Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 1000-1006

ON THE MECHANISM OF ACTION OF LYSOPHOSPHOLIPASE-TRANSACYLASE FROM RAT LUNG

G.Paul H. van Heusden and Henk van den Bosch

Biochemistry Laboratory,Padualaan 8,3508 TB Utrecht
The Netherlands

Received August 22, 1979

SUMMARY

Lysophospholipase-transacylase from rat lung catalyzes the transfer of palmitate from 1-palmitoyl-*sn*-glycero-3-phosphocholine to water and to another molecule of 1-palmitoyl-*sn*-glycero-3-phosphocholine.Incorporation of palmitate into phosphatidylcholine is restricted to palmitate donated by lysophosphatidylcholine,free palmitate cannot be esterified to lysophosphatidylcholine by the enzyme.Experiments in the presence of $H_2{}^{18}O$ and mass spectrometric analysis of the reaction products show that ^{18}O is incorporated into the released palmitate but not into the transesterification product phosphatidylcholine.This proofs that the hydrolytic reaction proceeds by O-acyl cleavage.Furthermore,the results strongly suggest that transfer of palmitate to lysophosphatidylcholine occurs through an intermediary covalent acyl-enzyme complex.

INTRODUCTION

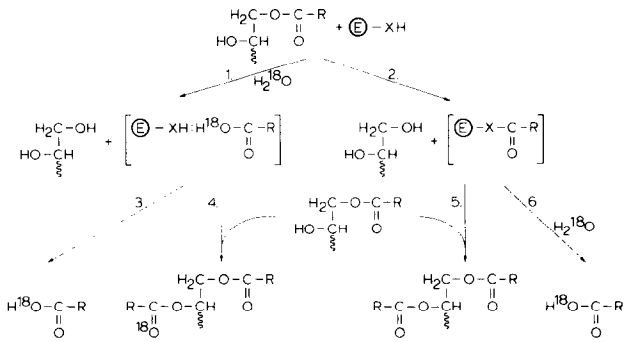
Previously,we reported (1) on the purification of an enzyme preparation from rat lung cytosol which catalyzed the conversion of lysophosphatidylcholine into glycerophosphocholine,fatty acid and phosphatidylcholine. It was concluded from several lines of evidence that all three products were formed by a single enzyme (1,2).To explain the formation of these products a reaction mechanism was proposed in which the enzyme formed an intermediary acyl-enzyme complex (scheme 1,reaction 2) from which the acylgroup was transferred to either water (reaction 6) to yield glycerophosphocholine and fatty acid or to a second molecule of lysophosphatidylcholine (reaction 5) to give glycerophosphocholine and phosphatidylcholine. In the present paper the reaction mechanism was investigated in more detail.Incubations were carried out in the presence of $H_2{}^{18}O$ to distinguish between the occurrence of a non-covalent (reaction 1) and a covalent (reaction 2) acyl-enzyme intermediate.

MATERIALS AND METHODS

MATERIALS

1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphocholine and radioactive fatty acids were obtained from NEN,Boston,USA.1-Acyl-*sn*-glycero-3-phosphocholines containing unlabelled or ^{3}H-labelled fatty acids were prepared by phospho-

0006-291X/79/191000-07$01.00/0



Scheme 1. Possible pathways for acyltransfer by lysophospholipase-transacylase.

lipase A_2 degradation of the corresponding diacylphosphatidylcholines synthesized from the $CdCl_2$-adduct of *sn*-glycero-3-phosphocholine (Koch-Light, Colnbrook,England) as described previously (3).1,2 Dipalmitoylphosphatidylcholine and palmitic acid,used for mass spectrometry,were obtained from Fluka AG,Switzerland and BDH,Poole,England,respectively.$H_2{}^{18}O$ (99 atom $\%{}^{18}O$) was purchased from Norsk Hydro,Oslo,Norway.

3-Hexadecanoylthio-propanol-1-phosphocholine (thiodeoxylysophosphatidylcholine) was a gift of Dr.A.J.Aarsman.5,5'-Dithiobis(2-nitrobenzoic acid) was obtained from Aldrich,Beerse,Belgium.

Silica gels and magnesium silicate (Florisil TLC) were products of Merck,Darmstadt,GFR.

Lysophospholipase-transacylase from rat lung was purified as described earlier (1).

METHODS

Incubations in the presence of $H_2{}^{18}O$. 1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphocholine (1 µmol,20000 dpm) was incubated for 60 min. at 37°C with 4.5 µg enzyme in 0.3 ml 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 ml $H_2{}^{18}O$ (99 atom %).The lipids were extracted (4) and isolated by TLC on silicagel 60HR plates containing 2% (w/w) Florisil.The plate was developed in the first direction with petroleum ether (b.p. 40-60°C)/diethylether/formic acid (60:40:1.5,v/v) and in the second direction with $CHCl_3$/CH_3OH/conc.NH_4OH/H_2O (90:54:5.5:5.5,v/v).The phosphatidylcholine and free fatty acid spots were scraped and the lipids eluted from the silicagel with $CHCl_3$/CH_3OH (1:4,v/v).The eluates contained about 85 nmol phosphatidylcholine and 250 nmol fatty acid,respectively.

Synthesis of ^{18}O-labelled phosphatidylcholine. [^{18}O]-Palmitic acid was prepared by hydrolysis of 5 µl palmitoylchloride in 0.1 ml $H_2{}^{18}O$ (99 atom %) and 10 µl 0.5 N HCl in normal water during 3 h at room temperature. The lipids were isolated by Bligh and Dyer extraction (4),dried overnight in vacuo above P_2O_5.Palmitic acid was reconverted into palmitoylchloride with oxalylchloride as described by Daubert et al. (5).The ^{18}O-labelled palmitoylchloride was used to acylate the $CdCl_2$-adduct of 2 µmol of 1-palmitoyl-*sn*-glycero-3-phosphocholine under the conditions described by Baer and Buchnea (6).The phosphatidylcholine was purified by two-dimensional TLC as described above.Mass spectra (fig.2B) showed that the phosphatidylcholine consisted for 35% of molecules with a single ^{18}O label.

Analytical methods. Mass spectra were recorded with a AEI MS 902 Mass Spectrometer under the following conditions: ionizing voltage,70 eV; emission current 300 µA and chamber temperature 250 °C using a direct insertion probe.

Protein was determined by a modified Bradford procedure (7) as described earlier (2).

Attempts to isolate the acyl-enzyme complex. Purified enzyme (24 µg, about 0.4 nmol) was incubated with 40 nmol 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (spec.act. 25000 dpm/nmol) in 0.1 ml 0.1 M potassium phosphate buffer (pH 6.5) for 1 min. at 37°C.The reaction was stopped in one of the following ways: addition of 20 µl 1% SDS;addition of 35 µl 0.1 N HCl; addition of 0.2 ml 0.1 N HCl in CH_3OH or heating for 1 min. at 100 °C. Thereafter,the mixtures were immediately frozen in CO_2/acetone and lyophilized.The lyophilized samples were used for SDS-polyacrylamide gel electrophoresis according to Shapiro et al. (8).The gels were stained (9) and sliced to determine the radioactivity distribution in the gels.

RESULTS AND DISCUSSION

Table I shows results obtained when the lysophospholipase-transacylase was incubated with palmitoyl-labelled lysophosphatidylcholine in the absence or presence of differently labelled palmitate.As can be seen from exp.A the amount of [^{3}H]palmitate recovered in phosphatidylcholine (0.8 picomol) is at least 8000-fold less than the incorporation of [^{14}C]palmitate residues from lysophosphatidylcholine (6.8 nmol).Similar results were obtained when the radioactive labels were reversed.Also,preincubation of

Table I

LACK OF INCORPORATION OF FREE FATTY ACIDS

Exp.	Substrate	Free palmitate	Palmitate recovered in phosphatidylcholine from	
			Lyso-PC (nmol)	Free palmitate (pmol)
A.	^{14}C lyso-PC	-	6.9	0.0
	^{14}C lyso-PC	^{3}H palmitate	6.8	0.8
	^{3}H lyso-PC	-	11.1	0.0
	^{3}H lyso-PC	^{14}C palmitate	16.1	0.9
B.	^{14}C lyso-PC	-	13.7	1.4
	^{14}C lyso-PC	^{3}H palmitate	10.0	1.0
	^{3}H lyso-PC	-	7.3	1.5
	^{3}H lyso-PC	^{14}C palmitate	6.2	3.3

In exp.A the incubation mixtures consisted of 200 nmol lyso-PC (1-[1-^{14}C]palmitoyllyso-PC,100 dpm/nmol or 1-[9,10-$^{3}H_2$]palmitoyllyso-PC, 808 dpm/nmol),5µg enzyme and 50 µ mol potassium phosphate buffer (pH 6.5) in 0.3 ml.When indicated 15 nmol of palmitate ([1-^{14}C]palmitate,15000 dpm/nmol or [9,10-$^{3}H_2$]palmitate,60000 dpm/nmol) was present.In exp.B the enzyme was preincubated for 10 min at 37°C in the presence or absence of 15 nmol labelled palmitate before addition of 200 nmol of the indicated lyso-PC in 0.1 ml water.After 10 min incubation at 37°C the lipids were extracted (4) and separated by TLC (10).The phosphatidylcholine was eluted and quantitatively transferred to scintillation vials for radioactivity measurements in a Packard TriCarb Model 3320 scintillation spectrometer with external standard quench correction.Lyso-PC=lysophosphatidylcholine.

the enzyme in the presence of free palmitate before addition of a differently labelled palmitoyllysophosphatidylcholine resulted in the exclusive formation of phosphatidylcholine with palmitoyl groups derived from the lysophosphatidylcholine substrate.The trace amounts of free palmitate label recovered in the phosphatidylcholine apparently can be ascribed to inadequate overlap corrections in the dual-label radioactivity measurements. These results indicate that only acylgroups released by the enzyme from lysophosphatidylcholine can be transferred to a second molecule of lysophosphatidylcholine,presumably from an acyl-enzyme intermediate.The acylgroup in this complex is not freely miscible with the added free palmitate.

Two different acyl-enzyme complexes can be envisaged (scheme 1),i.e. a non-covalent complex formed in the hydrolysis reaction 1 or a covalent complex produced by the attack of a nucleophilic group -XH of the enzyme on the acylester bond in the substrate (reaction 2).When the reaction is carried out in the presence of $H_2{}^{18}O$,both dissociation of the former complex or hydrolysis of the covalent complex will yield free fatty acid with identical atom % excess ^{18}O as present in the water.However,an analysis of the phosphatidylcholine product can give information on the mechanism of its formation.As indicated in scheme 1,a reaction pathway involving a non-covalent acyl-enzyme complex (reactions1 and 4),due to the equivalence of the oxygen atoms in the carboxylgroup,should result in the appearance of phosphatidylcholine molecules having ^{18}O to a percentage which equals half the atom % excess of ^{18}O in the water.In contrast,if the reaction proceeds via a covalent acyl-enzyme intermediate (reactions 2 and 5) no ^{18}O will be incorporated in the phosphatidylcholine product.

Fig. 1B shows the mass spectrum of palmitate released enzymatically from palmitoyllysophosphatidylcholine in the presence of $H_2{}^{18}O$.A reference spectrum of commercial palmitate is given in fig.1A.The following carboxylgroup containing fragments were observed: *m/e* 101,115,129,143,157,171,185, 199,213,227 and 256.The latter peak corresponds to the molecular ion.Loss of a CH_3-CH_2-fragment results in the peak at *m/e* 227.The other fragments are formed by subsequent losses of CH_2-groups.In the spectrum of the enzymatically released palmitate (fig. 1B) all these peaks are accompanied by a second peak at two mass units higher,demonstrating the presence of ^{18}O.The ^{18}O content of this palmitate amounted to 31.4 ± 0.6 atom % excess,in good agreement with the 33.0 atom % excess ^{18}O in the water used in the incubation mixture.This result proofs that the hydrolysis proceeds by O-acyl cleavage.The same conclusion was reached by Wells (11) for the hydrolysis of dipalmitoylphosphatidylcholine by *Crotalus adamanteus* venom phospholiase A_2 in the presence of $H_2{}^{18}O$.This site of cleavage in phospholipase A_2

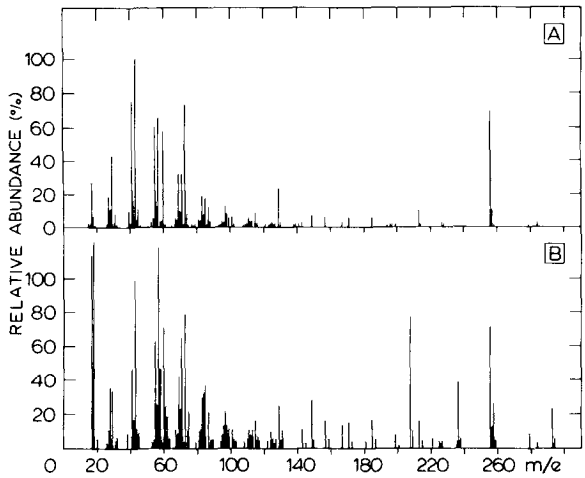


Figure 1. Mass spectra of control palmitic acid (A) and palmitic acid released from 1-palmitoyl-*sn*-glycero-3-phosphocholine by lysophospholipase-transacylase in the presence of $H_2{}^{18}O$ (B).

catalyzed reactions has recently been confirmed by Aarsman and van den Bosch (12).Using substrate analogs containing an acylthioester bond these authors demonstrated that the thiolgroups released during phospholipase A_2 catalyzed hydrolysis were exclusively present in the alkyl leaving group and not in the fatty acid.Similar experiments have been done with lysophospholipase-transacylase and 3-hexadecanoylthio-propanol-1-phosphocholine as substrate.The reaction products,i.e.3-thio-propanol-1-phosphocholine and palmitic acid can easily be separated by solvent extraction according to Bligh and Dyer (4).In a typical experiment the homogeneous phase obtained from the incubation mixture contained 70 nmol of released thiolgroups and 97% of these were recovered in the methanol/water phase.This result demonstrates that hydrolysis of 3-hexadecanoylthio-propanol-1-phosphocholine proceeds via an S-acyl cleavage mechanism,in agreement with the O-acyl cleavage mechanism deduced from the hydrolysis of 1-palmitoyl-*sn*-glycero-3-phosphocholine in the presence of $H_2{}^{18}O$.

Fig. 2C shows the mass spectrum of the phosphatidylcholine which is formed by the lysophospholipase-transacylase from 1-palmitoyl-*sn*-glycero-3-phosphocholine in the presence of $H_2{}^{18}O$.For comparison,the spectra of commercially obtained 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (fig. 2A) and of synthetically prepared 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine containing a ^{18}O-label in the acylester bond at the *sn*-2-position (fig. 2B) are given.In agreement with reported phosphatidylcholine spectra (13) the interpretation of the spectra in fig.2 is as follows.The peak at *m/e* 550 corresponds to a fragment caused by loss of the phosphocholine moiety and

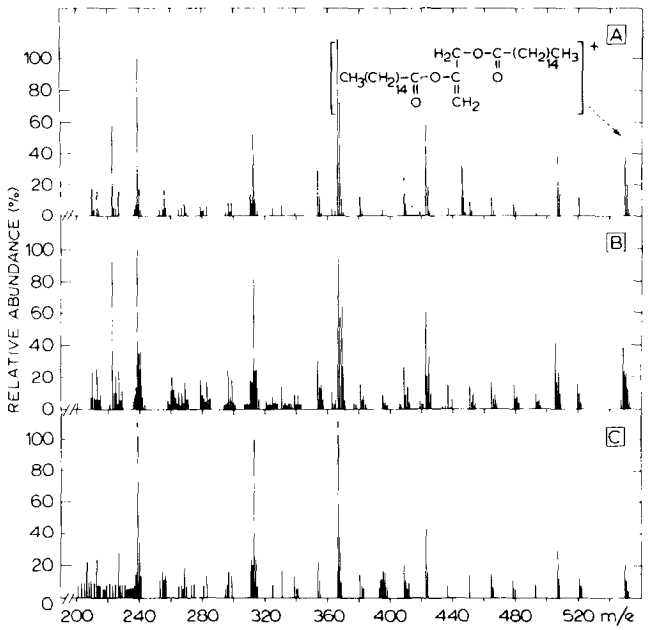


Figure 2. Mass spectra of control 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (A); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine containing a ^{18}O label in the acylester bond at the *sn*-2-position (B) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine synthesized by lysophospholipase-transacylase from 1-palmitoyl-*sn*-glycero-3-phosphocholine in the presence of $H_2{}^{18}O$ (C).

a H-atom.Subsequent release of CH_3-$(CH_2)_n$-fragments with n=1 to 12,gives rise to peaks with *m/e* ratios of 521,507,493,479,465,451,437,423,409,395,381 and 367.All fragments contain both acylester bonds and in case ^{18}O is present in the ester bond at the *sn*-2-position these peaks will be accompanied by peaks at *m/e* ratios two mass units higher.This is indeed seen in the reference spectrum of the synthetic ^{18}O-containing dipalmitoylphosphatidylcholine (fig. 2B).Such peaks are not observed in the spectrum of the dipalmitoylphosphatidylcholine synthesized by the lysophospholipase-transacylase in the presence of $H_2{}^{18}O$ (fig. 2C).This is interpreted to indicate that a palmitate residue is transferred from one lysophosphatidylcholine molecule to another through a covalent acyl-enzyme intermediate.

Recently,Antonov et al. (14) have studied acyltransfer reactions catalyzed by pepsin.Incubation of this proteolytic enzyme with L-leucyl-L-tyrosine amide led to the formation of L-leucyl-L-leucine in a transpeptidation reaction.When the reaction was carried out in $H_2{}^{18}O$ the pepsin catalyzed acyltransfer incorporated ^{18}O into the peptide bond of the L-leucyl-L-leucine transpeptidation product.The authors concluded from this result that no covalent acyl-enzyme complex is formed during pepsin catalysis.

The incorporation of ^{18}O from $H_2{}^{18}O$ in the palmitate released from palmitoyllysophosphatidylcholine by the lysophospholipase-transacylase proves that the enzyme does cause O-acyl cleavage.The most obvious mechanism compatible with O-acyl cleavage is formation of an acyl-enzyme intermediate.The lack of incorporation of ^{18}O in the phosphatidylcholine transesterification product provides strong evidence for a covalent acyl-enzyme intermediate,in which the acylgroup is attached to some group -XH on the enzyme.Presumptive,but not conclusive,evidence that -XH represents a serine hydroxyl is given by the observation that lysophospholipase-transacylase is readily inactivated by diisopropylfluorophosphate (2).Attempts to isolate the,albeit denatured,acyl-enzyme complex from incubations of the enzyme with 1- 1-^{14}C palmitoyl-*sn*-glycero-3-phosphocholine of high specific radioactivity have failed sofar,presumably because of low steady-state concentrations of this intermediate.

ACKNOWLEDGEMENTS

This work was carried out under the auspices of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) with financial aid of the Netherlands Foundation for Chemical Research (S.O.N.).The authors gratefully acknowledge the assistance of Dr.W.Heerma and Mr.C.Versluis, Department of Analytical Chemistry,in the Mass Spectrometry experiments.

REFERENCES

1. Brumley,G.,and van den Bosch,H.(1977)J.Lipid Res.18,523-532.
2. Vianen,G.M.,and van den Bosch,H.(1978)Arch.Biochem.Biophys.190,373-384.
3. Van den Bosch,H.,and van Deenen,L.L.M.(1965)Biochim.Biophys.Acta 106, 326-337.
4. Bligh,E.G.,and Dyer,W.J.(1959)Canad.J.Biochem.37,911-917.
5. Daubert,B.F.,Fricke,H.H.,and Longeneckers,H.E.(1943)J.Am.Chem.Soc.65, 2142-2144.
6. Baer,E.,and Buchnea,D.(1959)Can.J.Biochem.Physiol.37,953-959.
7. Bradford,M.M.(1976)Anal.Biochem.72,248-254.
8. Shapiro,A.L.,Vinuela,E.,and Maizel,J.V.(1967)Biochem.Biophys.Res.Commun.28,815-819.
9. Reisner,A.H.(1975)Anal.Biochem.64,509-516.
10. Skipski,V.,Peterson,R.,Sanders,J.,and Barclay,M.(1963)J.Lipid Res.4, 227-228.
11. Wells,M.A.(1971)Biochim.Biophys.Acta 248,80-86.
12. Aarsman,A.J.,and van den Bosch,H.(1979)Biochim.Biophys.Acta 572,519-530.
13. Klein,R.A.(1971)J.Lipid Res.12,123-131.
14. Antonov,V.K.,Ginodman,L.M.,Kapitannikov,Y.V.,Barshevskaya,T.N.,Gurova, A.G.,and Rumsh,L.D.(1978)FEBS Lett.88,87-90.