Biochimica et Biophysica Acta, 566 (1979) 152—156

BBA 68637

ACTIVATION OF GLUTAMINASE BY PHOSPHORIBOSYLPYROPHOSPHATE AND ITS INTERFERENCE WITH THE ASSAY OF PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE

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(Received July 21st, 1978)

Key words: Glutaminase; Amidophosphoribosyltransferase; Phosphoribosylpyrophosphate

Summary

Phosphate-dependent glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) from rat liver was found to be strongly activated by phosphoribosylpyrophosphate (*P*-rib-*PP*), the substrate of amidophosphoribosyltransferase (EC 2.4.2.14). Since the assay of the latter is based on the *P*-rib-*PP*-dependent conversion of glutamine to glutamate, the amidotransferase activities determined in crude tissue preparations were found to be too high. The interference of glutaminase, however, could be completely eliminated by its inactivation at 50°C. Amidotransferase was not affected by the heat treatment. Because of the increased rate of the glutamate formation at this temperature, the incubation time of the assay could be significantly reduced.

Introduction

Amidophosphoribosyltransferase (5-phosphoribosylamine:pyrophosphate phosphoribosyltransferase (glutamate-amidating), EC 2.4.2.14) is the proposed regulatory enzyme in de novo purine biosynthesis [1]. Altered activities and kinetic properties of the enzyme have been reported in neoplastic transformations [2—5] and gout [6]. Consequently, there is interest in the determination of this enzyme in crude tissue preparations. The enzyme is commonly determined by *P*-rib-*PP*-dependent glutamate liberation from glutamine. Since glutaminase can be considered as a major obstacle to the accurate measurement of amidophosphoribosyltransferase in crude tissue extracts [7] a blank value in the absence of *P*-rib-*PP* is usually established for glutamate formed by glutaminase [1—4]. However, glutaminase is known to be activated by a variety of phosphate esters [8]. Thus, the possibility of activation by *P*-rib-*PP* had to be

examined. Studies were performed to prevent interference by glutaminase with the amidophosphoribosyltransferase (hereafter termed amidotransferase) assay.

Methods and Materials

Enzymatic assays. Amido-*P*-rib-*PP*-transferase activity was measured by the *P*-rib-*PP*-dependent liberation of L-[^{14}C]glutamate from L-[^{14}C]glutamine (Amersham Buchler, Braunschweig, F.R.G.). The standard assay was performed as follows. 25 μl enzyme sample, containing 25 mM potassium phosphate buffer (pH 7.4), 60 mM β-mercaptoethanol and 5 mM $MgCl_2$ plus 15 μl 10 mM *P*-rib-*PP* (Sigma) (containing 33.5 mM $MgCl_2$ and 335 mM triethanolamine-hydrochloride buffer (pH 7.4) were preincubated for 10 min at 37°C. The reaction was started with 10 μl 100 mM L-[^{14}C]glutamine (0.125 Ci/mol). After 60 min at 37°C, the reaction was stopped with 5 μl 0.7 M perchloric acid. A 5-μl aliquot was spotted on No. 2043 b Mgl chromatography paper (Schleicher and Schüll, Dassel) and glutamate was separated from glutamine by paper electrophoresis at 80 V/cm in 0.16 M sodium acetate buffer (pH 3.9). After 30 min, the paper strips were dried at 120°C for 5 min, cut in the middle and both halves counted in a liquid scintillation counter.

Glutaminase was measured in 0.1 M triethanolamine-hydrochloride buffer containing 20 mM L-[^{14}C]glutamine (0.125 Ci/mol). The glutamate formed after 60 min at 37°C was estimated. Enzyme activities are expressed as nmol glutamate formed per min per ml.

Phosphoribosylpyrophosphate. *P*-rib-*PP* is unstable even in dry state. Thus, the actual content of *P*-rib-*PP* was estimated as previously described [9].

Enzyme purification. Amidotransferase: Rat liver was homogenized with 2 vols. 25 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 60 mM β-mercaptoethanol (buffer A) in a blender at 4°C. The 50 000 $\times g$ supernatant was rapidly brought to 50°C and maintained at this temperature for 20 min. The supernatant of the following centrifugation at 25 000 $\times g$ (30 min) was brought to 40% $(NH_4)_2SO_4$ saturation with saturated (4°C) $(NH_4)_2SO_4$ in buffer A, stirred for 20 min and centrifuged for 40 min at 25 000 $\times g$. The supernatant was adjusted to 55% $(NH_4)_2SO_4$ saturation. The precipitate was separated by centrifugation at 25 000 $\times$g for 40 min and dissolved in 60 mM β-mercaptoethanol until a specific conductance of 0.062 $ohm^{-1} \cdot cm^{-1}$ was obtained. This fluid was mixed with 1/4 vol. DEAE-Sephadex A 50 (equilibrated with buffer A containing 50 mM KCl (specific conductance 0.062 $ohm^{-1} \cdot cm^{-1}$)). The DEAE-Sephadex was pourced into a column and washed with 5 vols. equilibration buffer. The elution was performed with a linear gradient of 200—500 mM KCl in buffer A. The total volume of the gradient was 4 times that of the DEAE-Sephadex bed. Those fractions containing amidotransferase activity (specific conductance 0.116 to 0.2 $ohm^{-1} \cdot cm^{-1}$) were immediately concentrated by ultrafiltration (BM 500 ultrafiltration membrane (Berghof, Tübingen)) and stored in liquid N_2, after addition of glycerol up to 20%. No glutaminase activity was detectable in this enzyme preparation.

Glutaminase: Since no amidotransferase activity is found in mitochondria [10], sonicated, washed rat liver mitochondria were used as the enzyme preparation.

Results

In order to examine the possible activation of phosphate-dependent glutaminase by *P*-rib-*PP* and thus interference with the assay of aminotransferase, glutaminase activity in relation to the concentration of *P*-rib-*PP* and, for comparison, inorganic phosphate was determined. The activation by *P*-rib-*PP* was found to be considerably more pronounced than by inorganic phosphate (Fig. 1). At 5 mM, corresponding to the *P*-rib-*PP*-concentration in the standard assay of amidotransferase, the activation was 4.5-fold with *P*-rib-*PP*, while only 2.5-fold with phosphate. With 10 mM *P*-rib-*PP*, the same increase in glutaminase activity was found as with 200 mM phosphate.

However, glutaminase could be rapidly destroyed by heat treatment: at 50°C the enzyme was completely inactivated within less than 3 min.

To demonstrate clearly the interference of the *P*-rib-*PP*-dependence of the glutaminase activity with the standard assay of amidotransferase, the *P*-rib-*PP*-dependent conversion of glutamine to glutamate at 37°C was determined with a mixture of 30-fold purified amidotransferase and crude glutaminase with and without preincubation at 50°C for 5 min (Fig. 2).

Before preincubation, both glutamate-forming activities of the enzyme mixture were determined. The relationship between the rate of glutamate formation and *P*-rib-*PP* concentration did not reach saturation within the *P*-rib-*PP* concentration range investigated (upper curve). In contrast, normal saturation kinetics were obtained after preincubation at 50°C (lower curve). The maximum of the glutamate formation rate after preincubation corresponded with the amidotransferase activity added to the mixture, indicating that no loss of amidotransferase had occurred.

This was confirmed by the linearity of the amidotransferase assay at both 37°C and 50°C for at least 80 min in the presence of 30 mM β-mercaptoethanol (Fig. 3).

When the conversion of glutamine to glutamate in a phosphate-free

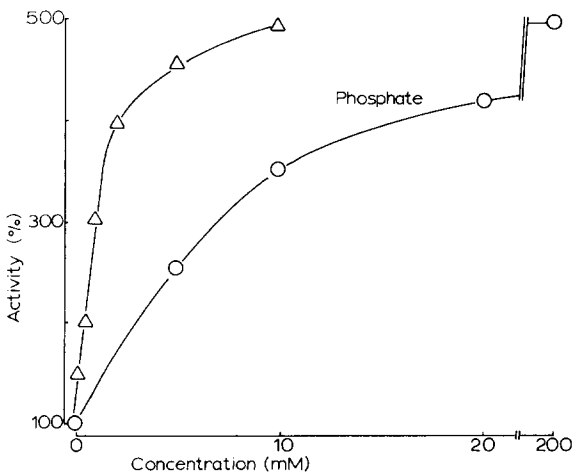

Fig. 1. Activation of glutamine from rat liver by inorganic phosphate (○———○), and *P*-rib-*PP* (△———△).

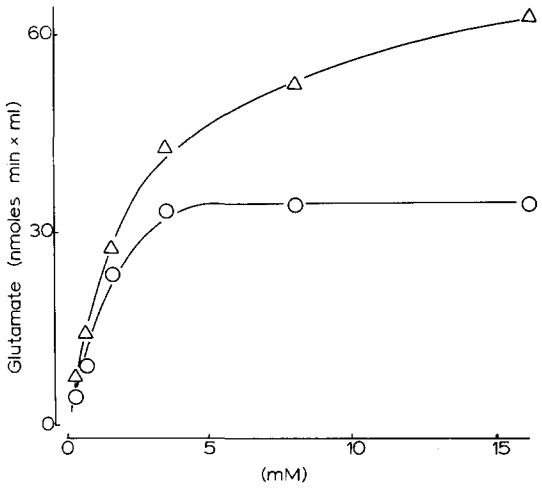

Fig. 2. Rate of glutamate formation by a mixture of 30-fold purified *P*-rib-*PP* amidotransferase (36.3 nmol glutamate/min per ml, 16.5 mM *P*-rib-*PP*, 5 mM inorganic phosphate) and crude glutaminase (30 mol glutamate per min per ml, 16.5 mM *P*-rib-*PP*, 5 mM inorganic phosphate) in relation to *P*-rib-*PP* concentration with, (○———○), and without (△———△) preincubation at 50° C for 5 min.

8000 × *g* supernatant from rat liver is measured in the absence of *P*-rib-*PP* and without preincubation at 50°C, (as is commonly done to correct for the glutaminse activity) as a rate of 1.0 nmol per min per ml was obtained. In the presence of 5 mM *P*-rib-*PP* the rate increase to 5.4 nmol glutamate/min per ml, giving an amidotransferase activity of 4.4 nmol glutamate/min per ml. However, when glutaminase was inactivated at 50°C, only 2.6 nmol glutamate/min per ml were formed in the presence of 5 mM *P*-rib-*PP*, now representing the correct amidotransferase activity. No correction has to be performed for non-enzymatic glutamate formation. This clearly shows, that the presence of 5 mM *P*-rib-*PP*

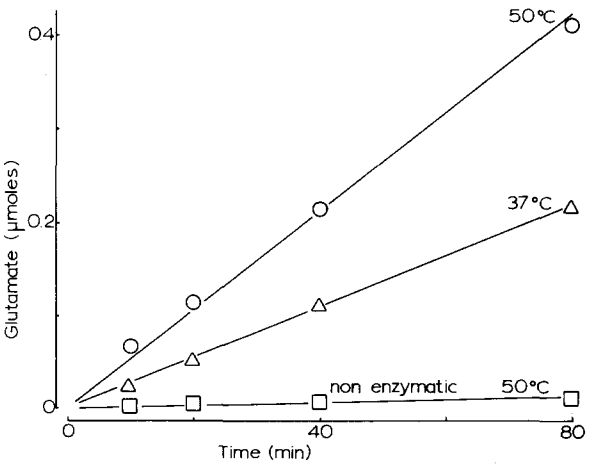

Fig. 3. Kinetic curves of amidotransferase at, (△———△), 37° C and, (○———○), 50° C. Obtained with the standard assay.

resulted in an activation of glutaminase from 1.0 to 2.8 nmol/min per ml. Thus the estimated amidotransferase activity would have been too high without prior heat denaturation of the interfering glutaminase.

Discussion

The observation that phosphate-dependent glutaminase from rat liver is strongly activated *P*-Rib-*PP* is in accordance with the activation of this enzyme from rat brain by a great number of phosphate esters [8]. As a consequence, this can interfere with the amidotransferase assay based on the *P*-rib-*PP*-dependent conversion of glutamine to glutamate. As shown by the present study, erroneous results may be obtained with crude tissue preparations still containing phosphate dependent glutaminase. Since glutaminase is activated by the substrate of the enzyme under investigation, the glutaminase activity determined in the absence of *P*-Rib-*PP* has to be considered as too low. Thus, this value does not represent a true blank value in the amidotransferase assay.

Furthermore, not only higher activities but also altered kinetic properties may be deduced since the v/s relationship is substantially modified in the presence of glutaminase. Thus, information about activities and properties of amidotransferase determined in intact cells or cell free extracts should be regarded with care, in particular, if, as is often reported, the assay is carried out in a phosphate free medium.

However, amidotransferase can be measured accurately, if the interfering glutaminase is eliminated by inactivation at 50°C. No time consuming high-speed centrifugation is necessary to precipitate the particular glutaminase. Moreover, since amidotransferase is stable at 50°C its determination can be performed at 50°C. Because of the increased rate of glutamate formation and its linearity with respect to time at this temperature, the incubation time of the assay can be drastically reduced.

Acknowledgement

This work was supported by the 'Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 76' and by the 'Fonds der Chemischen Industrie'.

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