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Synthesis of Docosahexaenoyl Coenzyme A in Human Spermatozoa

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ABSTRACT: The synthesis of docosahexaenoyl coenzyme A (22:6-CoA) was studied in a long-chain fatty acid: CoASH ligase (AMP)-enriched fraction from human spermatozoa and was compared to palmitoyl CoA (16:0-CoA) synthesis. The pH optimum for 22:6 activation was 8.4, which was identical to the value obtained with 16:0. The K_m for ATP was 0.5 mM when 22:6 was the acyl substrate; however, when 16:0 was incubated with the ligase preparation, the K_m for ATP was 2.9 mM. When CoASH was varied and 22:6 was the fatty acyl acceptor, a pattern of negative cooperativity was observed. This was confirmed by a downwardly concave double-reciprocal plot, a Hill coefficient of 0.63, and an R_s in excess of 150. The Hill coefficient with 16:0 and CoASH was 0.94. Palmitic acid was demonstrated to be a competitive inhibitor of 22:6-CoA synthesis. Based upon these data, we conclude that the kinetics of spermatozoan ligase are complex, and, in addition, these data support the hypothesis that 22:6 may regulate ligase activity, and therefore free fatty acid utilization, in sperm.

Key words: Docosahexaenoic acid, palmitic acid, coenzyme A, long-chain fatty acid: CoASH ligase (AMP), human spermatozoa, enzyme kinetics.

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Long-chain fatty acid: CoASH ligase (AMP) (E.C. 6.2.3.1), also referred to as fatty acyl thiokinase or fatty acyl CoA synthetase, couples free fatty acids to coenzyme A (CoASH) at the expense of Mg-ATP hydrolysis to AMP and inorganic pyrophosphate. The ligase reaction appears to be the rate-limiting step for free fatty acid metabolism in a variety of mammalian tissues (Brindley and Ferrier, 1972; Jones et al, 1981; Normann et al, 1981).

We have extensively characterized the ligase from human spermatozoa and have noted that the saturated acyl substrate specificity in sperm is more restricted than the ligase activity found in whole liver homogenates (Jones and Plymate, 1986). The activity for sperm ligase with saturated fatty acids was found to peak with palmitic acid (16:0) and was otherwise limited to myristic (14:0) and stearic (18:0) acids. The K_m's for these fatty acids were essentially identical (approximately 4 μM) (Jones and Plymate, 1986). In contrast, hepatic ligase activity peaked with 14:0 and demonstrated a considerable capacity to activate lauric acid (12:0).

Unsaturated fatty acids can also serve as substrate for sperm ligase. With the exceptions of arachidonic acid (20:4) and docosahexaenoic acid (22:6), unsaturated fatty acids, regardless of chain length or number of double bonds, shared a K_m similar to that seen with the saturated fatty acids (Jones and Plymate, 1987). The K_m for 22:6 was considerably lower (1.2 μM), and 20:4 could not be activated in this preparation (Jones and Plymate, 1987). Additional studies have demonstrated that linolenic acid (18:3) and 18:0 were competitive inhibitors for palmitoyl CoA synthesis, whereas 22:6 was a noncompetitive inhibitor (Jones and Plymate, 1988). Thus, sperm, like other tissues (Marcel and Suzue, 1972; Groot and Hülsmann, 1973; Banis and Tove, 1974; Norman et al, 1981) appear to have a single enzyme that activates saturated and most unsaturated fatty acids. However, the specifics underlying docosahexaenoyl CoA (22:6-CoA) synthesis in human sperm have not been studied. The lower K_m and noncompetitive pattern of inhibition could reflect properties unique to 22:6, or, alternatively, it could indicate that there is a separate enzyme in sperm that activates 22:6. Accordingly, we conducted further studies to characterize the synthesis of 22:6-CoA in human sperm and compared it to 16:0 activation.

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Materials and Methods

Tissue Preparation

Human spermatozoa were obtained by masturbation from healthy young men. Spermatozoa were separated from the seminal plasma by centrifugation at 2,000 × *g* and washed in 75 mM NaCl, 75 mM KCl, 0.2 mM EDTA, and 20 mM Tris HCl (pH 7.4). Ligase activity was solubilized with Triton X-100 (final concentration 0.1%) as previously described (Jones et al, 1985), and the preparation derived from individual ejaculates was used in separate kinetic assays. This Triton preparation has a relatively low specific activity but does not contain activity of other acyl CoA-requiring enzymes (Choy et al, 1992; Jones and Plymate, unpublished observations).

Enzyme Assays

Ligase activity was measured using two different procedures. The first employed ^{3}H-G-CoASH (specific activity 3.7 Ci/mmol; New England Nuclear, Boston, Massachusetts) and was based on the radioligand–millipore filter technique developed by Polokoff and Bell (1975). The incubation medium consisted of 20 mM ATP, 20 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM CoASH, 380 mM Tris (2-amino-hydroxymethylpropane-1,3-diol) (pH 8.4), and up to 50 μM fatty acyl substrate. The assay conditions and product recovery have been previously reported (Jones et al, 1985). In the second assay, ^{14}C-1-22:6 (specific activity 56.9 mCi/mmol; New England Nuclear) was used as the radioligand, and the procedure was based on a liquid–liquid partition system to separate free from CoA thioesterified fatty acids (Bar-Tana et al, 1973). Because of high blanks due to the coincubation with Triton X-100 (Jones and Plymate, 1988), several modifications of the original technique were incorporated into this study. The composition of the incubation was identical to that used in the first procedure. The reaction was initiated by adding 50 μl of solubilized ligase (approximately 35 μg protein/assay), bringing the total volume to 0.4 ml. The mixture was incubated at 37°C in a shaking water bath for 5 minutes. The reaction was terminated with the addition of 100 μl albumin (1 mg/ml) (fatty acid poor, fraction V, Sigma Chemical Company, St. Louis, Missouri) followed by 3 ml chilled 0.3 M trichloroacetic acid (TCA). The tubes were placed on ice for 5 minutes and were later poured over millipore-type filters (pore size 0.45 μm). The filters were washed twice with 3 ml 0.3 M TCA. After the filters were dried, they were delipidated with 4 ml chloroform/methanol (2:1, v:v). The chloroform/methanol phase was taken to dryness under N_2 at 42°C, and the residue was resolubilized in 0.75 ml H_2O, 0.6 ml *n*-heptane, and 1.0 ml Doles reagent (isopropanol/*n*-heptane/0.5 M H_2SO_4; 40:10:1, v:v). The resulting aqueous phase was extracted four more times with 0.6 ml *n*-heptane to remove the nonthioesterified 22:6. An aliquot (1.0 ml) of the aqueous phase was counted in 10 ml Instagel (United Technologies, Packard, Downers Grove, Illinois). To determine enzyme blanks, the enzyme source was added at the end of the incubation and immediately terminated with albumin and 0.3 M TCA. Enzymatic rates were calculated as nanomoles acyl CoA formed/minute per mg protein. Each condition was performed in duplicate.

Authentication of 22:6-CoA

In order to authenticate the presumptive 22:6-CoA recovered with both assays, incubations with ligase were performed using ^{14}C-1-22:6 with unlabeled CoASH. The reaction was terminated with albumin, acid precipitated, and filtered. The filters were delipidated with chloroform:methanol, which was evaporated under N_2, and the residue was resolubilized in a minimal volume of ethanol. Heat-activated silica gel G plates were spotted with the resulting solution as well as with standards (^{3}H-CoASH, ^{14}C-22:6, and 22:6-CoA) and chromatographed using *n*-butanol/acetic acid/H_2O (5:2:3, v:v). The plates were developed by exposure to iodine vapor. The labeled spots were scraped, transferred to scintillation vials, and counted in Instagel. The standard for 22:6-CoA was synthesized according to the method of Bishop and Hajra (1980).

Kinetic Calculations

Kinetic constants were determined using both double-reciprocal plots and weighted analyses (Cleland, 1967). Inhibition constants were calculated using standard formulae (Segel, 1968). Additional transformations were performed according to Koshland (1970) and Levitzki and Koshland (1969).

Miscellaneous

Protein was determined with a dye affinity assay (Bio-Rad Laboratories, Richmond, California). All reagents, except those already specified and the solvents used in the synthesis of 22:6-CoA, which were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin), were obtained from Sigma.

Results

Thin-layer chromatography of the extracted incubation media compared to the ^{3}H-CoASH, ^{14}C-22:6, and 22:6-CoA standards demonstrated the presence of free 22:6 and the absence of unesterified CoASH. Because these incubations were not further separated, the contamination with free 22:6 was anticipated. The organically synthesized 22:6-CoA had an R_f (0.65) identical to that of the labeled 22:6 derivative obtained from these incubations.

The pH dependency of 22:6 activation is shown in Figure 1. Enzymatic activity peaked at pH 8.4.

The ATP curves for 22:6 and 16:0 are shown in Figure 2. Both plots were readily linearized by a double-reciprocal transformation. The K_m for ATP with 22:6 as the acyl substrate was 0.5 mM. On the other hand, with 16:0 as the fatty acid, the K_m for ATP was 2.9 mM. Essentially identical values were obtained using a weighted analysis. The maximum velocity was considerably higher with 16:0 (23.2 nmol palmitoyl CoA formed/minute per mg protein) than with 22:6 (1 nmol docosahexaenoyl CoA formed/minute per mg protein), which was consistent with our previous results (Jones and Plymate, 1987).

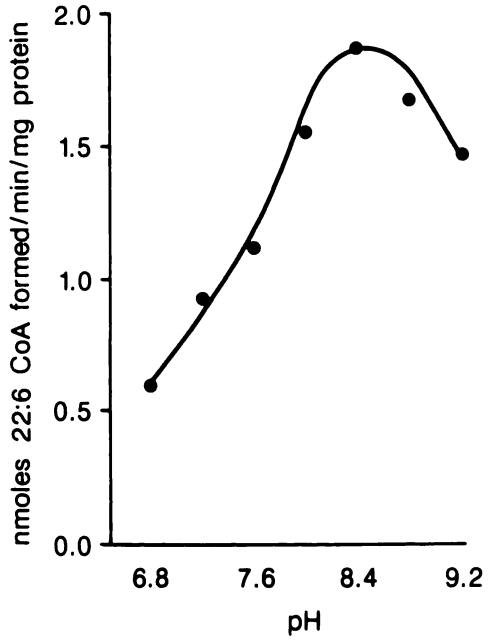

FIG. 1. pH curve. Sperm ligase activity was measured using ^{3}H-CoASH as the radioligand. The concentration of each substrate was saturating (20 mM ATP, 20 mM $MgCl_2$, 0.1 mM CoASH, and 50 μM 22:6), and the pH was varied from 6.8 to 9.2 with Tris. The values shown are the average of three experiments.

The CoASH saturation curve with 22:6 is illustrated in Figure 3. Lineweaver–Burk transformation of the data consistently resulted in a downwardly concave plot. The concentration of substrate that yielded one-half maximum velocity ($S_{0.5}$) was approximately 12 μM, and the R_s value was in excess of 150. The Hill plot for both 16:0 and 22:6 CoASH curves is shown in Figure 4. When 16:0 was the fatty acid substrate, the slope of the plot was 0.94; however, the slope obtained with 22:6 was 0.63.

The effects of 16:0 on 22:6 activation are shown in

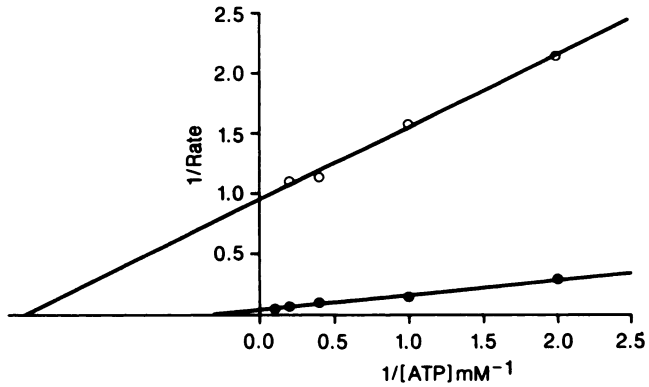

FIG. 2. ATP curve. Ligase activity was determined per Figure 1. With the exception of the ATP concentration, which was varied from 0.5 to 10 mM, all substrates were saturating. The concentration of both 16:0 (●——●) and 22:6 (○——○) was 50 μM. Each point represents the mean of two experiments.

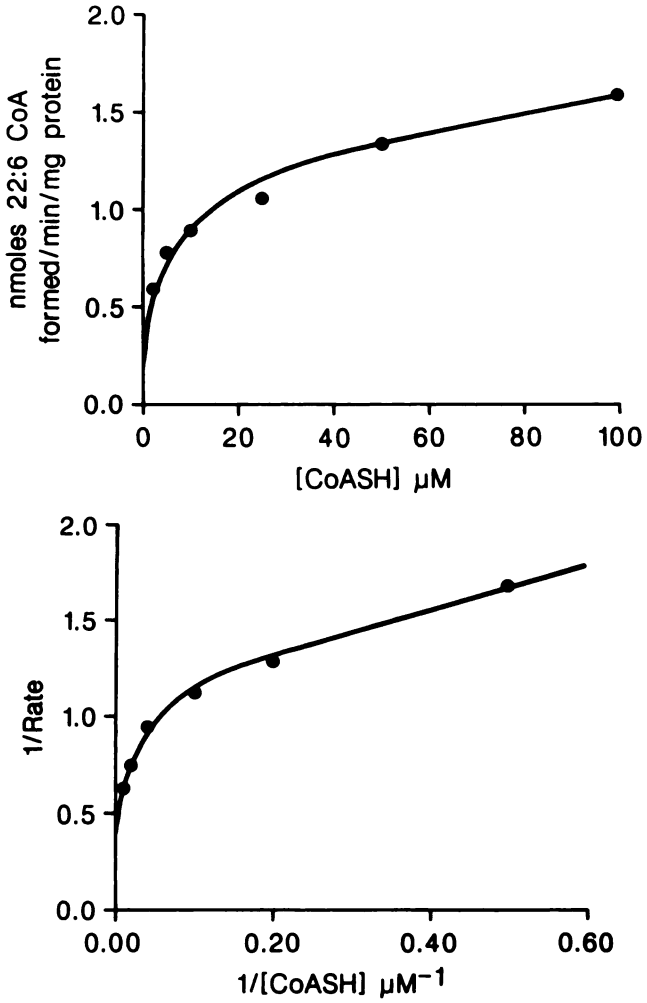

FIG. 3. CoASH curve. Incubation conditions were identical to those listed from Figure 1. The concentration of CoASH was varied from 2 to 100 μM. Each point is the mean of three experiments. (A) Saturation plot. (B) The double-reciprocal plot.

Figure 5. A competitive pattern of inhibition, as evidenced by a progressive increase in K_m and a lack of reduction in maximum velocity, was observed. The K_i for 16:0 was 7.4 μM. The K_m for 22:6 with these experiments was slightly higher than the value we had previously reported (2.8 vs. 1.2 μM) (Jones and Plymate, 1987). However, a similar elevation in K_m was noted in experiments using labeled 16:0; the K_m for 16:0 ranged from 5.5 to 10 μM in contrast to our previous observations of 4.0 to 4.4 μM (Jones and Plymate, 1986, 1987). Nonetheless, 22:6 consistently had a lower K_m than 16:0.

Discussion

In prior studies (Jones and Plymate, 1986, 1987, 1988) we have shown that both saturated and most unsaturated fatty acids are activated by a single enzyme in human

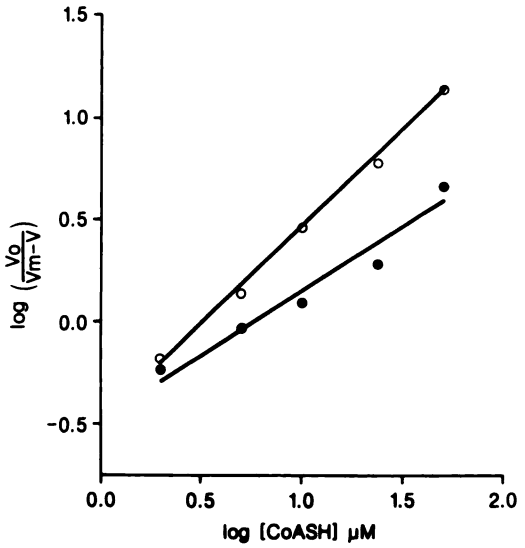

FIG. 4. Hill transformation of CoASH curves. The maximal velocity for both 16:0 (●——●) and 22:6 (○——○) was experimentally determined using the rate attained with 100 μM CoASH.

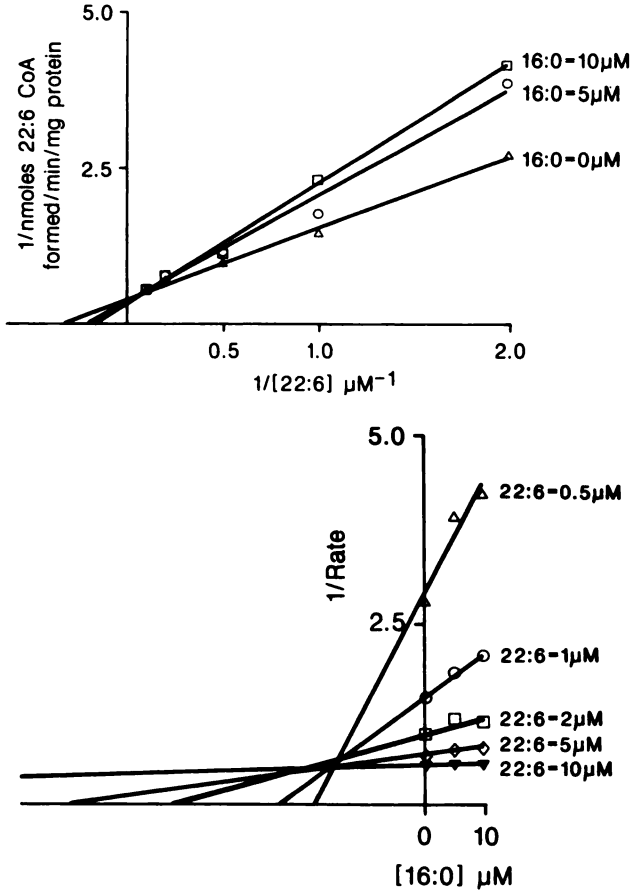

FIG. 5. Competition curves for 16:0 versus 22:6 activation. Ligase activity was measured using ^{14}C-1-22:6 as detailed in the Materials and Methods section. Each point represents the average of three experiments. **(A)** Lineweaver–Burk plot. **(B)** Dixon plot. The concentration of 22:6 was varied from 0.5 to 10 μM and the concentrations of 16:0 were 0, 5, and 10 μM. The remaining substrates were saturating as detailed in the legend to Figure 1.

spermatozoa. The exception, 22:6, demonstrated a lower K_m and a noncompetitive pattern of inhibition when compared to other fatty acyl substrates. This study was conducted to compare the kinetics of 22:6 activation with those of 16:0 in order to help clarify the possible interactions between these two fatty acids and to provide optimization for spermatozoal 22:6 activation.

The pH optimum for 22:6-CoA synthesis was identical to that previously obtained with 16:0 as substrate (Jones et al, 1985). Similar alkaline pH optima have been reported with ligase from rat liver mitochondria (Phillip and Parsons, 1979), microsomes (Pande, 1972), hepatic homogenates (Farstad et al, 1967), or brain microsomes (Reddy et al, 1984).

Despite the similarity in the pH optimum between 22:6 and 16:0, there were major differences in both the ATP and CoASH curves. With 22:6 as the acyl substrate, the K_m for ATP was considerably lower than the value obtained with 16:0, both in this study and as previously reported (Jones and Plymate, 1986). When the concentration of CoASH was varied and 22:6 was the acyl acceptor, the data could not be linearized by a double-reciprocal transformation. The resulting Lineweaver–Burk plot was downwardly concave, suggesting negative cooperativity (Levitzki and Koshland, 1969), which was confirmed by a slope in the Hill plot of less than 0.8 and an R_s value greater than 81. In contrast, CoASH kinetics were typical Michaelis–Menton when 16:0 was the fatty acid substrate. The finding of negative cooperativity implies interactions between enzyme subunits unique to CoASH and 22:6 because of the lack of deviation from linearity when either ATP or 22:6 were independently varied.

Negative homotropic kinetics are commonly seen in enzymology. With the exception of glyceraldehyde-3-phosphate dehydrogenase (Levitzki and Koshland, 1969), most enzymes that display negative cooperativity possess regulatory sites. There are several possible molecular mechanisms to explain negative cooperativity, but the hypothesis advanced by Koshland (1970) is that of ligand-induced conformational changes of the enzyme. In the case of human sperm ligase, it would appear that the negative effector is CoASH but only in the presence of 22:6.

Our observations that 16:0 is a competitive inhibitor of 22:6-CoA formation implies that both fatty acids share the same acyl substrate-binding site, and, if coupled with our previous studies determining that 22:6 or a metabolite of 22:6 is a noncompetitive inhibitor of palmitoyl CoA synthesis (Jones and Plymate, 1988), suggest the possibility that ligase has a second binding site that could be regulatory in nature. Alternatively, these data could be

explained by product inhibition (Plowman, 1972) due to 22:6-CoA; however, it would then be anticipated that 16:0, by virtue of its conversion to palmitoyl CoA, would also have demonstrated noncompetitive inhibition of 22:6-CoA synthesis.

In summary, our data provide some insight into the molecular regulation of ligase activity by 22:6. This model for the substrate-level regulation of ligase activity may explain the preferential accumulation of 22:6 into phospholipids of maturing epididymal spermatozoa (Evans and Setchell, 1979; Nikolopoulou et al, 1985) and into ejaculated human spermatozoa (Jones and Plymate, 1989).

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