Glycosylation of yeast aspartyl–tRNA synthetase

Affinity labelling by glucose and glucose 6-phosphate

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Several lines of evidence establish that the crystallizable aspartyl–tRNA synthetase from Baker's yeast contains some covalently bound glucose: (i) a positive staining of the enzyme was obtained after polyacrylamide gel electrophoresis followed by the concanavalin A-peroxidase test which is specific for glucose and mannose containing proteins; (ii) thin-layer chromatography and gas-liquid chromatography revealed the presence of glucose in enzyme hydrolysates; (iii) immunoaffinoelectrophoresis in agarose gels containing concanavalin A and antibodies raised against aspartyl–tRNA synthetase showed that the enzyme was able to precipitate entirely in the lectin. Finally incubation of the enzyme with [^{14}C]glucose or [^{14}C]glucose 6-phosphate led to the incorporation of radioactivity into trichloroacetic acid-precipitable protein. Indeed immunoprecipitation of [^{14}C]glucose-labelled aspartyl-tRNA synthetase with specific antibodies using the rocket method followed by autoradiography gave a radioactive peak. This last result also demonstrates the possibility of in vitro glycosylation of yeast aspartyl–tRNA synthetase.

Aminoacyl–tRNA synthetase *Aspartyl–tRNA synthetase* *Glycosylation* *Protein modification*

1. INTRODUCTION

Aminoacyl–tRNA synthetases have been extensively studied over the last 20 years from many viewpoints (review [1]). Structural studies have been carried out on many enzymes from various sources and several complete amino acid sequences have been established either from the protein itself [2] or through DNA cloning and sequencing [3–9]. Only a few cases of post-translational modifications of aminoacyl–tRNA synthetases have been reported. They concern essentially the phosphorylation of serine residues [10]. It has been shown in our laboratory [11] that aspartic acid could be covalently linked to yeast aspartyl–tRNA synthetase and that this attachment was induced by the enzyme in the absence of the cognate tRNA under conditions where the synthetase activates the amino acid into aspartyl–adenylate; i.e., in the presence of ATP and $MgCl_2$. Glycosylation of some aminoacyl–tRNA synthetases has also been suggested but only preliminary studies have been carried out so far in the cases of rat liver threonyl–tRNA synthetase [12] and chloroplast leucyl–tRNA synthetase from *Euglena gracilis* [13]. It has been reported [14,15] that the lysyl– and arginyl–tRNA synthetase complex isolated from rat liver contains carbohydrates but the chemical nature and amount of carbohydrates found raise some questions as to whether these enzymes can be considered genuine glycoproteins. Furthermore, the presence of a carbohydrate moiety in all mammalian aminoacyl–tRNA synthetases remains an open question and it is by no means established that such a feature represents a major difference between eukaryotic and prokaryotic enzymes. To answer these questions a systematic analysis of all available synthetases is obviously necessary. We have undertaken a study on several aminoacyl–tRNA synthetases purified from

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baker's yeast. We here report the results obtained with the crystallizable aspartyl–tRNA synthetase well characterized in our laboratory [16].

2. MATERIALS AND METHODS

2.1. *Enzyme*

Aspartyl–tRNA synthetase was purified from commercial baker's yeast (FALA, Strasbourg) harvested in the exponential growth phase as in [17] and adapted in [18] for large-scale preparations. The pure enzyme crystallizes [16] and exhibits a specific activity of 600–800 units/mg. One unit is that amount of enzyme which catalyzes the incorporation of 1 nmol aspartic acid into $tRNA^{Asp}$/min under the standard conditions at 37°C [18].

2.2. *Chemicals*

Ion-exchange resin AG 501-X8 (D) and agarose (standard low-M_r) were purchased from Bio-Rad Laboratories (Richmond CA). Thin-layer cellulose plates were from Macherel-Nagel (Polygram cel 400, 20 × 20 cm, Duren). Aniline phthalate spray reagent and precoated silica gel plates (HPTLC plates, für nano-DC, 10 × 10 cm) were obtained from Merck (Darmstadt). Concanavalin A and naphthoresorcinol were from Sigma (St Louis MO). Emulfogen BC 720 was from GAF (Paris). ^{14}C-Labelled D-glucose (300 mCi · mmol^{-1}) and D-glucose 6-phosphate (288 mCi · mmol^{-1}) were from the Commissariat à l'Energie Atomique, and Omnifluor was from New England Nuclear. All other chemicals were of analytical grade from Merck.

2.3. *Polyacrylamide gel electrophoresis*

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as in [19] in 11% acrylamide gel slabs of 1 mm thick. Protein samples were treated prior to electrophoresis for 3 min at 100°C in 50 mM Tris buffer, pH 6.8, containing 2% SDS, 2% β-mercaptoethanol, 15% glycerol and 0.001% Bromophenol blue. Glycoproteins were stained by the concanavalin A–peroxidase method as in [20].

2.4. *Thin-layer chromatography*

Protein samples were hydrolyzed with either 1 N HCl for 2 h or 2 N HCl for 4 h, at 100°C. Hydrolysates were deionised by passage through a column (1.6 × 9 cm) filled with a mixed-bed ion-exchange resin AG 501-X8(D). The column was eluted with bidistilled water and the eluates were evaporated to dryness. The resulting material was subjected to ascending thin-layer chromatography either on pre-coated silica gel plates with the solvent system ethyl acetate:propanol-1:propanol-2:water (8:5:1:1, by vol.) or on cellulose plates with the solvent system ethyl acetate:pyridine:water (2:1:2, by vol.). Sugars were visualised by spraying the dried plates with aniline phthalate or naphthoresorcinol [21] followed by heating at 110°C for 5–20 min depending on the reagent.

2.5. *Gas-liquid chromatography*

The sugar content was estimated by gas-liquid chromatography of the trifluoroacetate derivatives obtained after methanolysis of the protein [22].

2.6. *Immunoelectrophoresis and affinoelectrophoresis*

Rocket immunoelectrophoresis was performed as in [23]. Immuno-affinoelectrophoresis was carried out in gels containing specific antibodies (immunoelectrophoresis) and concanavalin A (affinoelectrophoresis) [24] which is a lectin specific for α-D-mannosyl, α-D-glucosyl and sterically similar residues. Ten ml of 1% agarose gel (w/v) in a 37.5 mM Tris/100 mM glycine buffer (pH 8.7) containing 0.8% Emulfogen were layered onto glass plates (10 × 10 cm). The same buffer without Emulfogen was put in the electrode compartments. Two agarose strips at right angles to the direction of migration were cut off the plate and were replaced by an agarose strip (2 × 3 cm) containing a specific antiserum and an agarose strip (2 × 3 cm) containing concanavalin A at a concentration of 1 mg/ml of agarose solution. Electrophoresis was carried out at 1.5 V/cm for 18 h. After electrophoresis the excess of immunoglobulins and concanavalin A was removed by pressing and washing the gel in a 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and in distilled water (2 × 24 h). For protein staining, the gel was dried in a stream of warm air and immersed for 5 min into a 0.25% (w/v) Coomassie brilliant blue solution. Finally the plate was destained in methanol:acetic acid:water (4:1:5, by vol.).

2.7. *Incorporation of [^{14}C]glucose and [^{14}C]glucose 6-phosphate*

Unless otherwise mentioned, the [^{14}C]glucose or [^{14}C]glucose 6-phosphate incorporation into aspartyl–tRNA synthetase was followed in 0.2 ml reaction mixtures containing 20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 50 μg purified enzyme and 4.5 μCi of radioactively-labelled sugar. After various incubation times at 4°C, to avoid enzyme denaturation, aliquots (10 μl) were transferred onto discs of Whatman 3MM filter paper, washed 3-times in 5% trichloroacetic acid at 0°C, twice in 95% ethanol solutions and finally in diethyl ether. The dried filters were placed in a solution of 4 g Omnifluor/l of toluene and counted for ^{14}C in an Intertechnique liquid scintillation counter.

As a control, identical experiments were carried out with aspartyl–tRNA synthetase alone or sugar-free reaction mixtures.

3. RESULTS

3.1. *Evidence for the presence of covalently bound sugars*

Yeast aspartyl–tRNA synthetase could be stained after polyacrylamide gel electrophoresis in the presence or absence of SDS either with Coomassie brilliant blue (protein staining) or with concanavalin A–peroxidase complex (detected by the chromogenic peroxidase substrate, diaminobenzidine) specific for glycoprotein containing mannose and glucose. This preliminary result suggested the presence of sugars in the enzyme but the co-migration of a contaminating glycoprotein at a very low level could not be entirely ruled out by such experiments.

To further establish the presence of covalently bound sugars in aspartyl–tRNA synthetase, immunoaffinoelectrophoresis of the enzyme was performed onto agarose plates containing two strips, the lower one with concanavalin A and the upper one with specific antibodies raised against yeast aspartyl–tRNA synthetase. Under the conditions described in section 2, no precipitation occurred in the upper strip containing the antibodies indicating that aspartyl–tRNA synthetase precipitated entirely in the lower strip as shown in fig.1A and that sugars were covalently linked to enzyme. A control experiment was carried out to verify that the en-

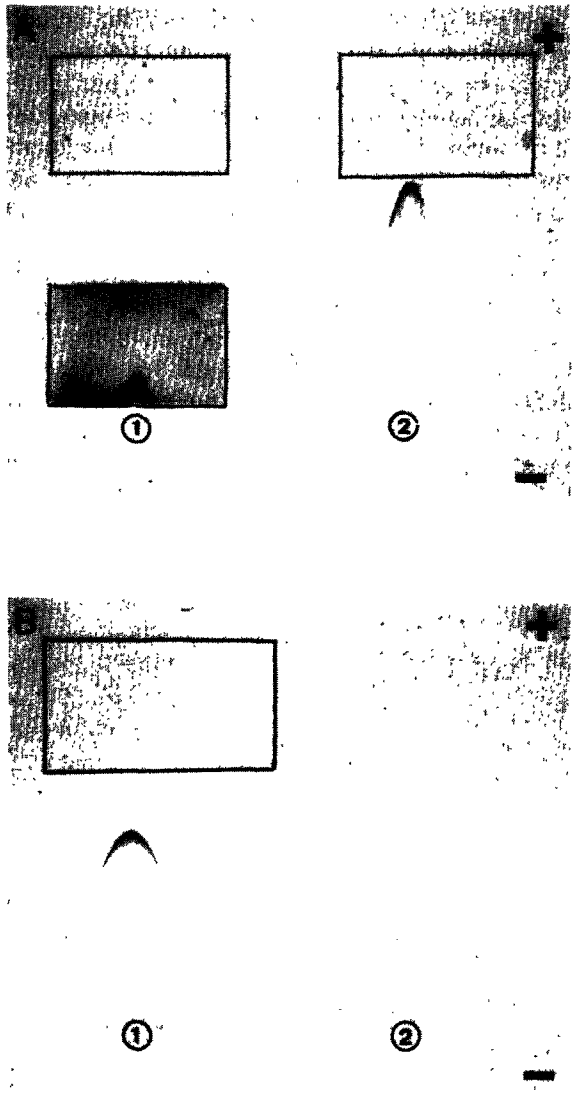

Fig.1. Immuno-affinoelectrophoresis of aspartyl–tRNA synthetase. (A) Well 1: the enzyme was electrophoresed in 1% agarose gels (w/v) containing two strips: the lower one with concanavalin A (1 mg/ml), the upper one with specific antibodies raised against yeast aspartyl–tRNA synthetase. No precipitation was observed in the upper strip containing the antibodies indicating that aspartyl–tRNA synthetase precipitated entirely in the lower strip. Well 2: precipitation occurred below the upper strip when the lower one was absent. (B) Well 1: control experiments showing the precipitation of aspartyl–tRNA synthetase below the strip containing its specific antibodies due to their electroendosmotic migration towards the cathode. Well 2: there is no precipitate at all when these antibodies are omitted. + and − refer to anode and cathode, respectively.

zyme precipitated in the presence of the specific antibodies when the strip containing concanavalin A was absent (fig.1A). In this case the precipitate was just below the upper strip due to the migration of the antibodies towards the cathode. This migration was caused by electroendosmosis. There was no precipitation at all when antibodies were absent (fig.1B).

The nature of sugars was determined by high performance thin layer chromatography (HPTLC). After exhaustive dialysis followed by hydrolysis with HCl, aspartyl–tRNA synthetase yielded free glucose mainly, if not exclusively, indeed no other neutral sugar could be visualised on the plates (fig.2). Glucose could be differentiated from galactose due to their different staining with the naphthoresorcinol reagent; indeed it yields a purple colour in contrast to the blue one given by

Fig.2. Chromatogram of sugars of the carbohydrate moiety of aspartyl–tRNA synthetase. Ascending thin-layer chromatography was carried out on a pre-coated silica gel plate using the solvent system ethyl acetate:propanol-1:propanol-2:water (8:5:1:1, by vol.). Numbers correspond to following spots: 1, mannose; 2, galactose; 3, glucose; 4, fucose; 5,6, aliquots of aspartyl–tRNA synthetase after exhaustive dialysis and hydrolysis at 100°C by 1 N HCl for 2 h and 2 N HCl for 4 h, respectively; 7, aliquot of the solution outside the dialysis bag containing the enzyme and treated by 2 N HCl for 4 h at 100°C.

Table 1

Carbohydrate analysis of aspartyl–tRNA synthetase as determined by gas-liquid chromatography

Carbohydrate	mol/mol enzyme
Ribose	0.03
Fucose	not detected
Galactose	0.05
Mannose	0.09
Glucose	1.97
N-Acetyl glucosamine	0.09
N-Acetyl galactosamine	0.04
Neuraminic acid	not detected

galactose or mannose. In all cases, blank experiments were carried out on aliquots of the solution outside the dialysis bag containing the enzyme to check for the absence of any glycopeptide or non-covalently bound sugar.

The results obtained by gas-liquid chromatography also show that significant amounts of glucose were present in the sample despite the prolonged dialysis performed prior to their methanolysis. The other sugars usually found in most glycoproteins were only present in trace amounts (table 1).

3.2. *In vitro glycosylation of yeast aspartyl–tRNA synthetase by labelling the enzyme with glucose or glucose 6-phosphate*

Aspartyl–tRNA synthetase was incubated at 4°C with [^{14}C]glucose or [^{14}C]glucose 6-phosphate and a 30-fold molar excess of sugar to determine whether the enzyme could be glycosylated in vitro. The amount of radioactivity incorporated into an acid-precipitable fraction was measured after 15 days: at this stage up to 20 mol of sugars (glucose or glucose 6-phosphate) were incorporated per mol enzyme. The covalent attachment of radioactive sugar to the aspartyl–tRNA synthetase was checked by immunoelectrophoresis. The antibodies raised against the enzyme display a radioactive peak of immunoprecipitation (rocket) with aspartyl–tRNA synthetase (fig.3). With both glucose and glucose 6-phosphate we also observed that this in vitro glycosylation could be accelerated by addition of a yeast crude extract to the purified enzyme (not shown). Nevertheless, the experiments performed with purified aspartyl–tRNA synthetase alone sug-

Fig.3. Rocket immunoelectrophoresis of aspartyl–tRNA synthetase against the corresponding antibodies. The enzyme was incubated with [^{14}C]glucose prior to the experiment and the radioactive peak of immunoprecipitation was detected by autoradiography using Fuji X-ray films.

gest that glycosylation can occur through a non-enzymatic mechanism.

4. DISCUSSION

Glycosylation of hemoglobins [25–28], albumin [29], human low density lipoprotein [30] and lens crystallins [31,32] have recently been reported. The covalent binding between glucose and protein involves free amino groups of the latter. In the case of hemoglobins, glycosylation occurs in vivo or in vitro by a condensation between glucose and the amino-terminal valine of the β-chains. The mechanism proposed in [25,33] is the formation of a Schiff base with the amino group of valine and an Amadori rearrangement to form an acid-stable ketoamine adduct.

The results obtained here are consistent with the presence of glucose covalently linked to yeast aspartyl–tRNA synthetase. Glucose is sometimes an external contaminant present in the biological preparations. This is why special precautions were taken such as exhaustive dialysis and blank experiments on buffers and solutions corresponding to the last dialysable fractions. Moreover, unlike the other yeast aminoacyl–tRNA synthetases tested, namely phenylalnyl–, arginyl–, valyl– and leucyl–tRNA synthetases, aspartyl–tRNA synthetase gave a positive and unambiguous answer as to the presence of covalently bound glucose. This enzyme is extensively studied in our laboratory and the elucidation of its amino acid sequence and 3-dimensional structure are in progress. The crystallisable aspartyl–tRNA synthetase has a dimeric structure made up of two identical subunits since there is only one amino- and one carboxy-terminal sequence (Reinbolt et al., personal communication). This is somewhat contradictory to the microheterogeneity (presence of several bands) observed by polyacrylamide gel electrophoresis and by isoelectrofocusing (Lorber et al., personal communication). When isolated, the different bands turned out to have the same amino- and carboxy-terminal sequences. This micro-heterogeneity could well be due, at least partly, to glycosylation of aspartyl–tRNA synthetase. Moreover, during prolonged dialysis a significant amount of enzyme precipitates. The insoluble material contains an amount of glucose higher than the soluble one. It may well be that glycosylation of aspartyl–tRNA synthetase enhances its ability to aggregate possibly through disulphide bond formation thus giving rise to high M_r species.

At present, the physiological significance of such a glycosylation is not clear. However this covalent attachment of glucose to aspartyl–tRNA synthetase is obviously very different from natural glycosylation occurring in glycoproteins.

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