Erythrocyte membrane acyl:CoA synthetase activity

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Received 5 August 1985; revised version received 10 October 1985

The presence of long-chain acyl:CoA synthetases in mammalian microsomes and mitochondria has been established previously [(1971) Biochim. Biophys. Acta 231, 32–47]. The presence of a plasma membrane-associated enzyme was investigated in human erythrocyte ghost plasma membranes, where an enzyme exhibiting high activity, and with a preferred substrate of 18 carbon chain length, was discovered. The results are consistent with the presence of a single enzyme. The effect of the degree of unsaturation of the fatty acid substrates was not as pronounced as that arising from the length of the carbon chain. The pattern of substrate preference of the enzyme was ω3 polyenoics > ω6 polyenoics > ω9 monoenoics > saturated fatty acids. This may relate to the similar substrate preference pattern exhibited by the fatty acyl desaturase enzymes. However, the role played by long-chain acyl:CoA synthetase in erythrocyte metabolism is uncertain, but may relate to the transportation of polyenoic fatty acids in the circulation.

Erythrocyte *Plasma membrane* *Acyl:CoA synthetase*

1. INTRODUCTION

The exact role of the plasma membrane long-chain acyl:CoA synthetase is not clear, however it may be associated with a mechanism whereby fatty acids are transported across the membranes [2]. It may also provide a means of retention of fatty acids within the cell, as the plasma membrane is impermeable to acyl:CoA but not to free fatty acids.

The requirement for the CoA thiol esters of fatty acids has long been established [3] and the metabolism of fatty acids is primarily that of these activated forms. The enzymes responsible for this activation step are the acyl:CoA synthetases (fatty acid:CoA ligases, EC 6.2.1.1-3), which can be subdividied into 2 classes depending on their requirement for specific nucleotide triphosphates [4]. The most metabolically active class are the acyl:CoA synthetase (AMP-forming) enzymes, which require ATP [5]. There are 3 enzyme species within this class relating to fatty acid substrate specificity. Short-chain acyl:CoA synthetases (EC 6.2.1.1) exhibit the highest activity with acetate as substrate [6], whilst medium-chain acyl:CoA synthetases (EC 6.2.1.2) have a preferred substrate of C7:0 chain length [7]. The long-chain acyl:CoA synthetase enzymes (EC 6.2.1.3) exhibit a much broader range of activity from C6:0 to C24:0 [8], however the preferred chain lengths are reported as C12:0–C16:0 [9]. In all tissues examined to date the highest activity of the mitochondrial enzyme has been with C16:0 as substrate [10], whilst both C12:0 and C16:0 have been reported as the preferred substrates of the microsomal fractions [11]. All work, until recently, has concentrated on these subcellular organelles [12].

A putative role for a plasma membrane-bound, or plasma membrane-associated, long-chain acyl:CoA synthetase had been postulated previously [13,18]. This possibility was investigated, initially in rat hepatocyte plasma membranes, and subsequently in other tissues. High levels of activity were obtained with the hepatocyte membranes, of the order of 3-times higher than those obtained with the erythrocyte as enzyme source, the pattern of activation however, was the same (Davidson and Cantrill, unpublished). Marker enzyme assays

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

(performed to check membrane purity) proved negative for microsomal and mitochondrial contamination. The erythrocyte ghost was chosen as a model plasma membrane source since it can be prepared uncontaminated by other subcellular organelles. The enzyme from this source activated saturated fatty acids, fatty acids with increasing degrees of unsaturation, and fatty acids from different polyenoic families. The chain lengths of the saturated fatty acids ranged from C12:0 to C24:0, the chain lengths of the monoenoics from C16:1ω9 to C24:1ω9, and the polyenoics from C18:2ω6 to C22:6ω3.

2. MATERIALS AND METHODS

Erythrocyte ghost membranes were prepared according to Dodge et al. [14], as modified by Hanahan and Ekholm [15]. Protein was assayed by the Bio-Rad procedure. Acyl:CoA synthetase activity was assayed by the method of Polokoff and Bell [16] as modified by Murphy and Spence [17], in which fatty acid substrates at 50 μM are reacted in the presence of 10 mM ATP and 8 mM Mg^{2+} with 50 μM [^{3}H]CoASH. Both of the above groups demonstrated that neither CoASH nor free fatty acid will adhere to the filters used in the assay, thus any radioactivity found stuck to the filters is attributable to acyl:CoA. All fatty acids were obtained from Sigma, St. Louis, USA. [^{3}H]CoASH (spec. act. 0.547 Ci/mmol) was obtained from New England Nuclear, Boston, USA. The HAWP filters required were supplied by Millipore, USA, and the Aquagel scintillant cocktail by Chemlab, Sandton, SA.

3. RESULTS

Table 1a–c shows the effects of varying the protein and fatty acid concentrations, and incubation time, respectively. Only the effects with palmitic acid as substrate are shown, but the other fatty acids used as reaction substrates exhibited a very similar pattern to C16:0. Table 2 shows the effects

Table 1

Comparison of the effects of varying fatty acid and protein concentrations, and incubation time, on the rate of activation of C16:0 (palmitic acid) with the long-chain acyl:CoA synthetase of human erythrocyte ghosts

	C16:0 (μM)			
	50 μM	25 μM	12.5 μM	6.25 μM
(a) nmol/min per mg protein	8.9 ± 0.38	8.6 ± 0.22	7.3 ± 0.36	4.6 ± 0.33
n	3	18	3	3
	Protein (mg)			
	0.1	0.05	0.025	0.0125
(b) nmol/min	0.70 ± 0.04	0.43 ± 0.02	0.23 ± 0.05	0.12 ± 0.04
n	3	18	3	3
	Time (min)			
	1	5	10	30
(c) nmol/mg protein	10.7 ± 1.96	42.9 ± 1.10	51.6 ± 1.86	53.8 ± 1.84
n	3	18	3	3

Table 2

Comparison of the maximal rates of activation of 22 fatty acids with the long-chain acyl:CoA synthetase of human erythrocyte ghosts relative to C16:0 (palmitic acid)

Chain length	Degree of unsaturation							
	0	1ω9	2ω6	3ω6	3ω3	4ω6	5ω3	6ω6
12 +ATP	6.02 ± 0.14[b]							
−ATP	1.28 ± 0.23							
14 +ATP	8.40 ± 0.26[a]							
−ATP	1.45 ± 0.07							
16 +ATP	8.58 ± 0.22	9.23 ± 0.17[b]						
−ATP	1.47 ± 0.08	1.54 ± 0.04						
17 +ATP	9.28 ± 0.16[b]							
−ATP	1.50 ± 0.07							
18 +ATP	10.11 ± 0.14[b]	11.78 ± 0.22[b]	12.14 ± 0.15[b]	12.21 ± 0.16[b]	12.56 ± 0.14[b]			
−ATP	1.72 ± 0.04	1.76 ± 0.03	1.89 ± 0.02		2.01 ± 0.03			
20 +ATP	9.7 ± 0.17[b]	9.83 ± 0.10[b]	10.15 ± 0.14[b]	10.31 ± 0.17[b]	10.62 ± 0.12[b]	9.67 ± 0.16[b]	10.21 ± 0.16[b]	
−ATP	1.51 ± 0.02	1.59 ± 0.04	1.76 ± 0.02			1.50 ± 0.03	1.81 ± 0.02	
22 +ATP	7.37 ± 0.19[b]	7.94 ± 0.15[b]						7.40 ± 0.20[b]
−ATP	1.36 ± 0.02	1.42 ± 0.03						1.41 ± 0.03
24 +ATP	6.41 ± 0.16[b]	7.40 ± 0.14[b]						
−ATP	1.31 ± 0.03	1.37 ± 0.02						

[a] $p<0.1$
[b] $p<0.005$

All *t*-tests calculated relative to 16:0 (palmitic acid), and for full reaction mixture assays only

The results obtained are expressed as nmol/min per mg protein of fatty acyl:CoA produced and are shown both with and without ATP in the reaction mixture in most instances

of both including and excluding ATP from the reaction mixture. The rates of activation of all the fatty acid substrates were drastically reduced when compared to the reaction rate with the full mixture, thus demonstrating the requirement for ATP in the reaction system. The results of the assay with the full reaction mixture are described below.

Table 2 shows the levels of activation with the C12–C24 saturated fatty acids as substrates. The substrate exhibiting the maximal rate of activation was stearic acid (C18:0) (10.11 nmol/min per mg protein), and the activation profile of all the saturated substrates approximated to a flattened bell-shaped curve. This probably indicates the presence of only one enzyme. The maximal activation of the monoenoic substrates was again with the 18-carbon moiety, oleic acid (*cis*-C18:1ω9) (11.78 nmol/min per mg protein). All the monoenoic substrates exhibited enhanced activation relative to their saturated counterparts (monoenoics approx. 9% higher). Table 2 also depicts the activation of the ω6 polyenoic fatty acids, i.e. linoleic, γ-linolenic, dihomolinoleic, dihomo-γ-linolenic, and arachidonic acids (*cis*-C18:2ω6, *cis*-C18:3ω6, *cis*-C20:2ω6, *cis*-C20:3ω6, and *cis*-C20:4ω6, respectively). There were slight differences between the two 18-carbon moieties (12.14 and 12.21 nmol/min per mg protein, with C18:2ω6 and C18:3ω6, respectively), and between the three 20-carbon compounds (10.15, 10.31 and 9.67 nmol/min per mg protein, with C20:2ω6, C20:3ω6 and C20:4ω6, respectively), however, these differences were not statistically significant. Within the ω6 polyenoics the only statistically significant differences related to chain length (12.14 and 12.21, 18 carbons, compared to 10.15, 10.31 and 9.67, 20 carbons) ($p<0.005$). The rates of activation of the ω3 polyenoics are shown and these exhibit a very similar pattern to that of the ω6 polyenoics. The fatty acids were α-linolenic, dihomo-α-linolenic, eicosapentaenoic, and docosahexaenoic acids (*cis*-C18:3ω3, *cis*-C20:3ω3, *cis*-C20:5ω3, *cis*-C22:6ω3, respectively). The difference between the rates of activation of the two 20-carbon fatty acids was significant (10.62 and 10.21 nmol/min per mg protein, for C20:3ω3 and C20:5ω3, respectively) ($p<0.005$), however they were not consecutive members of the ω3 family of compounds and thus the difference may not reflect a real effect. When one examines the effect of differences in chain length amongst the ω3 polyenoics, these were again highly significant ($p<0.005$) (12.56 nmol/min per mg protein for C18:3ω3 compared with 10.62 nmol/min per mg protein with C20:3ω3; 10.21 nmol/min per mg protein for C20:5ω3 compared with 7.40 nmol/min per mg protein with C22:6ω3). Table 3 illustrates the influence of both degree of unsaturation and position of unsaturation on the activity of the 18-carbon fatty acids. The order was α-linolenic $>\gamma$-linolenic $>$ linoleic $>$ oleic $>$ stearic, all differences being significant ($p<0.005$) except between γ-linolenic and linoleic (12.56, 12.21, 12.14, 11.78, 10.11 nmol/min per mg protein, respectively). The activation rates of the 20-carbon fatty acids are shown in table 4. The trend is similar to the rates of activation seen with the 18-carbon substrates (table 3). When one considers the chain-elongated homologues of the 18-carbon compounds the order of activation was dihomo-α-linolenic $>$ dihomo-γ-linolenic $>$ dihomolinoleic $>$ arachidoleic $>$ arachidic (10.62, 10.31, 10.15, 9.83, 9.17

Table 3

Comparison of the maximal rates of activation of all the 18-carbon fatty acids with the long-chain acyl:CoA synthetase of human erythrocyte ghosts relative to C18:0 (stearic acid)

Chain length	Degree of unsaturation							
	0	1ω9	2ω6	3ω6	3ω3	4ω6	5ω3	6ω6
18	10.11 ± 0.14	11.78 ± 0.22[a]	12.14 ± 0.15[a]	12.21 ± 0.16[a]	12.56 ± 0.14[a]			

[a] $p<0.005$

All *t*-tests calculated relative to 18:0 (stearic acid)

Table 4

Comparison of the maximal rates of activation of all the 20-carbon fatty acids with the long-chain acyl:CoA synthetase of human erythrocyte ghosts

Chain length	Degree of unsaturation							
	0	1ω9	2ω6	3ω6	3ω3	4ω6	5ω3	6ω6
20	9.17 ± 0.17	9.83 ± 0.10[a]	10.15 ± 0.14[a]	10.31 ± 0.17[a]	10.62 ± 0.12[a]	9.67 ± 0.16[a]	10.21 ± 0.16[a]	

[a] $p<0.005$

All t-tests calculated relative to 20:0 (arachidic acid)

nmol/min per protein), all differences being significant ($p<0.005$) except between dihomo-γ-linolenic and dihomolinoleic. The 2 most unsaturated fatty acids, arachidonic and eicosapentaenoic acids, showed lower rates of activation (9.67 and 10.21 nmol/min per mg protein, respectively) relative to their precursors, dihomo-α-linolenic and dihomo-γ-linolenic acids (10.31 and 10.62 nmol/min per mg protein). The maximal activity (10.62 nmol/min per mg protein) was, once more, exhibited by the trienoic ω3 compound.

4. DISCUSSION

The possibility of plasma membrane-linked long-chain acyl:CoA synthetases had been proposed in [13,18]. The results obtained clearly indicate the presence of such a long-chain acyl:CoA synthetase in human erythrocyte ghost plasma membranes. The activities are such as to compare favourably with results on other tissue plasma membranes (Davidson and Cantrill, unpublished). The erythrocyte exhibited activity similar to that of organelles investigated by other workers [19], and second only to that of the hepatocyte (Davidson and Cantrill, unpublished), and, of course, erythrocyte ghosts being plasma membrane only, this activity was directly attributable to such membranes. Morand and Aigrot [20] have recently shown that erythrocyte ghosts resealed with ATP and CoASH inside exhibited palmitoyl:CoA synthetase activity. They demonstrated that the enzyme was located on the inside of the membrane, and that it exhibited a requirement for both ATP and CoASH. Their results showed lower levels of activation than our preparations, but they were using intact ghosts with the full thickness of the membrane between the enzyme and substrate, whilst we used membrane fragments, thus the enzyme was in intimate contact with the substrates.

The preferred substrates reported elsewhere for microsomes were lauric (C12:0) and palmitic acids (16:0) [21] whilst that of mitochondria was palmitic acid (C16:0) [1], however the plasma membrane enzyme exhibits maximal activity with the 18-carbon substrates, this increasing with degree of unsaturation. The higher activity with C18 fatty acids compared with other substrates may reflect a greater need for the incorporation of these moieties within the phosphoglyceride structure of the membrane. The activity cascade ω3>ω6>ω9> saturate indicates a definite preference for the ω3 series polyenoics relative to the ω6, and these to the ω9 and the saturates. This follows exactly the substrate preference pattern exhibited by Δ-6-desaturase [22], and possibly Δ-5 and Δ-4-desaturase as well. It is possible, therefore, that the patterns of substrate preference of the long-chain acyl:CoA synthetases and the desaturases are related in some way.

The role of the enzyme in the erythrocyte membrane is debatable. It may fulfil the role of supplying activated fatty acids for the membrane phosphoglycerides [23], and thus help maintain fluidity and integrity. Although the activity may just be a vestigial remnant, it would seem too great for this to be the sole reason. Alternatively, the enzyme may fulfil a role in the provision of suitably activated fatty acids for incorporation into the lysocholine phosphoglyceride–choline phosphoglyceride cycle which occurs within the plasma membrane of the erythrocyte [24]. It has been

shown [25] that choline phosphoglyceride is the major metabolically active component of the erythrocyte plasma membrane lipids, and that cycling between choline phosphoglyceride and lysocholine phosphoglyceride is a relatively common process [26]. This process presents the opportunity for the selective accumulation of preferred fatty acids within the phosphoglycerides, and the possibility of their subsequent release when required to perform a function within a target tissue [27]. Thus the erythrocyte plasma membrane long-chain acyl:CoA synthetase may act as the first step in a fluid system for the selective supply of fatty acids required by other tissues.

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