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Evidence for a role of phosphofructokinase and tRNA in the polyribosome disaggregation of amino acid deficiency

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The activity of rabbit muscle phosphofructokinase was inhibited by transfer ribonucleic acid. This inhibition was reduced by inclusion of an aminoacyl-tRNA charging system. The results are discussed in terms of the loss of ATP in amino acid deprived cells and in the critical role of fructose 1,6-diphosphate in peptide chain initiation.

Phosphofructokinase; Transfer ribonucleic acid; Amino acid control

1. INTRODUCTION

Many cell types, other than reticulocytes, exhibit a disaggregation of their polyribosome structure during an amino acid deficiency [1]. This is interpreted as an inhibition of peptide chain initiation [1]. Under similar conditions the polyribosomes of reticulocytes are not disaggregated. Instead completed 'frozen' polyribosomes accumulate and are blocked in ribosomal travel along messenger-RNA at codon sites corresponding to the deficient amino acids [2–4].

It has been known for 20 years that amino acid deficiency in HeLa cells is accompanied with a 50% loss of the cells' ability to incorporate P^{32} into nucleoside triphosphates [5]. Subsequent studies have shown a loss in glycolytic capacity and that deprivation of either amino acids or glucose brings about a loss of ATP and similar disaggregation of polyribosomes [6]. Reticulocytes do not require glucose as an energy source for protein synthesis during short term anaerobiosis [7,8] because of their abundant supply of endogenous 2,3-diphosphoglycerate [9]. A block in glucose metabolism would therefore not result in the rapid loss of cellular ATP, and the resulting polyribosome disaggregation, as seen with most other cells.

Deacylated tRNA has been suspected as the cellular mediator for recognition of amino acid deficiency [10,11]. However, it was not active in blocking peptide chain initiation in cell free systems [12]. In this communication, evidence is presented which indicates that the block by such tRNA is manifested by inhibition of phosphofructokinase, the key enzyme regulator of glycolysis [13].

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2. MATERIALS AND METHODS

The assay used to follow the activity of rabbit muscle phosphofructokinase, PFK (EC 2.7.1.11) is based on conversion of the reaction product, fructose 1,6-diphosphate to α-glycerophosphate in the presence of aldolase (EC 4.1.2.13), triosephosphate isomerase (EC 5.3.1.1), α-glycerophosphate dehydrogenase (EC 1.1.1.8) and β-NADH. The rate of oxidation of NADH is followed spectrophotometrically at 340 nm. This method, originally proposed by Racker [14] has been used extensively by many investigators for assay of the enzyme in various cells and tissues [see (1975) Methods Enzymol. 42, 67–115 pt.C, and (1982) 90, 35–82 pt.E.].

All solutions were prepared in a medium, BESME, buffered by BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 0.05 M, pH 7.1 at 25°C, and containing dithiothreitol, 1 mM, fructose-6-phosphate, 0.2 mM, magnesium acetate 2 mM, and gentamycin 50 μg/ml. This solution was sterile filtered and stored at 4°C. The NADH was dissolved in the above medium so that an absorbancy of 1.33 at 340 nM was obtained in the final reaction volume. Rabbit muscle phosphofructokinase was obtained as a suspension of crystals in 1.4 M ammonium sulfate. The crystals were sedimented, a concentrated solution in BESME prepared and stored at −20°C. The three auxiliary enzymes indicated above were crystalline preparations in 2–3 M ammonium sulfate. The crystals were sedimented, dissolved in BESME and stored frozen. As suggested by Uyeda and Racker [15] no dialysis was performed because of activation of PFK by ammonium ion. The adequacy of these auxiliary enzymes in the assay system was tested by addition of fructose 1,6-diphosphate and obtaining an instantaneous drop in absorbancy. This was particularly evident under the inhibitory conditions to be described.

ATP (disodium salt) was dissolved in BESME to yield a concentration of 1–5 mM. The transfer ribonucleic acid, from rabbit liver, ranged in acceptor activity of 11–43 pmol per A_{260} unit. It was dissolved in BESME and its concentration estimated, based on A_{260} of 21.4 for 1 mg/ml and a nominal molecular weight of 25000. The

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amino acid supplement comprised the complete minimal essential mixture for tissue culture of essential and non-essential amino acids [16] neutralized and dissolved in BESME. The aminoacyl-tRNA synthetase was a crude preparation from rabbit reticulocytes kindly provided by Dr Dolph Hatfield of this Institute. All other components were products of the Sigma Chemical Co., St. Louis, MO, USA.

Stock phosphofructokinase solution was diluted with BESME at ambient temperature to a dilution which permitted measurable rates of NADH oxidation. The reaction was initiated by addition of PFK.

3. RESULTS

The activity of PFK supported by 2 mM ATP and inhibition of such activity at 5 mM is shown in Fig. 1. Also shown is the additional inhibition by micromolar concentrations of tRNA at the higher ATP concentration. Inhibition by tRNA was not observed at the lower ATP concentration. Such a role of inhibitory concentrations of ATP as a critical factor in promoting the efficacy of other inhibitors upon this allosteric enzyme has long been noted [13]. Indeed, Bridger and Henderson [17] have implied that intracellular PFK should be almost completely inhibited at the physiological concentrations of ATP.

During the course of this investigation it was observed that a concentration of ATP at 5 mM at times caused such a large inhibition that the additional effect of tRNA could not be ascertained. It was subsequently found that the auxiliary enzymes, aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase, increased PFK activity under these conditions, and then that ammonium sulfate, carried over with these enzymes, was all that was needed to increase phosphofructokinase activity in the presence of a high concentration of ATP. Since the ammonium ion is an activator which does not affect the ATP inhibition or binding [15,18,19], but increases the enzyme's affinity for fructose-6-phosphate, it may be assumed that PFK retains its allosteric form induced by high ATP. This increased activity opens the range where the additional inhibition by tRNA can be observed.

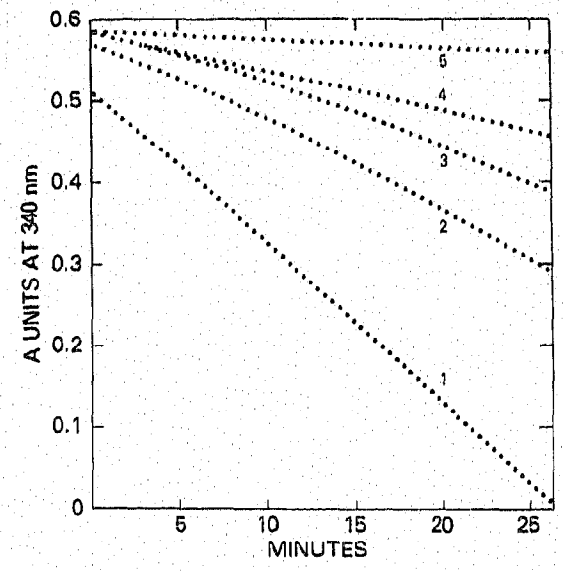

Fig. 1. After equilibration to ambient temperature a solution of PFK was added to cuvettes and rapidly mixed. The rate of NADH oxidation was determined as described in section 2 with (1) 2 mM ATP, (2) 5 mM ATP, (3) 5 mM ATP and 1 μM tRNA, (4) 5 mM ATP and 3 μM tRNA and (5) 5 mM ATP and 10 μM tRNA. Curve 3 was obtained from a replicate experiment where conditions for (2) and (4) were superimposable.

Table I

Protection of phosphofructokinase from tRNA inhibition by aminoacyl-tRNA synthetase and amino acids

	Percent activity
Phosphofructokinase	100
+ tRNA	48
+ tRNA + aminoacyl-tRNA synthetase	46
+ tRNA + aminoacyl-tRNA synthetase + amino acid mixture	96

After equilibration at ambient temperature, the assay for phosphofructokinase was initiated as indicated in Fig. 1, with an ATP concentration of 5 mM and a tRNA concentration of 9 μM. The amino acid mixture concentration was 7% listed as required for tissue culture [16], and by itself, had no effect on tRNA inhibition of phosphofructokinase activity.

Addition of a preparation of aminoacyl-tRNA synthetases and amino acids prevented the inhibition of PFK by tRNA (Table I), thus lending further support to the direct inhibition of uncharged tRNA on PFK activity.

4. DISCUSSION

Several reports in the literature indicate that uncharged tRNA does not directly inhibit peptide chain initiation. Thus Pain et al. [11] could find no evidence of an inhibitor of initiation complex formation in mixing experiments with extracts of Ehrlich ascites cells fed with or starved of lysine. Similar mixing experiments identified an inhibitor of globin chain initiation in extracts of reticulocytes fed with or starved of hemin [20]. In addition, no major defect could be observed in extracts of starved cells in the translation of exogenous mRNAs [11]. This might be anticipated if run-off and polyribosomal ribosomes were compared in a reconstituted system with an independent energy source. Subsequently, Austin et al. [12] reported that uncharged tRNA, oxidized with periodate to prevent recharging, did not directly inhibit peptide chain initiation, thus supporting the role of an indirect mechanism.

Austin and Clemens, in their review [1] point out that amino acid deficiency results in disaggregation of cellular polyribosomes before a drop in ATP as described by Smulson [5], Van Venrooij et al. [6] and Grummt and Grummt [21] becomes evident. Another, earlier sensor is required. The role of phosphorylated

sugars, particularly fructose 1,6-diphosphate, the product catalyzed by phosphofructokinase, has been recognized as having a role in peptide chain initiation for over a decade [22–25]. This activity may be mediated by stimulation of Met-tRNA$_f^{Met}$ binding to the 40 S native ribosomal subunit [22] without altering the rate of formation of the ternary complex, Met-tRNA$_f^{Met}$:initiation factor eIF-2:GTP [23]. Wu [24] suggested that its activity resides in a prevention of inhibition of such ternary complex formation by m-RNA and he and Suhadolnik [25] demonstrated a unique role of fructose 1,6-diphosphate among phosphorylated sugars in inhibition of met-tRNA$_f^{Met}$ deacylase, a controlling enzyme in peptide chain initiation.

Fodge and Rubin [26] have suggested that several stimulants of cell multiplication function through activation of PFK. This may represent the 'restriction point' of Pardee [27], a control site in metabolism where normal animal cells are blocked in division by an amino acid deficiency and enter a quiescent state. He presents evidence that malignant cells have lost their restriction point control. It is therefore of interest that Boscá and Corredor [28] report that unlike in liver, muscle, erythrocytes and platelets, phosphofructokinase is not the limiting enzyme of glycolysis in ascites tumor cells, and it may be inferred that this could be a cause for their continued, less restricted growth under deficiency conditions.

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