The Enzymatic Phosphorylation of Sphinganine[1,2]

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SUMMARY

A partially purified enzyme preparation from bovine kidney has been shown to catalyze the phosphorylation of sphinganine. The enzyme utilizes ATP as a phosphate donor and the product of the reaction has been identified as sphinganine-1-phosphate.

Recent studies of Keenan and Maxam (1), Stoffel et al (2), and Gatt and Barenholz (3) have shown that ATP or an ATP generating system is required for the degradation of sphinganine by cell-free preparations from rat liver. The products of the reaction have been identified as palmitaldehyde and phosphoryl ethanolamine. In addition, Stoffel et al (3) showed that synthetic sphinganine-1-phosphate is cleaved by rat liver mitochondrial preparations. The phosphorylation of the sphingolipid base, presumably the first step in sphingolipid base catabolism had not been demonstrated. This paper describes the enzymatic phosphorylation of sphinganine and some of the characteristics of this hitherto unreported reaction.

MATERIALS AND METHODS

Substrates and Other Compounds--The method of Glynn and Chapell (4) was employed for the preparation of γ-labeled AT^{32}P. Radioactive L-α-glycerophosphate was prepared by incubating glycerol and AT^{32}P with glycerokinase

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2. The nomenclature used in this communication conforms to the nomenclature suggested by the IUPAC-IUB Commission on Biochemical Nomenclature, thus sphinganine refers to the compound formerly called dihydrosphingosine.

(obtained from Sigma Chemical Company) and the product was isolated by ion-exchange chromatography as described by Bublitz and Kennedy (5). Sphinganine and ^{3}H-labeled sphinganine were prepared from beef brain sphingolipids and purified as previously described (6). Sphinganine-1-phosphate was synthesized according to the procedure of Weiss (7).

Enzyme preparation--Fresh frozen bovine kidneys were freed of adhering fat and homogenized in 9 volumes of cold phosphate buffer (pH 7.4, 0.05 M.) for 3 minutes in a Waring Blender. The homogenate was centrifuged at 44,000 x g for 1 hour and the precipitate was discarded. The clear, red supernatant was brought to 40% saturation with solid $(NH_4)_2SO_4$ while maintaining the pH at 7.4 with N NaOH. The $(NH_4)_2SO_4$ precipitate was collected by centrifugation and taken up in sufficient phosphate buffer to give preparations containing about 20 mg protein per ml. These enzyme preparations which usually contained about 70% of the total activity found in the homogenate represented an approximately 8 fold purification over the original homogenate. These preparations gradually lost activity over several days storage at 4°.

Assay of Enzyme Activity--Considerable difficulty was experienced in attempting to develop a quantitative assay for measuring the formation of phosphoryl sphinganine, since this compound is sparingly soluble in both polar and non-polar solvents. The method which was used for the studies reported here consisted of precipitating the trichloroacetic (TCA) insoluble material from the incubated sample with an equal volume of ice cold 10% TCA. The TCA precipitate was incubated for 30 minutes at 38° with 0.5 ml of 1 N methanolic KOH which converted the bulk of the lipid-soluble phosphorus to a water soluble form. The alkali treated sample was acidified with 0.45 ml of 2.2 N acetic acid and extracted with 1.0 ml of chloroform. Aliquots of the chloroform extract were plated and counted in a gas-flow counter. Highly reproducible data were obtained using this assay and the method was also checked by carrying samples of synthetic sphinganine-1-phosphate through a similar procedure and determining the recovery of the added phosphorus. It

was found that 60 ± 3% of the phosphoryl sphinganine could be accounted for in the chloroform extracts. In experiments in which the incorporation of ^{3}H-sphinganine was being measured, it was necessary to modify the assay procedure to remove the contaminating substrate. In these experiments, the TCA precipitate was extracted with 5.0 ml of acetone followed by 3 extractions with 5.0 ml portions of ether which had been equilibrated against concentrated NH_4OH. After a final extraction with 5.0 ml of acetone, the precipitate was saponified, acidified, and extracted with chloroform as described above. Aliquots of the chloroform extract were counted in a scintillation counter and the activity was corrected to disintegrations per minute through the use of internal standards. This modified procedure removed the great bulk of the contaminating ^{3}H-activity with only relatively small losses in sphinganine phosphate.

RESULTS

Characteristics of the Sphinganine Phosphorylating System--The effects of adding various factors and altering the incubation conditions were determined by following the incorporation of ^{32}P from AT^{32}P by the assay procedure described in the preceeding section. Relatively little radioactivity was found in the chloroform phase of the assay system if incubation was carried out in the absence of sphinganine. The amount of radioactivity incorporated into the product increased proportionately with both increasing enzyme concentration and time. Increasing the AT^{32}P concentration increased the rate of the reaction until a plateau was reached. The pH optimum of the reaction was 7.0 in phosphate buffer. Magnesium and fluoride both increased the amount of product.

Incorporation of ^{3}H-sphinganine--Although it was difficult to remove all the excess tritium-labeled substrate, it was found that a reproducible fraction of the total activity was present in a compound with the properties of sphinganine-1-phosphate. The results of one such experiment are presented in Table I. It was calculated from the data in this table that the ratio of tritium to phosphorus incorporated is 1.12. Ratios very near unity were also obtained in two other experiments. These values approximate the theo-

TABLE I

Incorporation of ^{32}P and ^{3}H-Sphinganine into Sphinganine Phosphate

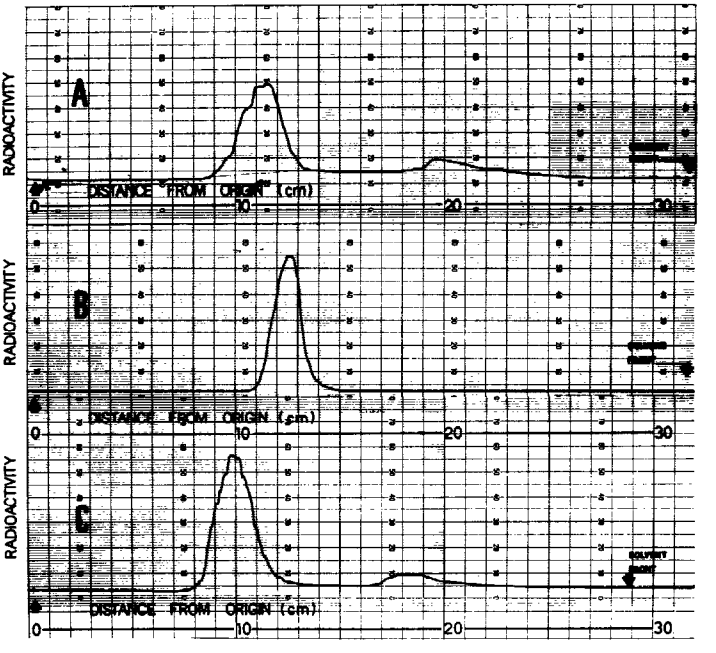
Additions	μMoles Labeled Compound in Chloroform Extract x 10^5	Net μMoles Labeled Compound in Chloroform Extract x 10^5
AT^{32}P, 0.20 μmole	2.02	--
AT^{32}P, 0.20 μmole + 0.06 μmole Sphinganine	40.7	38.8
-- 0.06 μmole ^{3}H-Sphinganine	20.5	--
ATP, 0.20 μmole + 0.06 μmole ^{3}H-Sphinganine	64.1	43.6

The reaction mixtures consisted of 10 μmoles of NaF, 10 μmoles of $MgCl_2$, 50 μmoles of phosphate buffer, pH 7.0, and 3.4 mg of enzyme preparation in a volume of 0.65 ml. In addition, each reaction vessel also contained 0.04 mg of Cutscum (isooctylphenoxylpolyoxyethanol, Fisher Chemical Company), or an 0.04 mg Cutscum suspension of sphinganine or labeled sphinganine as shown. Incubation was carried out for 10 minutes at 38°. All samples were subjected to TCA precipitation, acetone and ether extractions, saponification, acidification, and chloroform extraction as described in the text. The values shown represent the average of triplicates.

retical ratio which should be obtained for sphinganine phosphate.

Identification of the Radioactive Product--Although the properties of the compound produced enzymatically resembled those of synthetic sphinganine-1-phosphate, periodate oxidation was used to prove that the incorporated phosphoryl group was on the primary hydroxyl of the lipid base. Unlabeled sphinganine was incubated with AT^{32}P under the conditions described in Table I, but both the size of the incubated sample and the assay procedure were scaled up 5 times. Aliquots of the chloroform extract were taken to dryness and the dried residue was mixed with 4.0 ml of methanol and 1.0 ml of 0.1 N sodium periodate and incubated at 38^{o} with shaking for 90 to 100 minutes. At the end of this period, excess periodate was reduced with sodium sulfite and sufficient chloroform and water were added to give a biphasic system. After periodate oxidation almost all the radioactivity was found in the aqueous phase (under similar conditions we found that synthetic sphinganine-1-phosphate yields water soluble phosphorus and palmitaldehyde in the chloroform phase). Aliquots of the aqueous phase were chromatographed on Whatman No. 1 paper using the t-butanol-picric acid solvent of Hanes and Isherwood (8). L-α-glycerophosphate labeled with ^{32}P was also carried through periodate oxidation and chromatography and strips of the chromatograms were assayed for radioactivity in a Packard Strip Scanner. The results are shown in Fig. 1.

It is apparent from the results in Fig. 1 that both L-α-glycerophosphate and the radioactive product produced in the incubation of sphinganine with AT^{32}P give rise to the same periodate oxidation product. The R_f value of 0.36 to 0.40 which was found for the principal radioactive product corresponds to that reported by Rouser et al(9) and Loring et al (10) for glycoaldehyde phosphate. A mixture of the periodate oxidation product of α-glycerophosphate (glycoaldehyde phosphate) and that of the phosphorylated sphinganine periodate product were not separated. In addition to the major peak (over 90% of the total radioactivity), a minor peak was usually observed depending on the reaction conditions. This compound is probably an artifact of the reaction and



LEGEND TO FIGURE 1. The Chromatographic Separation of the Radioactive Products Formed by Periodate Oxidation.

A. Periodate treated reaction product.

B. Periodate treated P^{32}-labeled α-glycerol phosphate.

C. A mixture of A and B containing equal amounts of radioactivity.

Details of the procedure are described in the text. Strips of the chromatograms were scanned for radioactivity on a Packard Strip Scanner.

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has not been characterized. It is clear from the data in Fig. 1, however, that essentially all of the phosphate incorporated into the sphingolipid base must have been located on the primary hydroxyl group.

DISCUSSION

Previous studies (1,2,3) have shown that the in vitro degradation of sphinganine by an enzyme system from rat liver requires ATP. It has been postulated that the ATP is required for the phosphorylation of the primary

hydroxyl group of the lipid base prior to enzymatic cleavage, but this reaction had not been demonstrated. In preliminary studies we were able to show the phosphorylation of sphinganine by a rat liver preparation, but we found that spleen and kidney homogenates were approximately 100 times more active. Using a partially purified preparation from bovine kidney, we have demonstrated that the phosphoryl group from ATP is transferred to the primary hydroxyl group of the lipid base molecule.

It is not known whether the observed activity is due to an enzyme specific for sphinganine or represents a minor reaction of some other kinase which is not completely specific. Investigations are now in progress to further purify the enzyme and determine its properties and specificity.

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REFERENCES:

1. Keenan, R. W. and Maxam, A., Biochim. Biophys. Acta, 176, 348 (1969).
2. Stoffel, W., Sticht, G., and LeKim, D., Z. Physiol. Chem. 349, 1745 (1968).
3. Gatt, S. and Barenholz, Y., Biochim. Biophys. Res. Comm. 32, 588 (1968).
4. Glynn, I. M., and Chappel, J. B., Biochem. J., 90, 147 (1964).
5. Bublitz, C. and Kennedy, E. P., J. Biol. Chem. 211, 963 (1954).
6. Keenan, R. W., and Okabe, K., Biochemistry 7, 2696 (1968).
7. Weiss, B., J. Am. Chem. Soc. 79, 5553 (1957).
8. Hanes, C. S. and Isherwood, F. A., Nature 164, 1107 (1949).
9. Loring, H. S., Levy, L. W., Moss, L. K , and Ploeser, J., J. Am. Chem. Soc. 78, 3724 (1956).
10. Rouser, G., Berry, J. F., Marinetti, G., and Stotz, E., J. Am. Chem. Soc. 75, 310 (1953).