EVIDENCE FOR EXISTENCE AND A TENTATIVE IDENTIFICATION OF COENZYME IN YEAST THIAMINE PYROPHOSPHOKINASE

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SUMMARY: Thiamine pyrophosphokinase (E.C. 2.7.6.2.) from Saccharomyces cerevisiae was found to require the presence of a non-protein, non-metal compound for its activity. myo-Inositol was found capable of stimulating the kinase activity in the presumably resolved but otherwise crude sample of the enzyme. The hexytol was also found capable of inducing the enzyme in growing yeast cells. The cultured yeast cells, in which the kinase had been induced, were used as source of the enzyme for its purification. The compound that had been left adsorbed to the final column of DEAE-Sephadex was proved to have a coenzyme activity towards the enzyme and tentatively identified with myo-inositol 1-pyrophosphate. A sample of synthetic myo-inositol 1-pyrophosphate was made and its coenzyme activity was observed.

The so-called "cocarboxylase" had first been isolated from brewer's yeast in 1937 by Lohmann and Schuster (1), who also identified it with thiamine pyrophosphate (TPP) (2). In 1938, Lipschitz et al. demonstrated that TPP is synthetized from thiamine in the presence of brewer's yeast, hexose diphosphate, and a boiled extract of chicken liver (3). A number of papers on TPP biosynthesis in microorganisms (e.g. 4, 5) and animal tissues (e.g. 6-8) and on purifications and characterizations of thiaminokinase (e.g. 4-6, 8-10) have been published. It has been established by Greiling (11) and Shimazono et al. (12) that the so-called thiamine kinase from yeast catalyzes a one-step pyrophosphorylation of the substrate. Although Iwashima et al. demonstrated thiamine monophosphokinase in the soluble

fraction of E. coli (13), the latter enzyme has not been found in any other organisms or tissues.

The present study demonstrates the requirement of yeast thiamine pyrophosphokinase for a coenzyme and the tentative identification of the latter with myo-inositol 1-pyrophosphate.

MATERIALS AND METHODS

Fresh baker's yeast was purchased from Oriental Yeast Company (Kyoto, Japan). Actinomycin D, myo-inositol 2-monophosphate, sodium phytate, alcohol dehydrogenase, and pyruvate decarboxylase were obtained from Sigma Chemical Company. [^{14}C]Thiamine, labeled at the thiazole-2-C, 2 mC per millimole, was a gift from Takeda Pharmaceutical Industries (Osaka, Japan) by the courtesy to Prof. Fujiwara. Potato acid-phosphatase was from Boehringer Mannheim GmbH. myo-Inositol 2-monophosphate was also prepared by a partial hydrolysis of sodium phytate, and myo-inositol 1-pyrophosphate by a chemical phosphorylation of myo-inositol 1-monophosphate in ways as will be described elsewhere.

The enzyme was assayed either by manometric measurement of TPP (14) or by the method of Kaziro using coupling enzymes and NADH (15) or by a radio-assay method. The procedure of the radio-assay will be described elsewhere. The unit enzyme activity was defined as that producing one millimicromole of TPP per hour and the specific activity as units per mg protein. Protein was determined either by reading absorbance at 280 nm wave length with Hitachi spectrophotometer or according to the method of Lowry et al. (16).

RESULTS AND DISCUSSION

Samples of apo-pyruvate decarboxylase were prepared from baker's yeast according to the procedure described by Kay and Murfitt (14) for the purpose of assaying TPP manometrically. The original samples were highly contaminated with activities of thiamine pyrophosphokinase. To reduce the contamination, they were put through repeated treatments with calcium ace-

tate after adjustments of pH to 9.0 each time. The contamination was apparently reduced, however, when the extract of the precipitate that had been made with calcium acetate was added to the final sample, the latter resumed the kinase activity. This was also true when the extract was boiled at pH 7 for 30 min or heated to about 300° at dryness for a few sec. This implied that some heat-stable, therefore non-protein, factor(s) might contribute the kinase activity. To know what the factor(s) is (are), several authentic compounds were checked for a stimulatory effect on the kinase activity. Eventually, glutathione, cysteine, and myo-inositol were found to have some of the stimulatory effect. The first and the second compounds above were excluded from the potential factors because they must be stimulatory protecting the free sulfhydryl groups of the kinase. The third was repeatedly rechecked and was found to require a 30-min preincubation at 30° with ATP, Mg ions, and the sample of apo-pyruvate decarboxylase which evidently possessed little TPP and poor activity of the kinase but otherwise was quite crude. In other words, it was possible that myo-inositol be phosphorylated during the preincubation. Moreover, the stimulatory effect declined as the concentration of myo-inositol in the medium was raised above 1 mM, and the declination in part disappeared when the ATP concentration had been raised to 10 mM. These indicated that the hexytol might have to be phosphorylated before exhibiting a stimulatory effect on the kinase activity.

Taking a chance to confirm the speculation that a phosphate ester of myo-inositol could be co-thiamine pyrophosphokinase, an induction of the enzyme in growing baker's yeast with the free form of the hexytol was attempted. The procedure will be described elsewhere, but it was found that concentrations of the hexytol in the growth media at 0, 1.75, 2.0, 2.25, and 2.5 mM brought along the specific activities of the kinase in the crude extracts of the grown yeast of 3.8, 4.7, 13.2, 14.8, and 11.1, respectively. To see if these were indeed results of an enzyme induction, effects of cy-

cloheximide and actinomycin D were tested. The specific activities in the crude extracts of the yeast cells grown in the media that contained no myo-inositol, 2.25 mM myo-inositol, 2.25 mM myo-inositol plus 3.5×10^{-4} M cycloheximide, and 2.25 mM myo-inositol plus 8×10^{-7} M actinomycin D were 8.0, 32.3, 6.0, and 9.3, respectively. These data strongly indicate that the hexytol induces the enzyme in growing yeast cells and also that the former is the body of the postulated coenzyme of the latter.

From the grown yeast cells, in which the enzyme had been induced, the kinase was purified to a pure singularity as judged from ultracentrifugal analysis and acrylamide gel electrophoresis (Figg. 1 and 2). The procedure

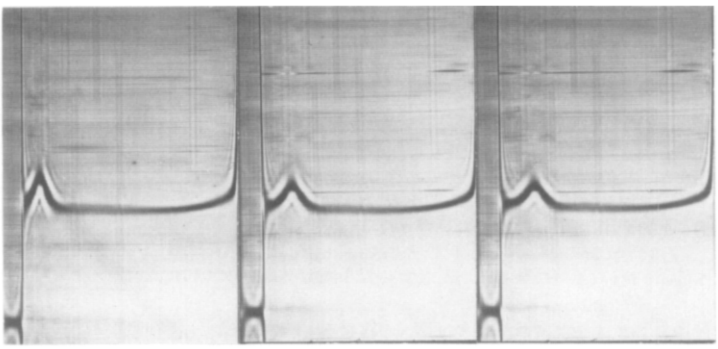

Fig. 1. Sedimentation patterns of the purified enzyme. Photographs were taken at 20, 36, and 44 min (from left to right) after the top speed had been attained. Rotor speed, 59,780 rpm; protein concentration, 1.4 mg per ml; temperature, 5°; buffer, 0.2 M potassium phosphate, pH 7.4, with 1 mM 2-mercaptoethanol and 0.001% phenylmethylsulfonyl fluoride; bar angle, 40°.

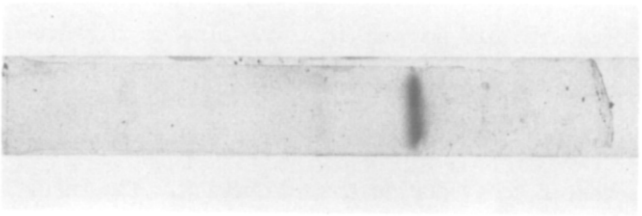

Fig. 2. Acrylamide gel electrophoresis of the purified enzyme. An aliquot of 27 µg of protein was applied to the gel. The gel was prepared in 0.05 M Tris-glycine buffer, pH 8.4, and the run (from right to left in the figure) was made at room temperature in a vertical column, 0.6 x 7.2 cm, for 90 min at 2 mA. Guide dye is visible about 1 cm left to the main band.

of the purification will be described elsewhere, but the eluates from the first and the third columns of DEAE-Sephadex, which had been used for the purification, manifested rather poor specific activities immediately after elutions. It was likely that the enzyme in the eluates had been resolved during the column chromatographies. For an experiment's sake, to convert the presumably resolved form of the enzyme to a holo form, the eluates of the first column were incubated with approximately 200 μg (*in toto*) of the sample of *myo*-inositol 2-monophosphate, which had been prepared from sodium phytate, at 4° for 15 days. The specific activities in the first-column eluates before and after the incubation were in the ranges of 78 - 210 and 4,900 - 16,900, respectively. The reason why the eluate from the second column of DEAE-Sephadex did not appear to be resolved may be because the sample of inositol phosphate had been added to the first eluate and the ion-exchanger in the second column was more or less saturated with the phosphate ester.

On the other hand, the third column was washed after the chromatography with 100 ml of 0.5 M KOH to collect the potential coenzyme, which presumably had been adsorbed to the ion-exchanger. The KOH-eluate was neutralized, centrifuged, and concentrated. An aliquot was incubated with the third-column eluate, which seemingly contained a resolved form of the enzyme, at 4° for 7 days. The specific activities measured in the solution before and after this incubation were 1,540 and 48,000, respectively.

These results strongly suggest that the eluates from the first and the third columns contained resolved forms of the enzyme and that the incubated sample of *myo*-inositol 2-monophosphate either contained the coenzyme or has partly been converted to it during the incubation. The results also support the presumption that the KOH-eluate from the third column contains the coenzyme.

Accordingly, the KOH-eluate was analysed in an attempt to identify the coenzyme. The results of analyses of the eluate in respect to the three

forms of phosphate according to Fiske and SubbaRow (17) were that there were very little, if any, free phosphate and 2.2 times as much total phosphate as acid-labile phosphate. They indicate a presence of pyrophosphate ester in the eluate. The question remained was what the compound that remains after removal of the acid-labile phosphate is. Based on the observations so far described, it was likely that what remains after a mild hydrolysis of the coenzyme is either myo-inositol 1-monophosphate or myo-inositol 2-monophosphate. In order to confirm and determine either one of the above two possibilities, the KOH-eluate was allowed to receive a spontaneous hydrolysis (the acid-labile phosphate is so labile that it has been nearly 50% hydrolysed during a week's storage at pH 7 and -20°) and applied to paper partition chromatographies according to the procedure described by Pizer and Ballou (18). Only one solvent system was employed because this system (isopropanol - ammonia) is the only one that gives satisfactory resolutions of inositol phosphates (18). Consequently, it turned out that the hydrolysed KOH-eluate contained a compound that was identical with myo-inositol 1-monophosphate as far as these chromatographies were concerned. The myo-inositol 1-monophosphate standard was prepared according to the procedure described by English et al. (19) from the authentic myo-inositol 2-monophosphate (Sigma).

To confirm the idea that the main body, i.e. the remainder after a total dephosphorylation, of the coenzyme is myo-inositol, the spontaneously hydrolysed KOH-eluate was further hydrolysed with potato acid-phosphatase according to the procedure of Benson (20). The compound in the final hydrolysate was identified with myo-inositol by the paper partition chromatography in the solvent system described above.

As another line of evidence for that the coenzyme of yeast thiamine pyrophosphokinase is myo-inositol 1-pyrophosphate, the chemically synthetized sample of the compound was incubated with the purified and resolved enzyme, i.e. the third-column eluate, at 4° for 6 days. The specific ac-

tivities of the kinase in the solution before and after the incubation were 803 and 9,100, respectively. The reason why the chemically synthetized sample did not raise the specific activity to the same level as the KOH-eluate had done may be because the purified and resolved enzyme had been stored at 2-4° for the period of time required for the chemical synthesis of the compound and had been in part denatured before it was incubated with the latter.

In conclusion, the coenzyme has been tentatively identified with myo-inositol 1-pyrophosphate.

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