Fatty acid structural requirements for activity of arachidonoyl-CoA synthetase

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Abstract We have examined the fatty acid substrate specificity of arachidonoyl-CoA synthetase from human platelet membranes. A variety of positional isomers and chain-length analogs of arachidonic acid [20:4(5, 8, 11, 14)] were synthesized, and assayed for their ability to inhibit arachidonoyl-CoA formation or to serve as substrates for the synthetase. The chain-length specificity of the synthetase for $\Delta^{8,11,14}$ trienoic fatty acids was $C_{19} > C_{18} = C_{20} \gg C_{21} > C_{22}$. Inhibition activity by positional isomers of arachidonate was 20:4(5, 8, 11, 14) $\simeq$ 20:4(6, 9, 12, 15) = 20:4(7, 10, 13, 16) $\gg$ 20:4(4, 7, 10, 13), however, V_{max} for arachidonate was greater than that for 20:4(6, 9, 12, 15). The enzyme apparently "counts" double bonds from the carboxyl terminus. As counted from the methyl terminus we found that several n-6,-9,-12 fatty acids were ineffective as inhibitors [18:3(6, 9, 12); 19:4(4, 7, 10, 13); 21:3(9, 12, 15)], whereas all methylene-interrupted tri- and tetraenoic fatty acids which contained Δ^8 and Δ^{11} double bonds were potent inhibitors. The Δ^{11} double bond was best associated with optimal inhibition: 20:3(5, 11, 14) had a lower K_i than 20:3(5, 8, 14). 13-Methyl-20:3(8, 11, 14) did not inhibit the enzyme. Partially purified enzyme from calf brain, depleted of nonspecific long-chain acyl-CoA synthetase, exhibited the same fatty acid specificity as crude platelet enzyme.—**Neufeld, E. J., H. Sprecher, R. W. Evans, and P. W. Majerus.** Fatty acid structural requirements for activity of arachidonoyl-CoA synthetase. *J. Lipid Res.* 1984. **25:** 288–293.

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Platelets and other cells that synthesize eicosanoids (prostaglandins, leukotrienes, and related compounds) specifically esterify arachidonic acid [20:4(5, 8, 11, 14)] into their membrane phospholipids. This specificity is conferred by an arachidonate-specific, long-chain acyl-CoA synthetase that prefers eicosanoid precursor fatty acids as substrates to palmitate, stearate, oleate, and linoleate (1). The arachidonoyl-CoA synthetase is responsible for high affinity uptake and esterification of eicosanoid precursors by human platelets (2) and by $HSDM_1C_1$ murine fibrosarcoma cells. A mutant $HSDM_1C_1$ line defective in high-affinity arachidonate uptake lacks the specific synthetase (3). The enzyme has also been found in sheep seminal vesicles (1), calf brain,[1] and rat brain (4).

Previous studies of the arachidonoyl-CoA synthetase (1, 2, 4) have not addressed the range of its specificity. Possible specificities include: *1*) a chain-length requirement only (≥20 carbons, for example); *2*) all tri- and tetraenoic fatty acids, regardless of double bond position or spacing; or *3*) very strict structural requirements, limited to those fatty acids that serve as eicosanoid precursors. The first two alternatives suggest a fairly general role for the enzyme. The third explanation implies that the enzyme exists specifically for eicosanoid precursor homeostasis, as previously suggested (1, 3). To distinguish these possibilities, we have examined the ability of a variety of polyunsaturated fatty acids differing in chain length and degree and position of unsaturation to serve as inhibitors of, and substrates for, the arachidonoyl-CoA synthetase of human platelets and calf brain.

METHODS

Fatty acids

Unlabeled oleic, linoleic, arachidonic, and dihomo-γ-linolenic acids were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). Eicosapentaenoic acid was from Sigma. [1-^{14}C]Arachidonic acid (55 μCi/μmol) and [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (100 Ci/mmol) were purchased from Amersham. [9,10-^{3}H]Oleic acid (10 Ci/mmol), and [1-^{14}C]linoleic, dihomo-γ-linolenic, and eicosapentaenoic fatty acids (all 55 μCi/μmol) were from New England Nuclear. All other labeled and unlabeled fatty acids were synthesized as described (5, 6). Purity of fatty acids was determined by gas–liquid chromatography of methyl esters on 10% SP2330 on Chromosorb W at 190°C with a carrier gas flow of 30 ml/min. When necessary, impurities were removed by high performance

[1] Laposata, M., and P. W. Majerus. Unpublished observations.

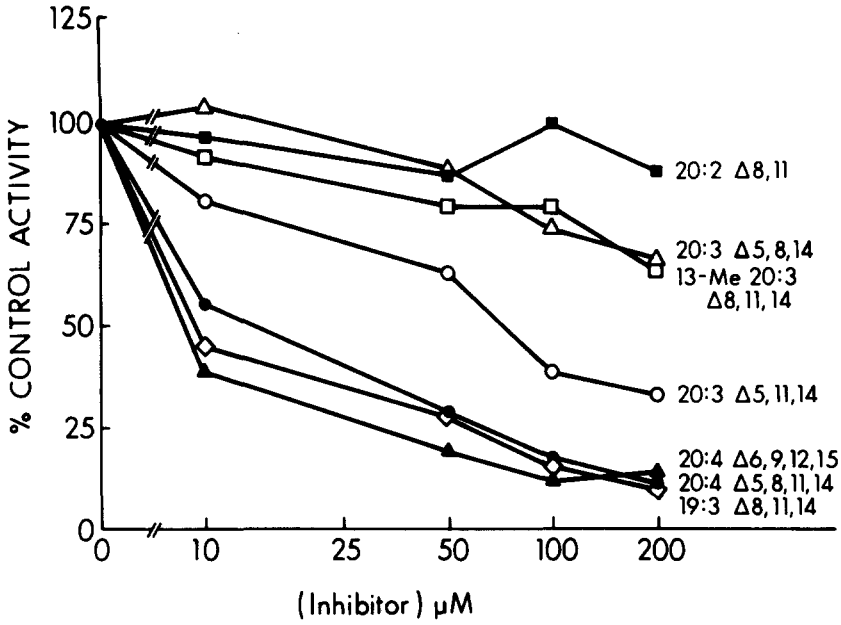

Fig. 1. Inhibition of [^{14}C]arachidonoyl-CoA synthesis by unlabeled fatty acids. [^{14}C]Arachidonate (1 μM) was incubated with platelet membranes (25 μg) and varying concentrations of unlabeled fatty acids as described (1). Reactions were stopped after 5 min. Results are expressed as percent of activity found in the absence of inhibitors (0.03 nmol/min per reaction mixture).

liquid chromatography on C-18 reverse phase columns as described (3, 6). 2-Mercaptoethanol was obtained from Eastman (Rochester, NY).

Enzyme preparations

Washed human platelets from aspirin-free donors were isolated (7) and suspended at 10^9/ml in pH 6.5 phosphate buffer plus 10 mM 2-mercaptoethanol. After sonication for 3 × 30 sec at 150 W in an ice-cooled tube with a Bronwill Biosonik instrument (Rochester, NY), membranes were pelleted by centrifugation at 50,000 *g* for 40 min. The pellets were resuspended at a concentration equivalent to 5×10^9 platelets/ml in pH 6.5 phosphate buffer and 10 mM 2-mercaptoethanol, and stored at −70°C for up to 6 weeks without loss of activity.

Preparation of calf brain enzyme

Fresh brain was obtained from a local slaughterhouse. All buffers used in the procedure contained 10 mM 2-mercaptoethanol. Twenty grams of tissue was disrupted with three strokes of a Potter-Elvehjem homogenizer in 100 ml of buffer (0.32 M sucrose, 0.01 M HEPES, pH 7.5), diluted in an equal volume of the same buffer, and centrifuged at 1000 *g* for 5 min. The supernatant solution was centrifuged at 48,000 *g* for 15 min, and the membrane pellet was resuspended to 5 mg of protein/ml in 0.02 M potassium phosphate, pH 7.4, with 0.12% Triton X-100. The mixture was brought to 1% NP-40 (Sigma), stirred 1 hr at 0°C, and centrifuged 1 hr at 48,000 *g*. The supernatant solution was stored at −20°C for 3 months without loss of activity.

Seven ml of solubilized enzyme was applied to a 15-ml column of hydroxylapatite (BioGel HTP, Bio-Rad) equilibrated in 20 mM KPO_4, pH 7.4, 1% NP40. The column was washed with 48 ml of the same buffer, and eluted with 45 ml of 0.083 M KPO_4^{2-}, pH 7.4, plus 1% NP-40, followed by a linear salt gradient from 0.083 to 0.3 M phosphate containing 1% NP-40 (90 ml). In this procedure, nonspecific acyl-CoA synthetase eluted at 0.083 M phosphate, while the peak activity of arachidonoyl-CoA synthetase eluted at 0.21 M phosphate.[2] Crude brain homogenate contained equal amounts of specific and nonspecific enzymes. The hydroxylapatite eluate was 15-fold enriched for arachidonoyl vs nonspecific acyl-CoA synthetase.

Enzyme assays

Long-chain acyl-CoA synthetase activity was measured as described by Wilson, Prescott, and Majerus (1). Solutions of inhibitory fatty acids (0.5 or 1 mM) were prepared immediately before use in 50 mM $NaHCO_3$ containing 0.1% Triton X-100. Substrates, inhibitors, and other components of each reaction mixture were combined and warmed to 37°C. Reactions were started by addition of enzyme.

[2] Laposata, M., E. L. Reich, and P. W. Majerus. Unpublished observations.

RESULTS

Inhibition studies

The structural requirements for substrates of arachidonoyl-CoA synthetase were determined by measuring the ability of various fatty acids to inhibit radiolabeled arachidonate incorporation into arachidonoyl-CoA, using as an enzyme source membranes from sonicated platelets. **Fig. 1** shows dose-response curves for some of the fatty acids tested in a representative inhibition experiment.

Three kinds of fatty acids are distinguished by their ability to compete with arachidonate in this type of assay. One group of fatty acids, including arachidonate itself, has a low I_{50} similar to the K_m of the enzyme for arachidonate (12 μM). A second group of fatty acids was unable to inhibit arachidonate esterification substantially, even at 200 μM. A few fatty acids (such as 20:3(5, 11, 14)[3] in Fig. 1) were intermediate inhibitors, with I_{50}'s from 5 to 20 times as high as the best inhibitors.

The results for all fatty acids tested are summarized in **Table 1.** For each fatty acid, an inhibition experiment of the type shown in Fig. 1 was performed. In the conditions used for the inhibition assays (1 μM [^{14}C]-arachidonate) with platelet membranes as an enzyme source, only the arachidonoyl-CoA synthetase should have been measured. This is true because the K_m for arachidonate of the specific enzyme is 12 μM (2) while the K_m for arachidonate with the nonspecific enzyme is 170 μM (1), and V_{max} for the specific enzyme is $\geq$2 times that for the nonspecific enzyme in platelet microsomes.

To be certain that the specificities observed were not a function of contaminating nonspecific enzyme, the inhibition experiments were repeated using a preparation partially purified from calf brain, as described in Methods, that is greatly enriched in arachidonoyl-CoA synthetase. Table 1 shows that the qualitative results obtained with this preparation were the same as with the platelet enzyme; that is, the rank order of inhibitory activity is identical. The apparent I_{50} values were all lower for detergent-containing partially purified brain enzyme than for platelet enzyme. We have found that apparent K_i and K_m values diminish as smaller amounts of enzyme are present in an assay mixture, presumably due to binding of fatty acid to the membranes themselves or to other proteins in the reaction mixture. The actual K_m of arachidonate for the arachidonoyl-CoA synthetase may be very low ($\leq$1 μM) (2). Several conclusions may be drawn from the data in Table 1.

TABLE 1. Apparent I_{50} or K_i values of several fatty acids for arachidonoyl-CoA synthetase

Inhibiting Fatty Acid	Enzyme Source: Platelet Membranes Apparent K_i (μM)	Enzyme Source: Calf Brain Extract I_{50} (μM)
18:1(9)	No inhib.	No inhib.
18:2(9, 12)	No inhib.	NT
18:3(6, 9, 12)	>200	NT
(9, 12, 15)	>200	NT
(8, 11, 14)	23	7
18:4(5, 8, 11, 14)	10	<5
19:3(8, 11, 14)	<10	<5
19:4(4, 7, 10, 13)	190	NT
20:2(8, 11)	No inhib.	No inhib.
20:3(5, 8, 11)	34[a]	NT
(7, 10, 13)	150	48
(8, 11, 14)	30[a]	NT
13 Methyl (8, 11, 14)	>200	140
(5, 8, 14)	>200	70
(5, 11, 14)	71	16
20:4(4, 7, 10, 13)	200	>200
(5, 8, 11, 14)	13	<5
(6, 9, 12, 15)	<10	<5
(7, 10, 13, 16)	<10	6
(8, 11, 14, 17)	14	<5
20:5(5, 8, 11, 14, 17)	8[a]	NT
21:3(9, 12, 15)	No inhib.	NT
(8, 11, 14)	125	15
21:4(8, 11, 14, 17)	13	<5
22:3(8, 11, 14)	>200	63
22:6(4, 7, 10, 13, 16, 19)	60	NT

Platelet enzyme experiments were carried out as described for Fig. 1. Calf brain reactions contained 25 μg of protein and were incubated for 1 min. The radiolabeled arachidonate concentration (S) was 1 μM. The relative efficacy of the inhibitors was the same for both enzymes sources. All I_{50} values were lower for the hydroxylapatite-purified brain enzyme, presumably because the preparation contained fewer fatty acid-binding contaminants. I_{50} and apparent K_i for enzymatic reactions are related by $I_{50} = K_i (1 + S/K_m)$ (20). K_m for the platelet enzyme is 12 μM. The K_m for the calf brain enzyme preparation used in this experiment was not determined, so values are reported as I_{50}. No inhib., <20% inhibition at 200 μM; NT, not tested.

[a] Data from ref. 2.

1. The enzyme prefers fatty acids of 18 to 20 carbons, but chain length is not the sole basis of specificity. Thus, 18:4(5, 8, 11, 14) and 19:3(8, 11, 14) were equally as good inhibitors as arachidonate, yet two C_{20} fatty acids—20:2(8, 11) and 20:4(4, 7, 10, 13)—were very poor inhibitors. For $\Delta^{8,11,14}$ trienoates, the chain length preference was $19 > 20 = 18 \gg 21 > 22$.

2. The enzyme "counts" double bonds from the carboxyl terminus. The inhibitory activity of fatty acids containing $\Delta^{8,11,14}$ unsaturation was much greater than that of fatty acids with double bonds at the n-6, -9, -12 positions in fatty acids of 18, 19, and 21 carbons (for C_{20}, the notation 20:3(8, 11, 14) is identical to n-6,9,12). For ex-

[3] 20:3(5, 11, 14) is equivalent to 20:3 $\Delta^{5,11,14}$.

ample, 18:3(6, 9, 12) (n-6) and 21:3(9, 12, 15) (n-6) were inactive as inhibitors, while 18:3(8, 11, 14) (n-4) and 19:3(8, 11, 14) (n-5) were active. While 20:4(6, 9, 12, 15) (n-5) inhibits the synthetase as well as arachidonate, it is not as active as a substrate (see below).

3. Δ^{11} Unsaturation is necessary but not sufficient for optimal activity. Our previous report (2) demonstrated that 20:3(5, 8, 11) and 20:3(8, 11, 14) were equally good substrates for the enzyme. To determine whether the Δ^8 or Δ^{11} double bonds, or both, were necessary and sufficient for substrates, we used the diene 20:2(8, 11) and two novel arachidonate analogs missing internal double bonds, 20:3(5, 11, 14) and 20:3(5, 8, 14). Fig. 1 shows that 20:2(8, 11) was completely inactive as an inhibitor. Thus, the $\Delta^{8,11}$ structure is not sufficient for recognition by arachidonoyl-CoA synthetase. While 20:3(5, 8, 14) was a very poor inhibitor, 20:3(5, 11, 14) was considerably more active (Fig. 1). Hence, the Δ^{11} unsaturation, in a triene or tetraene, seems to be necessary for optimal activity.

4. A 13-methyl substituent abolishes inhibitory activity of 20:3(8, 11, 14). Do and Sprecher (8) have shown that 13-Me 20:3(8, 11, 14) is a very poor substrate for cyclooxygenase, presumably because the methyl group blocks access of the enzyme to the 13-hydrogen, which is abstracted as part of the reaction mechanism. We found that this fatty acid failed to inhibit arachidonoyl-CoA synthetase (Table 1). This suggests that the substrate binding site of the enzyme fits closely around the middle part of the fatty acid, even though the alkenyl chain is not directly involved in the reaction.

5. Δ^4 Unsaturation in arachidonate positional isomers diminishes inhibitory activity. The rank order of inhibition among the tetraenoic positional isomers was 20:4(5, 8, 11, 14) = 20:4(6, 9, 12, 15) = 20:4(7, 10, 13, 16) = 20:4(8, 11, 14, 17) = 21:4(8, 11, 14, 17) $\gg$ 20:4(4, 7, 10, 13) and 19:4(4, 7, 10, 13). In light of this finding, it was surprising that 22:6(4, 7, 10, 13, 16) was a relatively good inhibitor (Table 1). It may be that the large number of double bonds in this molecule allowed it to attain a configuration suitable to the enzyme despite the Δ^4 unsaturation.

Radioactive analogs as substrates

In addition to the inhibition studies described above, ^{14}C-labeled analogs (most but not all of the fatty acids listed in Table 1) were assayed directly for esterification.

In general, good inhibitors were good substrates (V_{max} and K_m approximately equal to arachidonate) and poor inhibitors were inactive as substrates. However, there were

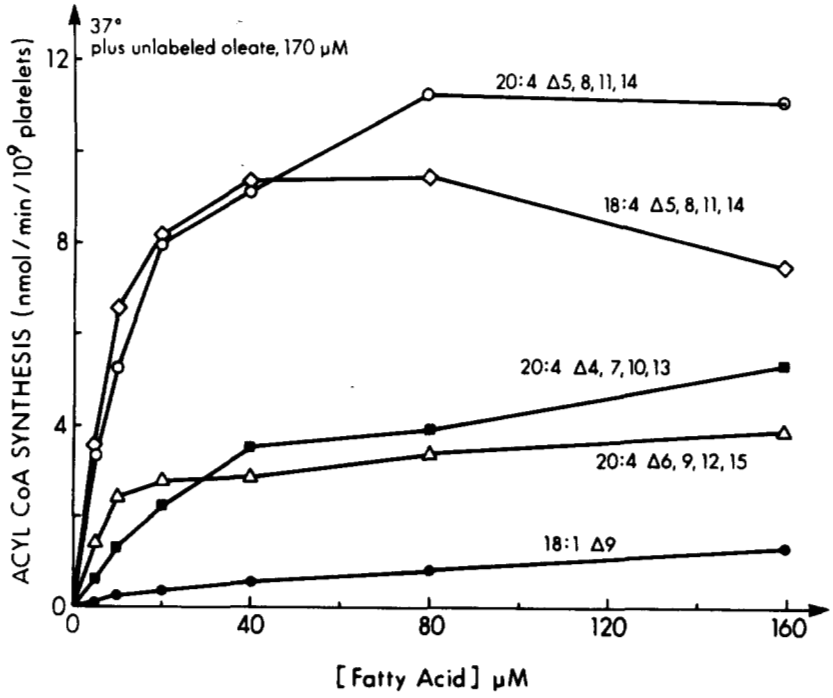

Fig. 2. Acyl-CoA synthesis rate vs concentration of radiolabeled fatty acid. Platelet membranes (25 μg) were incubated with various concentrations of radiolabeled fatty acid for 5 min. In each reaction mixture 170 μM unlabeled oleate was present to inhibit nonspecific acyl-CoA synthetase as described (1). Incorporation of labeled oleate, 18:1(9), into product therefore represents a "blank": the amount of activity expected even in the absence of arachidonoyl-CoA synthetase.

some exceptions. **Fig. 2** shows an experiment in which arachidonate esterification was compared to esterification of 18:4(5, 8, 11, 14), 20:4(6, 9, 12, 15) (both good inhibitors) and 20:4(4, 7, 10, 13) and oleate (both poor inhibitors). As expected, 18:4(5, 8, 11, 14) was as good a substrate as arachidonate. However, the potent inhibitor 20:4(6, 9, 12, 15) had the same K_m as arachidonate, but V_{max} only one-third as great. As expected, 20:4(4, 7, 10, 13) had a higher K_m and lower V_{max}.

DISCUSSION

Prior to the present experiments, it was suggested that arachidonoyl-CoA synthetase is required for eicosanoid precursor homeostasis in cells that produce these mediators (1–4), but there was not enough information to determine whether this was the primary function of the synthetase. The data presented here support this hypothesis. Among common fatty acids that occur in mammals, only arachidonate, dihomo-γ-linolenate, eicosapentaenoate, and docosahexaenoate can serve as substrates. Additionally, 20:3(5, 8, 11), the Mead fatty acid of essential fatty acid deficiency (9), which can serve as a lipoxygenase substrate (10), is active as a substrate, although the essential fatty acids, linoleate and γ-linolenate [18:3(6, 9, 12)], are not. This exceptional fatty acid specificity parallels the specificities previously described for those of cyclooxygenase (11–13) and some lipoxygenases (10, 14), although it is not identical.

Cyclooxygenase apparently counts double bonds from the methyl terminus. In the 1960's the research group at Unilever showed that (n-6) fatty acids[4] of 19–21 carbons (and 20:3 (n-7) to a smaller degree) were active substrates for sheep seminal vesicle cyclooxygenase (11, 13), whereas 18, 19, 21, and 22 carbon fatty acids including double bonds at $\Delta^{8,11,14}$ were poorer substrates (12, 13). [However, platelet cyclooxygenase has been reported (16) to be quite active against the n-7 acid, 21:4(5, 8, 11, 14).] Cyclooxygenase may also recognize and cause hydroxylation of two (n-6) fatty acids that cannot be converted to prostaglandins, 20:2(11, 14) (17) and linoleate (18). Both cyclooxygenase and the arachidonoyl-CoA synthetase have strong preferences for Δ^{11} (n-9) unsaturation, and an unsubstituted 13 (n-7) carbon. Both exhibit relative preference for 20 carbon chain length.

A major goal of research on phospholipid metabolism has been to account for the high degree of fatty acid specificity exhibited during cellular lipid biosynthesis (reviewed in 19). Until recently, it was thought that long-chain acyl-CoA synthetase did not confer specificity to the process, since the purified enzyme from liver accepts nearly all long-chain fatty acids as substrates (14). This work demonstrates, to the contrary, that the so-called arachidonoyl-CoA synthetase is active with only a small number of specific fatty acids.

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[4] IUPAC-IUB rules for use of the [n-x] convention apply especially to double bonds. The 1976 recommendations (15) state "n is the number of carbon atoms in the chain, . . . and x is the (lower) locant of [a] double bond." Note that although n-6 and the old ω-6 nomenclature refer to the same double bond (Δ^{14} in arachidonate), they specify the 14- and 15-carbons, respectively. We also use [n-x] for specifying positions besides double bonds (e.g., the n-7 position refers to carbon 13 in arachidonate or 11 in linoleate).

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