Nonoxidative Ethanol Metabolism in Rabbit Myocardium: Purification to Homogeneity of Fatty Acyl Ethyl Ester Synthase†

Stanley Mogelson and Louis G. Lange*

ABSTRACT: Fatty acyl ethyl esters, previously identified in our laboratory as metabolites of ethanol in human and rabbit myocardium, arise from an esterification of free fatty acids with ethanol in the absence of ATP and coenzyme A. This study was designed to isolate and purify the enzyme(s) in rabbit myocardium that catalyze(s) this reaction. Enzyme activity in homogenates of rabbit myocardium, as assayed by the rate of synthesis of ethyl [^{14}C]oleate from 0.4 mM [^{14}C]oleic acid and 0.2 M ethanol, was 31 nmol/(g·h), and all of it was recovered in the 48400*g* supernatant. This soluble ethyl ester synthase activity bound to DEAE-cellulose at pH 8, and elution with a NaCl gradient (0–0.25 M) separated two enzyme activities accounting for 13 and 87% of recovered synthase activity. The major enzyme activity was then purified over 5000-fold to homogeneity by sequential gel permeation, hydrophobic interaction, and anti-albumin affinity chromatographies with an overall yield of 40%. Up to 45 μg of enzyme was present per g of myocardium. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed a single polypeptide with M_r 26 000, and gel permeation chromatography under nondenaturing conditions indicated a M_r of 50 000 for the active enzyme. Kinetic analyses using the purified enzyme indicated that greatest rates of ethyl ester synthesis were observed with unsaturated octadecanoic fatty acid substrates [V_{max} = 1.9 and 1.5 nmol/(mg·s) for linoleate and oleate, respectively], with lesser rates associated with palmitate, stearate, and arachidonate substrates [0.14, 0.03, and 0.35 nmol/(mg·s), respectively]. K_m's for these fatty acids were essentially identical and equal to 0.2 mM; substrate specificity with respect to alcohol chain length, however, resulted from varying K_m's for methanol, ethanol, 1-propanol, and 1-butanol, i.e., >1.4, 1.10, 0.53, and 0.07 M, respectively, while V_{max} was constant at approximately 1.5 nmol/(mg·s). The amino acid analysis of this synthase is not unusual and serves to distinguish it from typical cholesterol esterases. Moreover, under conditions in which the enzyme is maximally active with respect to ethyl ester synthesis, it did not hydrolyze cholesterol oleate. Thus, fatty acid ethyl esters are synthesized in myocardium primarily by a soluble dimeric enzyme comprised of two identical or nearly identical subunits (M_r 26 000). This enzyme, not described previously, esterifies free fatty acids with ethanol to produce a nonoxidative metabolite of ethanol that accumulates in vivo with potentially toxic effects.

Alcohol-induced heart disease is characterized by abnormal myocardial contractile function and energy metabolism, even in the absence of nutrient deprivation (Lochner et al., 1969; Segal et al., 1979). Alterations in myocardial lipid metabolism pursuant to chronic ethanol ingestion, including abnormal triglyceride homeostasis and fatty acid oxidation, were well described (Regan et al., 1966; Kikuchi & Kako, 1970; Kako et al., 1973), but the biochemical mechanisms by which these perturbations are engendered by ethanol have been largely unknown since no myocardial ethanol metabolism had been described. In recent studies, ethanol incorporation into a family of fatty acyl ethyl esters was documented to occur in isolated rabbit hearts perfused with ethanol, in rabbit heart homogenates incubated with ethanol, and in the myocardium of rabbits exposed to ethanol in vivo (Lange et al., 1981; Lange & Sobel, 1983a). Moreover, fatty acyl ethyl esters are now known to accumulate in human myocardium with a biological half-life in vivo that exceeds that of ethanol and in sufficient amounts to be potential mediators of metabolic derangements consequent to ethanol abuse (Lange & Sobel, 1983a). Such fatty acid ethyl esters induce mitochondrial dysfunction in vitro (Lange & Sobel, 1983b) and could account, in part, for the decrements in fatty acid oxidation noted to occur in alcohol-induced heart disease. Thus, this nonoxidative metabolism of ethanol may be a biochemical link between ethanol ingestion and perturbed myocardial lipid homeostasis with attendant pathobiological consequences.

The lipid precursor for esterification with ethanol to form fatty acyl ethyl esters in heart was identified as nonesterified fatty acid (Lange, 1982), and heat activation studies suggested that formation of fatty acyl ethyl esters from ethanol and nonesterified fatty acid is enzymatically mediated (Lange et al., 1981). The existence of an enzyme capable of effecting this reaction was established when purified pancreatic cholesterol esterase, an exocrine product, was found to catalyze the net synthesis of fatty acyl ethyl esters by using nonesterified fatty acid and ethanol as substrates in the absence of ATP and coenzyme A (Lange, 1982). We hypothesized that this or a similar enzyme was responsible for synthesis of fatty acyl ethyl esters in myocardium.

In order to examine this hypothesis, to study the relationships between the enzyme(s) responsible for fatty acyl ethyl ester synthesis in myocardium and cholesterol esterase, and to determine the factors that permit the thermodynamically unfavorable synthesis of fatty acyl ethyl esters from free fatty acids and ethanol, we have identified two enzymes in rabbit myocardium that catalyze fatty acyl ethyl ester synthesis and report here the purification to homogeneity of the enzyme accounting for the bulk of such synthesis, along with its properties. It is a dimeric enzyme of M_r of 50 000 that is present in myocardium at a concentration of up to 45 μg/g wet wt of heart. In the accompanying paper (Mogelson et al., 1984), we report kinetic analyses that help clarify the biochemical mechanisms by which this enzyme catalyzes fatty acyl ethyl ester synthesis independently of coenzyme A and ATP and physical properties of fatty acyl ethyl esters that contribute to their accumulation in myocardium.

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Materials and Methods

Materials. All reagents were the highest commercially available grade. [^{14}C]Oleic acid (59.9 Ci/mol), [^{3}H]oleic acid (5.7 Ci/mmol), [^{14}C]linoleic acid (56 Ci/mol), [^{14}C]arachidonic acid (58.4 Ci/mol), [^{14}C]stearic acid (57.6 Ci/mol), and [^{14}C]palmitic acid (55 Ci/mol) were purchased from Amersham.

Ethyl [^{3}H]oleate was synthesized in 70% yield by acid-catalyzed esterification of [^{3}H]oleic acid in ethanol (Smith, 1939). A total of 5 mCi of [^{3}H]oleic acid was dissolved in 100 mL of ethanol, and the solution was bubbled with HCl gas for 20 min. The ethanol was evaporated under reduced pressure, and residual lipids were dissolved in acetone. Ethyl [^{3}H]oleate was then isolated by preparative thin-layer chromatography on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid (75/5/1) (Lange et al., 1981). Its specific radioactivity was 5.7 Ci/mmol, and it was greater than 98% radiopure by thin-layer chromatography (<1% free oleic acid).

Enzyme Assay. Rates of fatty acyl ethyl ester synthesis were determined by incubating samples containing enzyme with 0.4 mM [^{14}C]oleate (20 000 dpm/nmol) and 200 mM ethanol in 60 mM sodium phosphate buffer, pH 7.2, in a total volume of 0.17 mL in capped vials at 37 °C. At the end of the incubation interval, the reaction was terminated by the addition of 2 mL of cold acetone containing a known amount of ethyl [^{3}H]oleate and 0.6 μmol of carrier ethyl oleate. Volumes were reduced by evaporation under a stream of nitrogen at 37 °C, and residual lipids in acetone were chromatographed on silica OF plates (Analabs, North Haven, CT) developed with petroleum ether/diethyl ether/acetic acid (75/5/1) (Lange et al., 1981). After visualization of lipids with iodine vapor, fatty acyl ethyl ester spots were scraped, and the lipid was eluted with acetone and assayed for radioactivity. ^{14}C counts were adjusted for yield as determined by recovery of ^{3}H, and after subtraction of blanks, results were expressed as nmoles of fatty acyl ethyl ester formed per milliliter per hour. Assays for enzymatic synthesis of fatty acyl ethyl esters were linear with respect to expended time (up to 30 min) and added enzyme (up to 0.02 mg/mL). Since the assay does not utilize solvent alcohols or mineral acid, there is no generation of fatty acid ethyl ester by either transesterification between solvent alcohol and lipid esters (Gordon et al., 1970) or acid-catalyzed synthesis from solvent alcohol and fatty acid (Smith, 1939).

Homogenates. Rabbits (15) were stunned by a blow to the head and their rapidly excised hearts placed into ice-cold 0.01 M Tris-HCl,[1] pH 8.0, containing 1 mM 2-mercaptoethanol (buffer A). Blood was removed by flushing the vascular space retrogradely from the aorta with cold buffer A. Ventricular myocardium (30–40 g) was trimmed free of fat and connective tissue, minced, and homogenized in 8–10 volumes of cold buffer A with a Polytron PT-20 homogenizer at a half-maximal setting for 30 s. Homogenate was centrifuged at 15000*g* for 10 min. The sediment was washed once with half the original volume of buffer A, and the pooled supernatants were centrifuged at 48400*g* for 1 h. This supernatant was taken as the soluble fraction. After each centrifugation, any floating fat cake was removed by aspiration. All centrifugations were performed at 4 °C. Protein was estimated by the Lowry method (Lowry et al., 1951) with bovine serum albumin as standard.

Anion-Exchange Chromatography. The soluble fraction (200 mL) was dialyzed overnight against buffer A and then applied to DEAE-cellulose (Whatman DE-52; 15 × 2.5 cm) equilibrated with buffer A at 4 °C. The resin was washed at a flow rate of 0.5 mL/min until the effluent absorbance at 280 nm was less than 0.1, and then proteins retained by the column were eluted with a linear gradient of sodium chloride (0–0.25 M) in a total volume of 300 mL of buffer A, with 2.5-mL fractions collected.

Gel Permeation Chromatography. Fractions from the preceding step having an activity greater than 2 nmol/(mL·h) were pooled, and the protein in the pooled fractions was concentrated at 4 °C by addition of solid ammonium sulfate (70% saturation). The resultant precipitate was sedimented by centrifugation at 48400*g* for 15 min and redissolved in 2–3 mL of 10 mM Tris–PO_4, pH 7.0, containing 50 mM sodium phosphate, pH 7.0, and 1 mM 2-mercaptoethanol (buffer B). The protein was applied to Sephacryl S-200 (Pharmacia; 1.5 × 100 cm) equilibrated with buffer B, and ascending chromatography was performed at 4 °C at a flow rate of 4 mL/h; 2.5-mL fractions were collected. Calibration of the column for molecular weight determination was performed by determining the elution volume of proteins of known molecular weight, including aldolase (M_r 158 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and α-chymotrypsinogen A (M_r 25 000). Elution of protein standards was monitored by absorbance at 280 nm. The partition coefficient, K_{av}, for each standard was then calculated as $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the protein, V_0 is the void volume, and V_t is the column volume. K_{av} for fatty acyl ethyl ester synthase was determined from the elution volume of enzyme activity assayed as described above. Enzyme molecular weight was then calculated from the plot of ln M_r vs. K_{av}.

Hydrophobic Interaction Chromatography. Fractions from the gel column containing enzyme activity greater than 2 nmol/(mL·h) were pooled and applied at 0.5 mL/min to octyl-Sepharose CL-4B (Pharmacia; 1.0 × 4.0 cm) equilibrated with buffer B at 4 °C. Nonbound and loosely bound proteins were removed by washing the resin with buffer B containing 0.1% sodium cholate (w/v). Elution of enzyme was then performed with a linear gradient of sodium cholate (0.1–0.5%) in 100 mL of buffer B. Fractions of 2 mL were collected.

Anti-Albumin Antibody Affinity Chromatography. The IgG fraction of anti-rabbit albumin sheep immune serum (Miles Laboratories, Elkhart, Indiana), precipitated with 45% saturated ammonium sulfate and fractionated by chromatography on DEAE-cellulose (Fahey & Terry, 1967), was coupled for 2 h to CNBr-activated Sepharose 4B (Pharmacia) in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M sodium chloride. The resultant resin was treated with 0.2 M glycinamide, pH 8, for 1 h and then washed with 0.1 M sodium acetate, pH 4, containing 0.5 M sodium chloride. The Sepharose–anti-albumin antibody conjugate was washed with the coupling buffer and then equilibrated with buffer B. Fractions eluting from octyl-Sepharose having ethyl ester synthase activity greater than 2 nmol/(mL·h) were pooled and applied to the Sepharose–anti-albumin antibody conjugate (0.9 × 6 cm). Subsequent elution was performed with buffer B, with 2-mL fractions collected, and those containing more than 0.01 net A_{280} unit were pooled.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed as described by

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; ADH, alcohol dehydrogenase; MEOS, microsomal ethanol-oxidizing system; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Purification of Fatty Acid Ethyl Ester Synthase[a]

step	total protein (mg)	total act. (nmol/h)	sp act. [nmol/(mg·h)]	purification (x-fold)	yield (%)
homogenate[b,c]	4725	754			
major		592	0.125	1	100
minor		163	0.034		
cytosol[b,c]	1029	1012			
major		921	0.895	7	148
minor		91	0.088		
DEAE-cellulose chromatography[c]					
major	117	285	2.43	19	48
minor	80	73	0.908		
S-200 chromatography	26	206	7.9	63	35
octyl-Sepharose chromatography	0.57	250	439	3510	42
anti-albumin affinity chromatography	0.35	236	674	5400	40

[a] Data are averaged from two representative preparations. [b] Major and minor activities in homogenate and cytosol were calculated from the proportions of major and minor activities as determined after DEAE-cellulose chromatography. [c] Samples were dialyzed against 10 mM Tris–PO_4 and 50 mM sodium phosphate, pH 7.2, containing 1 mM 2-mercaptoethanol before assays for enzyme activity.

Laemmli (1970). Gels containing 4.5 (stacking gel, pH 6.8) and 12.5% (separating gel, pH 8.8) acrylamide were run at 80 (stacking) and 150 V (separating) after which they were fixed and stained with silver (Merrill et al., 1981) or with 0.2% Coomassie Brilliant Blue. Enzyme subunit molecular weight was calculated from a plot of R_f vs. ln M_r determined for each gel with polypeptide standards of known molecular weight [phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soy bean trypsin inhibitor (20 100), and α-lactalbumin (14 400)].

Amino Acid Analysis. Analyses were performed by the Protein Chemistry Facility of Washington University School of Medicine with the generous assistance of Dr. Greg Grant (Spackman et al., 1958). Samples were hydrolyzed in evacuated sealed ampules for 24, 36, and 72 h in 6 N HCl at 110 °C and analyzed with a Waters programmable gradient HPLC with a Waters cation-exchange amino acid column. Detection was by fluorescence with *o*-phthalic dicarboxaldehyde. Values for threonine and serine were obtained by extrapolation to zero time of hydrolysis (Moore & Stein, 1963); cysteine and methionine were determined after performic acid oxidation (Hirs, 1967). Proline was detected after continuous post-column infusion of hypochlorite.

Results

Isolation and Purification. Fatty acyl ethyl ester synthase activity was measured in dialyzed rabbit myocardial homogenates and 48400*g* supernatant fractions. (Identical results were obtained with 100000*g* supernatant fractions). The rate of synthesis of ethyl [^{14}C]oleate by homogenates of rabbit ventricular myocardium under standard assay conditions (0.4 mM [^{14}C]oleic acid, 200 mM ethanol, pH 7.2, 37 °C) was 31.2 ± 4.7 nmol/(g·h) (mean ± SD, n = 3). Of this activity, 148 ± 30% (n = 3) was recovered in the soluble fraction. Application of this soluble fraction of heart homogenate to DEAE-cellulose at pH 8 resulted in binding of all of the ester synthase activity. Subsequent elution with a linear gradient of sodium chloride (0–0.25 M) separated two peaks of enzyme activity accounting for 13 and 87% of recovered activity, respectively, and together 50–70% of activity initially present (Figure 1 and Table I). No additional synthetic activity was recovered by further elution of the resin with buffer A containing up to 0.5 M sodium chloride. Further studies have utilized the major enzyme, accounting for the bulk of myocardial fatty acyl ethyl ester synthetic capability.

Protein in the eluate from anion-exchange chromatography containing fatty acyl ethyl ester synthase activity was concentrated by ammonium sulfate precipitation and applied to

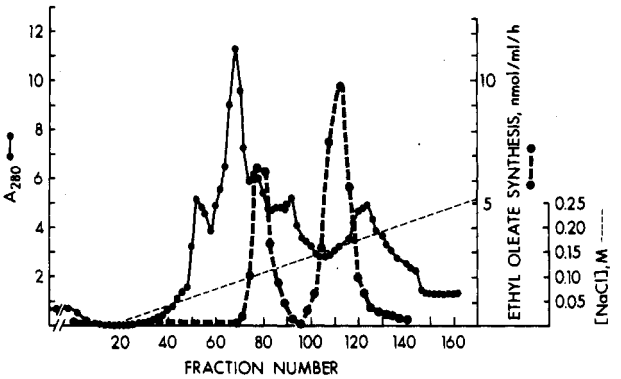

FIGURE 1: DEAE-cellulose chromatography. The soluble fraction from rabbit myocardium was applied to DEAE-cellulose (2.5 × 15 cm) equilibrated with 10 mM Tris-HCl, pH 8.0, and 1 mM 2-mercaptoethanol, and retained proteins were eluted in a NaCl gradient, 0–0.25 M (– –). Elution of protein is indicated by the solid line, and the elution of fatty acyl ethyl ester synthase activity is indicated by the heavy broken line, as assayed as described under Materials and Methods. Flow was 0.5 mL/min; 2-mL fractions were collected.

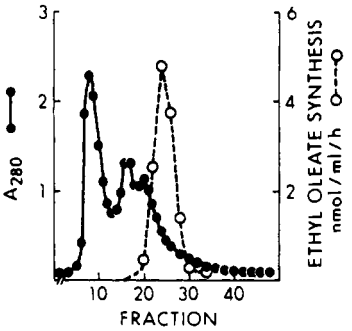

FIGURE 2: Gel permeation chromatography. Protein, in 2 mL of buffer containing 10 mM Tris–phosphate, 50 mM sodium phosphate, pH 7.2, and 1 mM 2-mercaptoethanol, was applied to Sephacryl S-200 (100 × 1.5 cm) equilibrated with the same buffer and eluted in the ascending direction. Eluent was collected in 2-mL fractions at 4 mL/h and monitored for protein (A_{280}, ●) and fatty acyl ethyl ester synthase activity (○).

Sephacryl S-200 for gel permeation chromatography. A single peak of activity eluted (Figure 2), with a 75% recovery of activity and a purification factor of 3–4. Comparison of the elution volume of active enzyme with the elution volumes of molecular weight standards indicated that the M_r of fatty acyl ethyl ester synthase is 50 000 (K_{av} = 0.26).

Fractions from the Sephacryl S-200 column containing enzyme activity greater than 2 nmol/(mL·h) were pooled and applied to octyl-Sepharose CL-4B for hydrophobic interaction chromatography (Figure 3). Nonbound and loosely bound

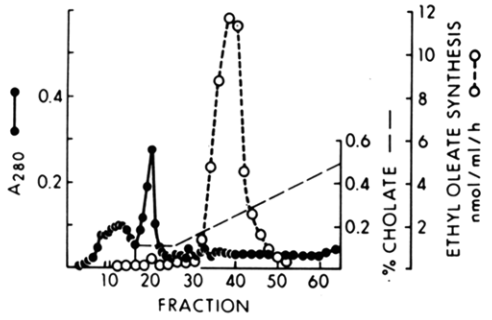

FIGURE 3: Hydrophobic interaction chromatography. Protein was applied to octyl-Sepharose CL-4B (1 × 4 cm) equilibrated with 10 mM Tris–phosphate, 50 mM sodium phosphate, pH 7.2, and 1 mM 2-mercaptoethanol; loosely bound proteins were eluted by washing with 0.1% cholate (w/v) in the same buffer (fractions 17–25), and retained proteins were eluted in a gradient of cholate, 0.1–0.5% (fractions 26–63). Eluent was monitored for protein (A_{280}, ●) and enzyme activity (○). Flow was 0.5 mL/min with 2-mL fractions collected.

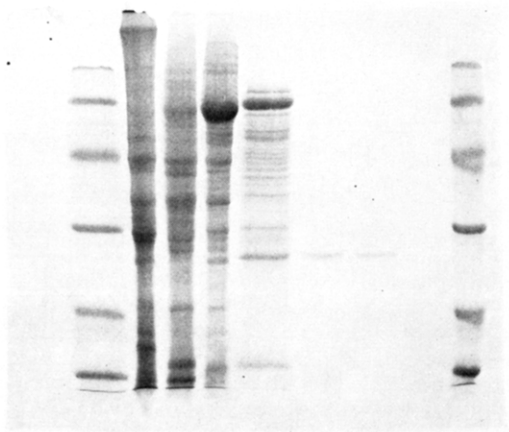

FIGURE 4: SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 12.5% gels. From left to right, the lanes contained (1 and 9) molecular weight standards (see Materials and Methods), (2) homogenate, (3) soluble fraction, (4) DEAE-cellulose pool, (5) Sephacryl S-200 pool, (6) octyl-Sepharose CL-4B pool, and (7) the final product after anti-albumin affinity chromatography; lane 8 is blank. This gel was stained with Coomassie Brilliant Blue; similar results were noted after silver staining.

proteins were removed by washing the resin with buffer B containing 0.1% sodium cholate. After elution with a linear gradient of sodium cholate (0.1–0.5%), fatty acyl ethyl ester synthase emerged at a sodium cholate concentration of approximately 0.2% (w/v). More than 95% of the activity initially applied to octyl-Sepharose was recovered in this fashion with an overall purification of 55-fold. SDS-PAGE (Figure 4) showed that the product of this hydrophobic interaction chromatography contained two polypeptides, fatty acyl ethyl ester synthase (R_f 0.66) and albumin (R_f 0.24). Albumin was then selectively removed from the preparation by passage over an anti-albumin antibody affinity column. Subsequent assay for fatty acyl ethyl ester synthase activity indicated full retention of enzyme activity, and SDS–PAGE of the preparation demonstrated the presence of a single polypeptide with a M_r of 26 000 (Figure 4).

A summary of the purification of fatty acyl ethyl ester synthase from rabbit myocardium is provided in Table I. The product was purified 5400-fold with an apparent overall yield of 40%. Recovery of total activity at each step of the purification generally exceeded 50%, indicating that the purified enzyme represents the major fatty acyl ethyl ester synthase activity in myocardium. Since albumin inhibits synthesis of

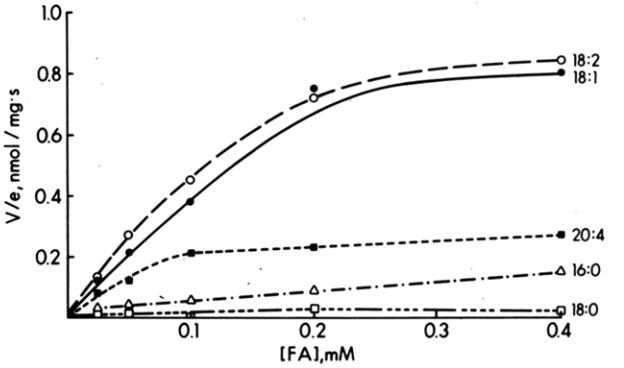

FIGURE 5: Velocity vs. substrate concentration plots for formation of fatty acyl ethyl esters from selected [^{14}C]fatty acids and 1 M ethanol. Rates of incorporation of [^{14}C]palmitate (△), [^{14}C]stearate (□), [^{14}C]oleate (●), [^{14}C]linoleate (○), and [^{14}C]arachidonate (■) into ethyl esters were determined at selected fatty acid concentrations. Purified fatty acid ethyl ester synthase, 3 μg/mL, was incubated in 60 mM sodium phosphate (pH 7.2) containing 1 M ethanol and ^{14}C-labeled fatty acid at 37 °C for 30 min. Product was extracted into acetone and quantitated as described under Materials and Methods.

ethyl esters in the assay in a concentration-dependent fashion (data not shown), the yields determined for assays of homogenates, cytosols, and the products of DEAE-cellulose and S-200 gel chromatographies (which copurify albumin and the synthase) may be underestimated in comparison to assays of samples with less concentrations of albumin. The final calculated yield of 40% may therefore be apparently augmented due to underestimation of activity as assayed in the starting myocardial homogenate.

SDS–PAGE indicated that the product was comprised of a single polypeptide having a M_r of 26 000. These results demonstrate that the major fatty acyl ethyl ester synthase activity in rabbit myocardium is a soluble dimeric enzyme with a M_r of approximately 50 000. On the basis of a specific activity of 700 nmol/(mg·h) (as assayed in the presence of 0.2 M ethanol and 0.4 mM oleic acid) and rates of homogenate ethyl oleate synthesis, we calculated that myocardium contains approximately 45 μg of major fatty acid ester synthase/g of myocardium. Since this value may be an overestimate due to albumin inhibition of homogenate activity assays, a minimal estimate was also calculated from the weight of purified enzyme protein obtained from the starting wet weight of myocardium. This was found to be 12 μg of synthase/g of myocardium.

Properties. Since the fatty acid composition of fatty acyl ethyl esters in myocardium formed in vivo is very different from that of the donor fatty acid pool, nonesterified fatty acids, or that of phospholipids and triglycerides (Lange, 1982), we studied the specificity of the synthase with respect to fatty acid chain length and saturation. Enzyme was incubated with ^{14}C-labeled fatty acids representing the major fatty acids in heart, i.e., palmitate, stearate, oleate, linoleate, and arachidonate, in the concentration range 0.025–0.4 mM, and in the presence of 1 M ethanol, to determine rates of ethyl ester formation. The results (Figure 5) show that the synthase possesses selectivity with respect to fatty acid chain length and saturation. Maximal rates of ethyl ester synthesis (V_{max}), determined from double-reciprocal plots, were approximately 10-fold greater for linoleate and oleate substrates than for arachidonate, stearate, and palmitate, i.e., 1.91 and 1.51 compared to 0.35, 0.03, and 0.14 nmol/(mg·s), respectively. The binding affinities of fatty acyl ethyl ester synthase for the selected fatty acid substrates, determined as K_m from the 1/velocity vs. 1/substrate concentration plots, are essentially

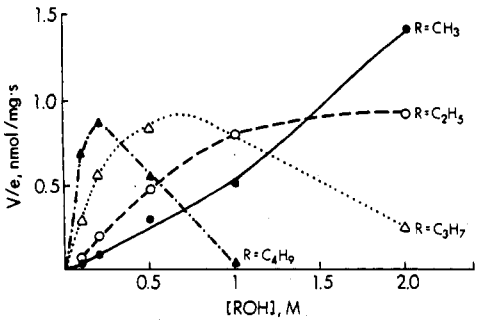

FIGURE 6: Velocity vs. substrate concentration plots for formation of fatty acyl alcohol esters from 0.4 mM [^{14}C]oleate and selected alcohols. Rates of fatty acyl alcohol ester synthesis were determined for methanol (●), ethanol (○), 1-propanol (△), and 1-butanol (▲) at alcohol concentrations between 0.1 and 2.0 M. Otherwise, conditions were as described in the legend to Figure 5.

the same for all five fatty acids and equal to 0.2 mM.

The substrate specificity with respect to alcohol chain length was also investigated by incubating enzyme with methanol, ethanol, 1-propanol, and 1-butanol in concentrations from 0.01 to 2 M in the presence of 0.4 mM [^{14}C]oleic acid (Figure 6). In contrast to the selectivity toward fatty acids, which was manifest as variations in V_{max}, the results with different unbranched alcohols showed selectivity expressed as increased binding affinity toward longer chain alcohols, with little change in observed maximal velocities. Respective K_m's for methanol, ethanol, 1-propanol, and 1-butanol were >1.3, 1.10, 0.53, and 0.07 M, with corresponding maximal velocities of >1.42, 1.55, 1.90, and 1.20 nmol/(mg·s) as determined from 1/velocity vs. 1/substrate concentration plots. Additionally, 1-butanol and 1-propanol demonstrated inhibition of alcohol ester synthesis at concentrations greater than 5 times K_m.

In order to define potential catalytic properties in common with pancreatic cholesterol esterase, we assayed the purified synthase for hydrolytic activity toward cholesterol oleate. Enzyme (4 μg/mL) was incubated with 42 μM cholesteryl [^{14}C]oleate incorporated into phosphatidylcholine vesicles in 60 mM sodium phosphate, pH 7.2, both in the presence and absence of 2 mM sodium taurocholate for 1 h at 37 °C, and hydrolysis of cholesteryl-^{14}C-oleate was assayed as described by Brecher et al. (1977). Under these conditions, there was no detectable hydrolysis of cholesteryl [^{14}C]oleate. On the other hand, this enzyme was fully active in catalyzing the synthesis of fatty acid ethyl esters as described above.

The results of amino acid analyses are given in Table II. The data are averaged from three analyses of separate preparations of fatty acyl ethyl ester synthase. The distribution of amino acids shows a predominance of acidic residues (aspartic and glutamic) vs. basic residues (arginine, lysine, and histidine) in the ratio 50:29, a finding consistent with our observation of a pI of approximately 4.9 (unreported data) for the synthase determined from activity elution from chromatofocusing systems.

Table II: Amino Acid Analysis

	residues/polypeptide	
	FAEE synthase[a]	sterol hydrolase[b]
Asp	23	27
Thr	10	15
Ser	17	24
Glu	32	24
Pro	10	16
Gly	22	26
Ala	20	21
Cys	22	4
Val	18	16
Met	6	1
Ile	8	11
Leu	16	16
Tyr	5	0
Phe	6	9
His	10	5
Lys	13	13
Arg	7	12
Trp		

[a] Averaged from three analyses, based on M_r of 25 000. [b] Normalized to M_r 25 000. Data from Gallo (1981).

Discussion

The accumulation of fatty acyl ethyl esters in myocardium following ethanol ingestion is a consequence of the enzymatic esterification of free fatty acid with ethanol (Lange et al., 1981; Lange, 1982). We have identified the presence in the soluble fraction of myocardial homogenates of two enzymes capable of catalyzing fatty acyl ethyl ester formation and have purified to homogeneity the enzyme accounting for the bulk of rabbit myocardial fatty acyl ethyl ester synthetic capability. It is a dimeric protein with a M_r of 50 000, comprised of two similar if not identical monomers of approximately M_r 26 000, and it is a prevalent enzyme in myocardium comprising 12–45 μg of protein/g of myocardium.

Some of our earlier observations of fatty acid ethyl ester synthesis in myocardium may reflect the catalytic properties of this synthase. For example, accumulated ethyl esters in perfused rabbit hearts exposed to ethanol are predominantly unsaturated species, with ethyl esters of linoleic, oleic, and arachidonic acids accounting for 43, 23, and 10%, respectively, of total fatty acyl ethyl esters (Lange et al., 1981). This pattern of chain lengths and saturations for fatty acyl ethyl esters apparently reflects the fatty acid substrate specificity of fatty acid ethyl ester synthase (Figure 5), a specificity remarkable for unsaturated octadecanoic fatty acids. The kinetic data indicate that this specificity is a consequence of greater rates of product formation for saturating concentrations of linoleic and oleic acid substrates than for palmitic, stearic, and arachidonic acids. This observation may be interpreted to indicate different free energies of activation for the transition state between substrates and products, with lower transition-state energies for linoleic acid and oleic acid substrates. In contrast, specificity with respect to alcohol chain length (Figure 6) is an expression of varying binding affinities, with the tightness of enzyme–alcohol binding increasing in relation to alcohol chain length (up to butanol). The decline in velocity observed with increasing concentrations of 1-propanol and 1-butanol may involve restricted access of the enzyme to the fatty acid substrate as the size and hydrophobicity of the alcohol substrate increases, but other as yet undefined mechanisms of inhibition may exist.

Comparison with the chain lengths and saturations of fatty acids in other myocardial lipids shows that the pattern of fatty acids in fatty acyl ethyl esters is virtually the same as that of endogenous cholesterol esters but different from the distribution of fatty acid species in nonesterified fatty acids and triglycerides. Indeed, this relationship was one of the pieces of evidence that suggested that the similar fatty acid compositions of fatty acyl ethyl esters and cholesterol esters may reflect substrate selection by the same enzyme, i.e., cholesterol esterase (Lange, 1982). In this regard, it is worth noting the structural and catalytic properties shared by classical cholesterol esterase and fatty acid ethyl ester synthase. Cholesterol esterase is a soluble enzyme that in its active form is an aggregate of apparently identical subunit polypeptides (Hyun

Table III: Properties of Ethanol-Metabolizing Enzymes

enzyme	subcellular localization	subunit composition no.	subunit composition M_r	pH optimum	cofactors	K_m for EtOH (M)
FAEE synthase	soluble	2	26 000	6–7	none known	2×10^{-1}
I-ADH[a]	soluble	2	40 000	10–12	NAD^+	2×10^{-3}
II-ADH[b]	soluble	2	40 000	10–11	NAD^+	2×10^{-5}
MEOS[c]	microsomes			7	NADPH, O_2	1×10^{-2}
catalase[d]	soluble	4	60 000	7–8	H_2O_2	

[a] Wagner et al., 1983. [b] Li et al., 1977. [c] Lieber & DeCarli, 1970. [d] Keilin & Hartree, 1945; Schonbaum & Chance, 1976.

et al., 1972; Calame et al., 1975). It catalyzes an equilibrium between an alcohol fatty acyl ester (cholesterol ester) and the free fatty acid and cholesterol. On the other hand, fatty acid ethyl ester synthase is also soluble and consists in its active form of a dimer of two apparently identical subunits. It too catalyzes an equilibrium between an alcohol fatty acyl ester (ethyl ester) and free fatty acid and alcohol.

Because of these analogies, we examined the newly purified synthase for further catalytic and structural properties in common with classical cholesterol esterase. Under conditions in which the purified synthase was fully active with respect to fatty acid ethyl ester synthesis, it did not catalyze the hydrolysis of cholesterol esters incorporated into phosphatidylcholine vesicles. Moreover, SDS–PAGE demonstrated different molecular weights for the subunits comprising purified synthase and exocrine cholesterol esterase (Hyun et al., 1972; Gallo, 1981). Comparison of the amino acid compositions of the synthase and pancreatic cholesterol esterase reveals unequivocal differences. Table II shows the amino acid composition of bovine pancreatic cholesterol esterase (Gallo, 1981), which, for comparison to fatty acid ethyl ester synthase, has been normalized to M_r 25 000. This comparison indicates specific differences in composition, especially in the contents of serine, glutamine, cysteine, arginine, and methionine. Moreover, cholesterol esterase, a larger protein than the synthase, entirely lacks tyrosine, whereas the synthase has five tyrosines per polypeptide. Thus, these results support the conclusion that fatty acyl ethyl ester synthase and exocrine cholesterol esterase are independent proteins. It is conceivable that the enzyme reported here may possess cholesterol esterase activity under as yet unidentified conditions or that sterol ester hydrolysis and ethyl ester synthesis are both catalytic activities of the minor fatty ethyl ester synthase. That different proteins may share cholesterol esterase activity is a possibility, as suggested by the observation that cholesterol esterases solubilized from rat aorta, adrenal gland, and liver do not cross-react with anti-rat pancreas cholesterol esterase serum (Gallo, 1981).

Fatty acyl ethyl ester synthase described here does not appear to be the same as previously described nonspecific esterases. Comparison with other reported esterases (Hayase & Tappell, 1969; Haugen & Suttie, 1974; Ikeda et al., 1977; Lexow et al., 1983) indicates major differences in structure and subcellular localization between nonspecific esterases and this synthase; amino acid analyses of these esterases are not available. Horse and rodent esterases are predominantly microsomal enzymes, and purified esterases have been found to have subunit M_r values of at least 57 000. In contrast, fatty acyl ethyl ester synthase is a soluble enzyme, with a subunit M_r of 26 000. Since fatty acyl ethyl esters do not occur spontaneously in nature in mammalian tissues but accumulate in myocardium as well as other tissues only after ethanol ingestion or exposure, the in situ effect of this enzyme is the net synthesis of fatty acyl ethyl ester, and this product accumulates. We have therefore used the term "synthase" to indicate an enzyme forming a carbon–oxygen bond between nonesterified fatty acid and ethanol in the absence of triphosphate cofactors. The term fatty acyl ethyl ester synthase denotes the importance of the in vivo product of the enzyme and its synthetic capabilities.

Biochemical inquiries into the chemical and metabolic properties of ethanol that determine the pathological consequences of its ingestion have focused on enzymes of ethanol oxidation, since oxidation to acetaldehyde has hitherto been the only known in vivo metabolic fate of ethanol. Three enzyme systems catalyzing the oxidation of ethanol have been characterized: alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol-oxidizing system (MEOS). Salient characteristics of these systems are given in Table III.

Human liver ADH has been purified to homogeneity (Lange et al., 1976), and subsequently, different genetically coded forms were distinguished by their electrophoretic behavior and kinetic properties (Li et al., 1977; Bosron et al., 1983; Wagner et al., 1983). Human hepatic alcohol dehydrogenases are dimeric zinc metalloenzymes comprised of subunits of approximately M_r 43 000, associated with 4 mol of zinc/mol of dimer. A new classification for these enzymes recently has been proposed on the basis of biochemical characteristics that distinguish them (Vallee & Bazzone, 1983; Bosron et al., 1983). Thus, I-ADH is inhibited by 4-methylpyrazole and migrates cathodically on electrophoresis at pH 8.2. II-ADH and III-ADH are less sensitive to pyrazole inhibition and have different electrophoretic properties. These enzymes have, in addition, different kinetic properties and substrate specificities (Table III). The microsomal ethanol-oxidizing system (Lieber & DeCarli, 1970) differs from ADH in subcellular localization, pH optimum, cofactor requirements, and kinetic properties (Table III), but it has never been purified to homogeneity. Hepatic catalase has also been shown to oxidize ethanol (Keilin & Hartree, 1945; Schonbaum & Chance, 1976), in a reaction sequence in which O_2 reduction to H_2O_2 is coupled with the catalase-catalyzed oxidation of ethanol to yield acetaldehyde and water. It is generally held that the contribution of the catalase pathway to total ethanol oxidation is minor compared to the proportion of ingested ethanol that is oxidized by alcohol dehydrogenase and that the MEOS pathway is more important during chronic alcohol consumption. Thus, rates of elimination of ethanol in man have been interpreted in terms of the known kinetic properties of alcohol dehydrogenases (Li et al., 1977).

The possibilities for relating the deleterious effects of alcohol ingestion to the chemical properties of its products of degradation are now expanded by the identification here of a nonoxidative metabolic fate for ethanol, catalyzed by either of two soluble enzymes, and the accumulation of fatty acid ethyl esters in potentially pathobiological concentrations. Elucidation of the properties of these enzymes and assessment of their contribution to total ethanol metabolism will clarify their role, if any, in determining the pathological consequences of ethanol ingestion. Moreover, multiple forms of fatty acid ethyl

ester synthase may exist since two enzyme activities are separable by DEAE-cellulose chromatography, both of which appear to have similar molecular weights. Elucidation of the relationship of the two activities separated by DEAE-cellulose chromatography, e.g., isoenzymes and their relative contributions to total ethanol metabolism in heart and other organs, awaits purification of the lesser activity and determination of its kinetic properties and substrate specificities.

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