Remarkably Broad Substrate Tolerance of Malonyl-CoA Synthetase, an Enzyme Capable of Intracellular Synthesis of Polyketide Precursors

Nicola L. Pohl,[†] Marcus Hans,[†] Hwan Young Lee,[‡] Yu Sam Kim,[‡] David E. Cane,[§] and Chaitan Khosla*,[||]

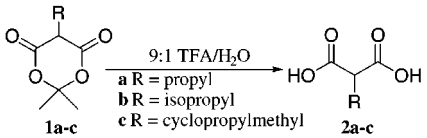
Departments of Chemical Engineering and Chemistry and Biochemistry, Stanford University Stanford, California 94305-5025 Department of Biochemistry, College of Science Protein Network Research Center Yonsei University, Seoul, Korea 120-749 Department of Chemistry, Brown University Providence, Rhode Island 02912

Received August 1, 2000

Polyketides are a structurally complex class of natural products with therapeutic and agrochemical utility.[1] Polyketide backbones are generated by the repetitive decarboxylative condensation of simple malonic acid derivatives by large multifunctional proteins called polyketide synthases (PKSs). These modular enzymes can be genetically modified to biosynthesize new "unnatural" natural products.[2] Notwithstanding the spectacular diversity of natural and engineered polyketides, however, the potential structural diversity of these molecules is seriously limited by the relatively small number of building blocks that are naturally available for polyketide biosynthesis within a cell. The ability to regioselectively incorporate new, orthogonally reactive functional groups into a polyketide scaffold has important implications for investigations into PKS mechanisms as well as for the medicinal exploitation of polyketides.

Acyltransferase domains within PKSs are responsible for selecting malonyl-CoA or its analogues for each round of condensation. The two most common metabolically available substrates are malonyl-CoA and methylmalonyl-CoA, although biosynthetic pathways for a few other α-carboxylated CoA thioesters are also presumed to exist.[3] A common biosynthetic strategy for the formation of malonyl- or methylmalonyl-CoA entails the carboxylation of acetyl- or propionyl-CoA, respectively. However, harnessing this pathway to extend the in vivo pool of α-carboxylated CoA thioesters would require an enzyme that would carboxylate a wide variety of CoA-linked acids with substituents directly at the reactive site. Recently, an alternative pathway for malonyl-CoA biosynthesis has been discovered in *Rhizobium trifolii*, in which exogenous malonate is imported via a membrane-bound dicarboxylate transporter protein, and is directly activated into malonyl-CoA by an ATP-dependent malonyl-CoA synthetase.[4] Heterologous expression of these two genes in a recombinant strain of *Streptomyces coelicolor* which produces 6-deoxyerythronolide B has been shown to result in dramatic improvements in macrolide productivity,[5] indicating that this precursor pathway can also utilize methylmalonate in addition to malonate. We have therefore investigated the substrate specificity of malonyl-CoA synthetase from *R. trifolii*. As shown below, the enzyme has remarkable tolerance for a variety of C-2 substituted malonic acids, making it an attractive catalyst for the in vivo or in vitro formation of building blocks for polyketide synthesis.

Scheme 1. Synthesis of Malonic Acid Derivatives



An important consideration in the exploitation of malonyl-CoA synthetase is the availability of suitable 1,3-dicarboxylic acid substrates. Although malonic acid derivatives can be accessed by alkylation of a malonic acid diester by an electrophile and subsequent base-catalyzed saponification,[6] this synthetic protocol requires tedious isolation procedures to recover high yields of dicarboxylic acids. On the basis of our previous experience with the synthesis of *N*-acetyl cysteamine monothioesters of malonic acid via ring-opening of Meldrum's acid,[7] we reasoned that in addition to being an activating group, the isopropylidene ketal could also be viewed as a protecting group that could be cleaved to avoid aqueous product extractions. Ultimately, a mixture of trifluoroacetic acid and water[8] were chosen. Deprotection took place in minutes at ambient temperature, and the reagents and reaction byproducts were volatilized to leave the desired diacid in almost quantitative yield.[9] Several alkylated malonic acids were obtained in this manner (Scheme 1).

Malonyl-CoA synthetase has previously been shown to convert malonic acid and CoASH to malonyl-CoA with hydrolysis of ATP to AMP and diphosphate via a malonyl-AMP intermediate.[10] While malonic acid and methylmalonic acid were converted to their corresponding monothioesters, acetate, propionate, or succinate were not. This suggested that the 1,3-diacid functionality was crucial for enzymatic activity.

To probe the molecular recognition features of malonyl-CoA synthetase, ethyl-, propyl-, allyl-, isopropyl-, dimethyl-, cyclopropyl-, cyclopropylmethylene-, cyclobutyl-, and benzyl-malonate were either purchased or prepared, and assayed in the presence of the enzyme, ATP, and CoASH as described previously.[11,12]

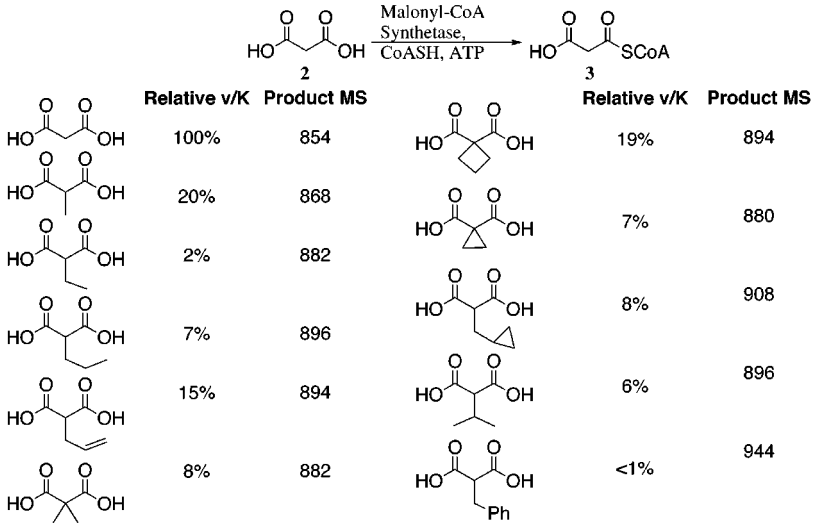
[†] Department of Chemical Engineering, Stanford University.
[‡] Department of Biochemistry, College of Science, Protein Network Research Center, Yonsei University.
[§] Department of Chemistry, Brown University.
[||] Departments of Chemistry and Biochemistry, Stanford University.

(1) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465.
(2) Khosla, C.; Gokhale, R.; Jacobsen, J. R.; Cane, D. E. *Annu. Rev. Biochem.* **1999**, *68*, 219.
(3) (a) Kim, Y. S.; Chae, H. Z. *Biochem. J.* **1991**, *273*, 511. (b) Kakavas, S. J.; Katz, L.; Stassi, D. *J. Bacteriol.* **1997**, *179*, 7515. (c) Motamedi, H.; Shafiee, A. *Eur. J. Biochem.* **1998**, *256*, 528. (d) Wu, K.; Chung, L.; Revill, W. P.; Katz, L.; Reeves, C. D. *Gene* **2000**, *251*, 81.
(4) An, J. H.; Kim, Y. S. *Eur. J. Biochem.* **1998**, *257*, 395−402.
(5) Lombo, F.; Pfeifer, B.; Leaf, T.; Ou, S.; Kim, Y. S.; Cane, D. E.; Licari, P.; Khosla, C. *Biotech. Prog.* in press (2001).
(6) See for example: Patterson, F. L. M.; Buchanan, R. L.; Dean, F. H. *Can. J. Chem.* **1965**, *43*, 1700−1713.
(7) Pohl, N. L.; Gokhale, R. S.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **1998**, *120*, 11206−11207.
(8) Christensen, J. E.; Goodman, L. *Carbohydr. Res.* **1968**, *7*, 510−512.
(9) *Procedure for deprotection of isopropylidene-protected malonic acid derivatives.* Isopropyl Meldrum's acid (**1b**) (200 mg, 1.07 mmol) was treated with water (0.2 mL) and trifluoroacetic acid (1.8 mL). The reaction mixture was allowed to sit at ambient temperature for 15 min. Solvent was removed under reduced pressure. Diethyl ether (5 mL) was added to the resulting oil and then removed under reduced pressure to leave a white amorphous solid (**2b**) (154 mg, 1.05 mmol, 98%). **2b**: (400 MHz ^{1}H NMR, DMSO-d_6) δ 2.94 (d, J = 8.8 Hz, 1 H), 2.2−2.1 (m, 1 H), 0.93 (d, J = 6.7 Hz, 6 H); (100 MHz ^{13}C NMR, DMSO-d_6) δ 171.3, 60.0, 29.0, 21.4. **2c**: (400 MHz ^{1}H NMR, DMSO-d_6) δ 3.27 (t, J = 7.3 Hz, 1H), 1.63 (dd, J = 7.3 Hz, 2 H), 0.92 (m, 2 H), 0.38 (m, 2 H), 0.08 (m, 2 H); (100 MHz ^{13}C NMR, DMSO-d_6) δ 171.8, 53.0, 34.3, 9.8, 5.2.

(10) Kim, Y. S.; Kang, S. W. *Biochem. J.* **1994**, *297*, 327−333.
(11) Stock solutions of the diacids (pH 6−7) were made using aqueous sodium hydroxide. A direct spectrophotometric assay method was used as previously reported.[11] This method is based on the measurement of the increase in absorbance at 232 nm by the formation of thioester bond of malonyl-CoA. The incubation mixture for this assay contains (in micromoles) potassium phosphate buffer, pH 7.2, 100; sodium malonate-derivative, 10; $MgCl_2$, 2; ATP, 0.4; coenzyme A, 0.2; and enzyme (0.9 μg) and water in a total volume of 1.0 mL. Control reaction mixture contains 10 μmol of malonate instead of sodium malonate-derivatives. The rate of increase in absorbance at 232 nm is recorded by a spectrophotometer equipped with 30 °C circulator. The molar extinction coefficient of thioester bond of malonyl-CoA at 232 nm is 4500 M^{-1} cm^{-1}.
(12) Kim, Y. S.; Bang, S. K. *Anal. Biochem* **1988**, *170*, 45−49.

10.1021/ja0028368 CCC: $20.00
Published on Web 05/26/2001

Table 1.



Surprisingly, the enzyme converted all these diacids into their corresponding monothioesters (Table 1). Moreover, except for benzylmalonate, in all cases the bimolecular rate constants for product formation were greater than 1% that for the natural substrate, malonate. As a point of reference, the k_{cat} and K_M for malonate are $19 \pm 0.3\ s^{-1}$ and $208 \pm 6\ \mu M$, respectively, whereas the k_{cat} for a typical PKS is in the range of $1\ min^{-1}$. Therefore, rate constants equivalent to 1% that for the natural substrate should be adequate to ensure nonlimiting generation of a desired substrate in vivo.

The above results suggest that malonyl-CoA synthetase has broad tolerance toward unnatural diacid substrates, especially those with hydrocarbon side chains. Moreover, the enzyme does not appear to have a fixed angle requirement for the two carboxylate functionalities, as both cyclobutyl dicarboxylic acid and cyclopropyl dicarboxylic acid were turned over. Most interestingly, the enzyme can handle substrates such as allylmalonate and cyclopropylmethylenemalonate which, if incorporated into polyketide backbones, could provide functional handles for regioselective modifications.

As mentioned above, acyltransferase domains in PKS modules are the principal determinants of extender unit selectivity. These domains can strongly discriminate against alternative extender units available in a cell.[13] To test whether PKS modules might be able to process these unusual extender units, methylmalonyl-CoA, ethylmalonyl-CoA, and propylmalonyl-CoA were synthesized using malonyl-CoA synthetase and assayed against the terminal module of the 6-deoxyerythronolide B synthase using (2*S*,3*R*)-2-methyl-3-hydroxy-pentanoyl-*S*-*N*-acetylcysteamine as the source of primer units.[14] Mass spectrometric analysis of overnight incubations of each reaction mixture revealed the expected triketide lactone product.[15] This assay could not be used for accurate kinetic analysis. However, under saturation conditions (0.5 mM for methylmalonyl-CoA, 5 mM for other thioesters), the relative product yields could be estimated at the end of the incubation period. The molar ratios of products derived from methylmalonyl-CoA, ethylmalonyl-CoA, propylmalonyl-CoA and malonyl-CoA were 100:8:6:<0.1, respectively. Thus, although this methymalonyl-CoA specific module can discriminate against both smaller and bulkier substituents at the α-carbon, it appears to have greater tolerance toward unnatural precursors that are ordinarily not available in a cell.

In summary, the *R. trifolii* malonyl-CoA synthetase provides an attractive route to expand the lexicon of precursors available for polyketide biosynthesis. Parenthetically, we note that several orthologs of this enzyme have recently been reported in the genomic literature with approximately 30% sequence identity;[16] these enzymes may have complementary substrate preferences to the enzyme described here. Since PKS modules appear to have modest tolerance toward some of these unnatural precursors, coexpression of PKSs with slightly altered acyltransferase selectivity and malonyl-CoA synthetase could yield polyketides with unnatural functional groups.

Acknowledgment. This work was funded by NIH Grants to C.K. (CA66736) and to D.E.C. (GM22172) and Grants from the Korea Science and Engineering Foundation (97-05-01-06-01-5) to Y.S.K. N.L.P. is the recipient of a National Institute of General Medical Studies Postdoctoral Fellowship (1 F32 GM19540-02). We thank Allis Chien at the Stanford Mass Spectrometry Laboratory for carrying out the mass spectrometric measurements.

JA0028368

(13) For example, the methylmalonyl-CoA-dependent acyltransferase domain from the terminal module of the 6-deoxyerythronolide B synthase is 360-fold more selective toward its natural substrate than toward malonyl-CoA (Lau, J. Ph.D. Thesis, Stanford University, 2000).

(14) Assay conditions are described in Wu, N.; Kudo, F.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2000**, *122*, 4847.

(15) Observed masses (ESI-MS): 172.0, 185.1, and 199.0 for the methylmalonyl-, ethylmalonyl- and propylmalonyl-derived β-ketolactones, respectively. In addition, tandem MS analysis of the molecular ions yielded the expected $M-H_2O$ species. (described in Weissman, K. J.; Kearney, G. C.; Leadlay, P. F. and Staunton, J. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2103−2108).

(16) For example, see: (a) Fuldam, M.; Heinz, E.; Wolter, F. P. *Mol. Gen Genet.* **1994**, *242*, 241−249. (b) Blobel, F.; Erdmann, R. *Eur. J. Biochem.* **1996**, *240*, 468−476. (c) Koo, H. M.; Kim, Y. S. *Arch. Biochem. Biophys.* **2000**, *378*, 167.