The Possibility of Involvement of "Cyclase" Enzyme of the Calditol Carbocycle with Broad Substrate Specificity in *Sulfolobus acidcaldarius*, a Typical Thermophilic Archaea

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The cyclopentane ring of calditol, a characteristic lipid molecule of *Sulfolobus*, a major thermophilic Archaea, was biosynthesized from glucose directly, and galactose was converted to calditol with a similar extent to glucose. This finding indicates the possibility of the "cyclase" enzyme of the calditol carbocycle and the involvement of catalytic oxidoreduction at the C-4 of glucose with broad specificity.

Thermophilic Archaea live under harsh conditions such as high temperatures (60 to 100 °C) and low pH (2 to 3). The lipid of this species is characteristic ether-bonded isoprenoidal macrocyclic, which consists of a double-face monolayer membrane.[1] This lipid is stable in acidic, high temperature environments. The genus *Sulfolobus* (*Sulfolobales*), one of the major groups of thermophilic Archaea, has a characteristic lipid known as calditoglycerolcaldoarchaeol (GDCT, **1**, Figure 1). The hydrophilic portion of **1** is a 5-membered carbocycle bonded with glycerol by ether linkage.[2] This carbocycle also contributes to tolerance of harsh living conditions.

The formation of abundant carbocyclic compounds from carbohydrates has been proven and the mechanistic implications of the biosynthesis of each compound have been discussed. Regarding biosynthesis of the cyclopentane ring from carbohydrates, the mechanistic implication of the cyclization mechanism of a number of secondary metabolites from several bacterial species has been shown.[3–6] Further, the cyclopentane ring, linked to bacteriohopanetetrol in *Zymomonas mobilis*, has shown to be biosynthesized from *N*-acetylglucosamine.[7]

Recently, Gambacorta et al.[8] and our group[9] performed biosynthetic studies of calditol **3**, revealing the occurrence of glucose cyclization in a 5-membered carbocycle with C–C bond formation at C-1 and C-5. The involvement of *myo*-inositol 1-phosphate synthase-like cyclase was also suggested. However, the key step of cyclization, the involvement of catalytic oxidoreduction was not shown directly, although the inversion of C-4 stereochemistry from glucose to calditol was needed for the reaction sequence.

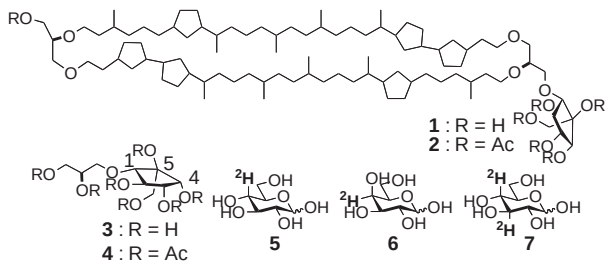

Figure 1. Structure of GDCT, calditol, and labeled substrates.

On the other hand, the central metabolism of *Sulfolobus* is a nonphosphorylative variant of the Entner–Doudoroff pathway.[10] Furthermore, the enzymes of this pathway have shown to possess "substrate promiscuity" that enables catalysis of glucose and galactose to a similar degree.[11] Observation of the fate of labeled glucose and galactose in C-4 during calditol carbocycle formation can clarify the key step of the unique carbocycle formation mechanism and enhancement of substrate promiscuity of glucose and galactose in *Sulfolobus*.

First, [4-^{2}H]glucose (**5**) and [4-^{2}H]galactose (**6**) were synthesized.[12,13] The double-labeled glucose [3,4-$^2H_2$]glucose (**7**) was synthesized according to the synthesis of **5**,[13] instead of through $NaBH_4$ reduction of the 4-ulose intermediate by NaB^2H_4. The chase experiment was performed as we reported recently.[9] *S. acidocaldarius* (JCM 9063, from RIKEN) was grown on medium with labeled glucose or galactose (1 g L^{-1}). GDCT (**1**) was isolated from the hydrolysate of the extracted lipid, converted to its acetate **2** and purified.

The assignment of signals at H-2 (δ 5.47), H-3 (5.35) and H-4 (5.43) of **2** were determined previously.[9] The NMR spectra

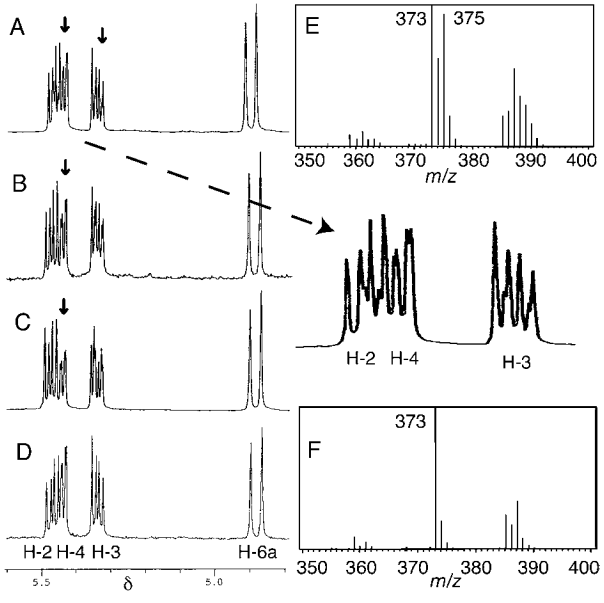

Figure 2. Partial NMR spectra of **2** and MS spectra of **4** obtained from deuterium-labeling experiment by each substrate, (NMR of **2**) A: [3,4-$^2H_2$]glucose (**7**), B: [4-^{2}H]galactose (**6**), C: [4-^{2}H]glucose (**5**) Inc, D: non-labeled calditol; (MS of **4**) E: [3,4-$^2H_2$]glucose (**7**), F: non-labeled calditol. The magnification of H-2,3,4 signals of spectrum A was also shown.

Table 1. ^{1}H NMR deuterium incorporation value at each hydrogen of calditol in labeling experiment

Precursor	Integral values		Incorporation
	H-2 and H-4	H-3	
[4-^{2}H]Glu (**5**)	1.65	1.03	35% (H-4)
[4-^{2}H]Gal (**6**)	1.73	0.99	27% (H-4)
[3,4-$^{2}H_2$]Glu (**7**)	1.62	0.65	38% (H-4), 35% (H-3)

[a]For the experiment of [2-^{2}H]glucose, no incorporation of deuterium was observed.[9] So, the incorporation of deuterium was estimated by assuming the incorporation of deuterium at H-2 was zero.

are shown in Figure 2, and the estimation of deuterium incorporation is presented in Table 1.

In the incorporation experiment using **5** (Figure 2, spectrum C), the H-4 in the carbocycle of calditol was apparently deuterated.[14] The deuterium incorporation from glucose was 35% as estimated by the integration of signals. Interestingly, the deuterium incorporation was 27% in the deuterium incorporation experiment with **6** (Figure 2, spectrum B), suggesting that galactose was also converted with similar extent to the carbocycle of calditol during the cyclization reaction.

Thus, three possibilities are suggested for the cyclization of calditol carbocycles. a) The putative cyclization enzyme (calditol carbocycle cyclase, "CCCase"), which has relatively broad substrate specificity at the C-4 of glucose, might exist in this reaction. b) Galactose was converted to glucose (with UDP-Galactose 4-epimerase) very rapidly, and the converted glucose was successively cyclized to the carbocycle. c) We also considered degradation of galactose and glucose to C-3 components such as pyruvate and dihydroxyacetone and reconstruction to glucose.

Next, we examined whether epimerization between glucose and galactose at C-4 or degradation of galactose (and glucose) to C-3 components and reconstruction of glucose could mitigate the labeling of deuterium at the C-4 of glucose/galactose. The double-labeling experiment of [3,4-$^{2}H_2$]glucose (**7**) suggested no extinct loss of deuterium at the C-4 of glucose compared with the C-3 from NMR observation (Figure 2, spectrum A). Further, intact incorporation of double-labeled glucose to calditol was also suggested from the EI-MS spectrum of **4** which was degraded and prepared from **7**.[15] The EI-MS of **4** from [3,4-$^{2}H_2$]glucose showed charactaristic fragment **8** (Chart 1) m/z 373 (non-labeled) and 375 (two deuterium inc.) almost same intensities while m/z 374 (one deuterium inc.) was about a half of the intensities at m/z 373 and 375. (Figure 2, E). That means the major part of doubly labeled glucose was incorporated to calditol with directly. These results may suggested the possibility of a), the involvement of cyclization enzyme of calditol carbocycles at the biosynthetic pathway.

The reaction mechanism (Figure 3), the involvement of catalytic oxidation–reduction in the C-4 OH group resembles to *myo*-inositol 1-phosphate synthase, dehydroquinate synthase,[16] 2-deoxy-*scyllo*-inosose synthase[17,18] and deoxysugar biosynthe-

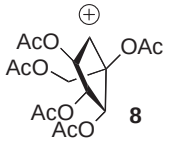

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Chart 1.

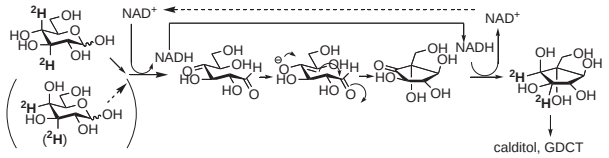

Figure 3. Putative *Sulfolobus* calditol carbocycle "cyclase" (CCCase) reaction mechanism.

sis.[19] Substrate promiscuity may also be expressed at the "cyclization" enzyme responsible for cyclization of calditol from glucose. Unique substrate recognition was observed with enzymes in the non-phosphorylative Entner–Doudoroff pathway in *Sulfolobus*,[11] and *Thermoplasma acidophilum*.[20] However, neither the substrate of the reaction nor the product produced just after cyclization have so far been detected in calditol carbocycle biosynthesis. Elucidation of the intermediate enzyme activity during the "cyclization" of calditol carbocycle will be important in understanding the details of the cyclization mechanism.

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