and Abramowitz, A. (1967) Anal. Biochem. 21, 273-278.
17. McGeer E.G., and McGeer P.L. (1973) in Metabolic Inhibitors IV, 45-105, Hochster, Kates and Quastel, eds., Academic Press.
18. Koe, B.K. (1967) Med. Pharmacol. Exp. 17, 129-138.
19. Fuller, R.W., Snoddy, H.D., Wolen, R.L., Coburn, S.P., and Sirlin, E.M. (1972) Adv. in Enzyme Regulation 10, 153-167, Weber, ed., Pergamon Press.
20. Prichard, J.W., and Guroff, G. (1971) J. Neurochem. 18, 153-160.
21. Wapnir, R.A., Hawkins, R.L., Stevenson, J.H., and Bessman, S.P. (1970) Biochem. Med. 3, 397-403.
22. Antonas, K.N., Coulson, W.F., and Jepson, J.B., (1974) Biochem. Soc. Transactions 2, 105-107.
23. Ayling, J.E., Helfand, G.D., and Pirson, W.D. (1974) Enzyme (in press).