32P-labeling of bovine tryptophanyl-tRNA synthetase with \([32P]\)Pyrophosphate

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Abstract

The covalent derivative of the tryptophanyl-tRNA synthetase obtained under the action of \(32P\) contains one mole of the covalently bound pyrophosphate (or 2 moles of orthophosphate) per mole of dimeric enzyme. Dephosphorylation with alkaline phosphatase causes practically no changes of enzymatic activity although the enzyme loses its ability to bind PPi.

Introduction

One of the important approaches for investigation of enzymes consists of isolation of their covalent complexes with substrates or products. Recently, a covalent derivative of beef pancreas tryptophanyl-tRNA synthetase was detected, isolated and characterized (1–3). In this paper we describe for the same protein, the preparation and some properties of the other enzyme form containing a covalently attached pyro- (or ortho) phosphate residue.

Materials and methods

\([32P]\)Pyrophosphate (disodium salt, 84 Ci/mole) was purchased from Radiochemical Centre, Amersham; thin-layer plates F1440/PEI/LS254 were from Schleicher and Schüll, \(E. coli\) alkaline phosphatase and yeast inorganic pyrophosphatase were from Worthington. The sources of other materials were the same as reported earlier (4). The purity of \(32P\) was checked by TLC on PEI-cellulose sheets in 0.75 M KH2PO4, pH 3.5. After chromatography, the sheet was cut into 0.2 cm pieces and the radioactivity counted. Only those preparations which contained no more than 1% of polyphosphates and 1% of orthophosphate were used. In some experiments pyrophosphate preparations were preliminarily purified on DEAE-cellulose.

Tryptophanyl-tRNA synthetase (molecular weight 120 000 daltons, a dimer of \(\alpha_2\) type) homogeneous after SDS-polyacrylamide gel electrophoresis was obtained as described (4, 5). Tryptophan-specific tRNAs from yeast and beef liver were kind gifts of Dr. G. Keith (Strasbourg) and Dr. V. Scheinker (this laboratory) respectively.

Enzyme activities were measured as described for tryptophanyl-tRNA synthetase (4), alkaline phosphatase (6) and inorganic pyrophosphatase (7).

Tryptophanyl-tRNA synthetase (1.4 \(\cdot\) 10\(^{-5}\) M) was incubated for 5 min at 37 °C with \([32P]\)pyrophosphate (3 \(\cdot\) 10\(^{-3}\) M) in 0.02 M tris \(\cdot\) HCl (pH 7.5), 2 \(\cdot\) 10\(^{-4}\) M ME. The amount of \(32P\)-radioactivity bound to the protein was determined in aliquots after precipitation with 3–5% TCA. After 5 min at 4\(^{\circ}\), the precipitates formed were transferred to nitrocellulose filters (AUFS,
Chemapol, 24 mm), washed 5 times with 10-ml portions of cold water, dried, and counted in an Intertechnique SL-30 spectrometer. Otherwise, the incubation mixture was passed at 4°C through a Sephadex G-50 fine column (0.8 × 22 cm) equilibrated with 0.02 M tris HCl (pH 7.5) and 0.2 mM ME. The elution rate was 2 ml/hour, the fractions were collected and counted according to Cherenkov (8). In some cases, the same procedure was performed in the presence of 1% SDS. In experiments with nonradioactive Pi and PPi, their concentrations were 10 and 30 mM, respectively.

The 32P-labeled enzyme (5 μM) was incubated after gel filtration with inorganic pyrophosphatase (0.1 mg/ml, 1:10 v/v) and Mg2+ (10 mM) as described (9) or in the presence of Zn2+ (1 mM) in 20 mM tris HCl (pH 7.0) as described (10). The amount of 32P-label that remained bound to the protein was determined after TCA precipitation of the protein as described above.

Dephosphorylated tryptophanyl-tRNA synthetase was released from alkaline phosphatase on a DEAE-cellulose column (0.5 × 3 cm) equilibrated with 20 mM tris HCl (pH 7.5), 0.02 mM ME and 0.2 mM EDTA. Elution was done with a linear gradient of NH4Cl (0–0.2 M, total volume 12 ml) in the same buffer at a flow rate of 2.5 ml per hour.

Results

When tryptophanyl-tRNA synthetase preparations were incubated with a 20–200-fold molar excess of 32PPi or 32PPi-Mg2+, with subsequent precipitation of the protein (using a 3% TCA), the time-independent binding of the 32P-label to the protein was observed (Fig. 1). The binding with native tryptophanyl-tRNA synthetase had two plateaus which corresponded to ~0.5 and ~1.0 mole of PPi (or ~1 and ~2 moles of Pj) per dimeric molecule of the enzyme. The 32P-labeled protein was obtained also after gel filtration through a Sephadex G-50 column under native and denatured conditions. The level of binding was the same as in TCA precipitation experiments.

The 32P-label was retained on the protein after storage for 5 days at 4°C in tris HCl (pH 7.5) and was not split after boiling in SDS for 2 min. When the 32P-labeled enzyme was treated with inorganic pyrophosphatase under the conditions of hydrolysis of PPi (9) or pyrophosphopeptides in the presence of Zn2+ (10), the amount of the protein-bound radioactivity did not change.

Preincubation of the enzyme with non-radioactive PPi followed by 32PPi treatment yielded fully nonradioactive protein. The same treatment with orthophosphate did not prevent 32P-labeling of the protein with 32PPi as well as treatment with tryptophan (0.2 mM), ATP-Mg2+ (5 mM) or AMP (1 mM).

The 32P-labeled protein isolated after gel filtration completely retained its enzymatic activity. The kinetic parameters of the enzyme in the activation of L-tryptophan or aminoacylation of tRNAyeast remained practically unchanged after 32P-labeling (Table 1). The study of the influence produced by various ligands on the stability of 32P-labeled protein has shown that low molecular weight substrates and tRNA under the given conditions do not release the 32P-label from the protein (Table 2). However, the 32P-label could be removed nearly quantitatively by E. coli alkaline phosphatase treatment (95% in 5 min). The dephosphorylated enzyme after careful separation from alkaline phosphatase by means of DEAE-cellulose chromatography retains nearly 80% of its initial enzymatic activity; however, it is unable to incorporate again the 32P-label from 32PPi under the above mentioned conditions.

Discussion

The stoichiometric incorporation of the 32P-label from 32PPi into beef tryptophanyl-tRNA synthetase is caused by the formation of a covalent bond