The Mechanism of Enzymic Formation of Homogentisate from *p*-Hydroxyphenylpyruvate[1]

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Abstract: The mechanism for the enzymic formation of homogentisate from *p*-hydroxyphenylpyruvate has been studied by carrying out the reaction in $^{18}O_2$ and in $H_2{}^{18}O$. Gas chromatography–mass spectrometry of the bis-trimethylsilyl ether of the methyl ester of the formed homogentisate has established that oxygen from $^{18}O_2$ is incorporated *both* in the new hydroxyl and in the carboxyl. The isolated homogentisate contained ^{18}O in only 30% of the new hydroxyl groups as a result of an exchange with water during the enzymic reaction, presumably in a hydrated quinonoid structure. It is proposed that incorporation of both atoms from an O_2 molecule into the homogentisate occurs through the formation of an intermediate cyclic peroxide and that the oxygen reduction occurs through the oxidative decarboxylation of the α-keto side chain in *p*-hydroxyphenylpyruvate. The mechanism is thus analogous to that proposed for 2-ketoglutarate dependent hydroxylations.

Formation of homogentisate from *p*-hydroxyphenylpyruvate is catalyzed by the enzyme *p*-hydroxyphenylpyruvate hydroxylase, which has been classified as a monooxygenase on the basis of data from experiments with isotopic oxygen reported by Mason and coworkers.[2] They concluded that 1 atom of molecular oxygen and 2 atoms of oxygen from water were incorporated into homogentisate. Crandall[3] suggested that the new hydroxyl arises from molecular oxygen and that reducing equivalents are obtained by dehydrogenation of the hydrated carbonyl in *p*-hydroxyphenylpyruvate. For the group of oxygenases requiring 2-ketoglutarate as cofactor we have recently proposed a reaction mechanism according to which a peroxide is formed between the substrate to be hydroxylated and the α-keto acid.[4] For γ-butyrobetaine hydroxylase—an enzyme which belongs to this group—we have shown the incorporation of molecular oxygen into the succinate formed during the reaction from 2-ketoglutarate, as is required by the proposed mechanism.[5] A similar mechanism may be formulated for the conversion of *p*-hydroxyphenylpyruvate (I) to homogentisate (VIII) (Scheme I).

Scheme I. Proposed Reaction Mechanism for the Enzymic Formation of Homogentisate from *p*-Hydroxyphenylpyruvate

A metal ion–oxygen complex might attack the carbanion of *p*-hydroxyphenylpyruvate[6] (II) with the formation of a peroxide ion (III). Nucleophilic attack on the α-carbon of the side chain yields a cyclic peroxide (IV). Decarboxylation results in the intermediate V, the carboxymethyl side chain of which migrates to the ortho position to yield VII by a mechanism similar to that in the so-called NIH shift.[7] Intermediate VII may stabilize either by elimination of a proton or by isomerization to a quinonoid species (not shown in Scheme I).

Experimental results which support this mechanism have been obtained by carrying out the enzymic reaction in $^{18}O_2$ gas and in $H_2{}^{18}O$.

Results

Incubations in $^{18}O_2$. Table I shows the percentage distribution of ions—after correction for the presence

(1) This work was supported by grants from the Swedish Medical Research Council (13 X 585), from Alfred Österlunds Stiftelse, and from Stiftelsen Gustaf och Thyra Svenssons Minne.
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(3) D. J. Crandall in "Oxidases and Related Redox Systems," T. E. King, H. S. Mason, and M. Morrison, Ed., Wiley, New York, N. Y., 1965, p 263.
(4) E. Holme, G. Lindstedt, S. Lindstedt, and M. Tofft, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 2, 29 (1968).
(5) B. Lindblad, G. Lindstedt, M. Tofft, and S. Lindstedt, *J. Amer. Chem. Soc.*, 91, 4604 (1969).
(6) The reaction has been formulated with the keto form of *p*-hydroxyphenylpyruvate. The enzyme phenylpyruvate keto–enol isomerase (EC 5.3.2.1) present in liver catalyzes an equilibrium between the keto and enol forms of *p*-hydroxyphenylpyruvate. So far no physiological role can be ascribed to this enzyme, but one cannot exclude the possibility that the enol form is substrate for the hydroxylase.
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Table I. Presence of Oxygen-18 in Bistrimethylsilyl Ether of the Methyl Ester of Enzymically Formed Homogentisate and Its Fragments[a]

	$^{18}O_2$				$H_2{}^{18}O$	
	Expt 1		Expt 2			
m/e	^{18}O atoms	%	^{18}O atoms	%	^{18}O atoms	%
M (326–330)	0	1.04	0	1.07	0	1.91
	1	69.7	1	72.0	1	31.8
	2	29.3	2	26.9	2	66.3
M − 15 (311–315)	0	3.02	0	4.09	0	2.41
	1	66.7	1	69.9	1	31.9
	2	30.2	2	26.0	2	65.7
M − 73 (253–255)	0	71.9	0	70.0	0	29.8
	1	28.1	1	30.0	1	70.2

[a] The values have been calculated from the mass distribution in the mass spectra and corrected for the presence of ^{16}O in the $^{18}O_2$ gas and in the $H_2{}^{18}O$ used in the incubations.

of $^{16}O_2$ in the $^{18}O_2$—in the mass spectrum of enzymically formed homogentisate. The ions *m/e* 326, 328, and 330 correspond to the molecular ions, M, M + 2, and M + 4, *i.e.*, molecular ions with 0, 1, or 2 atoms of ^{18}O per molecule. The ions at *m/e* 311, 313, and 315 arise from the loss of a methyl group in a trimethylsilyl group in molecules with 0, 1, or 2 atoms of ^{18}O. The ions resulting from the loss of the side chain appear at *m/e* 253 (M − 73) and 255 (M − 73 + 2).

The two experiments carried out in $^{18}O_2$ gas gave similar figures for the incorporation of ^{18}O, *i.e.*, the nuclear fragment contained 1 atom of ^{18}O in 28.1 and 30.0% of the molecules (M − 73 + 2). Since 96–99% of the fragments with the side chain contained 1 *or* 2 atoms of ^{18}O the side chain must have been labeled to the same extent. When authentic homogentisate was incubated in the complete system (minus *p*-hydroxyphenylpyruvate) in an $^{18}O_2$ atmosphere, no incorporation of ^{18}O occurred.

Incubations in $H_2{}^{18}O$. The distribution of ^{18}O between the nuclear hydroxyl and the carboxyl was deduced from the same fragments as in the $^{18}O_2$ experiments, and corrected for ^{16}O in the water. Of the formed molecules 70% contained isotopic oxygen in the new nuclear hydroxyl (M − 73 + 2, Table I). Only about 2% of the ions containing the side chain were unlabeled and thus the carboxyl was labeled in more than 95% of the molecules. When homogentisate (5 μmol) was incubated in $H_2{}^{18}O$ in the complete system (minus *p*-hydroxyphenylpyruvate) less than 0.5% of the molecules incorporated ^{18}O. However, when *p*-hydroxyphenylpyruvate (10 μmol) was incubated in the complete system the reisolated *p*-hydroxyphenylpyruvate contained 1 atom of ^{18}O in 82% of the molecules.

Discussion

The characteristic feature of the reaction mechanism which we have suggested is the incorporation of one atom of oxygen from molecular oxygen into the carboxyl of the enzymically formed homogentisate. In this respect our mechanism differs from those earlier proposed.[3,8] The experimentally found distribution of molecules with different labeling is shown in Scheme II,

(8) A. H. Soloway, *J. Theoret. Biol.*, 13, 100 (1966).

Scheme II. Distribution of Labeled Molecules after Enzymic Reaction in $^{18}O_2$ and $H_2{}^{18}O$

$^{18}O_2$	OH, $CH_2C^{18}OOH$, ^{18}OH ~30%	OH, $CH_2C^{18}OOH$, OH ~70%	OH, CH_2COOH, OH <5%
$H_2{}^{18}O$	OH, $CH_2C^{18}OOH$, ^{18}OH ~70%	OH, $CH_2C^{18}OOH$, OH ~30%	OH, CH_2COOH, OH <3%

which illustrates the fact that more than 95% of the new molecules derive one atom in the carboxyl from the gas phase. The fact that one oxygen from water is incorporated into the carboxyl is not at variance with the suggested mechanism, since α-keto acids exchange the ketonic oxygen with water, as was also established in a control experiment with *p*-hydroxyphenylpyruvate.

In our, as well as in other, mechanisms the new hydroxyl oxygen originates from molecular oxygen. As illustrated in Scheme II only 30% of the molecules contained ^{18}O in the new hydroxyl in $^{18}O_2$ experiments. It was important therefore to establish if a loss of oxygen could have occurred subsequent to the initial incorporation. The experiment in which the reaction was carried out in $H_2{}^{18}O$ resulted in a 70% exchange, *i.e.*, a figure which fully accounts for the low labeling in the $^{18}O_2$ experiments. This exchange may occur in a quinonoid structure in equilibrium with intermediate VII. An exchange of oxygen in quinones with water has been reported.[9,10] We have formulated the reaction with a quinonoid structure as the molecular species which is attacked by a metal-oxygen complex (II in Scheme I). The occurrence of such intermediates in electrophilic aromatic substitution has been discussed, by, among others, Cannell.[11,12]

The results of the present study have established that hydroxylation in the aromatic ring and oxidative decarboxylation of the α-keto side chain occur in a concerted reaction in the formation of homogentisate from *p*-hydroxyphenylpyruvate. Thus, this reaction and the α-ketoglutarate-dependent hydroxylation of proline to hydroxyproline,[13,14] of lysine to hydroxylysine,[14,15] of γ-butyrobetaine to carnitine,[4,16,17] of thymine to 5-hydroxymethyluracil,[18,19] and of 5-hydroxymethyluracil to 5-formyluracil[20] are examples of the same type of reaction. In common for these reactions is the requirement for a metal ion and the stimulation by catalase and by reductants such as ascorbate, isoascorbate, or 2,6-dichlorophenolindophenol. The mechanistic studies carried out with γ-butyrobetaine hydroxylase[5] and with *p*-hydroxyphenylpyruvate hydroxylase clearly indicate that ascorbate or other reductants are not directly involved in the hydroxylation reaction; they probably act by maintaining the metal ion or sulfhydryl groups in the enzyme in the reduced state.

The results of the present study raise some questions of nomenclature. Thus, the systematic name for the enzyme studied is *p*-hydroxyphenylpyruvate, ascorbate: oxygen, oxidoreductase (hydroxylating), EC 1.14.2.2, *i.e.*, it belongs to the class oxidoreductases acting on paired donors with incorporation of oxygen into one donor (hydroxylases). We suggest the name *p*-hydroxyphenylpyruvate:oxygen oxidoreductase (hydroxylating). The enzyme would then belong to class 1:13, oxidoreductases, acting on single donors with incorporation of oxygen (oxygenases). The present nomenclature is, however, not well suited for classification of the α-ketoglutarate-dependent enzymes mentioned above; it would seem desirable to include all enzymes acting by the same mechanism into the same group.

Experimental Section

Chemicals. Isotopic oxygen (90 and 99% ^{18}O) and ^{18}O-enriched water (98% $H_2{}^{18}O$) were obtained from Miles Laboratories, Inc., Elkhart, Ind. Homogentisic acid and *p*-hydroxyphenylpyruvic acid were obtained from Sigma Chemical, Co., St. Louis, Mo. *p*-Hydroxyphenylpyruvic acid, twice recrystallized from ethyl acetate-light petroleum (bp 40-60°), was converted to the potassium salt by the addition of slightly less than the theoretical amount of 1 *M* potassium hydroxide in 99.5% ethyl alcohol. The precipitated potassium *p*-hydroxyphenylpyruvate was filtered off and washed several times with 99.5% ethyl alcohol. The dried salt was stored dry at −20°. Reduced glutathione was purchased from B.D.H., Ltd., Poole, England; 2,6-dichlorophenolindophenol and 2,2′-dipyridine were obtained from E. Merck A.G., Darmstadt, West Germany; hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories Inc., State College, Pa. All other reagents and solvents were of analytical grade and purchased from local stores.

***p*-Hydroxyphenylpyruvate Hydroxylase.** An acetone powder of rat liver was prepared as described by Taniguchi and Armstrong.[21] The dried acetone powder was extracted at +4° for 2 hr with a 50 m*M* potassium-sodium phosphate buffer at pH 6.5 in $H_2{}^{16}O$. In the $H_2{}^{18}O$ experiment the potassium-sodium phosphate buffer was prepared in $H_2{}^{18}O$. The extracted acetone powder was centrifuged at 3000 rpm for 10 min and the supernatant was used as the source of *p*-hydroxyphenylpyruvate hydroxylase.

Incubations. The incubation mixtures for the $^{18}O_2$ experiments contained *p*-hydroxyphenylpyruvate hydroxylase from 240 mg of rat liver acetone powder (see above), potassium *p*-hydroxyphenylpyruvate (35 μmol), 2,6-dichlorophenolindophenol (1.05 μmol), reduced sodium glutathione (70 μmol), 2,2′-dipyridine (7 μmol), and potassium-sodium phosphate buffer pH 6.5 (350 μmol) in a total volume of 7.0 ml. The reaction mixtures *minus enzyme* were flushed with argon for 5 min, whereafter the enzyme was added; the incubation mixtures were quickly frozen in liquid N_2 and connected to a vacuum manifold in which air was replaced by $^{18}O_2$ gas. The incubations were carried out at a partial pressure of 150 mm at 37° for 90 min after which the reaction mixtures were acidified with dilute sulfuric acid to pH 1-2, salt saturated by the addition

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(21) K. Taniguchi and M. D. Armstrong, *J. Biol. Chem.*, **238**, 4091 (1963).

of solid sodium chloride, and extracted three times with 15 ml of ethyl acetate and three times with 15 ml of peroxide-free diethyl ether. The extractions were completed within 1 hr and the organic phases were dried, first with anhydrous sodium sulfate at room temperature for 1 hr, then with anhydrous magnesium sulfate for 6 hr at $-20°$. Homogentisic acid was converted to the bistrimethylsilyl ether of the methyl ester as described in a previous publication.[22]

Incubation in $H_2{}^{18}O$ was carried out in a total volume of 0.25 ml and in an atmosphere of air. Otherwise the conditions were the same as for the $^{18}O_2$ experiments in the enzymic reaction.

Gas-Liquid Chromatography-Mass Spectrometry. The bistrimethylsilyl ether of the methyl ester of homogentisic acid was analyzed on an instrument for combined gas chromatography-mass spectrometry[23] (LKB Model 9000, LKB Produkter, Stockholm, Sweden).

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(23) Initial work was performed on an instrument which had been designed at the Department of Medical Biochemistry, University of Gothenburg. We extend our thanks to Professor S. Stenhagen for help in this phase of the work.

Determination of D- and L-Amino Acid Residues in Peptides. Use of Tritiated Hydrochloric Acid to Correct for Racemization during Acid Hydrolysis[1]

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Abstract: A method for measuring the racemization of some amino acids during the acid hydrolysis of peptides is described. The peptide is hydrolyzed in tritiated HCl and the radioactivity of each amino acid is measured with a flow-cell scintillation counter attached to the amino acid analyzer. The method was tested with free L-amino acids; the samples were heated in tritiated HCl and the amount of enantiomer formed was calculated from the amount of tritium incorporated. This value was compared with the actual amount of D isomer found chromatographically after the sample was coupled with an L-amino acid *N*-carboxyanhydride; the resulting diastereoisomeric dipeptides were separated on an amino acid analyzer. The incorporation of tritium was proportional to the amount of D isomer formed within a range of 1% for alanine, valine, isoleucine, leucine, serine, threonine, lysine, arginine, methionine, and proline. The other amino acids tested (aspartic acid, glutamic acid, phenylalanine, tyrosine, and histidine) have exchangeable hydrogen atoms in the side chains and hence this technique is not applicable to them. As shown by proton nmr analysis of samples heated in deuterium chloride, the α-hydrogen atom of an α-amino acid is not removed in strong acid as readily as the α-hydrogen atom of an aliphatic carboxylic acid such as isobutyric acid. This may be a result of the absence of the positively charged conjugate acid of the α-carboxylic acid group which is energetically not favored when a protonated α-amino group is present. Analysis of synthetic L-bradykinin showed that D-serine, D-proline, and D-arginine (0.1–2.8%) present in the acid hydrolysate were formed by racemization during the hydrolysis. An acid hydrolysate of natural bacitracin A contained D-leucine (6.8%) and D-alloisoleucine (15.7%) which were formed from the corresponding L residues during the hydrolysis. In general, the amount of D-amino acid found in excess of that formed by racemization during acid hydrolysis provides a measure of the D isomer in the peptide.

Racemization of amino acids occurs during acid hydrolysis of a peptide[2] and the amount of this racemization must be determined before the configurations of the amino acids in either a synthetic or a natural peptide can be rigorously established. Treatment of the corresponding free amino acids with hot acid has been employed as a control for measurement of the racemization of amino acid residues during acid hydrolysis of a peptide.[3,4] However, this control may not always be adequate because the amino acid residues in some sequences of a peptide may be subject to increased racemization during hydrolysis; for example, in bacitracin A Ile-1 is completely epimerized during the hydrolysis,[5] and the phenylalanyl residue in L-Phe-L-Ser is racemized more than free L-phenylalanine during hydrolysis.[3]

When the stereochemical purity of a synthetic L-peptide is being evaluated by a study of an acid hydrolysate of the material, the natural L-peptide is the best control for correction for the racemization which occurs during the hydrolysis. When the natural material is not available for comparison, it has been difficult to establish unequivocally the configurations of the amino acids in the synthetic peptide because there has not been a method for measuring increased racemization of some amino acid residues during acid hydrolysis. The same consideration applies to the examination of a natural peptide for D- and L-amino acid content.

Since racemization occurs concomitant with removal of the α-hydrogen atom of an amino acid, incorporation of tritium should provide a convenient means for

(1) This study was supported in part by a grant from the National Institutes of Health. A preliminary report has been communicated (J. M. Manning and A. Marglin, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 29, 902 (1970)). The Fischer convention is used to designate the configurations of the amino acids.

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