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Properties and Specificities of Sphingosine Kinase from Blood Platelets

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Summary: This communication describes the intracellular distribution of sphingosine kinase in human and pig thrombocytes (platelets), its properties and specificities. The enzyme is soluble and present in the cytoplasm. It is ATP-dependent; UTP is only half as active as ATP, while CTP and GTP are ineffective as phosphate donors. The kinase exhibits a pH optimum around 7.0 and the optimum chain length for saturated long chain bases is 14 to 16 carbon atoms. It has the highest affinity for D(+)*erythro*-4*t*-sphingenine followed by D(+)*erythro*- and L(−)*threo*-sphinganine. The K_m values for these and the inhibition by L(−)*erythro*- and D(+)*threo*-sphinganine were determined. The enzyme is stable at −18°C and can be lyophilized without appreciable loss of enzymic activity.

Isolierung, Eigenschaften und Spezifitäten der Sphingosin-Kinase aus Thrombozyten

Zusammenfassung: Es werden Isolierung, Eigenschaften und Spezifitäten der Sphingosin-Kinase aus Thrombozyten beschrieben. Das Enzym befindet sich im Cytosol und ist ATP-abhängig. UTP ist halb so wirksam wie ATP, während CTP und GTP als Cofaktoren gegenüber dem Kontrollwert zu keiner Steigerung der Phosphorylierung führen. Es zeigt sich ein pH-Optimum bei 7,0 und ein Kettenlängen-Optimum für Basen mit 14 und 16 C-Atomen in der Reihe der gesättigten langkettigen Basen. Die höchste Affinität besitzt das Enzym für D(+)*erythro*-4*t*-Sphingenin. Die K_m-Werte für D(+)*erythro*-4*t*-Sphingenin, D(+)*erythro*-Sphinganin und L(−)*threo*-Sphinganin sowie die Hemmung durch die L(−)*erythro*- und D(+)*threo*-Isomeren wurden bestimmt. Das Enzym ist bei −18°C stabil und kann ohne Verlust der enzymatischen Aktivität lyophilisiert werden.

In the preceding publication[1] we demonstrated that human and pig platelets contain a very active sphingosine kinase, which phosphorylates the primary alcohol group of the different long chain bases. This reaction initiates the degradation of the long chain bases[2–7]. In this paper we report the results of further studies on the intracellular distribution, properties, kinetics and stereospecificities of the enzyme.

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Enzymes:

Sphingosine kinase, ATP: sphingosine-1-phosphotransferase
(EC 2.7.1.?, not yet listed)
(Sphingosine stands for all natural long chain bases)

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Results

Intracellular distribution

Human platelets were disrupted for fractionation studies by the glycerol load hypotonic lysis technique of Barber *et al.*[8], and pig platelets were lysed by repeated freezing and thawing. A 100000 × g supernatant, a membrane fraction[8,9] and a granular fraction[9] were isolated and their specific activities determined. The valuable technique described before[1], which separates the free long chain base (unreacted substrate) from the 1-phosphate ester by ion exchange chromatography, allowed a rapid and reproducible assay. The intracellular distribution of the enzyme activity is given in Table 1.

Table 1. Intracellular distribution of sphingosine kinase activity in human platelets.

Cell fraction	Activity [mU × (mg prot.)$^{-1}$]
Membranes	0.077
Granules	0.033
100000 × *g* supernatant	0.450

Each assay mixture, in which radioactive long chain bases were used as substrates, was also analyzed by radio thin-layer chromatography for product formation. An example is given in Fig. 1. Increasing amounts of enzyme from the platelet homogenate caused a linear increase of product formation as shown for [3-^{3}H]4*t*-sphingenine 1-phosphate (Fig. 2).

On ammonium sulfate fractionation, the enzyme activity precipitated at 20 to 50% saturation. The specific activity of the 100000 × *g* supernatant was determined at 0.49 mU/mg, that of the 0–20% $(NH_4)_2SO_4$ fraction at 0.07 mU/mg, that of the 20–50% fraction at 0.56 mU/mg, and that of the 50–100% fraction at 0.10 mU/mg. The 100000 × *g* supernatant was free of sphingenine 1-phosphatase, which is present in the 100000 × *g* sediment. There was therefore no hydrolysis of substrate, which would have lowered the substrate concentration and thus the product formation.

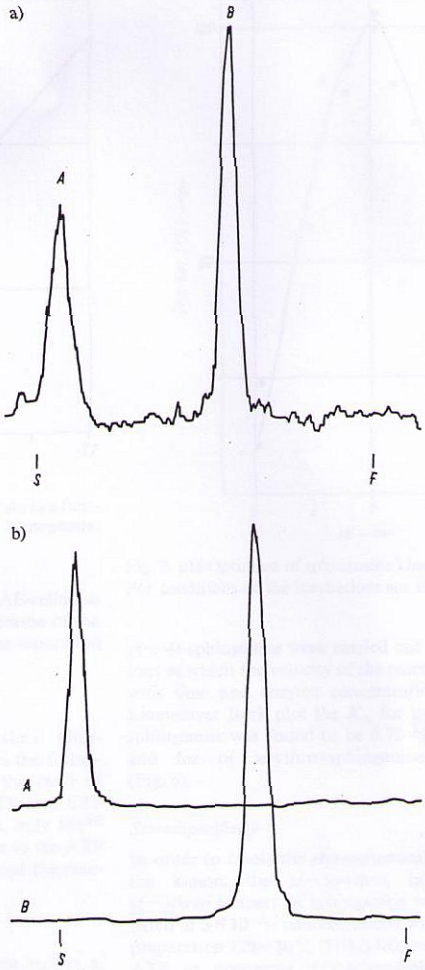

Fig. 1. Radio thin-layer chromatogram of extracted radioactive compounds after incubation of sphingenine with 100000 × *g* supernatant of human platelets in the presence of ATP.

a) Before and b) after ion exchange separation. A: sphingenine 1-phosphate, B: sphingenine. Solvent system: chloroform/methanol/water 65:25:4. For details see Experimental[1].

[8] Barber, A. J. & Jamieson, G. A. (1970) *J. Biol. Chem.* **245**, 6357–6365.

[9] Marcus, A. J., Zucker-Franklin, D., Safir, L.B. & Ullmann, H. L. (1966) *J. Clin. Invest.* **45**, 14–18.

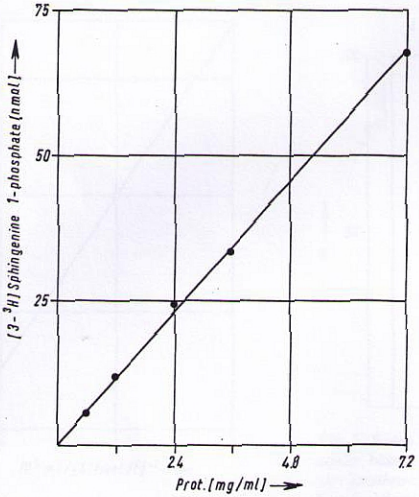

Fig. 2. Formation of sphingenine 1-phosphate as a function of enzyme concentration in platelet homogenate. For conditions see Experimental.

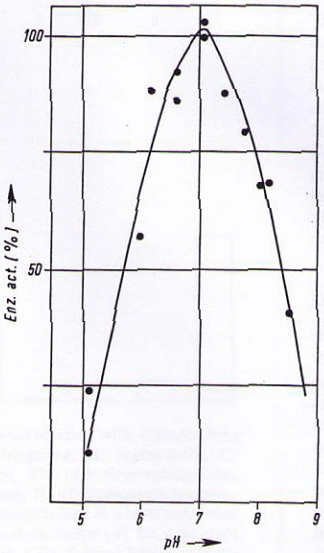

Fig. 3. pH-Optimum of sphingosine kinase. For conditions of the incubations see Experimental.

Attempts to purify the enzyme by DEAE-cellulose or Sephadex chromatography failed because of the rapid loss of enyzmic activity under the separation conditions used.

Cofactor and ion requirements

At 3.5 to 7.0×10^{-3} M ATP gave maximal phosphorylation, UTP was less effective (in the formation of 4*t*-sphingenine 1-phosphate, the ratio of ATP:UTP activity was 2:1), and GTP and CTP had no effect. Of the bivalent cations, only $Mg^{2\oplus}$ and $Mn^{2\oplus}$ at concentrations equimolar to the ATP stimulated the reaction. EDTA inhibited the reaction.

pH Optimum

In sodium phosphate and Tris maleate buffers at different pH values, the optimum of the kinase reaction was observed at pH 7.0. The phosphorylation yields were identical in the two buffers (Fig. 3).

Kinetic studies

Incubations with increasing concentrations of [3-³H]D(+)*erythro*-sphinganine and [3-³H]D(+)*erythro*-4*t*-sphingenine were carried out under conditions at which the velocity of the reaction was linear with time and enzyme concentration. From the Lineweaver Burk plot the K_m for D(+)*erythro*-4*t*-sphingenine was found to be 0.72×10^{-4} M (Fig. 4) and for D(+)erythro-sphinganine, 1.0×10^{-4} M (Fig. 6).

Stereospecificity

In order to assess the stereochemical properties of the kinase, the L(−)*erythro*, D(+)*threo* and L(−)*threo* isomers of sphinganine were also incubated at 3×10^{-4} M concentrations with the enzyme preparation (20 − 50% $(NH_4)_2SO_4$ saturation) and ATP as coenzyme. L(−)*threo*-Sphinganine was phosphorylated to a significant extent, whereas the L(−)*erythro* and D(+)*threo* isomers did not undergo significant phosphorylation. Fig. 5 shows the reaction rates of 4t-sphingenine, sphinganine, L(−)*erythro*-, D(+)*threo*-, L(−)*threo*- and 4D-hydroxysphinganine. The K_m for L(−)*threo*-sphinganine, derived from the Lineweaver Burk plot,

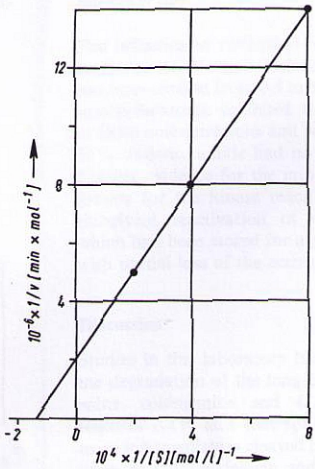

Fig. 4. Determination of K_m-value of 4*t*-sphingenine. $K_m = 0.72 \times 10^{-4}$M.

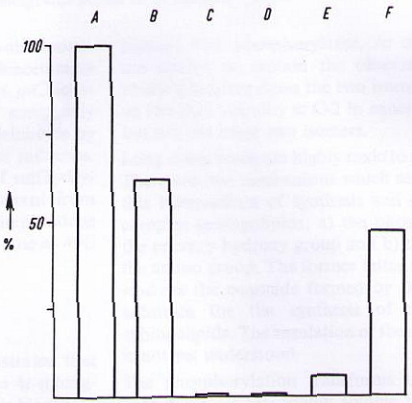

Fig. 5. Rates of the kinase reaction with different long chain bases. A: 4*t*-sphingenine, B: sphinganine, C: L(−)*erythro*-sphinganine, D: D(+)*threo*-sphinganine, E: L(−)*threo*-sphinganine, F: 4*D*-hydroxysphinganine. Each incubation mixture contained in a total volume of 1.0 ml: 100 μmol Tris maleate buffer pH 7.0, 0.30 μmol long chain base, 7 μmol ATP, 7 μmol $MgCl_2$, 1.5 mg protein. The yield of [3-^{3}H]D(+)*erythro*-sphingenine 1-phosphate formed was 60 nmol × h^{-1}. This value was taken as reference (100%).

was found to be 10^{-3}M. The L(−)*erythro*-, D(+)*threo*- and L(−)*threo*-sphinganine isomers are competitive inhibitors of the kinase. They all inhibit the reaction up to 50% at concentrations between 0.35 and 3.6×10^{-4}M. This is demonstrated in Fig. 6 for L(−)*erythro*-sphinganine as an example, the K_I of which is 1.62×10^{-4}M.

Chain length specificity

In a series of experiments homologous [3-^{3}H]DL-*erythro*-sphinganines of chain length C_{14}, C_{16}, C_{18} and C_{20} were incubated under optimal conditions with the 100000 × *g* supernatant as enzyme source. If the chain lengths and reaction rates are plotted semilogarithmically, the reaction rates increase in a linear fashion from C_{14} to C_{20}-DL-*erythro*-sphinganines. These results may well reflect the solubility of the substrates, which decreases with chain length.

Stability of the enzyme

The enzyme, present in the 100000 × *g* supernatant, was stable at −18°C for at least three weeks without any loss of activity. When this enzyme preparation or the dialyzed 20–50% $(NH_4)_2SO_4$ precipitate was stored at 4°C, a linear decrease of the enzymic activity was observed, which could not be prevented by 10 mM mercaptoethanol. On lyophilization the enzymic activity was almost completely retained.

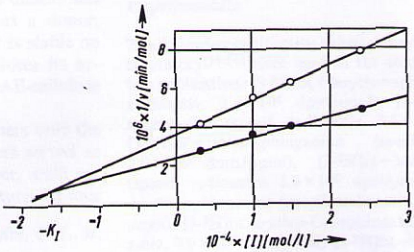

Fig. 6. Determination of K_I of L(−)*erythro*-sphinganine in the sphingosine kinase reaction by the Dixon plot with D(+)*erythro*-sphinganine as substrate. Substrate concentration: ○ = 2×10^{-4}M; ● = 5×10^{-5}M. $K_I = 1.62 \times 10^{-4}$M.

The influence of sulfhydryl reagents *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetamide has been studied from 0.5 to 3.0×10^{-3} M. *p*-Chloromercuribenzoate inhibited the kinase completely at these concentrations and *N*-ethylmaleinimide by 50%. Iodoacetamide had no significant influence. Further evidence for the importance of sulfhydryl groups for the kinase reaction is apparent from thioglycol reactivation of enzyme preparations which had been stored for a period of time at 4°C with partial loss of the activity.

Discussion

Studies in this laboratory have demonstrated that the degradation of the long chain bases 4*t*-sphingenine, sphinganine and 4*D*-hydroxysphinganine requires ATP, and that sphinganine 1-phosphate as an intermediate is cleaved by a membrane-bound lyase to palmitaldehyde and ethanolamine phosphate[2,3,4]. A subsequent search for the kinase revealed its wide distribution in different organs such as blood platelets[1,5], bovine kidney[7], rat liver[10] and *Tetrahymena pyriformis*[11]*; except in the latter organism, the enzyme is present in the soluble fractions. The phosphate esters of naturally occurring sphingosine bases such as sphinganine, 4*t*-sphingenine and 4*D*-hydroxysphinganine are formed[5]. Their structures were determined in previous studies[5,6]. Since the kinase is present as an enzyme in the cytosol of thrombocytes, it seemed promising to further characterize this enzyme with regard to its properties and specificity. It was found that ATP is by far the best phosphate donor, and UTP also functions to some extent as a donor, whereas GTP and CTP are inactive. It is stable on ammonium sulfate fractionation but loses its activity during chromatography on DEAE-cellulose and different Sephadex columns.

Of the four synthetic sphinganine isomers only the D(+)*erythro* and the L(−)*threo* isomers served as substrates. In studies *in vivo*, however, with rat liver[12] and BHK-cells** in tissue culture, all four isomers were phosphorylated. At the moment we are unable to explain the observation that the kinase phosphorylates the two isomers which have an identical chirality at C-2 in experiments *in vitro*, but not the other two isomers.

Long chain bases are highly toxic to animal cells**. There are two mechanisms which serve to detoxify this intermediate of synthesis and degradation of complex sphingolipids: a) the phosphorylation of the primary hydroxy group and b) the acylation of the amino group. The former initiates degradation, whereas the ceramide formed by the latter is the substrate for the synthesis of more complex sphingolipids. The regulation of these two processes is not yet understood.

The phosphorylation transforms the long chain base, which is reasonably soluble in aqueous salt solutions, into an extremely insoluble product. The phosphorylated long chain bases are then integrated into the membrane system of the endoplasmic reticulum due to their insolubility in water. Since the endoplasmic reticulum contains the lyase (aldolase), the product of the kinase reaction will be cleaved into the corresponding long chain aldehyde and ethanolamine phosphate. Both are then utilized for the synthesis of complex phospholipids[13].

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Experimentals

The following radioactive substrates synthesized in this laboratory[12,14] were used in the studies described in this publication: [3-^{3}H]D(+)*erythro*-sphinganine (specif. radioactiv. 3.4×10^6 dpm/μmol), [3-^{3}H]L(−)*erythro*-sphinganine (spezif. radioactiv. 3.4×10^6 dpm/μmol), [3-^{3}H]D(+)*threo*-sphinganine (specif. radioactiv. 3.0×10^6 dpm/μmol), [3-^{3}H]L(−)*threo*-sphinganine (specif. radioactiv. 3.0×10^6 dpm/μmol), [3-^{3}H]D(+)*erythro*-sphingenine (specif. radioactiv. 4.35×10^6 dpm/μmol), [3-^{3}H]DL-*erythro*-C_{14}-sphinganine (specif. radioactiv. 7.9×10^6 dpm/μmol) [3-^{3}H]DL-*erythro*-C_{16}-sphinganine (specif. radioactiv. 26.7×10^6 dpm/μmol), [3-^{3}H]DL-*erythro*-C_{18}-sphinganine (specif. radioactiv. 3.4×10^6 dpm/μmol), [3-^{3}H]DL-*erythro*-C_{20}-sphinganine

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** Stoffel, W. & Zerhusen, M. (1973) *this J.* in preparation.

[13] Stoffel, W. (1970) *Chem. Phys. Lipids* **5**, 139−158.

[14] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 1561−1569.

(specif. radioactiv. 3.5×10^6 dpm/μmol), [3-^{14}C;4,5-$^3H_2$]4*D*-hydroxysphinganine (specif. radioactiv. 1×10^5 dpm ^{14}C/μmol).
All coenzymes were purchased from Boehringer Mannheim GmbH.

Preparation of human and pig platelets. Human or pig blood was collected in siliconized vessels (Siliconöl WS 60, Wacker Chemie, München) with acid citrate-dextrose (USP formula A) as coagulant (67.5 m*l* for 450 m*l* blood). A platelet-enriched plasma was obtained by centrifugation at $840 \times g$ for 7 min at room temperature in siliconized glass tubes. In a second centrifugation at $680 \times g$ for 10 min, the rests of the contaminating erythrocytes and leucocytes were sedimented in specially shaped tubes[1]. The supernatant, which contained the platelets, was carefully removed from the erythrocyte-leucocyte pellet and the platelets were sedimented at $2400 \times g$ for 20 min at 4°C. The sediment was washed twice with 0.02M sodium phosphate buffer pH 7.0, 0.1M in NaCl and 0.04M in glucose. The washed platelets could be stored at −18°C without loss of enzymic activity for several weeks. They were contaminated by less than 1 erythrocyte/10^4 platelets and 1 leucocyte/5×10^4 platelets.

Platelet fractionation. A platelet homogenate was prepared from a frozen suspension of platelets by thawing at 20°C for 30 min under hypotonic conditions (0.02M sodium phosphate buffer pH 7.4). The homogenate was centrifuged for 60 min at $100000 \times g$ and the cytosol obtained as the supernatant fraction.
A membrane preparation from human platelets was isolated according to Barber and Jamieson[8] after disruption of the platelets by the glycerol load hypotonic lysis technique.
The method could not be adapted to the fractionation of pig platelets. These were lysed by freezing and thawing and subjected to a linear sucrose gradient (30−60%). Granules were prepared according to Marcus *et al.*[9]. Protein was measured by the method of Lowry *et al.*[15].
Incubations. The long chain bases (see substrates) were dissolved in a small volume of absolute ethanol. The standard incubation mixture contained in a total volume of 1 m*l*: 0.3 μmol substrate, 7 μmol ATP, 7 μmol $MgCl_2$, 1 to 7 mg protein and 80 μmol Tris maleate or sodium phosphate buffer, pH 7.0. The incubation at 37°C was stopped after 60 min by the addition of 2 m*l* acetone/methanol 1:1 (v/v). The protein precipitate was extracted twice with 1 m*l* acetone/methanol/water 2:1:1. All reactions were carried out in duplicate.

The enzymatic preparation of radioactive 4*t*-sphingenine 1-phosphate on a preparative scale was carried out under the following conditions: 12 μmol [3-^{3}H]D-*erythro*-4*t*-sphingenine (11.4 μCi/μmol) and 119 μmol ATP, neutralized with 1N NaOH were allowed to react in a total volume of 17.0 m*l* incubation mixture containing 119 μmol $MgCl_2$, 170 μmol 2-mercaptoethanol, 8.5 mg Triton X-100, 1.2 mol Tris maleate buffer pH 7.0 and 100 mg protein from $100000 \times g$ supernatant of pig platelets at 37°C for 6 h in a 240 m*l* round bottom flask. The yield of [3-^{3}H]4*t*-sphingenine 1-phosphate was 45%. It was raised to 70% by the addition of 80 mg of lyophilized supernatant protein and a further 9 h incubation. The reaction was stopped by lyophilization. The lyophilized material was stirred overnight with 50 m*l* of chloroform/methanol 2:1 at room temperature. After centrifugation, the residue was extracted four times with n-butanol/acetic acid/water 6:2:2 at 50°C. The combined extracts were concentrated under reduced pressure and then loaded onto a 1.0×20.5 cm column of silicic acid.
Neutral lipids were eluted with 100 m*l* chloroform, and the unreacted long chain bases (3.0×10^7 dpm) with 110 m*l* of acetone/methanol 9:1. The reaction product, [3-^{3}H]4*t*-sphingenine 1-phosphate, was eluted with 200 m*l* of n-butanol/acetic acid/ water 6:2:2 and appeared as a sharp band after the void volume had passed the column.

Analytical procedures. The quantitative separation of the long chain bases and their 1-phosphate esters was carried out as described before[1]. Radioactivity was measured in a Packard Tricarb liquid scintillation counter, Model 3380. Radio thin-layer chromatography was carried out on a Packard 7021 scanner or a Berthold scanner LB 2722. Silica gel H plates (0.2 mm) were used. Solvent systems: chloroform/methanol/water 65:25:4; n-butanol/acetic acid/water 60:20:20.
Radio-gas chromatography of long chain aldehydes was carried out on 15% EGS on Chromosorb. The radioactive bands were collected discontinously. The phosphate esters of the long chain bases were identified as follows: 1) radio thin-layer chromatography in two solvent systems (see analytical procedures), 2) enzymatic hydrolysis with platelet phosphatase of the $100000 \times g$ sediment[1] and rat liver microsomal phosphatase prepared according to Siekevitz[16], 3) periodate oxidation of the phosphate ester or the free base obtained after enzymatic hydrolysis of the phosphate ester bond with subsequent quantitation of the long chain aldehydes by gas chromatography.

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