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EFFECTS OF LIPIDS ON THE ACTIVITY OF FERROCHELATASE

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Summary

Removal of lipids from submitochondrial particles or detergent-solubilized mitochondrial preparations of rat liver resulted in a 90% loss of ferrochelatase (protochemeferro-lyase, EC 4.99.1.1) activity. The addition of either a fatty acid or phospholipid restored enzyme activity; the extent of reactivation being correlated with the degree of unsaturation of the fatty acid or acyl chain and independent of the polar head group of the phospholipid. Arrhenius plots of the ferrochelatase activities of submitochondrial particles and detergent-solubilized mitochondrial preparations showed transition temperatures of 37 and 28.5°C, respectively. Ferrochelatase of submitochondrial particles or detergent-solubilized preparations had an absolute requirement for Ca^{2+}. The ferrous salt of oxalic acid, a Ca^{2+} chelator, was a very poor substrate for these preparations. In contrast, ferrochelatase activities of fatty acid- or lipid-supplemented acetone extracts of these preparations were not dependent on the presence of Ca^{2+} and ferrous oxalate served as substrate for these extracts.

Introduction

Ferrochelatase (protoheme ferro-lyase, EC 4.99. 1.1), which is a membrane-bound enzyme, catalyzed the incorporation of Fe^{2+} into protoporphyrin IX to form protoheme [1—5]. The precise mechanism by which ferrochelatase facilitates combination of the two substrates is unknown. It has been suggested [6—8] that phospholipids are involved in the function of the enzyme since the enzymic reaction is stimulated by organic solvents and enzyme activity can be restored in lipid-free extracts by the addition of phospholipid. Phospholipids are known to play a role in the function of a number of enzymes, either by modifying the conformation of the enzyme, or by facilitating the binding of substrates to the enzyme. The exact mechanism of action of phospholipid in the ferrochelatase reaction is uncertain but the studies reported here suggest that it might affect enzyme structure since (i) the activation of ferrochelatase by phospholipid was independent of the nature of the charge of the polar head

group, (ii) only phospholipids which contained unsaturated acyl chains acted as cofactors of ferrochelatase, and (iii) Arrhenius plots of ferrochelatase activity were segmented, the transition temperatures being dependent on the state of the non-polar environment.

Materials

Protoporphyrin IX dimethyl ester, mesoporphyrin IX dimethyl ester, bovine heart cardiolipin and *cis*-linoleic acid were supplied by Sigma Chemical Co. Distearoyl-L-α-phosphatidylcholine, dioleoyl-L-α-phosphatidylcholine and dilinoleoyl-L-α-phosphatidylcholine were obtained from Supelco Inc. Stearic acid, oleic acid, β-γ-dipalmitoyl-L-α-phosphatidylcholine and bovine phosphatidylserine were products of Calbiochem. Linolenic acid was obtained from Hormel Foundation and Sephadex G-25 was purchased from Pharmacia Fine Chemicals.

Methods

Preparation of enzyme extracts. Male Wistar rats (220 g each), which had been starved for 24 h, were killed and their livers removed immediately and chilled in cold buffer containing 5 mM Tris · HCl, pH 7.5, and 0.25 M sucrose. Mitochondria were prepared from homogenates of rat liver by the method of Hogeboom [9].

Submitochondrial particles were prepared by resuspending the mitochondrial pellets in two volumes of 50 mM Tris · HCl, pH 7.5, and subjecting the suspension to ultrasonic vibration for 5 min at 20 kHz using a Bronwill Biosonik Oscillator cooled by a rapidly flowing stream of cold water. The sonically disrupted suspension was centrifuged at 10 000 × *g* for 10 min. The supernatant was removed and recentrifuged at 100 000 × *g* for 1 h. The pellet was resuspended in two volumes of 50 mM Tris · HCl, pH 7.5, and designated the submitochondrial particle preparation.

The detergent-solubilized extract was prepared by resuspending the mitochondrial pellets in two volumes of 50 mM Tris · HCl, pH 7.5, containing 0.8% KCl and 1% sodium cholate and subjecting the suspension to ultrasonic vibration for 5 min at 20 kHz. After incubation in an ice bath for 30 min, mitochondrial debris was removed by centrifugation at 20 000 × *g* for 20 min. The supernatant was recentrifuged at 176 000 × *g* for 1 h in a Spinco 60 Ti rotor. The resulting high speed supernatant constitutes the detergent-solubilized preparation.

Dialyzed enzyme preparations were obtained by dialyzing aliquots of enzyme preparation for 20 h against two changes of 20 mM Tris · HCl, pH 7.5, containing 20 mM 2-mercaptoethanol and either 0.1 or 1.0 mM EDTA.

Lipid-depleted enzyme preparations were obtained by a modification of the acetone extraction procedure described by Lester and Fleischer [10]. 2 vols. of cold 90% aqueous acetone were added in drops to an enzyme preparation at 0° C. After stirring for 20 min, the protein was collected by centrifugation at 10 000 × *g* for 10 min. The pellet was spread on Whatman No. 50 filter paper and dried for 2 h at 4° C. The acetone powder was redissolved in two volumes

of 50 mM Tris · HCl, pH 7.5, and designated the acetone extract or lipid-depleted preparation.

Preparation of porphyrins. The dimethyl esters of protoporphyrin IX and mesoporphyrin IX were hydrolyzed with 7.0 M HCl for 5 h at room temperature in the dark. The acid was evaporated under reduced pressure and the free porphyrin was dissolved in 0.01 M KOH containing 20% ethanol to make a stock solution of 1.5 mM which was stored at −20°C.

Assay of ferrochelatase. Ferrochelatase activity was assayed by the pyridine hemochrome procedure essentially as described by Porra and Ross [11]. The reaction mixture (2 ml) contained: 100 mM Tris · HCl, pH 7.5, 10 mM cysteine, 0.1 mM $FeSO_4$, 0.1 mM proto- or mesoporphyrin IX, and the enzyme preparation. One unit of ferrochelatase activity is defined as the amount which catalyzes the formation of 1 nmol of heme from either proto- or mesoporphyrin IX and Fe^{2+} in 1 h under standard assay conditions. Specific activity is expressed as units per mg of protein.

Protein determination. Protein content was measured by the method of Lowry et al. [12] using crystalline bovine serum albumin (Fraction V) as standard.

Results

Effect of fatty acids on ferrochelatase activity in lipid-depleted preparations

Lipid depletion of detergent-solubilized mitochondrial preparations resulted in a marked decrease in the level of ferrochelatase activity. Enzyme activity could be restored in the acetone extracts by the addition of a fatty acid (Table I). The effectiveness of the various fatty acids tested was in the following order, linolenic acid (18 : 3) > linoleic acid (18 : 2) > oleic acid (18 : 1) > stearic acid (18 : 0). None of the fatty acids had any effect on ferrochelatase activity in the detergent-solubilized preparations prior to acetone extraction.

TABLE I

EFFECT OF FATTY ACIDS ON FERROCHELATASE ACTIVITY

Each suspension of fatty acid was prepared by adding 0.2 ml of 0.5 M Tris · HCl, pH 7.5, to the fatty acid and agitating the suspension on a Vortex mixer for 10 min in the presence of glass beads (0.45—0.5 mm diameter) [13]. Protoporphyrin IX (0.8 ml, 0.1 mM) was then added and incorporated into the lipid phase and this was followed by the addition of 0.5 ml of resuspended acetone extract obtained from a detergent-solubilized enzyme preparation. The reaction was started by the addition of 0.5 ml of 0.4 mM $FeSO_4$. Assay conditions were as described in Methods. The concentration of fatty acid in each assay was 10 mM

Fatty acid (10 mM)	Specific activity (units/mg protein)
None	1.00
Steaic acid (0) *	3.00
Oleic acid (1)	4.12
Linoleic acid (2)	5.94
Linolenic acid (3)	6.12

* Number of double bonds per molecule.

Effect of phospholipids on ferrochelatase activity in lipid-depleted preparations

In addition to fatty acids, phospholipids also activated ferrochelatase in lipid-free preparations (Table II). The extent of activation of ferrochelatase in acetone extracts of detergent-solubilized preparations by pure synthetic phosphatidylcholine vesicles was correlated with the degree of unsaturation of the acyl chains, thus, dilinoleoyl phosphatidylcholine > dioleoyl phosphatidylcholine > distearoylphosphatidylcholine. Submersion of the reaction mixtures in an ultrasonic bath for 1 min did not affect the degree or pattern of activation of ferrochelatase indicating that the differences observed were not due to differences in the state of dispersion of the phospholipids (cf. ref. 7). Again, the addition of phospholipid to the detergent-solubilized and sonicated enzyme preparations prior to acetone extraction had no affect on enzyme activity.

A comparative study of several commercially available phospholipids, which differed greatly in fatty acid composition and polar head groups, indicated that the nature of the polar head group was not a significant determinant of ferrochelatase activity (Table III). In all cases, the extent of activation of ferrochelatase in the acetone extracts of detergent-solubilized preparations by these phospholipids was decreased markedly in the presence of 10% cholesterol (Table III). As shown in Table IV, the degree of activation of ferrochelatase by pure dipalmitoylphosphatidylcholine decreased with increasing concentrations of cholesterol at 45°C and increased with increasing concentrations of cholesterol at 22.5°C.

Evidence for a lipid-determined activation of ferrochelatase

Ferrochelatase activity of submitochondrial particles and detergent-solubilized preparations was maximal at 45°C. Arrhenius plots for the temperature range between 25 and 60°C of the submitochondrial and detergent-solubilized preparations showed definite inflection points at 37 and 28.5°C, respectively (Fig. 1), suggesting that the transition temperature is dependent on the state of the non-polar environment of the enzyme. The activation energy (E) for ferrochelatase of submitochondrial particles was 21 500 cal/mol below 37°C and 4530 cal/mol above 37°C, and for ferrochelatase of detergent-solubilized preparations it was 49 400 cal/mol below 28.5°C and 4570 cal/mol above 28.5°C.

TABLE II

EFFECT OF PURE PHOSPHOLIPIDS ON FERROCHELATASE ACTIVITY

The suspensions of phospholipids were prepared in the same manner as the fatty acid suspensions described in Table I. Assays were performed under standard conditions as described in Methods with mesoporphyrin IX as the porphyrin substrate. An acetone extract of a detergent-solubilized preparation was used as the source of enzyme. The concentration of phospholipid in each assay was 1 mM.

Phospholipid (1 mM)	Specific activity (units/mg protein)
None	6.70
Distearoylphosphatidylcholine	7.72
Dioleoylphosphatidylcholine	8.14
Dilinoleoylphosphatidylcholine	10.09

TABLE III

EFFECT OF CHOLESTEROL ON THE ACTIVATION OF FERROCHELATASE BY PHOSPHOLIPIDS

The suspensions of phospholipids were prepared in the same manner as the fatty acid suspensions described in Table I. Assays were performed under standard conditions as described in Methods with protoporphyrin IX as the porphyrin substrate. An acetone extract of a detergent-solubilized preparation was used as the source of enzyme. The concentration of cholesterol in each assay was 10% (w/v).

Compound	Specific activity (units/mg protein)
None	1.57
Phosphatidylserine	2.47
Phosphatidylserine + cholesterol	1.63
Phosphatidylcholine	2.69
Phosphatidylcholine + cholesterol	2.07
Cardiolipin	2.02
Cardiolipin + cholesterol	1.85

Effect of lipids on the calcium requirement of ferrochelatase

Ferrochelatase activity was stable to dialysis for 20 h against a buffer containing 0.1 mM EDTA, whereas dialysis against 1 mM EDTA or gel filtration through a Sephadex G-25 column equilibrated with 50 mM Tris · HCl buffer, pH 7.5, resulted in a total loss of enzyme activity (Table V). Activity was not restored by the addition of nucleotides or coenzymes ($FADH_2$, NADH, NADPH, ATP, succinate, fumarate), but the addition of 0.5 mM $CaCl_2$ to the assay mixture resulted in a complete reactivation of the enzyme. $MgCl_2$ (2 mM) effected a partial, but variable, reactivation of the enzyme.

The calcium requirement of ferrochelatase was also investigated in reconstituted lipid-free preparations. A detergent-solubilized enzyme extract and a submitochondrial preparation were each dialyzed for 20 h against 1 mM EDTA. An acetone extract was then prepared from each of the dialyzed preparations and the ferrochelatase activity reconstituted with linoleic acid. Both prepara-

TABLE IV

EFFECT OF CHOLESTEROL ON THE ACTIVATION OF FERROCHELATASE BY DIPALMITOYLPHOSPHATIDYLCHOLINE

The suspension of dipalmitoylphosphatidylcholine was prepared in the same manner as the fatty acid suspensions described in Table I. Standard assay conditions were used as described in Methods except that the incubations were carried out at 45 or 22.5°C, as indicated in the Table. Mesoporphyrin IX was used as the porphyrin substrate and an acetone extract of a detergent-solubilized preparation was used as the source of enzyme.

Cholesterol (%)	Specific activity (units/mg protein)	
Temperature:	22.5°C	45°C
0	2.77	5.50
10	2.88	4.43
25	3.23	3.57
50	3.27	2.10

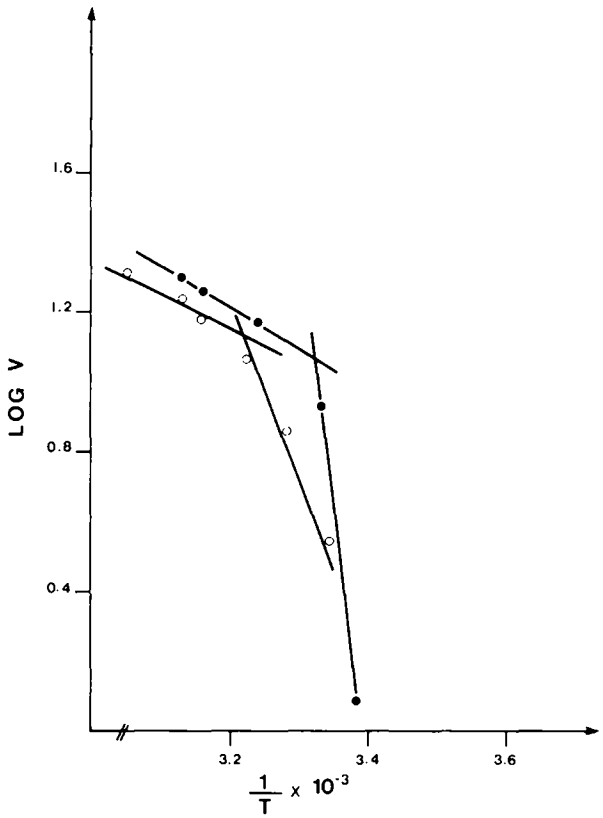

Fig. 1. Arrhenius plots of the effects of temperature on the activities of ferrochelatase of submitochondrial and detergent-solubilized preparations. Assays were performed under standard conditions as described in Methods except that the temperature of the incubation was varied. Protoporphyrin IX was used as the porphyrin substrate. The ordinate values are the log of nmol of protoheme formed per 1 h per mg of protein. ○, submitochondrial particle preparation; ●, detergent-solubilized preparation.

TABLE V

EFFECT OF DIVALENT CATIONS ON THE ACTIVITY OF FERROCHELATASE IN DIALYZED, DETERGENT-SOLUBILIZED PREPARATIONS

Samples of detergent-solubilized enzyme preparation were dialyzed for 20 h against buffer containing 0.1 mM or 1 mM EDTA. The samples were then assayed for ferrochelatase activity as described in Methods except for the addition of 0.5 mM $CaCl_2$ or 2 mM $MgCl_2$ as indicated in the table. The porphyrin substrate was mesoporphyrin IX.

Treatment	Specific activity (units/mg protein)
None	10.32
Dialyzed against 0.1 mM EDTA	10.21
Dialyzed against 1 mM EDTA	0.0
Dialyzed against 1 mM EDTA and assayed in presence of 0.5 mM $CaCl_2$	10.13
Dialyzed against 1 mM EDTA and assayed in presence of 2 mM $MgCl_2$	3.11

TABLE VI

EFFECT OF CALCIUM ON THE ACTIVITY OF FERROCHELATASE IN LINOLEIC ACID VESICLES

Detergent-solubilized and submitochondrial enzyme preparations were each dialyzed for 20 h against buffer containing 1 mM EDTA. An acetone extract was then prepared from each preparation and the ferrochelatase activity reconstituted with a suspension of linoleic acid as described in Table I. Standard assay conditions were used as described in Methods except for the addition of 0.5 mM $CaCl_2$ as indicated in the table. Protoporphyrin IX was used as the porphyrin substrate.

Treatment	Specific activity (units/mg protein)
Acetone extract of submitochondrial preparation + linoleic aicd	4.0
Acetone extract of submitochondrial preparation + linoleic acid + Ca^{2+}	5.1
Acetone extract of detergent-solubilized preparation + linoleic acid	6.1
Acetone extract of detergent-solubilized preparation + linoleic acid + Ca^{2+}	5.2

TABLE VII

EFFECT OF THE SOURCE OF IRON ON THE ACTIVITY OF FERROCHELATASE

Standard assay conditions were used as described in Methods except that the source of iron was varied as indicated in the table. The porphyrin substrate was protoporphyrin IX and the enzyme source was either a detergent-solubilized preparation or a linoleic acid-reactivated acetone extract of this preparation as indicated in the table.

Enzyme source	Iron source	Specific activity (units/mg protein)
Detergent-solubilized preparation	$FeCl_3$	3.93
	$FeSO_4$	4.93
	FeC_2O_4	1.90
Linoleic acid-reactivated acetone extract	$FeCl_3$	3.93
	$FeSO_4$	4.65
	FeC_2O_4	4.45

tions were assayed in the presence and absence of Ca^{2+}. The results (Table VI) indicated that Ca^{2+} was not required for activity when the enzyme was in a model lipid environment.

This conclusion was supported by the finding that the ferrous salt of oxalic acid, a chelator of divalent cations with a high affinity for Ca^{2+}, was a good substrate for ferrochelatase of acetone extracts reactivated with linoleic acid (Table VII), but was a very poor substrate for ferrochelatase of detergent-solubilized preparations.

Discussion

The studies described here indicate that the extent of activation of ferrochelatase by phospholipids is directly related to the number of double bonds in the acyl chain of the phospholipid. Since unsaturation breaks up the hydrophobic interaction leading to a more fluid hydrocarbon core our results suggest that ferrochelatase activity is dependent on a fluid hydrophobic phase. Cholesterol is known to increase membrane fluidity below the transition

temperature and to decrease the fluidity above the transition temperature [14]. Thus, the observation that ferrochelatase activity at 45°C, which is well above the transition temperature of 37°C, decreased with increasing concentrations of cholesterol whereas at 22.5°C enzyme activity increased with increasing concentrations of cholesterol is consistent with this interpretation.

It has been shown that hydration is correlated with the fluidity of the hydrocarbon core [15]. The effect of fluidity on ferrochelatase activity might, therefore, reflect the ability of water to penetrate the lipid phase or it might indicate a requirement for hydration of polar groups. Alternatively, the fluidity of the hydrophobic phase might facilitate favourable enzymic conformation or it might act by allowing more mobility within the membrane.

Studies of the calcium requirement of ferrochelatase indicate that it does not depend on the formation of a complex between enzyme, metal and substrates for activity since gel filtration of an enzyme preparation followed by lipid removal and reactivation gave an enzyme-lipid system which was independent of Ca^{2+}. Instead, it would appear that the effect of Ca^{2+} on ferrochelatase activity is mediated via an effect of the metal ions on the membrane. Ca^{2+} could affect the membrane in any of three ways; by acting as a chelator, by screening the surface charge of the phospholipid bilayer, or by altering the phase characteristics of the membrane. The first explanation is unsatisfactory because reconstitution of the lipid-depleted enzyme preparations occurred in the presence of monovalent ions which lack the ability to chelate. The second explanation seems unlikely for several reasons. First, it is difficult to reconcile with the 'electrostatic reaction' described by Lowe and Phillips [16]; second, it does not account for the Ca^{2+} requirement of ferrochelatase since in the model fatty acid and phospholipid environments polar group charges are balanced by monovalent ions; and third, it is not consistent with the findings of Sawada et al. [7] that the negative charge of the phospholipid head groups activates ferrochelatase. However, it is possible that the final argument applies only to the enzyme system in avian erythrocytes since our studies indicate that, in rat liver, the activation of ferrochelatase by phospholipid is independent of the particular charge on the polar group of the molecule. Although the third explanation applies to vesicles of pure phosphatidylglycerol and phosphatidylserine in which it has been demonstrated that 1 mM Ca^{2+} abolishes the phase transition between 0 and 70°C [17], it is not known whether Ca^{2+} plays a significant role in determining the phase characteristics of the native mitochondrial membrane which is comprised of 76% protein and a wide variety of phospholipids. Indeed, until now, it was not known that the phase characteristics of the membrane affected ferrochelatase activity. However, this seems probable since the inflection point at 37°C in the Arrhenius plot of ferrochelatase activity in submitochondrial particles was lowered to 28.5°C after disruption of the hydrophobic phase with cholate. This indicates that the transition results from alterations in the lipid environment of the enzyme. The possibility that Ca^{2+} effects an adequate environment in the native mitochondrial membrane for ferrochelatase by modifying the phase characteristics of the membrane is presently under investigation.

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