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Incorporation of Stereospecifically Deuteriated Tyrosines into Caldariellaquinone in *Sulfolobus acidocaldarius*

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Cells of *Sulfolobus acidocaldarius* were grown in a medium containing a series of stereospecifically deuteriated tyrosines and the incorporation of these labelled tyrosines into caldariellaquinone (CQ) was measured. The results show that the C-3 *pro-S* hydrogen of either L-or D-tyrosine is retained during the transformation of these tyrosines to the benzothiophene moiety of CQ. The incorporation of both D- and L-tyrosine into the cellular protein indicates that these cells can readily convert D-tyrosine into L-tyrosine.

Caldariellaquinone (CQ) is a unique benzothiophene quinone found only in species of extremely thermophilic and acidophilic archaebacteria, *i.e.*, *Sulfolobus* spp.,[1] *Desulfurolobus ambivalens*,[2] and in three strains of *Acidianus*.[3] The biosynthesis of CQ in *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* has been previously studied in our laboratory.[4] It was found that tyrosine serves as a precursor of CQ and that all carbons except C-1 of tyrosine are incorporated as a unit into CQ. Furthermore, it was demonstrated that only one hydrogen of the tyrosine was incorporated into CQ and that it originated from the C-3 methylene of the tyrosine. The stereospecificity for the incorporation of this hydrogen, however, was not established. We now report in this communication that it is the *pro-S* hydrogen of either L- or D-tyrosine that is incorporated into CQ (Fig. 1).

In order to study the stereospecificity of the incorporation of the C-3 hydrogen of tyrosine, four stereospecifically deuteriated isomers of tyrosine (Fig. 1), (3*S*)-L-[2-^{2}H, 3-^{2}H]- **1**, (3*R*)-D-[2-^{2}H, 3-^{2}H]- **2**, (3*S*)-D-[3-^{2}H]- **3** and (3*R*)-L-[3-^{2}H]-tyrosine **4**, were synthesised using the procedure described by Kirby.[5] The simultaneous synthesis of isomers **3** and **4** began from (*Z*)-α-benzoylamino-4-methoxy[3-^{2}H]cinnamic acid, which was reduced by hydrogenation in the presence of palladium to *N*-benzoyl-*O*-methyl[3-^{2}H]tyrosine, which was hydrolysed by 40% hydrogen bromide to give a mixture of tyrosine isomers **3** and **4**. Isomers **1** and **2** were synthesised in an analogous manner except that nonlabelled (*Z*)-α-benzoylamino-4-methoxycinnamic acid was hydrogenated with deuterium gas. Each pair of D-L isomers was resolved by treatment of its chloroacetyl derivative with carboxypeptidase A from bovine pancreas (Sigma).[5] The deuterium content of each sample was analysed from the mass spectral data of its *N*,*O*-bistrifluoroacetyl, butyl ester derivative.[6] Total deuterium content of the tyrosine was measured from the isotopic enrichment of the *m*/*z* 328 fragment ion ($M^+ - CO_2C_4H_9$) and deuterium specifically at C-3 was measured from the isotopic enrichment of the *m*/*z* 203 fragment ion ($M^+ - CH(NHCOCF_3)CO_2C_4H_9$). The enantiomeric purity of each sample was determined using the phenylalanine ammonium lyase assay.[7] In this assay, L-tyrosine is deaminated to *p*-hydroxycinnamic acid with the specific removal of the C-3 *pro-S* hydrogen of the tyrosine. Thus, each stereospecifically labelled L-tyrosine sample was incubated with phenylalanine ammonium lyase from *Rhodotorula glutinis* (Sigma) in Tris buffer (65 mmol dm^{-3}, pH 8.6) and the appearance of *p*-hydroxycinnamate was measured by the increase in absorption at 333 nm. The *p*-hydroxycinnamic acid was then isolated by extracting the acidified reaction mixture with ethyl acetate and its deuterium content was calculated from the isotopic en-

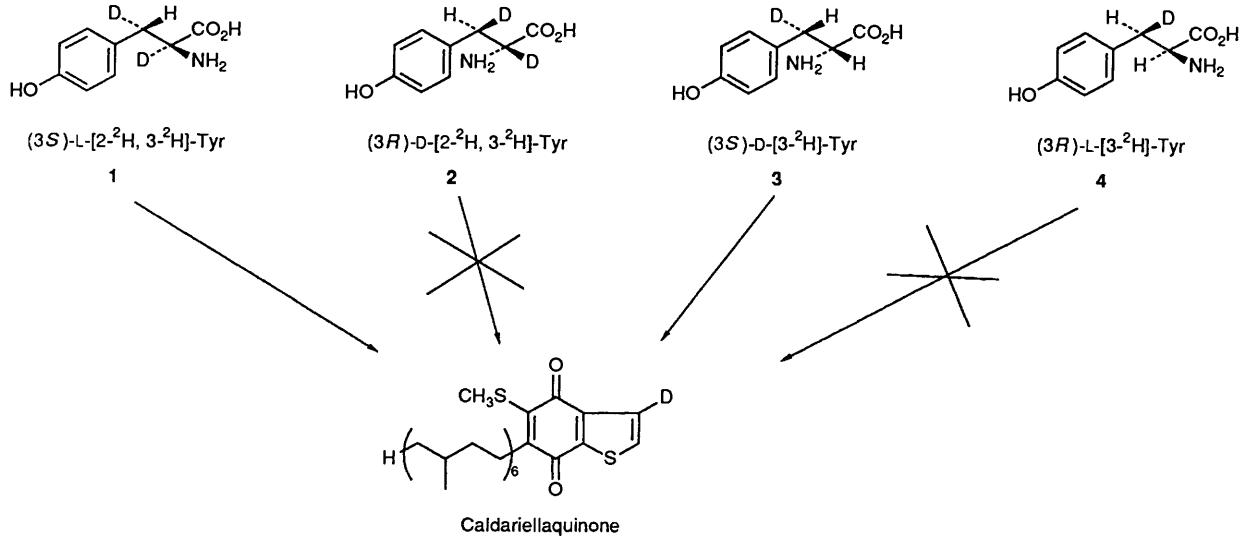

Fig. 1

Table 1 Incorporation of stereospecifically labelled tyrosines into CQ and cellular protein by *S. acidocaldarius*

Expt. Tyr. fed[c]	Distribution of ^2H in CQ[a] $^2H_0$	$^2H_1$	% Incorp.[d] into CQ	Distribution of ^2H in protein tyrosine[b] $^2H_0$	$^2H_1$	$^2H_2$	% Incorp.[e] into Tyr.
1. (3*S*)-L-[2-^{2}H, 3-^{2}H]-	56.7	43.4	55.2	47.6	49.1	3.3	52
	(82.9	100)[f]		(82.9	100	20.9)	
2. (3*R*)-D-[2-^{2}H, 3-^{2}H]-	90.5	9.5	7.7	67.3	32.5	0.1	34
	(100	54.8)		(100	65.9	8.7)	
3. (3*S*)-D-[3-^{2}H]-	(66.8	33.2	47.0	61.0	38.8	0.2	47
	(100	94.0)		(100	98.2	9.8)	
4. (3*R*)-L-[3-^{2}H]-	94.0	6.0	7.8	77.0	22.6	0.4	27
	(100	52.0)		(100	47.0	5.6)	

[a] The M^+ *m/z* 630 and the $M^+ + 1$ *m/z* 631 ions were used for the measurement of the isotopic distribution in the CQ. The measured normalised ion intensity for an unlabelled CQ sample was 100% and 44.3% for M^+ *m/z* 630 and 631, respectively. [b] The intensity data were obtained from the $M^+ - 101$ *m/z* 328 fragment ion of the *N,O*-bis(trifluoroacetyl)butyl derivative of the tyrosine. The measured normalised ion intensity for an unlabelled tyrosine derivative sample was 100, 17.6 and 0.0% for the *m/z* 328, 329 and 330, respectively. [c] Sample (1) contains 78.6% of (3*S*)-L-form and 16.4% of (3*R*)-L-form, sample (2) contains 78.6% of (3*R*)-D-form and 16.4% of (3*S*)-D-form, sample (3) contains 68.0% (3*S*)-D-form and 14.2% of (3*R*)-D-form, and sample (4) contains 68.0% of (3*R*)-L-form and 14.2% of (3*S*)-L-form. [d] The % incorporation of the indicated isomer into CQ. [e] The % incorporation of deuterium of C-3 tyrosine into protein-bound tyrosine. [f] Numbers in parentheses are the observed normalised ion intensities.

richment of the M^+ *m/z* 164 ion. The difference between the deuterium content of the *p*-hydroxycinnamic acid and the total deuterium content in the starting L-tyrosine was used to determine the stereochemical purity for the C-3 hydrogen of tyrosine. Since the enzyme only acts on L-tyrosine, the enantiomeric purity of the two D-isomers was assumed to be the same as the L-isomers since the synthesis and resolution methods used were the same. The two D-isomers treated with the enzyme did not produce any detectable amount of *p*-hydroxycinnamic acid, indicating no significant amount of L-isomer contamination in the samples.

Data on the deuterium content and enantiomeric purity of each sample are shown in the Table. Compounds **3** and **4** contain *ca.* 18% unlabelled molecules consistent with the extent of labelling of the [1-^{2}H]-*p*-anisaldehyde used in the synthesis. The labelled molecules were *ca.* 82.7% enantiomerically pure. This stereoimpurity resulted from racemization at the C-2 position of tyrosine during its chemical conversion into the chloroacetyl derivative prior to its resolution with carboxypeptidase.[8]

Four cultures of *Sulfolobus acidocaldarius* were grown for 3 days at 70 °C in a yeast extract medium[4] (100 cm^3), each supplemented with 45 mg of one of the stereospecifically deuteriated tyrosines. The distribution of ^2H in the CQ and in the protein-bound tyrosine in the cells was analysed by mass spectrometry as previously described.[4] The results (Table) show that the C-3 deuterium from all four tyrosine isomers were readily incorporated into the cellular protein, but only isomers **1** and **3** contributed a significant amount of deuterium into the CQ. These results indicate that it is the *pro*-3*S* hydrogen of either L- or D-tyrosine that is retained during the biosynthesis of CQ.

The quantitative incorporation of *pro*-3*R* or *pro*-3*S* hydrogen of the tyrosines into CQ was calculated from the deuterium incorporated in CQ and the enantiomeric purity of each isomer. Thus, for experiment 1 and 4, it was shown that the *pro*-3*S* isomer was incorporated into CQ to an extent of 55.8% and the *pro*-3*R* isomer was incorporated to an extent of $<0\%$. Similarly, it was shown from the incorporation data reported for experiment 2 and 3, that the *pro*-3*S* isomer of D-tyrosine was incorporated into CQ to an extent of 48.4% and the *pro*-3*R* isomer was incorporated to an extent of $<2\%$. Thus all of the deuterium incorporated into CQ were derived from the *pro*-3*S* hydrogen.

The stereospecificity of a number of biosynthetic processes involving the loss or retention of the prochiral C-3 centres of phenylalanine and tyrosine has been investigated.[9] Depending upon the specific reaction involved, either the *pro*-3*R*[7–9,10] or *pro*-3*S*[5,11] hydrogen could be retained during the process. However, since no clear pattern has emerged between the stereochemistry of the proton lost in these different reactions and a specific reaction type or mechanism, it is impossible to relate the observed loss of the *pro*-*S* hydrogen of tyrosine during formation of the benzothiophene ring of CQ with a specific reaction pathway. Thus, the true importance of this stereochemical information on CQ biosynthesis from tyrosine will only become apparent when the specific steps in the reaction are established.

A further interesting observation on the metabolism of tyrosine by *S. acidocaldarius* is that both D- and L-tyrosine are readily utilised by the cells. The utilisation of D-tyrosine by *S. acidocaldarius* is apparent from the data presented in the Table which show that both isomers are incorporated efficiently into both CQ and the cellular proteins. The efficient utilisation of both D- and L-tyrosine was also confirmed by the observation that cells grown on the yeast extract medium supplemented with 15 mg DL-tyrosine consume 87% of the added tyrosine. (This was established by quantitation of the difference in the free tyrosine present in the medium before and after cell growth by amino acid analysis.) If we assume that all tyrosine in proteins is L-tyrosine, then some mechanism must be in place to convert the D-tyrosine into L-tyrosine. At present, there are two mechanisms known to accomplish this conversion. One is the direct conversion of D-amino acids into L-amino acids *via* a racemase.[12–14] The other mechanism is the oxidation of D-amino acids to keto acids *via* D-amino acid oxidase,[12,14,15] D-amino acid dehydrogenase,[16] or transamination. Since other work has shown that the first step in the metabolism of D- and L-tyrosine by *S. acidocaldarius* is its conversion into *p*-hydroxyphenylpyruvate (*p*HPP) (unpublished results), this finding would support the involvement of this keto acid in the conversion of D- into L-tyrosine by these cells. The ready reversibility of the L-tyrosine into *p*-hydroxyphenylpyruvate is also apparent from the data presented in the Table which show that the (3*S*)-L-[2-^{2}H, 3-^{2}H]tyrosine is incorporated into cellular protein with almost complete loss of the C-2 deuterium, suggesting that all of the cellular tyrosine equilibrates with this keto acid.

It should be noted that the incorporation of both D- and L-tyrosine was also observed during the biosynthesis of plastoquinones and tocopherol in higher plants.[17] It was suggested that *p*HPP was the first intermediate of the pathway and that it was generated from either D- and L-tyrosine by either a dehydrogenase or an oxidase. The *p*HPP was then oxidatively converted into homogentisic acid, which serves as a common

intermediate to plastoquinones, tocopherols and probably CQ.[4]

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