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Determination of the Solution Structure of Malonyl-CoA by Two-Dimensional Nuclear Magnetic Resonance Spectroscopy and Dynamical Simulated Annealing Calculations

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In order to understand the initial interaction of the substrates malonate, ATP, and CoA with malonyl-CoA synthetase, the catalytic product malonyl-CoA was characterized by NMR spectroscopy and molecular modeling. To assign proton and carbon chemical shifts, two-dimensional ^{1}H-^{1}H DQF-COSY and ^{1}H-^{13}C HMBC experiments were used. The structure of malonyl-CoA in the solution phase was determined based on distance constraints from NOESY and ROESY spectra. The structures were well-converged around the pantetheine region with the pairwise RMSD value of 0.08 nm. The solution structure exhibited a compact folded conformation with intramolecular hydrogen bonds among its carbonyl and hydroxyl groups. These findings will help us to understand the initial interaction of malonate and CoA with malonyl-CoA synthetase.

Keywords: Malonyl-CoA, Malonyl-CoA Synthetase, NMR, Simulated annealing.

Introduction

Malonyl-CoA synthetase (MCS) is a polypeptide of 504 residues with a molecular weight of about 54 kDa. MCS catalyzes the formation of malonyl-CoA from malonate and CoA by ATP hydrolysis (Kim and Chae, 1991), and shows high substrate specificity for malonate, ATP, and CoA (Kim and Chae, 1991). It has been suggested that MCS plays a critical role in nitrogen flow together with malonamidase (Kim and Chae, 1990; Kang and Kim, 1996). MCS was first purified and characterized from *Bradyrhizobium japonicum* (Kim and Chae, 1991). This enzyme was also purified and characterized from *Rhizobium trifolii* (Kim *et al.*, 1993). In addition, we reported that the steady-state kinetic mechanism of malonyl-CoA synthetase was similar to the ordered Bi Uni Uni Bi Ping Pong Ter Ter system based on initial-velocity and product-inhibition studies (Kim and Kang, 1994). The K_m values for the substrates, malonate, CoA, and ATP, were measured as 200 μM, 87 μM, and 33.3 μM, respectively. Chemical modification studies have shown that malonyl-CoA synthetase in *Rhizobium trifolii* requires arginine, histidine, and lysine residues for biological activity (Lee and Kim, 1993a; 1993b). In addition, it has been suggested that a tryptophan residue participates in the CoA binding of MCS in *Rhizobium trifolii* (Lee and Kim, 1993c). However, no detailed structural studies of the substrates of MCS have been reported yet. Lee and Sarma (1975) reported the rough conformations of both CoA and its derivatives based on J-coupling constants. Very recently, the solution conformation of CoA complexed to chloramphenicol acetyltransferase was reported (Barsukov *et al.* 1996). It was suggested that the adenosine region demonstrated as quite a compact conformation, while the pantetheine moiety was quite extended. However, there was still no detailed information about the solution conformation of malonyl-CoA. Here, we present the solution structure of malonyl-CoA based on two-dimensional nuclear magnetic resonance (NMR) spectroscopy and simulated annealing calculations.

Materials and Methods

Sample preparation Malonyl-CoA was purchased from Sigma (St. Louis, USA). For NMR experiments, 2 mg or 4 mg of malonyl-CoA was dissolved in 500 μl of 90% H_2O/10% $^{2}H_2O$ or 99.9% $^{2}H_2O$ solution.

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NMR spectroscopy All NMR experiments were performed on either a Bruker DMX600 or an AMX500 spectrometer in quadrature detection mode equipped with a SGI INDY computer. Most of the data was acquired at 30°C, and pulsed-field gradient (PFG) techniques combined with water-gated pulse sequence or presaturation were used for solvent suppression. Temperature was calibrated using a methanol standard. One-dimensional ^{31}P and ^{13}C spectra of malonyl-CoA were collected. Two-dimensional NOESY experiments (Jeener *et al.* 1979) with mixing times of 600 – 800 ms and double quantum-filtered (DQF) COSY spectra (Rance *et al.* 1983) were also collected. ROE mixing times of 50 – 250 ms were used for rotating frame nuclear overhauser enhancement spectroscopy (ROESY). All NMR data were recorded in phase-sensitive mode using the time-proportional phase incrementation method (Marion *et al.* 1983) with 2,048 data points in the acquisition domain and 256 or 128 in the time domain. Heteronuclear multiple-bond-correlated (HMBC) spectroscopy data was also collected for the purpose of carbon resonance assignments.

All data were transferred to an SGI Indigo^2 workstation and processed using nmrPipe (Biosym/Molecular Simulations Inc., San Diego,USA) or Bruker XWIN-NMR software. (Bruker Instruments, Rheinstetten, Germany)

Molecular modeling calculations Solution structures of malonyl-CoA were calculated using hybrid distance geometry and the dynamical simulated annealing procedures (Nilges *et al.*, 1988a; 1988b; 1988c; Driscoll *et al.*, 1989) with small modification (Lee, W. *et al.*, 1994) on X-PLOR 3.851 (Biosym/ Molecular Simulations Inc., San Diego, USA). The initial structure of malonyl-CoA was generated by adding the malonate moiety to the sulfonyl end of CoA. The topology and parameter files used for the modeling calculations were modified for the chemical structure of malonyl-CoA. Interproton distances for constraints were derived from both ROESY and NOESY experimental data. NOEs were converted to three distance ranges, 0.18–0.27 nm, 0.18–0.33 nm, and 0.18–0.50 nm, corresponding to NOE intensity. In the case of methyl and methylene protons, the upperbound distances were set to 0.1 nm longer for pseudoatom correction. In addition, vicinal coupling constants of the ribose ring and pantetheine protons were measured. However, these coupling constants did not offer much information for the structure calculations.

Results and Discussion

NMR resonance assignments Proton and carbon resonances of malonyl-CoA were easily assigned from the combined use of HMBC and DQF-COSY spectra (Table 1). Malonyl-CoA consists of ADP, pantetheine, and a malonate, as shown in Fig. 1. These three groups were easily distinguished according to their characteristic chemical shifts (Fig. 2). Methyl and aliphatic proton resonances show up at 0–3 ppm, and the protons of the ribose ring resonate around 4–6 ppm. The protons on the adenine moiety have larger chemical shifts in the downfield region due to ring current effects. The protons of the ribose ring were initially assigned from DQF-COSY spectra. These protons were identified at 4.7 ppm which is close to the solvent signal. In the pantetheine moiety, two CH_2-CH_2 groups showed similar patterns in the DQF-COSY spectrum. Each methylene group is coupled with its adjacent methylene, but no coupling network is observed because they are linked to each other with peptide bonds which block the transfer of magnetization by large coupling constants among carbonyl and amide groups.

Table 1. Proton and carbon chemical shift of malonyl-CoA.

Proton	Chemical Shift (ppm)
HA1	6.23
HA2	4.90
HA3	4.87
HA4	4.61
HA5	4.27
HP1′	3.86
HP1″	3.59
HP2′	0.91
HP2″	0.75
HP3	4.04
HP5	3.47
HP6	2.46
HP8	3.35
HP9	3.05
HP11	2.21
NH1P	8.1
NH2P	8.22
H2	8.41
H8	8.67
NH2	6.8

Carbon	Chemical shift (ppm)
C2	141
C4	143.5
C5	59
C6	146
C8	114
CA1	82
CA2	68
CA3	78
CA4	136
CA5	143
CP1	32
CP2	66.5
CP2′	16
CP2″	12
CP3	68.5
CP4	169
CP5	30
CP6	40
CP7	168
CP8	22
CP9	32
CP10	190
CP11	25
CP12	210

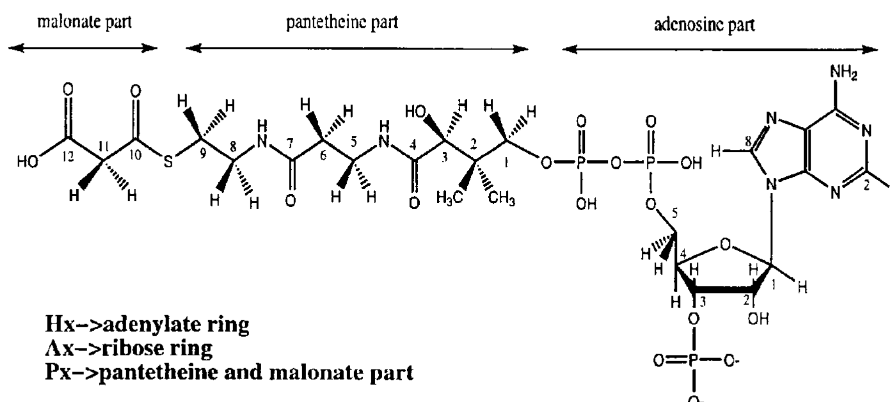

Fig. 1. Molecular structure of malonyl-CoA. Atoms are named as three groups which are adenine, ribose, and pantetheine, respectively. For atoms in the pantetheine moiety, 'P' was labeled with a number.

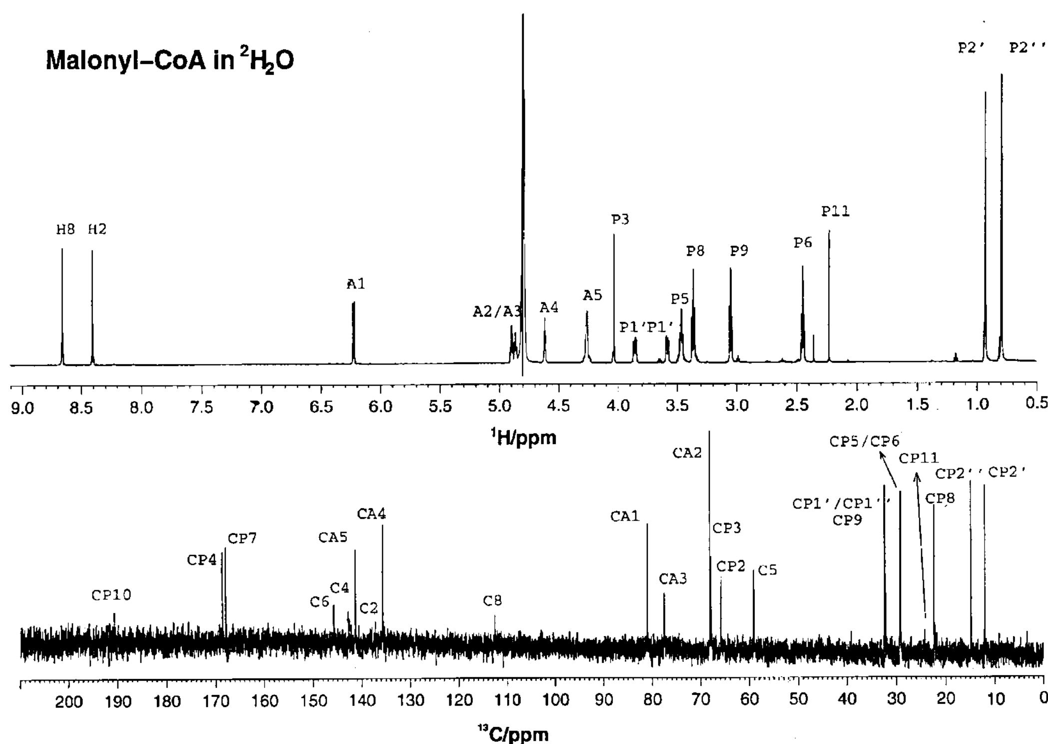

Fig. 2. Proton and carbon 1D spectra of malonyl-CoA at 600MHz in ^2H_2O solvent. These spectra were collected on a Bruker DMX600 spectrometer at 30°C.

Therefore, they were assigned by NOE correlation with HP3 and the adjacent group as well as the lowfield shifting of HP6. The rest of the proton resonances were assigned based on their standard chemical shifts as well as volume integrals of proton resonances. HP3 and HP11 showed no correlation in the DQF-COSY spectrum. HP3 is near to the hydroxyl group, which is an electron attractor and causes a downfield shift of HP3. HP11 is close to neighboring carbonyl groups which shift protons to a low field. Therefore, HP11 shifts to the low field. Carbon chemical shifts were assigned from the HMBC spectrum based on proton resonances (Fig. 4). The resonances of carbon directly linked to proton nuclei were easily identified since the one-bond coupling constant between carbon and proton nuclei is 140Hz. As a result, the coherence peaks were widely splitted doublets. The rest of the quaternary carbons were also assigned from the connectivities of the HMBC spectrum resonance. The ^{31}P-1D spectrum shows one

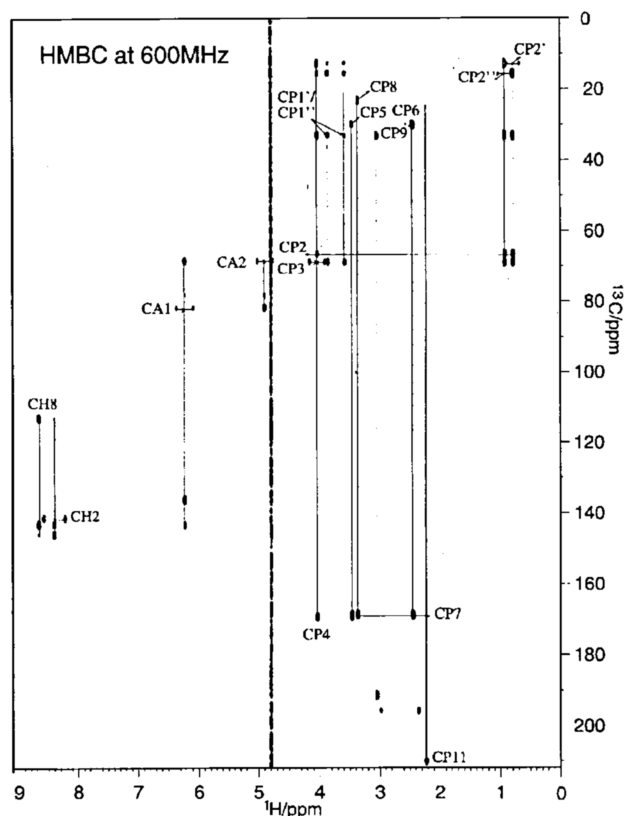

Fig. 3. HMBC spectrum of malonyl-CoA at 600MHz. This spectrum shows the through-bond connectivity between the proton and carbon nuclei.

singlet at 1 ppm and two doublets at –11 ppm and –11.7 ppm (data not shown). Since these doublets indicate that two phosphorus atoms are coupled to each other, we were able to assign all phosphorus resonances.

Solution structure of free malonyl-CoA From both ROESY and NOESY spectra, a total of 40 distance constraints were obtained (Fig. 5). Fifty structures were initially calculated using the experimental constraints. Among the structures of malonyl-CoA, fourteen final structures were selected for further analysis. The pairwise RMSD value including hydrogen is 0.20 nm and for the highly-conserved pantetheine part is 0.08 nm. The solution structure exhibited a compact folded conformation as shown in Fig. 6A. Our final structures agreed with previous work based on J-coupling constant information and chemical shift data of adenine by low field NMR study (Lee and Sarma, 1975). Interestingly, the pantetheine moiety was very well-conserved. Since no NOEs were observed for the malonate moiety, the malonate region was showed to be very flexible. These flexibilities could be explained by solution structures, since the malonate is located at the end of malonyl-CoA. In addition, there are two thioester bonds which are freely rotatable.

Some NOEs were observed between protons of the ribose ring and pantetheine acid moiety indicating that malonyl-CoA has a folded conformation. The compact structure of malonyl-CoA is mainly caused by the CP5-CP6 bond in the pantetheine moiety as shown in Fig. 6B.

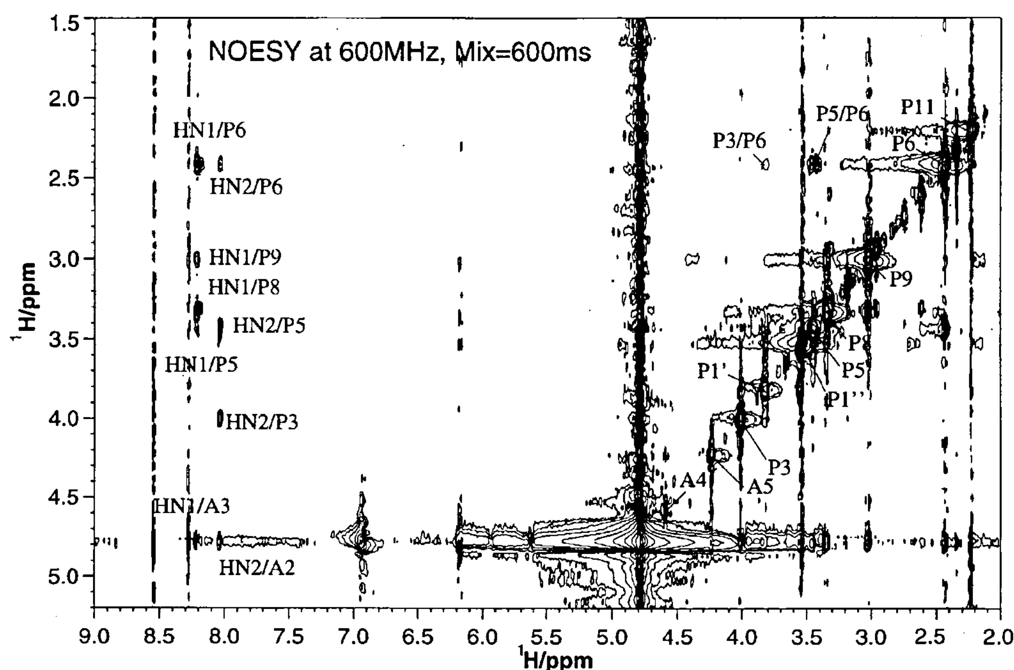

Fig. 4. 600MHz NOESY spectrum of malonyl-CoA with mixing time of 600 ms. The spectrum shows NOEs protons between neighboring groups. These peaks were also observed in the ROESY spectrum.

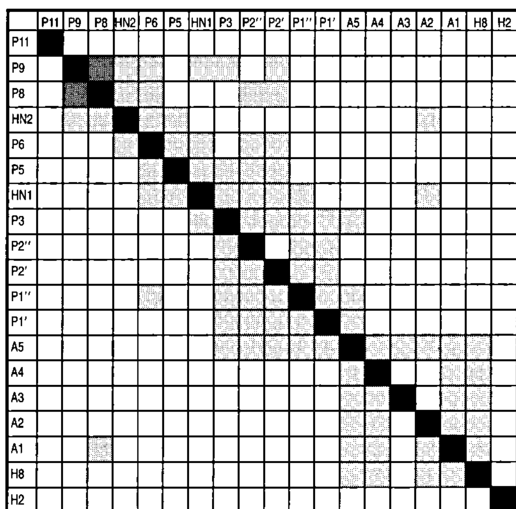

Fig. 5. NOE/ROE diagram of malonyl-CoA. The observed ROE and NOE are summarized in the diagram. The sequence of atoms is arranged by their relative bonding distance. Off-diagonal squares denote medium and long range NOEs.

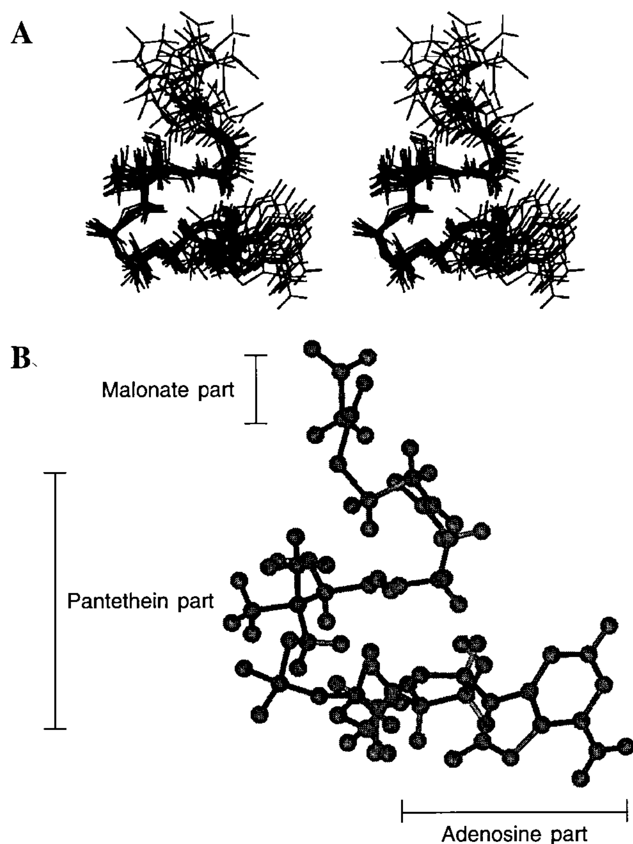

Fig. 6. Solution structures of malonyl-CoA. The ensemble structures consisting of 14 structures from 50 calculated (<SA>$_k$) structures were displayed. A. The pantetheine moiety was well conserved, but the malonyl part showed a high degree of flexibility. B. Ball and stick model of the REM average structure displaying the general topology of malonyl-CoA.

There are two CH_2-CH_2 groups in the pantetheine part, CP5-CP6 and CP8-CP9. The conformation of the CP8-CP9 bond was determined to be trans, which is the most-preferred form. However, the CP5-CP6 bond showed a gauche conformation, which is less preferred. In malonyl-CoA, there are two peptide bonds that are connected with CP5-CP6 in the pantetheine moiety. Since their NH and CO groups are potent hydrogen bond donors or acceptors, their interaction with charged groups could be a major driving force for its folded conformation. Unfortunately, there were no intramolecular hydrogen bonds observed in our simulated annealing structures. However, when the average structure was energy-minimized without any constraints, possible hydrogen bonds were observed. Therefore, we think that the hydrogen bonds of malonyl-CoA are not strong enough to be identified at room temperature. The temperature coefficients of amide protons were –5.8 ppb (Δppm/ΔT*10^3) for NH1P and –7.5 ppb for NH2P when temperature was changed from 283K to 303K. In particular, since the molecular size of malonyl-CoA is small, charged atoms would most likely be solvent-exposed. In our calculated structures, the relative orientation of the two peptide bonds in malonyl-CoA are parallel to each other. The pyrophosphate moiety is also exposed to solvent, having no steric hindrance with other bulky groups, so it may attribute to the initial interaction of malonyl-CoA with MCS. It has also been suggested that coenzyme A exists in a fast-exchanging conformation between linear and folded forms in aqueous solution (Lee and Sarma, 1975). Since the biosynthetic mechanism of malonyl-CoA formation by malonyl-CoA synthetase is still not well understood, our solution structure should help identify possible binding modes and the initial interaction of substrates to the malonyl-CoA synthetase.

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