BBA 66264

THE EFFECTS OF SOME NUCLEOSIDES AND DITHIOTHREITOL ON THE STABILITY AND ACTIVITY OF LIVER PHENYLALANINE HYDROXYLASE

CLARK BUBLITZ

Department of Biochemistry, University of Colorado School of Medicine, Denver, Colo. 80220 (U.S.A.)

(Received November 10th, 1970)

SUMMARY

Phenylalanine and 2-amino-4-hydroxy-5,6-dimethyltetrahydropteridine, but not oxygen, protect phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating), EC 1.14.3.1) from thermal inactivation. Several nucleosides also protect the enzyme from inactivation by heat. Most, but not all, of the nucleosides and related compounds which protect the enzyme also inhibit the activity of the enzyme. Adenosine is a linear competitive inhibitor of the enzyme with respect to the tetrahydropteridine cofactor. The inhibition constant for adenosine is 12.2 mM; the dissociation constant for the adenosine–enzyme complex is 2.7 mM as measured by the ability of adenosine at several concentrations to protect the hydroxylase from slow inactivation under the conditions of the assay.

Dithiothreitol has a dual effect on the stability of the enzyme. It labilizes the enzyme to thermal inactivation at low concentrations of adenosine or phenylalanine. However, at high concentrations of adenosine or phenylalanine dithiothreitol stabilizes the enzyme. Several other sulfhydryl compounds affect the stability of the enzyme like dithiothreitol. On the other hand, most chelators without a sulfhydryl group do not alter the stability of the enzyme. Some possible implications of these findings are discussed.

INTRODUCTION

Although the elegant work of KAUFMAN[1] demonstrated the stoichiometry and cofactor requirement of the reactions catalyzed by phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.3.1), much remains to be learned about the mechanism of the reaction. GUROFF *et al.*[2] have presented strong experimental support that the mechanism must be compatible with the "NIH shift". More recently STORM AND KAUFMAN[3] have suggested that all three substrates are bound to the enzyme prior to the reaction and that an unspecified peroxide is an intermediate in the reaction. However, little is

known about possible functional groups on the enzyme beyond the suggestion of MITOMA[4] that a metal ion may be involved.

During an investigation of liver phenylalanine hydroxylase, it was noted that adenosine both inhibits the activity of this enzyme as well as protects the enzyme from inactivation by heat under certain conditions. This paper describes a study of the effect of nucleosides and substrates on the stability and activity of this enzyme.

MATERIALS AND METHODS

The following compounds were obtained from the sources indicated: adenosine, inosine, D-ribose, 5'-GMP, adenine, 5'-AMP, 3',5'-AMP, ADP, ATP, cytosine, cytidine, uridine, 2',3'-CMP, 5'-CMP, thymidine, cytosine arabinoside, and cytosine-5-carboxylic acid (Sigma); CTP, dithiothreitol, β-mercaptoethylamine (Calbiochem); 2,2'-dipyridyl (Fisher); 8-hydroxyquinoline, sulfosalicylic acid, 4,5-dihydroxy-m-benzenedisulfonic acid (Eastman); potassium ethyl xanthate (K. and K.). Recrystallized[5] 2-amino-4-hydroxy-5,6-dimethylpteridine (Aldrich) was reduced catalytically[6]. After the catalyst was filtered off, the filtrate was dried in a vacuum desiccator over NaOH. The product was then dissolved in water, divided into several portions and stored at $-20°$ (see ref. 7). Reduction was always quantitative as determined by titration of the product with 2,6-dichlorophenolindophenol[8]. The author is grateful to the following workers for their samples of rare nucleosides: purineribonucleoside (Dr. G. B. Brown, Sloan-Kettering Institute for Cancer Research, New York); 7-β-ribofuranosylguanine (Dr. K. I. Imai, Tekeda Chem. Ind., Ltd., Osaka, Japan); 2-aminoadenosine (Dr. J. A. Montgomery, Southern Research Institute, Birmingham, Alabama); 6-*N*-methyladenosine (Dr. L. B. Townsend, University of Utah, Salt Lake City, Utah). In order to dissolve the nucleosides and analogous compounds, it was sometimes necessary to heat the solutions. Phenylalanine hydroxylase was prepared from rat liver through the second ammonium sulfate step of KAUFMAN[9].

Phenylalanine hydroxylase activity was determined as previously described[10]. Tyrosine was measured by the method of UDENFRIEND AND COOPER[11]; protein was determined by the biuret test[12] with bovine serum albumin (Sigma) as standard. The inhibition constant was determined using the program of CLELAND[13] for linear competitive inhibition.

RESULTS AND DISCUSSION

Specificity of nucleosides and related compounds in protecting the enzyme from thermal inactivation

Table I shows that several nucleosides and analogous compounds protect the enzyme from denaturation by heat. Under the conditions used adenosine, purineribonucleoside, and 6-*N*-methyladenosine are most effective in stabilizing the enzyme. Deoxyadenosine and 7-β-ribofuranosylguanine are somewhat less effective; adenine, 3'-AMP, and 5'-AMP are only marginally effective. All other compounds tested at 10 mM, which were inosine, D-ribose, 5'-GMP, 3',5'-AMP, ADP, ATP, propylene glycol, cytosine carboxylate, cytosine arabinoside, thymidine, 2-deoxycytidine, CTP, CMP, uridine, and cytosine were without effect. Guanosine is at least

TABLE I

SPECIFICITY OF NUCLEOSIDES AND RELATED COMPOUNDS IN PROTECTING THE ENZYME FROM INACTIVATION BY HEAT IN THE PRESENCE OF DITHIOTHREITOL

Solutions containing 40 μmoles Tris (pH 8.0), 2 μmoles dithiothreitol, 0.45 mg protein and the components indicated below in a volume of 1.0 ml were divided into 2 equal portions. One of these was incubated in a capped test tube at 54° for 10 min and then chilled immediately in an ice bath; the other portion was kept in an ice bath. Hydroxylase activity in 0.40-ml aliquots was then determined.

Expt.	*Compound added*	*Concn. (mM)*	*Recovery of enzyme after heat treatment (%)*
I	None	—	0
	Adenosine	10	94
	2-Dexoyadenosine	10	54
	Adenine	10	11
	3′-AMP	10	7
	5′-AMP	10	7
II	None	—	1
	Adenosine	10	81
	Cytidine	10	74
III	None	—	9
	Adenosine	10	102
	Purineribonucleoside	10	105
	7-β-Ribofuranosylguanine	8.5	29
IV	None	—	1
	Adenosine	10	92
	Guanosine*	10	30
	6-*N*-Methyladenosine	10	93

* Guanosine came out of solution during the assay of hydroxylase.

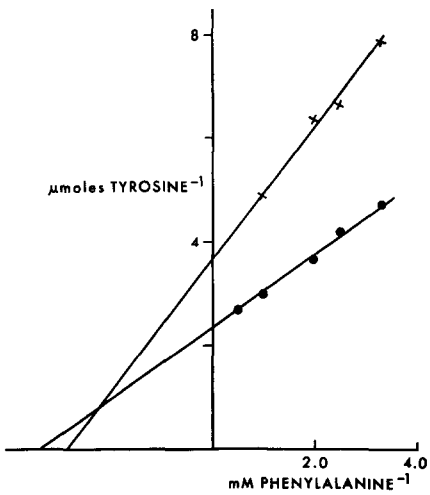

Fig. 1. Inhibition by adenosine at several concentrations of phenylalanine. The conditions used are those described in the text except that phenylalanine varied as was indicated. 0.25 mg protein was used. ×—×, with 15 mM adenosine, ●—●, without adenosine.

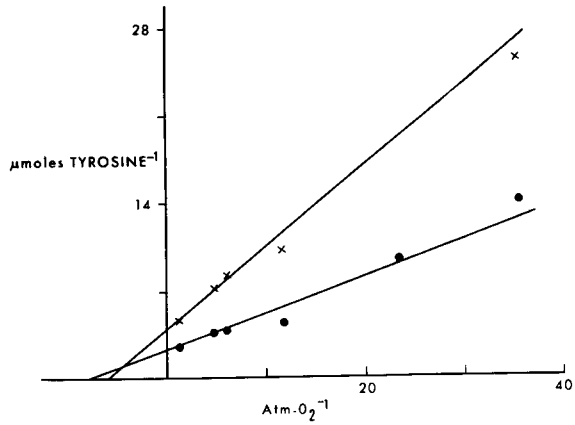

Fig. 2. Inhibition by adenosine at several concentrations of oxygen. The conditions used are those described in the text except that the reactions were carried out in Warburg flasks. The flasks were equilibrated for 10 min with mixtures of nitrogen and oxygen. The reaction was begun by addition of phenylalanine from the sidearm. ×—×, with 15 mM adenosine; ●—●, without adenosine. 0.15 mg portein was used.

as effective as 7-β-ribofuranosylguanine in protecting the enzyme; however, a more direct comparison of its effectiveness was not made because of its low solubility.

The effect of adenosine on the reaction

Since the data in Table I indicate that adenosine is bound to the enzyme, it was of interest to determine if this compound affects the activity of the enzyme. It was found that adenosine is a competitive inhibitor with respect to tetrahydropteri-

TABLE II

SPECIFICITY OF INHIBITION BY NUCLEOSIDES AND RELATED COMPOUNDS

The test system contained the components of the standard assay system except that 80 nmoles tetrahydropteridine were used. The additions were as indicated; 0.18 mg protein was added

Additions	*Amount (μmoles)*	*Tyrosine formed (nmoles)*
None	—	248
Adenosine	7	184
Adenosine	11	149
Adenosine	20	124
2-Dexoyadenosine	20	201
Adenine	20	82
ATP	20	214
Cytidine	20	198
Cytosine arabinoside	20	169
Purineribonucleoside	20	181
2-Aminoadenosine	11	196
6-*N*-Methyladenosine	20	169
Guanosine*	20	189

* Guanosine precipitated from solution during the assay of hydroxylase.

dine. Examination by Cleland's computer programs showed that the best overall fit for the data (using 5, 10, 15 mM adenosine) is provided by the program for linear competitive inhibition. The K_i for adenosine is 12.2 ± 0.1 mM. Consistent with this is the observation that a secondary plot of the slopes against inhibitor concentration for adenosine is linear. Inhibition by adenosine is non-competitive with respect to phenylalanine or oxygen (Figs. 1 and 2). The high K_i (12.2 mM) of adenosine and the observation that phosphorylated derivatives of adenosine are less effective inhibitors argue against any significant role for the "adenylate energy charge"[14] in controlling this reaction.

Specificity of inhibition by nucleosides and related compounds

Generally, the compounds which protect the enzyme from heat inactivation (Table I) also inhibit the reaction (Table II). Adenosine, deoxyadenosine, adenine, cytidine, purineribonucleoside, guanosine, and 6-*N*-methyladenosine inhibit the reaction as well as protect the enzyme. 2-Aminoadenosine inhibits the reaction some;

TABLE III

THE ABILITY OF SUBSTRATES TO PROTECT THE ENZYME FROM HEAT INACTIVATION

Solutions of 1.0 ml were prepared containing 40 μmoles Tris (pH 8.0), 2 μmoles dithiothreitol, the components indicated below and 1.05 mg enzyme which was previously filtered through a column of Sephadex G-25 equilibrated with 0.1 M Tris (pH 8.0). Tubes were made anaerobic by shaking them under a stream of moist N_2 for 12 min. Aliquots (0.5 ml) were transferred to another set of test tubes. The original set of tubes was capped and then placed in a water bath at 55° for 10 min. They were then chilled in an ice bath. Aliquots (0.20 ml) were assayed for hydroxylase activity. Air was the source of oxygen.

Additions	*Amount (μmoles)*	*Tyrosine formed (nmoles)*		*Recovery of enzyme after heat treatment (%)*
		after heat treatment	*Before heat treatment*	
None		0	259	0
O_2		0	296	0
Phenylalanine	2.0	260	331	79
H_4Pt	0.4	82	293	28
O_2 + Phenylalanine	2.0	172	306	56
O_2 + H_4Pt	0.4	90	242	37
Phenylalanine + H_4Pt	2.0 0.4	338	366	92
O_2 + Phenylalanine + H_4Pt	2.0 0.4	308	364	88

its ability to protect the enzyme was not tested. However, adenine, cytosine arabinoside and possibly ADP and ATP inhibit the reaction without protecting the enzyme. On the other hand, 7-β-ribofuranosylguanine and possibly 3'-AMP and 5'-AMP protect the enzyme without inhibiting the reaction. The following compounds produced less than 10% inhibition: inosine, ribose, GMP, 5'-AMP, ADP, propylene glycol, cytosine, uridine, 2',3'-CMP, 5'-CMP, CTP, thymidine, cytosine carboxylic acid (tested at 20 mM); 7 mM 7-β-ribofuranosylguanine; 4 mM 3',5'-AMP.

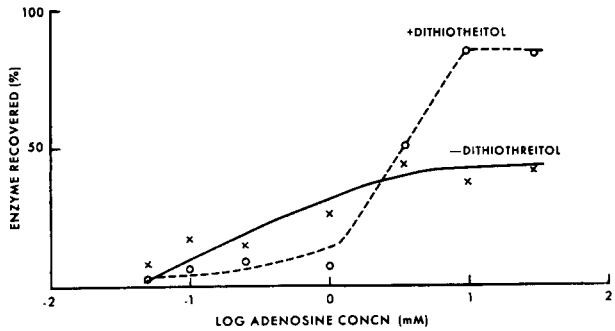

Fig. 3. Stability of the enzyme to heat inactivation at several concentrations of adenosine in the presence and absence of dithiothreitol. Solutions of 1 0 ml containing 1.17 mg enzyme previously filtered through a column of Sephadex G-25 equilibrated with 0.1 M Tris (pH 8.0), 40 μmoles Tris (pH 8.0) and the indicated components were divided into two equal portions. One portion was kept in an ice bath; the other portion was incubated in capped tubes for 10 min at 58° before being chilled in an ice bath. Hydroxylase activity in 0 4 ml aliquots of the heated and unheated preparations were compared. Where indicated 2 mM dithiothreitol was added during the heat treatment.

The effect of substrates on the stability of the enzyme to heat

Since adenosine, a competitive inhibitor with respect to 2-amino-4-hydroxy-5,6-dimethyltetrahydropteridine, protects the enzyme from inactivation by heat, the effects of the three substrates alone and and in various combinations on the stability of the enzyme to heat inactivation were measured (Table III). It was necessary in this experiment to add dithiothreitol to the enzyme solution before heat treatment in order to maintain the tetrahydropteridine in its fully reduced state and to minimize the effect of peroxide formed during aerobic oxidation of the tetrahydropteridine[1,10,15]. Both the tetrahydropteridine and phenylalanine significantly protect the enzyme

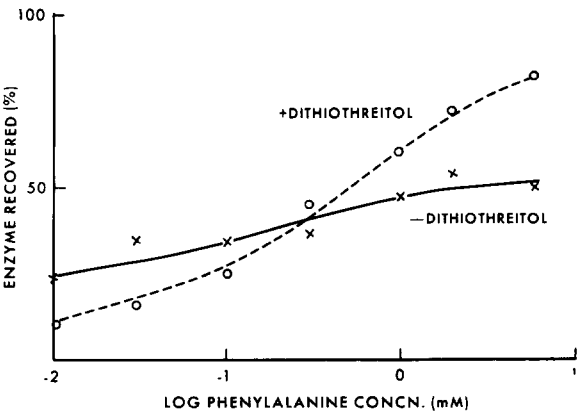

Fig. 4. Stability of the enzyme to heat inactivation at various concentrations of phenylalanine in the presence and absence of dithiothreitol. Solutions of 1.0 ml containing 1.17 mg enzyme previously filtered through a column of Sephadex G-25 equilibrated with 0.1 M Tris (pH 8.0), 40 μmoles Tris (pH 8.0) and the indicated components were divided into two equal portions. One portion was incubated in capped tubes for 10 min at 56° and then chilled in an ice bath; the other portion was kept in an ice bath. Hydroxylase activity in 0.40 ml of the heated and unheated samples were compared. Where indicated 2 mM dithiothreitol was added during the heat treatment.

from inactivation by heat but oxygen is without effect. These results show that under the conditions used either substrate may bind directly to the enzyme in the absence of any of the other substrates.

The effect of dithiothreitol on the stability of the enzyme to inactivation by heat at various concentrations of adenosine or phenylalanine

A preliminary observation not shown suggested that dithiothreitol protects the enzyme from thermal inactivation only in the presence of phenylalanine or adenosine. Accordingly, the stability of the enzyme was studied at several concentrations of adenosine (Fig. 3) or phenylalanine (Fig. 4) in the presence and absence of the dithiol. The data show that the dithiol inactivates the enzyme at low concentrations of adenosine or phenylalanine; however, dithiothreitol stabilizes the enzyme at high concentrations of either ligand. These results suggest that the dithiol reacts at two different sites on the enzyme. The simplest, though not the only, explanation is that the site at which the dithiol interacts to stabilize the enzyme is available in the absence of ligand. However, the site at which the dithiol labilizes the enzyme is unavailable when either ligand binds to the enzyme.

The stability of the enzyme to heat at various concentrations of dithiothreitol in the presence of adenosine

The data in Table IV show that in the presence of adenosine the enzyme is most stable to inactivation at 2 mM dithiothreitol. Concentrations of 6 mM dithiothreitol or higher inactivate the enzyme even in the cold.

The stability of the enzyme at various concentrations of adenosine under conditions of the assay

The data in Tables I and II indicate that most of the compounds which protect

TABLE IV

EFFECT OF VARYING THE CONCENTRATION OF DITHIOTHREITOL ON THE STABILITY OF THE ENZYME TO HEAT IN THE PRESENCE OF ADENOSINE

Solutions of 1.0 ml containing 40 μmoles Tris (pH 8.0), 0.01 M adenosine, the indicated amounts of dithiothreitol, and 1.17 mg enzyme which had been filtered through a column of Sephadex G-25 equilibrated with 0.1 M Tris (pH 8.0) were divided into two equal portions. One portion was kept in an ice bath. The other portion was incubated for 10 min in a water bath at 58° and then chilled in an ice bath. The hydroxylase activity in 0.4 ml of the heated and unheated solutions were determined.

Dithiothreitol (μmoles)	*Tyrosine formed (nmoles)*		*Recovery of enzyme after heating (%)*
	After heat treatment	*Before heat treatment*	
0	90	324	28
0.2	84	309	27
0.5	60	312	19
1.0	229	364	63
2.0	233	330	71
6.0	90	190	47
15.0	0	0	

the enzyme also inhibit the reaction. However, adenine and cytosine arabinoside inhibit without protecting the enzyme and 7-β-ribofuranosylguanine protects but does not inhibit. These data suggest possible separate binding sites for protection and inhibition. On the other hand, a comparison of the two sets of data is inconclusive because they were obtained under different experimental conditions. A more direct evaluation may be made by comparing the binding constants for one ligand in inhibiting the reaction and in protecting the enzyme from inactivation. MILDVAN AND LEIGH[16] have shown that the binding constants for a ligand as measured by its

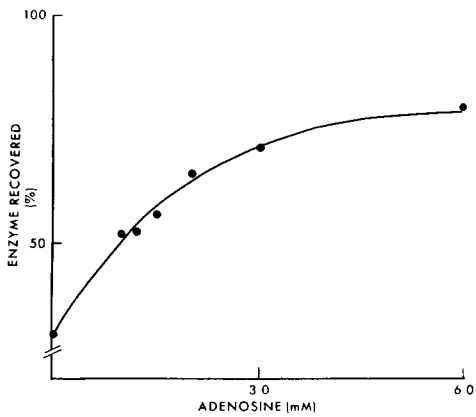

Fig. 5. Recovery of hydroxylase after prolonged incubation under conditions of assay at several concentrations of adenosine. The first incubation was at 25° for 5 h. The system contained 50 μmoles Tris (pH 7.2), 5 μmoles dithiothreitol, 0.48 mg enzyme which was filtered through a column of Sephadex G-25 equilibrated with 0.1 M Tris (pH 7.2) and the indicated amounts of adenosine in a total volume of 0.5 ml. At the conclusion of the first incubation, sufficient adenosine was added so that all tubes contained 3 μmoles. The enzyme was then assayed by the addition of 5 μmoles dithiothreitol, 50 μmoles Tris (pH 7.2), 2 μmoles phenylalanine and 0.4 μmole 2-amino-4-hydroxy-5,6-dimethyl-tetrahydropteridine. The second incubation was at 25° for 20 min in a total volume of 1.0 ml.

ability to protect an enzyme from slow inactivation is in good agreement with binding constants obtained by other methods. However, an enzyme dissociation constant of 2.7 mM is estimated from the data in Fig. 5 which measures the ability of several concentrations of adenosine to protect the enzyme under the conditions of the assay for hydroxylase activity. This dissociation constant is significantly less than the inhibition constant for adenosine (12.2 mM).

There appear to be two plausible explanations for the difference in adenosine–enzyme dissociation constants as determined by inhibition and protection. One possibility is that adenosine is bound to the enzyme at separate sites in the two tests. An alternative explanation rests on the differences in experimental conditions in the two tests. Phenylalanine which is present only during the test for inhibition may induce a change in the enzyme[17] which alters the binding site for adenosine. Efforts to distinguish experimentally between these two explanations by measuring the K_i's for adenosine at various concentrations of phenylalanine were unsuccessful.

Comparison of the ability of chelating agents and sulfhydryl compounds to alter the stability of the enzyme to thermal inactivation

The effects of dithiothreitol on the stability of the enzyme are likely due either to its reducing ability or to its ability to chelate a metal ion. Accordingly, the effectiveness of several chelators and other sulfhydryl compounds to replace dithiothreitol were determined under the conditions in which dithiothreitol stabilizes the enzyme and those in which it labilizes the enzyme to thermal inactivation. The addition of one of several chelators except 4,5-dihydroxy-*m*-benzene-disulfonate to the enzyme in the presence of adenosine appears if anything to sensitize the enzyme to thermal

TABLE V

THE EFFECT OF CHELATING AGENTS AND SULFHYDRYL COMPOUNDS ON THE STABILITY OF THE ENZYME TO HEAT IN THE PRESENCE OF ADENOSINE

Solutions of 0.50 ml containing 0.28 mg protein, 40 μmoles Tris (pH 8.0), 0.15 M adenosine and the additions listed in the Table were prepared. Portions (0.25 ml) of the samples were transferred to a set of tubes which were capped before being placed in a water bath at 58° for 10 min. They were then chilled in ice. Aliquots (0.20 ml) of the heated and unheated samples were assayed for hydroxylase activity.

Addition	*Concn. (mM)*	*Tyrosine formed (nmoles)*		*Recovery of enzyme after heat treatment (%)*
		After heat treatment	*Before heat treatment*	
None	—	55	140	39
2,2′-Dipyridyl	1	24	99	24
8-Hydroxyquinoline	1	10	55	18
Sulfosalicylic acid	1	47	145	32
Potassium ethyl xanthate	1	21	153	14
4,5-Dihydroxy-*m*-benzene-disulfonic acid	1	110	171	64
EDTA	2	19	81	23
Thioglycolate	5	135	154	88
β-Mercaptoethanol	5	150	145	103
β-Mercaptoethylamine	5	37	117	35
Dithiothreitol	2	111	149	75

inactivation (Table V). On the other hand, all sulfhydryl compounds tested except β-mercaptoethylamine stabilize the enzyme in the presence of adenosine. These results suggest that chelation of a metal ion by dithiothreitol is not primarily responsible for its stabilization of the enzyme in the presence of adenosine.

Similarly, the data in Table VI suggest that the ability of dithiothreitol to inactivate the enzyme in the absence of adenosine appears to be due to some property of the sulfhydryl group other than its ability to chelate. In the absence of adenosine, two chelators, α,α′-dipyridyl and 8-hydroxyquinoline stabilize the enzyme from thermal inactivation. Sulfosalicylic acid, ethyl xanthate and EDTA do not appreciably affect the stability of the enzyme under these conditions. However, 4,5-dihydroxy-*m*-benzenedisulfonic acid labilizes the enzyme some. On the other hand, all sulfhydryl compounds tested noticeably sensitize the enzyme to thermal inactivation. Thus, dithiothreitol alters the stability of the enzyme by two means, both of which are probably due primarily to the properties of the sulfhydryl group other

TABLE VI

THE EFFECT OF CHELATING AGENTS AND SULFHYDRYL COMPOUNDS ON THE STABILITY OF THE ENZYME TO HEAT IN THE ABSENCE OF ADENOSINE

Solutions (1.0 ml) containing 40 μmoles Tris (pH 8.0), 0.45 mg protein, and the additions given below were divided into two equal portions. One portion was kept in an ice bath; the other portion was incubated at 52° for 10 min and then chilled in ice. Aliquots of 0.4 ml were assayed for hydroxylase activity.

Addition	*Concn. (mM)*	*Tyrosine formed (nmoles)*		*Recovery of enzyme after heat treatment (%)*
		After heat treatment	*Before heat treatment*	
None		222	364	64
2,2'-Dipyridyl	1	77	74	104
8-Hydroxyquinoline	0.2	227	202	112
Sulfosalicylic acid	1	284	378	75
Potassium ethyl xanthate	1	271	378	72
4,5-Dihydroxy-*m*-benzene-disulfonic acid	1	151	357	42
EDTA	2	249	359	70
Thioglycolate	5	65	319	20
β-Mercaptoethanol	5	161	402	40
β-Mercaptoethylamine	5	138	392	35
Dithiothreitol	2	150	363	41

than to its chelating ability. The anomolous behavior of 4,5-dihydroxy-*m*-benzene-disulfonic acid may be due to its possible properties as a reductant[18].

ACKNOWLEDGMENTS

The author is grateful to Dr. Dwain D. Hagerman for calculating the kinetic data, to Dr. Albert S. Mildvan for his suggestions, and to Mr. T. R. Parkhill for his expert technical assistance. This work was supported by Grants AM-10708 and SR-05357 from the U.S. Public Health Service, National Institutes of Health.

REFERENCES

1 S. KAUFMAN, in O. HAYASHI, *Oxygenases*, Academic Press, New York, 1962, p. 127.
2 G. GUROFF, J. W. DALY, D. M. JERINA, J. RENSON, B. WITKOP AND S. UDENFRIEND, *Science*, 158 (1967) 1524.
3 C. B. STORM AND S. KAUFMAN, *Biochem. Biophys. Res. Commun.*, 32 (1968) 7881.
4 C. MITOMA, *Arch. Biochem. Biophys.*, 60 (1956) 476.
5 C. K. CAIN, M. F. MALLETTE AND E. C. TAYLOR, JR., *J. Am. Chem. Soc.*, 68 (1946) 1998.
6 A. POHLAND, E. H. FLYNN, R. G. JONES AND W. SHIVE, *J. Am. Chem. Soc.*, 73 (1951) 3247.
7 A. TIETZ, M. LINDBERG AND E. P. KENNEDY, *J. Biol. Chem.*, 239 (1964) 4081.
8 S. KAUFMAN, *J. Biol. Chem.*, 234 (1959) 2677.
9 S. KAUFMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 809.
10 C. BUBLITZ, *Biochim. Biophys. Acta*, 191 (1969) 249.
11 S. UDENFRIEND AND J. R. COOPER, *J. Biol. Chem.*, 196 (1952) 227.
12 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 447.
13 W. W. CLELAND, *Nature*, 198 (1963) 463.

14 D. E. ATKINSON, *Biochemistry*, 7 (1968) 4030.
15 K. H. NIELSON, *European J. Biochem.*, 7 (1969) 360.
16 A. S. MILDVAN AND R. A. LEIGH, *Biochim. Biophys. Acta*, 89 (1964) 393.
17 D. E. KOSHLAND, JR., *J. Cellular Comp. Physiol.*, 54 (1959) 235.
18 J. B. CONANT AND L. F. FIESER, *J. Am. Chem. Soc.*, 46 (1924) 1858.