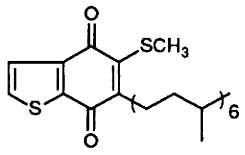
Biosynthesis of the Methylthio Side Chain of Caldariellaquinone

Dan Zhou and Robert H. White
Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 USA

Both the methyl and sulphur groups of the methylthio portion of caldariellaquinone (CQ), a benzothiophenquinone-containing quinone present in *Sulfolobus acidocaldarius*, have been shown to arise from methionine. Mass spectral analysis of the CQ isolated from cells grown in the presence of [^{34}S-*methyl*-$^{2}H_3$]-L-methionine clearly showed, however, that the methylthio group of CQ is not derived as an intact unit from the methylthio group of methionine.

The methylthio group is found in a wide assortment of natural products. The most important of these is methionine, an essential amino acid. Other natural products that contain the methylthio group include dimethylthiomethane and the methyl and methylthio esters of 3-thiomethyl propionate which represent the odorous components of the truffle,[1] the pineapple,[2] and human urine after the ingestion of asparagus,[3] respectively. Important metabolites which contain the thiomethyl group include urothion, a metabolite of the molybdenum cofactor,[4] and the *S*-methylcytokinins, which are modified bases in transfer RNAs.[5] The methylthio group is found in a metabolite of the fungicide pentachloronitrobenzene[6] and it is increasingly being found in metabolites of drugs.[7–9] It also forms part of the structure of CQ (I), the first described example of a naturally occurring, sulphur-containing quinone.[10]

The methylthio group in a metabolite could be generated by one of two completely different mechanisms. In the first mechanism, sulphur is transferred to the acceptor molecule to



(I)

generate a thiol which is subsequently methylated to the methylthio group, whereas, in the second mechanism, the methylthio group of methionine is transferred as an intact unit to the acceptor molecule. The first route is used in the biosynthesis of methionine where the sulphur of cysteine is transferred *via* cystathionine to homocysteine which is methylated to methionine in a methyltetrahydrofolate-dependent reaction.[11] A mechanism in many ways analogous to that used to generate the methylthio group of methionine also appears to operate in the methylthiolation of the drug 1-allyl-3,5-diethyl-6-chlorouracil in rabbits[9] and in the methylation of the fungicide pentachloronitrobenzene in *Tetrahymena thermophila*.[7] In both cases, evidence indicates that these compounds are first converted into a glutathione or cysteine conjugate[12,13] which is then cleaved by a cysteine conjugate β-lyase to generate the free thiol. Methylation of the resulting thiols by *S*-adenosylmethionine (SAM) generates the methylthio-containing metabolites.

Evidence for the transfer of the intact methylthio group of methionine to an acceptor molecule is weak, although, several authors have proposed it as a mechanism in the methylthiolation of drugs.[14,15] This could occur by the cleavage of the methionine to methanethiol followed by the addition of the methanethiol to the acceptor molecule. The enzymatic cleavage of methionine to methanethiol is well-documented and is known to occur by two separate metabolic pathways. In the first, the methionine is deaminated and then dethiomethylated with the release of methanethiol.[16,17] In the second, the methionine is deaminated and dethiomethylated simultaneously to methanethiol by the enzyme L-methionine γ-lyase.[18] No evidence for the addition of methanethiol to any acceptor molecule is presently available, however.

After considering the above points, it was decided to determine the metabolic origin of the methylthio group in CQ and to determine whether this group comes from methionine as an intact unit. This was accomplished by growing cells of *Sulfolobus acidocaldarius* with different labelled methionines and then measuring the incorporation of label into the biosynthesised CQ as previously described.[19] Growth of cells with [*methyl*-$^2H_3$]-L-methionine (50 mg/100 ml) and subsequent mass spectral analysis of the CQ isolated after the end of log phase growth showed that 74% of the molecules had incorporated a C^2H_3 group into the thiomethyl of the CQ. In a similar experiment, the cells were grown with [^{35}S]-L-methionine (11.6×10^8 cpm/mmol, 10 mg/100 ml) and, from the specific activity of the isolated CQ (7.1×10^8 cpm/mmol), it was calculated that 0.61 mol equiv. of sulphur from the methionine was incorporated into the CQ. Since CQ contains two sulphur atoms, these results show that, on average, the sulphur from the methionine was incorporated to an extent of 30% into each sulphur site. (Since the incorporation into the individual sulphur positions was not determined, one cannot determine from this data what the actual incorporation was at each site.)

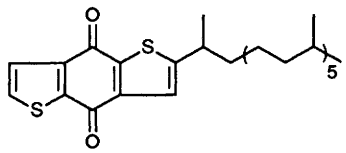
These observations show that the methyl of the methylthio group is derived from methionine and, due to the observed incorporation of sulphur from methionine, they suggest the possibility that the sulphur of the methylthio group may be derived as an intact unit from the methionine.

In order to test this idea, cells were grown with L-methionine (50 mg/100 ml) in which 34.1% of the molecules contained [^{34}S-*methyl*-$^2H_3$]-L-methionine.* If the thiomethyl group was incorporated into CQ as an intact unit and if no sulphur was incorporated into the benzothiophene ring from the methionine, then CQ molecules with an intact $^{34}SCD_3$ unit would be the only labelled product observed. This labelled product would be detected by an increase in the intensity of the $M^+ + 5$ ion in the mass spectrum of the CQ. Alternatively, if the sulphur and methyl groups of the methionine were incorporated as separate units, then the CQ would be found to contain only a single ^{34}S or a single CD_3. These labelled products would be detected by an increase in the intensities of the $M^+ + 2$ and $M^+ + 3$ ions of the CQ. The CQ isolated from this experiment was observed to contain 21.5% of the molecules with ^{34}S, 12.6% with a deuteriated methyl group, and only 7.4% with both an ^{34}S and a deuteriated methyl group, clearly indicating that the thiomethyl group was not derived as an intact unit from the methionine. (The methods used to calculate the isotopic incorporations from the mass spectral data have been previously described.[19]) By solving for the values in the equation $(a + b)(c + d)(x + y)$ that generate the observed isotopic pattern, it was found that the two sulphur sites are labelled to an extent of 10.6 and 22.3% and that the methyl group is labelled to an extent of 20.7%. [In this equation, $b/(a + b)$ is the atom % labelling at one sulphur site with ^{34}S, $d/(c + d)$ is the atom % labelling at the other sulphur site with ^{34}S, and $y/(x + y)$ is the mol % labelling of the thiomethyl group with C^2H_3.] Since only 34.1% the methionine was labelled, then, from these values, it is easily calculated that the methyl group of the methionine was incorporated to an extent of 60.6% and that the average value for sulphur incorporation into the two sites was 48.2%.

In order to ensure that the methionine in the cells still contained an intact methylthio group, the isotopic distribution of the cellular methionine was measured.[20] The results of this measurement revealed that 10.43% of the molecules contained one ^{34}S and that 21.2% of the molecules contained both an ^{34}S and a deuteriated methyl group, thereby indicating that some of the cellular methionine had been metabolised to ^{34}S-homocysteine and resynthesised from this labelled homocysteine using a nonlabelled methyl group. These results do show, however, that the major portion of the methionine was utilised by the cells without scrambling of the methyl–sulphur bond.

* [^{34}S-*methyl*-$^2H_3$]-L-Methionine was prepared by treating L-α-benzamido-γ-chlorobutyrate (1 mmol) with [^{34}S-*methyl*-$^2H_3$]methanethiol (1 mmol) dissolved in ethanol (5 ml) containing sodium ethoxide (1 mmol). The mixture was heated at 80 °C for 3 h after which the resulting ester was saponified with aqueous sodium hydroxide and the amide hydrolysed with 6M HCl. The resulting [^{34}S-*methyl*-$^2H_3$]-L-methionine was isolated from the acid hydrolysis mixture by adsorption onto Dowex-50 and elution with aqueous ammonia. The resulting labelled methionine was mixed with unlabelled methionine and the final product crystallised from aqueous ethanol. Mass spectral analysis of the final product, as the butyl trifluoroacetyl derivative, showed the methionine to contain 34.1% of the molecules with [^{34}S-*methyl*-$^2H_3$]-L-methionine. (The isotopic incorporation of the methionine was measured from the *m/z* 301 (M^+) and the *m/z* 61 CH_3S ions in the mass spectrum of the methionine as previously described.[20]) Ethyl L-α-benzamido-γ-chlorobutyrate was prepared by treating a solution of *N*-benzoyl L-homoserine lactone in ethanol with HCl as described by Hill and Robson.[22] *N*-Benzoyl L-homoserine lactone was prepared by treating L-homoserine lactone with benzoyl chloride in basic aqueous solution. [^{34}S-*methyl*-$^2H_3$]Methanethiol was prepared in a 73% yield by treating a solution of $^{34}S_8$ (34 mg) dissolved in tetrahydrofuran (1.5 ml) with a 1M solution of C^2H_3MgI (99 + atom % ^{2}H) in diethyl ether (2 ml). The mixture was stirred at room temperature for 30 min after which the solvents were evaporated and the resulting Mg salt was dissolved in ethanol for reaction with the chloride.

The above results prove that the thiomethyl group of CQ is not incorporated as a unit from methionine, even though both the sulphur and methyl group are found to be derived from methionine. The above evidence indicates that the first step in the formation of the methylthio group in CQ is the generation of a thiol which is then methylated, most likely by SAM, to CQ. In support of the intermediacy of this thiol is the recent discovery and characterisation of a new quonine in *S. solfataricus* that contains a benzo[1,2-*b*;4,5-*b*']dithiophene-4,8-quinone ring (II).[21] The second thiophene ring would be formed by the addition of a 5' thiol into a 2,3 unsaturated demethylated CQ precursor.



(II)

Acknowledgements

We thank Kim Harich for running the mass spectra and Linda D. White for editing the manuscript. This work was supported by National Science Foundation grant DMB-8718216.

References

1 A. Fiecchi, M. G. Kienle, and A. Scala, *Tetrahedron Lett.*, 1967, **18**, 1681.
2 J. O. Rodin, D. M. Coulson, R. M. Silverstein, and R. W. Leeper, *J. Food Sci.*, 1966, **31**, 721.
3 R. H. White, *Science*, 1975, **189**, 810.
4 M. Goto, A. Sakurai, K. Ohta, and H. Yamakami, *Tetrahedron Lett.*, 1967, **45**, 4507.
5 W. J. Burrows, D. J. Armstrong, F. Skoog, S. M. Hecht, J. T. A. Boyle, N. J. Leonard, and J. Occolowitz, *Biochemistry*, 1969, **8**, 3071.
6 R. Fall and S. E. Murphy, *J. Am. Chem. Soc.*, 1984, **106**, 3033.
7 A. Bergman, I. Brandt, Y. Larsson, and C. A. Wachtmeister, *Chem.-Biol. Interactions*, 1980, **31**, 65.
8 R. Kaul, G. Kiefer, and B. Hempel, *Chemosphere*, 1981, **10**, 929.
9 R. Pal and G. Spiteller, *Xenobiotica*, 1982, **12**, 813.
10 M. De Rosa, S. De Rosa, A. Gambacorta, L. Minale, R. H. Thomson, and R. D. Worthington, *J. Chem. Soc., Perkin Trans. 1*, 1977, 653.
11 M. Flavin, in 'Metabolism of Sulfur Compounds,' ed. D. M. Greenberg, Academic Press, New York, 1975, vol. 7, p. 457.
12 M. Tateishi, S. Suzuki, and H. Shimizu, *J. Biol. Chem.*, 1978, **253**, 8854.
13 H. Tomisawa, S. Suzuki, S. Ichihara, H. Fukazawa, and M. Tateishi, *J. Biol. Chem.*, 1984, **259**, 2588.
14 J. R. DeBaun, E. C. Miller, and J. A. Miller, *Cancer Res.*, 1970, **30**, 577.
15 I. C. Calder, M. J. Creek, and P. J. Williams, *Chem.-Biol. Interactions*, 1974, **8**, 87.
16 J. Ruiz-Herrera and R. L. Starkey, *J. Bacteriol.*, 1969, **99**, 544.
17 W. Segal and R. L. Starkey, *J. Bacteriol.*, 1969, **98**, 908.
18 H. Tanaka, N. Esaki, and K. Soda, *Biochemistry*, 1977, **16**, 100.
19 D. Zhou and R. H. White, *J. Bacteriol.*, 1989, **171**, 6610.
20 R. H. White, *Anal. Biochem.*, 1981, **114**, 349.
21 V. Lanzotti, A. Trincone, A. Gambacorta, M. De Rosa, and E. Breitmaier, *Eur. J. Biochem.*, 1986, **160**, 37.
22 E. M. Hill and W. Robson, *Biochem. J.*, 1936, **30**, 248.

Paper 0/01178I
Received 19*th March* 1990
Accepted 1*st May* 1990