ALIPHATIC MOLECULES (C-6 TO C-8) CONTAINING DOUBLE OR TRIPLE BONDS AS POTENTIAL PENICILLIN SIDE-CHAIN PRECURSORS

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Three different hexenoyl-CoA derivatives (*trans*-2-hexenoyl-CoA, *trans*-3-hexenoyl-CoA and *trans-trans*-2,4-hexadienoyl-CoA), two octenoyl-CoA (*trans*-2-octenoyl-CoA, *trans*-3-octenoyl-CoA) and 2-octynoyl-CoA were tested as substrates of the enzyme acyl-CoA: 6-Aminopenicillanic acid acyltransferase (AT) from *Penicillium chrysogenum*. Only *trans*-3-hexenoyl-CoA and *trans*-3-octenoyl-CoA were recognized by AT and efficiently converted into penicillin F and octenoylpenicillin, respectively. The *Km* values for these substrates were 0.6 and 0.5 mM, suggesting that the affinity of AT for these molecules is similar to that reported for phenyl acetyl-CoA, octanoyl-CoA and hexanoyl-CoA (0.5, 0.6, and 1 mM, respectively). The absence of enzymatic activity shown by AT with the other acyl-CoA derivatives tested is due to the different position of the double or triple bond(s) in their aliphatic chains. The influence of the free rotation round the bond C-2–C-3 and possibility of planar conformation in such molecules and the importance in the formation of the enzyme-substrate complex is discussed.

Acyl-CoA: 6-Aminopenicillanic acid (6-APA) acyltransferase (AT) catalyzes the last step of penicillin biosynthesis in *Penicillium chrysogenum*[1~3]. AT recognizes as substrates 6-APA and different acyl-CoA derivatives, thereby generating the corresponding penicillins (F, DF, K, V, G and X)[4]. The specificity of the enzyme for both substrates (6-APA and acyl-CoA) is quite different. Whereas AT accepts many different molecules as side chain precursors of penicillins, 6-APA cannot be replaced "*in vitro*" by similar structural molecules (7-aminocephalosporanic and 7-aminodeacetoxycephalosporanic acids)[1]. The broad specificity shown by AT with acyl-CoA variants might explain why *P. chrysogenum* is able to synthesize many different penicillins "*in vivo*"[3~5]. We have previously reported that this enzyme can accept as substrates acyl-CoA variants in which either the CoA or the acyl moieties have been modified. We have recently shown that a substrate in which CoA is replaced by glutathione or a glutamyl cycle derivative (cysteinyl-glycine) can be efficiently used by this enzyme[6]. Furthermore, the aliphatic chains corresponding to natural penicillins[2] can be replaced by rigid chains (aromatic or others) with the particular requirements that: a) their total length should lie within that of C-6 to C-8 and b) there should be an acetyl-*S*-residue linked to any ring structure[6]. However, the influence of other properties of the penicillin side-chain precursors (such as rigidity or volume) on their recognition as substrates by AT has still not been reported. To clarify this, we studied the enzymatic conversion into penicillins of several acyl-CoA variants with different degrees of rigidity (hexanoyl-CoA, *trans*-2-hexenoyl-CoA, *trans*-3-hexenoyl-CoA, *trans-trans*-2,4-hexadienoyl-CoA, octanoyl-CoA, *trans*-2-octenoyl-CoA, *trans*-3-octenoyl-CoA, 2-octynoyl-CoA) or volumes (cyclohexyl-acetyl-CoA, cyclopentylacetyl-CoA) but ranging between 6 to 8 carbon atoms in length. Their ability to be used as substrates by AT is reported and discussed.

Materials and Methods

Materials

trans-2-Hexenoic acid; *trans*-3-hexenoic acid *trans-trans*-2,4-hexadienoic acid; cyclohexylacetic acid and cyclopentylacetic acid were supplied by Aldrich-Chemie (Germany). *trans*-2-Octenoic acid; *trans*-3-octenoic acid and 2-octynoic acid were from Lancaster Synthesis Ltd. (UK). Hexanoyl-CoA, octanoyl-CoA and CoA were purchased from Sigma Chemical Company (U.S.A.). Benzylpenicillin potassium salt (1,590 U/mg) was from Antibioticos S. A. León (Spain). The β-lactamase from *Bacillus cereus* was from Difco (U.S.A.). All other products were of analytical grade.

Microorganisms

The strain of *P. chrysogenum* Wis 54-1255 used in the experimental work was obtained from the American Type Culture Collection (ATCC No. 28089). The fungus was grown and cultured as previously described[7].

Micrococcus luteus ATCC 9341 was used for the determination of penicillin by bioassay[8]. The sensitivity of the β-lactam antibiotic to β-lactamase was established by the loss of its antibacterial activity against *M. luteus*.

Purification of Acyl-CoA: 6-APA AT

The enzyme was assayed and purified as previously reported[3].

Synthesis of Acyl-CoA Derivatives

Acyl-CoA derivatives were synthesized by standard procedures from the acyl chlorides derivatives and coenzyme A[3]. Yields were higher than 85%.

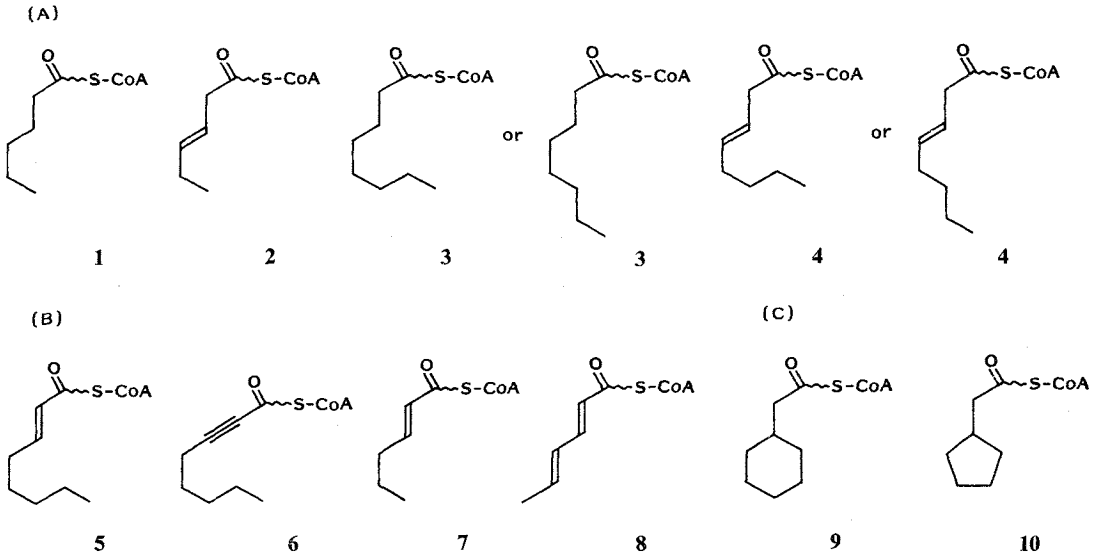
Results and Discussion

The importance of the carbon chain length on acceptance as a substrate by the acyl-CoA: 6-APA acyltransferase from *P. chrysogenum* (AT) has been established in earlier papers[2,3,6]. We have also shown that replacement of part of the aliphatic moiety by a ring (phenyl and thiophene) is accepted by AT, and generates the corresponding penicillins[3~6]. These results suggest that a partially rigid chain in the acyl moiety linked to CoA might help in forming of a steric conformation ideal for coupling the substrate molecule at the active site. To establish how critical the rigidity of the aliphatic chain might be, we assayed three different hexenoyl-CoA derivatives (*trans*-2-; *trans*-3- and *trans-trans*-2,4-hexadienoyl-CoA) two different octenoyl-CoA derivatives (*trans*-2-; and *trans*-3-octenoyl-CoA) and 2-octynoyl-CoA (Fig. 1) as hypothetical substrates for AT. These molecules are rigid structural variants of the natural substrates hexanoyl-CoA and octanoyl-CoA.

Incubation of pure AT with 6-APA and *trans*-3-hexenoyl-CoA or *trans*-3-octenoyl-CoA generated two molecules (penicillin F and octenoylpenicillin) that were active against *M. luteus* ATCC 9341 and sensitive to β-lactamase but the corresponding penicillins were not formed with *trans*-2-hexenoyl-, *trans-trans*-2,4-hexadienoyl-, *trans*-2-octenoyl- and 2-octynoyl-CoA (Table 1). The *Km* values calculated for *trans*-3-hexenoyl-CoA and for *trans*-3-octenoyl-CoA (0.6 and 0.5 mM, respectively) were similar to those reported for phenylacetyl-CoA (PA-CoA) (0.55 mM), for hexanoyl-CoA (1 mM), and for octanoyl-CoA (0.6 mM)[3]. These results indicate that the presence of a double bond at C-3 facilitates the recognition of the substrate by AT, whereas at C-2 it handicaps binding at the active site. Furthermore, when *trans*-2-hexenoyl-CoA, *trans-trans*-2,4-hexadienoyl-CoA, *trans*-2-octenoyl-CoA or 2-octynoyl-CoA (20 mM) were added to the reaction mixture together with PA-CoA, octanoyl-CoA or hexanoyl-CoA, the efficiency of benzylpenicillin, penicillin K or penicillin DF formation did not decrease (Table 1). These

Fig. 1. Structure of different acyl-CoA derivatives.

(A) Active molecules with free rotation round the bond C-2–C-3 and possibility of planar conformation of remaining part of the molecule: Hexanoyl-CoA (**1**), *trans*-3-hexenoyl-CoA (**2**), octanoyl-CoA (**3**), *trans*-3-octenoyl-CoA (**4**). (B) Inactive molecules with hindrance of free rotation: *trans*-2-octenoyl-CoA (**5**), *trans*-2-octynoyl-CoA (**6**), *trans*-2-hexenoyl-CoA (**7**), *trans-trans*-2,4-hexadienoyl-CoA (**8**). (C) Inactive molecules without possibility of planar conformation: Cyclohexyl-acetyl-CoA (**9**), cyclopentylacetyl-CoA (**10**).



data suggest that the compounds do not act as inhibitors of the natural reaction and that they do not bind to the enzyme.

The different enzymatic behavior shown by AT with hexenoyl-CoA and octenoyl-CoA derivatives having the double bond at different positions does not seem to be caused by the length of the carbon chain of the acyl moieties[3,6]. However, it could be argued that due to the presence of double bond(s), the total length of the carbon chain in the first class of molecules (C-6), would be slightly lower than in hexanoyl-CoA. This could mean that in this kind of substrate the molecular size of the acyl-moiety would be lower than the minimal value required by AT (C-6)[2,3,6]. Although such an explanation would account for the inability of *trans-trans*-2,4-hexadienoyl-CoA to be used as a substrate, it does not explain the difference between 2-hexenoyl and 3-hexenoy-CoA; only the latter was enzymatically *trans* formed into a penicillin (F) by AT. We suggest that the utilization or not of these C-6 to C-8 molecules as penicillin side chain precursors is governed by the special mobility of certain atoms. Thus, in the acyl-CoA variants tested, the mobility of the carbon atoms is maximal in hexanoyl-CoA and in octanoyl-CoA, decreasing with the presence and number of double bonds (Fig. 1). The carbon atoms affected by the rigidity of this bond (included on the same plane) differ according to the position of the first linkage in each compound (Fig. 1). Thus, for *trans*-2-hexenoyl-CoA, *trans*-2-octenoyl-CoA and 2-octynoyl-CoA maximal mobility occurs at C-5 and C-6 in the first, and at C-5, C-6, C-7 and C-8 in the other two compounds, whereas in *trans*-3-hexenoyl-CoA and *trans*-3-octenoyl-CoA, higher mobility occurs at C-1 and C-6 in the first compound and at C-1, C-6, C-7, and C-8 in the second. In *trans-trans*-2,4-hexadienoyl-CoA all the carbon atoms are in the same plane and their mobility is therefore lower than in the preceding compounds. We suggest that C-1 is the carbon atom for which mobility is important. It is unlikely to be C-6 since, although

Table 1. Formation of different penicillins by incubating AT with 6-APA and different hexenoyl and octenoyl-CoA derivatives.

	β-Lactam antibiotics evaluated as benzyl-penicillin (μg/ml)	Sensitivity to β-lactamase		β-Lactam antibiotics evaluated as benzyl-penicillin (μg/ml)	Sensitivity to β-lactamase
PA-CoA (1 mM)	1.8	+	Cyclohexylacetyl-CoA (20 mM) (g)	0	−
Hexanoyl-CoA (2 mM)	1.6	+	Cyclopentylacetyl-CoA (20 mM) (h)	0	−
trans-2-Hexenoyl-CoA (20 mM) (a)	0	−	PA-CoA (1 mM) + (a)	1.8	+
trans-3-Hexenoyl-CoA (20 mM) (b)	1.7	+	PA-CoA (1 mM) + (c)	1.8	+
trans-trans-2,4-Hexadienoyl-CoA (20 mM) (c)	0	−	Hexanoyl-CoA (2 mM) + (a)	1.6	+
			Hexanoyl-CoA (2 mM) + (b)	1.5	+
Octanoyl-CoA (2 mM)	2.0	+	PA-CoA (1 mM) + (d)	1.8	+
trans-2-Octenoyl-CoA (20 mM) (d)	0	−	PA-CoA (1 mM) + (f)	1.8	+
			Octanoyl-CoA (2 mM) + (d)	2.0	+
trans-3-Octenoyl-CoA (1 mM) (e)	1.9	+	Octanoyl-CoA (2 mM) + (f)	1.9	+
			PA-CoA (1 mM) + (g)	1.8	+
2-Octynoyl-CoA (20 mM) (f)	0	−	PA-CoA (1 mM) + (h)	1.8	+

Km calculated for *trans*-3-hexanoyl-CoA and for *trans*-3-octenoyl-CoA were 0.6 and 0.5 mM, respectively.

C-6 is outside the plane in *trans*-2-hexenoyl-CoA and in *trans*-3-hexenoyl-CoA, only the first compound *trans*-2 is not used as a substrate by AT. Moreover, AT catalyzes the conversion of PA-CoA (without free mobility at C-6, see Fig. 1) into benzylpenicillin.

It is also possible that the formation of the enzyme-substrate complex is affected by the free rotation of the C-2 atom in the substrate molecule. This would be consistent with the lack of activity shown by *trans*-2-hexenoyl-, *trans*-2-octenoyl-, 2-octynoyl- and *trans-trans*-2,4-hexadienoyl-CoA (see Table 1). However, neither cyclohexylacetyl-CoA nor cyclopentylacetyl-CoA (Fig. 1) were used as substrates by AT (see Table 1), even though the carbon length of these compounds lies within the C-6 to C-8 limits[9,10] and the rotation on C-2 is similar to that of the corresponding carbon of PA-CoA. Accordingly, we suggest that, although the free rotation at C-2 could be an important property, the volume of the substrate represents the true limitation in these cases.

Concluding Remarks

From the above data it can be inferred that, although the carbon length (C-6 to C-8) of the acyl-moiety of the substrate is important for AT recognition, mobility at C-1, free rotation at C-2 and the volume of the side-chain precursor are also characteristics that determine which molecules can be efficiently converted into penicillins. We have shown that AT can accept as substrates, PA-CoA, phenoxyacetyl-CoA and thiophene acetyl-CoA[3] which are molecules containing a rigid and plane acyl-moiety and can also accept molecules (hexanoyl-CoA, heptanoyl-CoA and octanoyl-CoA)[2] which are not planar but that could acquire an appropriate spatial conformation. The presence of double or triple bonds at C-2 in the latter could hinder the steric adaptability of such molecules at the enzymatic active site and, therefore, cause the lack of catalysis.

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References

1) LUENGO, J. M.; J. L. IRISO & M. J. LÓPEZ-NIETO: Direct evaluation of phenylacetyl-CoA: 6-Aminopenicillanic acid acyltransferase of *Penicillium chrysogenum* by bioassay. J. Antibiotics 39: 1565~1573, 1986
2) LUENGO, J. M.; J. L. IRISO and M. J. LÓPEZ-NIETO: Direct enzymatic synthesis of natural penicillins using phenylacetyl-CoA: 6-APA phenylacetyl transferase of *Penicillium chrysogenum*: Minimal and maximal side chain length requirements. J. Antibiotics 39: 1754~1759, 1986
3) ALONSO, M. J.; F. BERMEJO, A. REGLERO, J. M. FERNÁNDEZ-CAÑÓN, G. GONZÁLEZ DE BUITRAGO and J. M. LUENGO: Enzymatic synthesis of penicillins. J. Antibiotics 41: 1074~1084, 1988
4) MUKHERJEE, B. B. & B. K. LEE: Antibiotics, isolation, separation and purification. *In* Journal of Chromatography Library. *Eds.*, M. J. WEINSTEIN & G. H. WAGMAN, pp. 566~577, Springer-Verlag, 1978
5) LUENGO, J. M. & M. A. MORENO: Separation by high performance liquid chromatography of penicillin with C_4 to C_{10} aliphatic side-chains. Anal. Biochem. 164: 559~562, 1987
6) FERRERO, M. A.; A. REGLERO, J. MARTÍN-VILLACORTA, J. M. FERNÁNDEZ-CAÑÓN & J. M. LUENGO: V. Biosynthesis of benzylpenicillin (G), phenoxymethylpenicillin (V) and octanoylpenicillin (K) from glutathione *S*-derivatives. J. Antibiotics 43: 684~691, 1990
7) LUENGO, J. M.; G. REVILLA, J. R. VILLANUEVA & J. F. MARTÍN: Lysine regulation of penicillin biosynthesis in low-producing and industrial strains of *Penicillium chrysogenum*. J. Gen. Microbiol. 115: 207~211, 1979
8) LUENGO, J. M.; M. T. ALEMANY, F. SALTO, F. RAMOS, M. J. LÓPEZ-NIETO & J. F. MARTIN: Direct enzymatic synthesis of penicillin G using cyclases of *Penicillium chrysogenum* and *Acremonium chrysogenum*. Bio/Technology 4: 44~47, 1986
9) MARTIN-VILLACORTA, J.; A. REGLERO & J. M. LUENGO: IV. Acyl-CoA: 6-APA acyltransferase of *Penicillium chrysogenum*: Studies on substrate specificity using phenylacetyl-CoA variants. J. Antibiotics 42: 1502~1505, 1989
10) LUENGO, J. M.: Recent advances in the enzymatic synthesis of penicillins. *In* Progress in Industrial Microbiology. Vol. 27. *Eds.*, M. E. BUSHELL & K. GRAFE, pp. 315~332, Elsevier, 1989