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Biosynthesis of Caldariellaquinone in *Sulfolobus* spp.

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The biosynthesis of caldariellaquinone (CQ) was studied in species of *Sulfolobus* by measuring the incorporation of stable isotopically labeled tyrosines into CQ. By feeding a series of tyrosines labeled with deuterium or ^{13}C and then measuring the extent and position at which label was incorporated into CQ by mass spectrometry, it was shown that more than 95% of the label was incorporated into the benzo[*b*]thiophen-4,7-quinone moiety of CQ. From the labeling experiments, it is concluded that the benzo[*b*]thiophen-4,7-quinone is derived as an intact unit from all of the carbons of tyrosine except C-1.

Isoprenoid quinones, constituents of bacterial plasma membranes, play an important role in electron transport, oxidative phosphorylation, and possibly active transport (7). Two major structural groups of bacterial isoprenoid quinones, the naphthoquinones and benzoquinones, have been recognized. The naphthoquinones can be further divided into two main types, the phylloquinones and the menaquinones, on the basis of structural considerations. The benzoquinones also contain two main types, the plastoquinones and the ubiquinones.

In 1977, caldariellaquinone (CQ), the first example of a sulfur-containing quinone, was isolated from the extremely thermophilic and acidophilic bacterium *Caldariella acidophila* and was shown to be 6-(3,7,11,15,19,23-hexamethyltetracosyl)-5-methylthiobenzo[*b*]thiophen-4,7-quinone [CQ-6 (12H)] (Fig. 1a) (10). Recently, CQ has been identified as a major component of the quinone fraction in all known *Sulfolobus* spp. (8, 17, 31). *Sulfolobus* spp. also contain minor amounts of related quinones that all have a benzo[*b*] thiophen-4,7-quinone nucleus but differ in chain length (six to four isoprenoid residues), number of double bonds (one or two sites of unsaturation in the isoprenoid chain), and nature of the substituent at C-5 of the ring system (5-methyl group or 5-thiomethyl group) (31). (Moreover, a small amount of a new CQ analog, containing an additional thiophene ring, was recently discovered in *Sulfolobus solfataricus* [17].) Since menaquinone and ubiquinone are absent in *Sulfolobus* spp., CQ may function in their place in these organisms (8, 10).

The biosynthesis of biological quinones has been well reviewed (3, 4, 22, 23, 30). Since higher animals have lost their ability to synthesize aromatic rings from simple precursors, the biosynthesis of the quinone ring must rely solely on a dietary source, such as tyrosine and phenylalanine, for aromatic compounds. In contrast, microorganisms and many plants are able to synthesize aromatic compounds from such simple precursors as acetate or glucose via the polyketide or shikimic acid pathway. Tyrosine has been determined to be the precursor of many biologically active quinones in eucaryotes. During the biosynthesis of ubiquinones, tyrosine is metabolized to 4-hydroxybenzoic acid, which is then converted to the quinone ring of ubiquinone, with only the aromatic carbons of the tyrosine being incorporated. During the biosynthesis of plastoquinones, tocopherols, and tocopherolquinones in higher plants, the C-2 and the aromatic carbons of the tyrosine are incorporated into the quinone ring system via homogentisic acid (21, 36–38). In most eubacteria, 4-hydroxybenzoic acid, the precursor of ubiquinones as in eucaryotes, is formed directly from chorismic acid without the involvement of tyrosine (12, 13, 18). In one strain of *Aerobacter aerogenes*, it was found that tyrosine may serve as a source of 4-hydroxybenzoic acid, probably with 4-hydroxyphenylpyruvate as an intermediate (12, 18). The isoprenoid side chain of quinones in animals comes from acetate via mevalonate. The biosynthetic pathway to the isoprenoid side chain in some microorganisms also originates from mevalonate (4, 28, 29), but the origin of the mevalonate in eubacteria has not been determined (35). Here we report work toward establishing the biosynthetic pathway to CQ.

MATERIALS AND METHODS

Isotopically labeled compounds. L-[*aromatic*-^{18}O]tyrosine (79.1 atom% ^{18}O) was prepared from L-*p*-aminophenylalanine by using ^{18}O-labeled water (2.8 atom% ^{16}O, 1.8 atom% ^{17}O, and 95.4 atom% ^{18}O) as described by Eckert and Fiat (11). The labeled water was obtained from the Monsanto Research Corp. Mound Facility, Miamisburg, Ohio. DL-[3,3-$^2H_2$]tyrosine (94 atom% $^2H_2$) was prepared by the condensation of the sodium salt of ethyl acetamidocyanoacetate with [*methylene*-$^2H_2$]-4-methoxybenzyl bromide as previously described (39). DL-[2-^{13}C]tyrosine (99 atom% ^{13}C) was prepared in a similar manner but using diethyl [2-^{13}C]acetamidomalonate obtained from Sigma Chemical Co., St. Louis, Mo. L-[*aromatic*-$^{13}C_6$]tyrosine (99 atom% ^{13}C) was obtained from Cambridge Isotope Laboratories, Woburn, Mass. (The labeled tyrosine was diluted with unlabeled L-tyrosine to prepare a tyrosine sample in which 20 mol% of the tyrosines was labeled.) [U-$^2H_5$]homogentisic acid was prepared by the acid-catalyzed exchange of homogentisic acid lactone with $^2H_3PO_4$ as follows. Homogentisic acid lactone (69.2 mg) was dissolved in 4 ml of 85% $^2H_3PO_4$ (99 atom% deuterium) obtained from Sigma, and the mixture was heated at 90°C for 3 h. After the solution was cooled, 5 ml of ice water was added; most of the [U-$^2H_5$]homogentisic acid lactone precipitated and was collected by filtration. The remaining [U-$^2H_5$]homogentisic acid lactone was extracted with diethyl ether and purified by preparative thin-layer chromatography, using ethyl acetate-acetic acid (9:1 [vol/vol]). The total yield of the product was 58.1 mg. Analysis by mass spectrometry showed 7.3 atom% of the molecules with six deuterium, 75.6 atom% with five

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deuterium, and 17.1 atom% with four deuterium. The [U-$^2H_5$]homogentisic acid lactone (58.1 mg) was hydrolyzed in 0.3 ml of 1% sodium bisulfite and 0.3 ml of 1% sodium hydroxide solution for 1 h at 100°C, and the resulting homogentisic acid was extracted by diethyl ether after acidification with acetic acid. (Homogentisic acid lactone is partially hydrolyzed to homogentisic acid in the bacterial growth medium because of the acidity of the medium and the high temperature in which the cells are grown. Thus, hydrolysis of the [U-$^2H_5$]homogentisic acid lactone to the acid is not required.)

DL-[3,3-$^2H_2$]-2',5'-dihydroxyphenylalanine was prepared by the reaction of [*methylene*-$^2H_2$]-2',5'-dimethoxybenzyl bromide with diethyl acetamidomalonate as described by Shulgin and Gal (25). The [*methylene*-$^2H_2$]-2',5'-dimethoxybenzyl bromide was prepared from benzyl alcohol that had been prepared by $LiAl^2H_4$ reduction of 2',5'-dimethoxybenzoic acid (1).

Growth of bacterial strains. *S. solfataricus* 98-3, supplied by Tom Langworthy, University of South Dakota, Vermillion, and *S. acidocaldarias* (ATCC 33909) were grown at 70°C for 2 to 3 days in a pH 3.0 medium consisting of 2.0 g of yeast extract, 1.3 g of $(NH_4)_2SO_4$, 0.28 g of KH_2PO_4, 0.25 g of $MgSO_4$, 70 mg of $CaCl_2 \cdot 2H_2O$, and 10 ml of trace mineral solution (5) per liter of medium. The labeled compounds were added at the concentrations shown in Table 1, the pH was adjusted to 3.0 with sulfuric acid, and the medium was autoclaved.

The labeled tyrosine and the deuteriated 2,5-dihydroxyphenylalanine were all stable when incubated in the growth medium at 70°C and when autoclaved in the growth medium. Homogentisic acid, however, was found to undergo a slight decomposition when incubated in the growth medium at 70°C for 2 to 3 days and even more decomposition when autoclaved in the growth medium. This was readily observed by the development of a brown color in the medium. The decomposition resulting from autoclaving was eliminated by adding the homogentisic acid to the previously autoclaved medium at the time of inoculation. Because of the high temperature and low pH of the medium, which prevented the growth of the other bacteria, no contamination was ever observed with use of this procedure.

Isolation of CQ. The cells were harvested from the growth medium by centrifugation (10,000 × *g*) for 15 min, and the cell pellet was extracted for 30 min with CH_2Cl_2-CH_3OH (1:1 [vol/vol]) at 50°C. After centrifugation to remove the insoluble material, 0.5 volume of water was added to the supernatant. After shaking and centrifugation, the CH_2Cl_2 layer was separated and dried over anhydrous Na_2SO_4, and the CQ was purified by thin-layer chromatography (silica gel 60 F254; E. Merck, Darmstadt, Federal Republic of Germany), using the solvent system hexane-diethyl ether (8:2 [vol/vol]). The orange CQ spot (R_f = 0.38) was eluted with CH_2Cl_2 for mass spectrometric analysis by direct probe insertion.

Derivatization of CQ. The crude CQ fraction was derivatized by treatment with acetic anhydride and zinc dust as described by Campbell et al. (6) to produce the diacetate derivative of dihydrocaldariellaquinone. The diacetate derivative of CQ was then purified by thin-layer chromatography by using the solvent system described above.

Mass spectrometry of CQ. The mass spectrum of the isolated CQ shows an intense molecular ion at *m/z* 630, which allows for the measurement of the incorporation of stable isotopically labeled precursors into the entire molecule. The mass spectrum of CQ also has fragment ions at *m/z* 225 (Fig. 1b) and *m/z* 212, which allow for specific measurement of the label incorporated into the benzo[*b*]thiophenquinone portion of the molecule. Thus, the difference between the label incorporated into the entire molecule and that incorporated into the benzo[*b*]thiophenquinone-containing fragments represents the label incorporated into the isoprenoid portion of the molecule.

Two problems interfered with this straightforward measurement of label incorporation. The first was the occurrence of varying amounts of CQ in the cells that contained one site of unsaturation in the side chain. Since the procedure used for the isolation of CQ is unable to separate unsaturated from saturated CQ, a small amount of this unsaturated CQ was present in the samples from which the mass spectral data were obtained. The presence of unsaturated CQ was confirmed by the occurrence of its molecular ion at *m/z* 628 in the mass spectrum of the CQ. Since this molecule contains $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, and $M^+ + 4$ isotope peaks that interfere with measurement of the intensities of the required ions, their occurrence must be taken into consideration when the isotopic distributions are calculated. This was accomplished by assuming the normalized intensities of $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, and $M^+ + 4$ to be the same for the unsaturated and saturated CQ.

A more serious problem in measuring the incorporation of more than one ^2H or ^{13}C was the reduction (dismutation) of CQ in the mass spectrometer, resulting in the generation of the hydroquinone of CQ that had a molecular ion at *m/z* 632 (Fig. 1c). This reaction, which has been studied in other quinones, leads to variations in the intensity of the $M^+ + 2$ ion (2, 9, 33). The intensity of the $M^+ + 2$ ion varied from sample to sample in a seemingly random fashion and caused an ~20% error in the measurement of the intensity of the $M^+ + 2$ ion of CQ. This problem was solved by converting CQ to the hydroquinone diacetate derivative (Fig. 1d). The mass spectrum of this diacetate showed a molecular ion at *m/z* 716, an intense fragment ion at *m/z* 632, which corresponded to the hydroquinone, and a fragment ion at *m/z* 225 (Fig. 1). The intense *m/z* 632 ion allowed for the accurate measurement of isotope incorporation into the entire molecule of CQ without the interference caused by reduction in the mass spectrometer. By using this method and DL-[3,3-$^2H_2$]tyrosine, no multiple labeling of the CQ was observed (data not shown); therefore, tyrosine was not metabolized and incorporated into the side chain of CQ. Since the experiments reported here required the measurement of the incorporation of only a single ^2H or ^{13}C, all of the data were obtained from the mass spectra of the labeled CQ.

Analysis of isotope distribution in cellular proteins. The isotopic distribution of ^2H or ^{13}C in the protein-bound amino acids present in the cells was determined by gas chromatography-mass spectrometry of their *N*-(trifluoroacetyl) and *N,O*-bis(trifluoroacetyl) *n*-butyl esters as previously described (40).

RESULTS AND DISCUSSION

In a preliminary study on the biosynthesis of CQ by De Rosa et al. (10), both [1-^{13}C]- and [2-^{13}C]acetate were found to be incorporated solely into the isoprenoid side chain of the CQ. This pattern of acetate incorporation suggests that the isoprenoid chain is biosynthesized from acetate via the mevalonate pathway, as it is in plants and animals. This pattern of acetate incorporation is also consistent with the biosynthesis of isoprenoid lipids in other archaebacteria (16). On the basis of these preliminary data and from the structural similarities between CQ and plastoquinone and ubiqui-

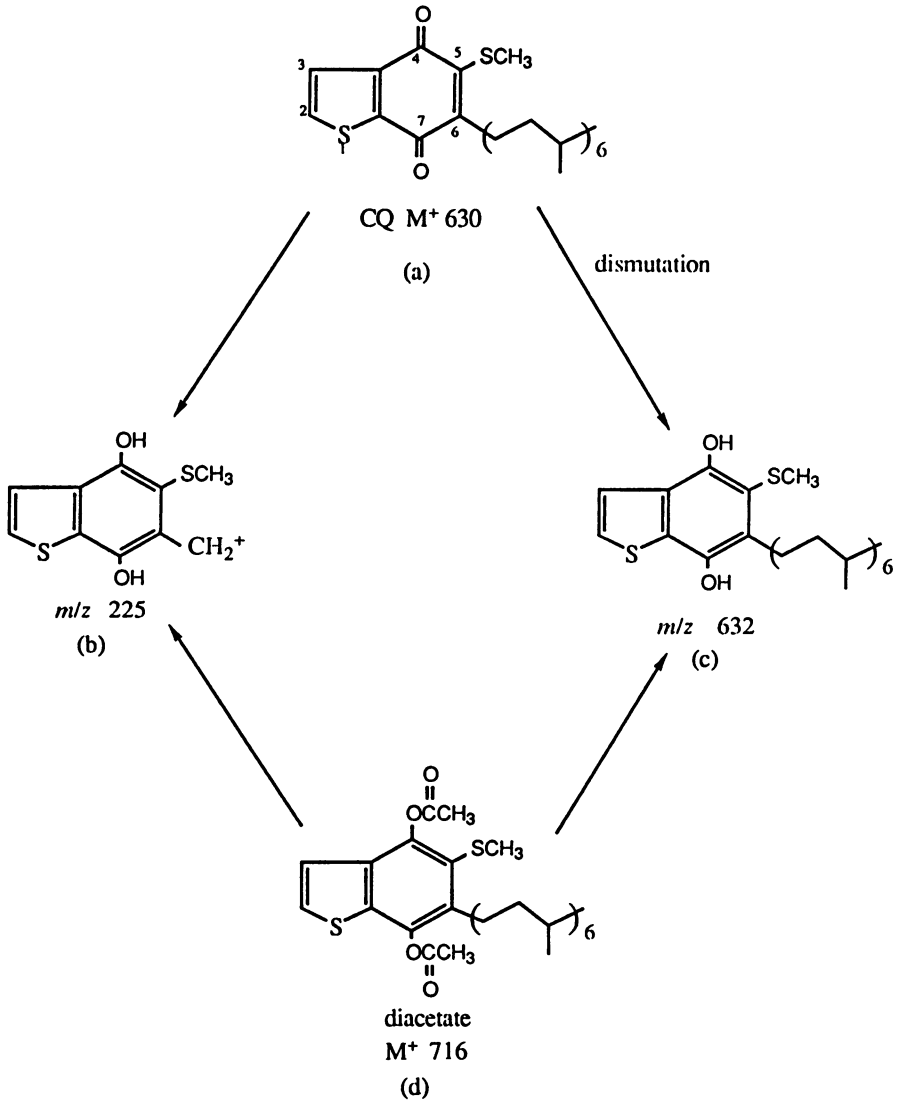

FIG. 1. Structures and mass spectral fragmentations of CQ and the diacetate derivative of dihydrocaldariellaquinone.

none, we suspected that CQ could be biosynthesized via the shikimic acid pathway, with tyrosine and homogentisic acid serving as likely intermediates.

The first indication that tyrosine was a precursor to the benzo[*b*]thiophene ring of CQ was the demonstration that DL-[2-^{13}C]tyrosine was readily incorporated into CQ. A single ^{13}C was incorporated from DL-[2-^{13}C]tyrosine into the *m/z* 225 fragment ion of the CQ to an extent of 66.3% and into the M^+ *m/z* 630 ion to an extent of 69.4% (Table 1, experiment 4a). (On the basis of the biosynthetic agreement presented below, it is assumed that this carbon is incorporated into the C-2 of the benzo[*b*]thiophene ring; however, this cannot be determined unequivocally by the mass spectroscopic method used here.) The difference between the label found in the intact molecule and that found in the aromatic portion of the ring can be explained by the incorporation of a small amount of ^{13}C from the tyrosine into the side chain.

That the aromatic ring of the tyrosine is incorporated as a single, intact unit into the six-member ring of the quinone was confirmed by growing cells in the presence of L-tyrosine in which 20% of the molecules contained L-[*aromatic*-$^{13}C_6$] tyrosine. Tyrosine labeled in this manner is used to allow for the detection of the degradation and resynthesis of the aromatic ring from its degraded products. If this were to occur, no intact C_6 unit would be observed. That the tyrosine was incorporated as a unit into both the cellular protein and the benzo[*b*]thiophene ring of CQ was confirmed by data (Table 1, experiment 2) showing, from both the *m/z* 225 and M^+ *m/z* 630 ions of the CQ and the *m/z* 260 ion of the *N,O*-bis(trifluoroacetyl) *n*-butyl tyrosine, that L-[*aromatic*-$^{13}C_6$]tyrosine was incorporated as an intact C_6 unit into both the protein and the benzo[*b*]thiophenquinone ring of the CQ. Since the extents of label incorporation for the *m/z* 225 ion of the CQ and the *m/z* 260 ion of the tyrosine derivative were both ~16%, and since only 20% of the tyrosine molecules were labeled, then ~80% of the tyrosine present in the cells must have been derived from the fed tyrosine. Furthermore, since no significant incorporation of ^{13}C units other than $^{13}C_6$ was observed, there must have been no significant metabolism of the tyrosine.

The only proton of tyrosine that could be retained in the benzo[*b*]thiophenquinone of CQ would have to arise from one of the C-3 protons of the tyrosine, since all of the other

protons are displaced in the transformation. This single proton would have to be incorporated at C-3 of the CQ. That this proton is, in fact, retained was confirmed by data (Table 1, experiment 4a) showing the incorporation of a single deuterium into the *m/z* 225 fragment and M^+ *m/z* 630 of the CQ isolated from cells grown with DL-[3,3-$^2H_2$]tyrosine. The extent of incorporation of deuterium originating from the deuteriated tyrosine, however, was much less than the extent of incorporation of ^{13}C originating from any of the ^{13}C-labeled tyrosines. This result would indicate that at some stage in the transformation of tyrosine to CQ, the protons originally on the C-3 of the tyrosine must exchange with the protons of the water. One intermediate that would be expected to exchange protons is *p*-hydroxyphenylpyruvate, which is derived reversibly from tyrosine by a transamination reaction. That this exchange does, in fact, occur was confirmed in that the tyrosine in the cellular protein was shown to contain only ~23% of the molecules with two deuterium and ~27% of the molecules with a single deuterium (Table 1, experiment 4a). If we assume that *p*-hydroxyphenylpyruvate has the same label distribution as does the cellular tyrosine and that the deuteriated tyrosine is incorporated to the same extent as are the ^{13}C-labeled tyrosines, then the decreased incorporation of deuterium from the deuteriated tyrosine can be completely accounted for by the exchange from the *p*-hydroxyphenylpyruvate.

Feeding of [^{18}O]tyrosine resulted in no observed incorporation of ^{18}O into CQ. Since the tyrosine in the cell protein was found to be labeled to an extent of 56%, the ^{18}O from the tyrosine must have been lost either during or after the formation of CQ. Exchange of the ^{18}O directly from the quinone, as has been observed in other quinones (24, 26), is the most plausible explanation for this result, especially when the required high growth temperature for these cells is considered.

The logical conclusion from these experiments is that all of the carbons of tyrosine except C-1 are incorporated as a unit into the benzo[*b*]thiophenquinone. This could occur by many different pathways (Fig. 2). In each of these pathways, C-2 and C-3 of the tyrosine supply the C-2 and C-3 of the benzo[*b*]thiophenquinone and the aromatic carbons supply the carbons of the six-member ring. An important consideration in determining which of the pathways is correct is whether homogentisic acid is an intermediate. Homogentisic acid is an established intermediate both in the degradation of tyrosine (34, 43) in microorganisms and in the biosynthesis of plastoquinones and tocopherols (36–38) in plants, but its function in *Sulfolobus* spp. is unknown. On the basis of the pattern of incorporation of tyrosine into CQ and the established biosynthetic pathway of plastoquinone (36–38), homogentisic acid was considered to be a likely intermediate in the biosynthesis of CQ from tyrosine. After transamination to hydroxyphenylpyruvate (Fig. 2, reaction a), the tyrosine would be metabolized to homogentisic acid by a well-known rearrangement catalyzed by *p*-hydroxyphenylpyruvate dioxygenase (Fig. 2, reaction b) (34). Further reaction could proceed by the conversion of the carboxylic acid of the homogentisic acid to an aldehyde, reaction with sulfide, and subsequent cyclization to form benzo[*b*]thiophen-4,7-quinone (Fig. 2, reaction c).

The possible involvement of homogentisic acid in the biosynthesis of CQ in *Sulfolobus* spp. was tested both by measuring the suppression of ^2H- or ^{13}C-labeled tyrosine incorporation into CQ by cells grown in the presence of homogentisic acid and by measuring the incorporation of [U-$^2H_5$]homogentisic acid into CQ (Table 1, experiments 1b,

TABLE 1. Incorporation of stable isotope-labeled compounds into CQ and cellular tyrosine[a]

Expt	Precursor (mg, ml of medium)	Distribution of ^2H or ^{13}C[b]											
		CQ						Tyrosine, *m/z* 203 or 260[c]					
		m/z 225[d]			*m/z* M^+ 630[d]								
		0	1	6	0	1	6	0	1	2	6		
1													
a	DL-[2-^{13}C]tyrosine (47, 100)	33.7 (45.2)	66.3 (100)		30.6 (44.0)	69.4 (100)		18.8 (22.3)	81.1 (100)	0 (18.0)			
b	DL-[2-^{13}C]tyrosine (47, 100) + homogentisic acid (50, 100)	44.0 (65.7)	56.0 (100)		39.3 (50.3)	60.7 (100)		30.1 (39.9)	69.9 (100)				
2	L-[*aromatic*-$^{13}C_6$]tyrosine[e] (25, 50)	83.3 (100)	0.0 (20.2)	16.7 (26.6)	85.0 (100)	0.8 (45.2)	14.2 (16.7)	82.5 (100)	1.0 (19.6)	0 (2.5)	16.4 (20.1)		
3	[U-$^2H_5$]homogentisic acid (40, 80)	98.1 (100)	1.9 (25.6)		98.7 (100)	1.3 (45.6)		ND[f]					
4													
a	DL-[3,3-$^2H_2$]tyrosine (100, 200)	80.2 (100)	19.8 (49.3)		79.4 (100)	20.6 (70.2)		49.3 (100)	27.6 (66.2)	23.1 (53.3)			
b	DL-[3,3-$^2H_2$]tyrosine (100, 200) + homogentisic acid (50, 200)	80.3 (100)	19.7 (49.2)		77.3 (100)	22.7 (73.7)		47.7 (100)	28.9 (71.2)	23.7 (57.0)			

[a] The cells used were *S. solfataricus* in experiments 4a and b and *S. acidocaldarius* in all other experiments.

[b] Numbers in parentheses are the observed normalized ion intensities.

[c] The intensity data were obtained from *m/z* 260 of the *N,O*-bistrifluoroacetyl *n*-butyl derivative of the tyrosine in all experiments except 4, in which case intensities from *m/z* 203 were used. The measured normalized ion intensities for an unlabeled tyrosine derivative sample were 100, 18.4, 2.95, and 0.0% for the *m/z* 260, 261, 262, and 266 ions and 100, 10.2, 0.8, and 0.0% for the *m/z* 203, 204, 205, and 208 ions, respectively.

[d] The measured normalized ion intensities for an unlabeled sample of CQ were 100, 24.6, and 0.3% for the *m/z* 225, 226, and 231 ions and 100, 44.3, and 0.0% for the *m/z* 630, 631, and 636 ions, respectively.

[e] Sample contained 20% of the molecules with [*aromatic*-$^{13}C_6$]tyrosine (99 atom % ^{13}C).

[f] ND, Not determined.

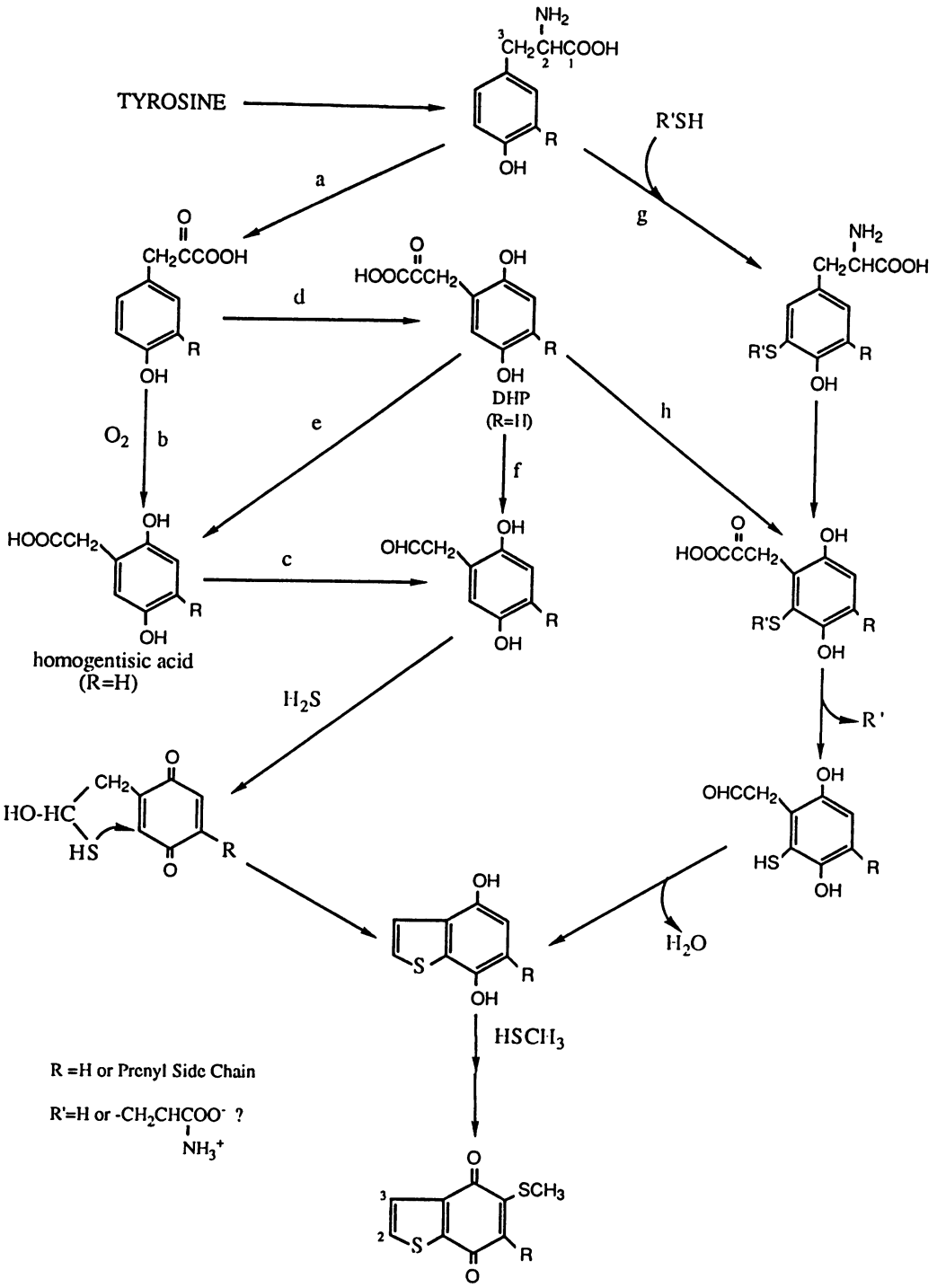

FIG. 2. Possible pathways for the biosynthesis of CQ from tyrosine. DHP, 2,5-Dihydroxyphenylpyruvate.

4b, and 3). No suppression of [3,3-$^2H_2$]tyrosine incorporation into CQ by homogentisic acid was observed in experiment 4b, whereas about 10% suppression of [2-^{13}C]tyrosine incorporation by homogentisic acid was observed in experiment 1b. In experiment 3, label from the [U-$^2H_5$]homogentisic acid was not incorporated into CQ. Measurement of the incorporation of deuterium from homogentisic acid into the cellular amino acids by gas chromatography-mass spectrometry showed less than 1% incorporation into glycine, serine, alanine, leucine, isoleucine, phenylalanine, glutamic acid, threonine, valine, proline, and aspartic acid. Thus, the 10% reduction in tyrosine incorporation into CQ by homogentisic acid observed in experiment 1b did not appear to result from the conversion of homogentisic acid to CQ but from the decreased utilization of the labeled tyrosine. This was confirmed by the decrease in ^{13}C in the tyrosine present in the cells from 81.1 to 69.9%. These negative results do not prove or disprove that homogentisic acid is an intermediate in CQ

biosynthesis, since the cell may simply be unable to take up the homogentisic acid.

Since all of the carbons of tyrosine except C-1 are incorporated into CQ, the rearrangement of the carbon structure that occurs during the conversion of *p*-hydroxyphenylpyruvate to homogentisic acid is a critical step in CQ biosynthesis. However, if homogentisic acid is not an intermediate, this rearrangement must occur in some other manner. This created a problem. We attempted to resolve this problem by considering the mechanism of homogentisic acid synthesis from *p*-hydroxyphenylpyruvate. The enzyme catalyzing this reaction has been found to act as a dioxygenase, with one of the oxygens of dioxygen being incorporated into the 2 position of the homogentisate and the other being incorporated into the carboxylic acid portion of the molecule (15, 19, 20, 32). This reaction, however, could easily be broken down into two separate steps. In the first step (Fig. 2, reaction d), an oxidative rearrangement would convert *p*-hydroxyphenylpyruvate to 2,5-dihydroxyphenylpyruvate. This compound, in turn, could be converted to homogentisate by an oxidative decarboxylation (Fig. 2, reaction e). In fact, this two-step oxidation has been observed by Yuasa et al. (44) in tyrosine metabolism in *Aspergillus sojae*. If the first oxidative rearrangement is followed by a nonoxidative decarboxylation (Fig. 2, reaction f), the 2,5-dihydroxyphenylpyruvate could be converted to the 2,5-dihydroxyphenylacetylaldehyde. This resulting aldehyde would then be converted to a thiol by a reaction sequence recently discovered in the biosynthesis of coenzyme M and component B (40–42). Oxidation of the hydroquinone to the quinone would allow for the addition of the thiol to the quinone to generate a dihydrothiophene ring. Dehydrogenation of the C-2–C-3 bond would then produce the benzothiophenquinone nucleus. Addition of the isoprenoid side chain and the thiomethyl group would complete the biosynthesis of CQ (Fig. 2).

Feeding of [3,3-$^2H_2$]-2′,5′-dihydroxyphenylalanine, which would be expected to undergo a transamination to the keto acid, however, did not lead to incorporation of deuterium into CQ. Thus, either 2,5-dihydroxyphenylalanine is not a precursor to homogentisic acid or the compound is not taken up by the cells.

There are, of course, many other possible pathways whereby the required carbons of tyrosine could be incorporated into CQ. Some of these pathways are outlined in Fig. 2. Each pathway requires an oxidative rearrangement of the original tyrosine carbon structure in order to generate a compound that can be transformed into the benzo[*b*]thiophen-4,7-quinone structure of CQ. Pathways not shown in the figure but considered possible were tested by growing cells with labeled compounds that could be intermediates in the biosynthesis and by measuring the incorporation of label into CQ by using mass spectrometry. The compounds tested were 2-(4-hydroxyphenyl)ethanol, 2-(4-hydroxyphenyl)ethanethiol, 2-(2,5-dihydroxyphenyl)ethanol, and 2-(2,5-dihydroxyphenyl)ethanethiol. In each case, the thiol compounds completely inhibited cell growth at a concentration of 0.5 mg/ml, which prevented them from being tested as precursors to CQ by this method. The alcohols had little or no effect on cell growth at a concentration of 0.5 mg/ml but were not found to be incorporated into CQ.

A possible explanation for why none of the intermediates listed above, including homogentisic acid, are incorporated into CQ is that the isoprenoid side chain is introduced at an early step in the biosynthesis and that the formation of the benzo[*b*]thiophene ring occurs at a later step in the biosynthesis. This early addition of the side chain does, in fact, occur during the biosynthesis of ubiquinones, in which *p*-hydroxybenzoic acid is reacted with isoprenyl pyrophosphate to form 2-polyprenylphenol in procaryotes and 3,4-dihydroxy-5-polyprenylbenzoate in eucaryotes. The resulting prenylated phenols are then modified to the final ubiquinones (22).

The formation of any sulfur-containing derivative of tyrosine early in the biosynthesis would also explain why none of the labeled compounds tested were incorporated. This formation would occur if the sulfur is introduced at an early stage of the biosynthesis, as shown in reaction g or h of Fig. 2. This idea is based on the biosynthesis of melanins, in which cysteine sulfur is introduced into the aromatic ring of tyrosine at an early stage in melanin formation (14, 27).

The available information on the biosynthesis of CQ is so limited that it is difficult to compare the biosynthesis of CQ in the archaebacteria with the biosynthesis of other quinones in other organisms. What can be said with certainty at this time is that the aromatic portion of CQ in archaebacteria is derived from tyrosine, as is the aromatic portion of ubiquinones, plastoquinones, and tocopherols in eucaryotes.

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