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Uncharged tRNA-phosphofructokinase interaction in amino acid deficiency*

Review Article

M. Rabinovitz

NIH, Bethesda, Maryland, U.S.A.

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Summary. When the tRNA of mammalian cells is incompletely charged due to amino acid deficiency or by analogs which cannot be activated, many metabolic events become limited. This rapid demise of cell function appears to be due to the inhibition of phosphofructokinase (PFK) by uncharged tRNA (FEBS Lett 302: 113 (1992)). Charged tRNA has been shown to be "sequestered within the protein synthetic machinery", (Negrutskii, B. S. and Deutscher, M. P. (1992) Proc Natl Acad Sci USA 89: 3601) and would therefore be removed from an inhibitory role. Besides the direct demonstration that tRNA inhibits PFK in an assay regarded as indicative of its control mechanism, several reports in the literature support this model. These include 1) The rapid onset of inhibition of glycolysis and glucose uptake by intact cells upon amino acid deficiency and the similar lesion at the 43S ribosomal subunit on glucose or amino acid deprivation. 2) The recognition that unusually high concentrations of cAMP required to stimulate protein synthesis in energy depleted or gel filtered lysates correlates with its action on PFK as an analog of the positive effector, adenosine-5′-monophosphate. 3) The often repeated observation that the product of PFK activity, fructose-1,6-diphosphate, is a stimulant of protein synthesis (see Jackson, R. J., et al. (1983) Eur J Biochem 131: 289). This diphosphate has been shown to be the proximate effector binding to eIF-2B, the guanine nucleotide exchange factor (Singh, L. P. Arror, A. R. and Wahba, A. J. (1994), FASEB J. 8: 279) which by releasing GDP bound to the inactive GDP:eIF-2 complex, permits the factor to initiate a new peptide chain. The above information supports the view that the block at the G1 restriction point in the cell cycle of normal cells brought about by amino acid deprivation is a result of inhibition of protein synthesis through the phosphofructokinase-uncharged tRNA mechanism. This is consistent with

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observations in the literature that tumor and transformed cells, which are more resistant to this block (Pardee, A. B., Proc Natl Acad Sci USA 71: 1286–1291 (1974)) have a higher phosphofructokinase activity or higher levels of fructose-1,6-diphosphate.

Keywords: Amino acid deficiency – Uncharged tRNA – Phosphofructokinase – Fructose-1,6-diphosphate – G1 Restriction point

Since the pathway of glycolysis has been established for many decades, the possibility that amino acid availability for protein synthesis could regulate this process has not been a topic of major consideration. Yet reports have been made from time to time that in organized systems, as opposed to cell-free extracts, glycolysis is diminished under conditions of amino acid deprivation.

Thus, in a cell culture of rat fibroblasts transfected with *ras* or *myc* oncogenes which had high rates of aerobic glycolysis, Racker et al. (1985) reported that high concentrations of the unmetabolizable amino acid, methylaminoisobutyric acid and others, inhibited glycolysis. They recognized that this was due to transinhibition of transport, in effect blocking the uptake of essential amino acids by the cells. A related inhibition of glycolysis and glucose uptake due to deprival of essential amino acids was reported by Van Venrooij et al. (1972) with the use of Ehrlich ascites cells in culture. They reported that deficiency of either glucose or several essential amino acids caused a similar disaggregation of polyribosomes to monoribosomes and ribosomal subunits, indicating a block in peptide chain initiation. A similar observation, again with Ehrlich ascites cells in culture was made by Pain and Henshaw (1975) who reported that deprivation of either glucose or lysine resulted in the same molecular lesion, a disaggregation of polyribosomes and a decrease in the amount of the small ribosomal subunit containing initiator Met-tRNA. The block in glycolysis coupled to an inhibition of glucose uptake suggested that the inhibition might be at the site of phosphofructokinase for, as Bloxham and Lardy state in their review on this enzyme (Bloxham and Lardy, 1973) (with references omitted here).

"A metabolic crossover point* at the level of phosphofructokinase has been demonstrated in a variety of situations. Starvation, alloxan diabetes (equivalent to insulin withdrawal), or exposure to fatty acids leads to a decrease in glycolysis which is associated with a rise in glucose-6-P and fructose-6-P concentrations and a fall in fructose diphosphate concentration. This effect can be demonstrated in the perfused heart, skeletal muscle and kidney cortex slices and clearly indicates that the decrease in glycolysis is related to a decrease in phosphofructokinase activity". This increased glucose-6-phosphate level would serve to block glycolysis by inhibiting

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* A metabolic crossover point is defined as a point in the sequence of enzymatic reactions, such as glycolysis, where an inhibition is made evident by an increase in an enzyme's substrate and a decrease in the product of its activity.

hexokinase, the enzyme involved in its formation which is very sensitive to product inhibition.

In this review evidence is presented which has been obtained from all levels of mammalian biological organization, including the intact animal, cell culture, cell lysates and highly purified enzymes that phosphofructokinase and the product of its activity, fructose-1,6-diphosphate play a single key role in the control of protein synthesis. In addition, support is given for this mechanism as the basis for the G1 block of cell cycle progression which takes place in amino acid deficiency. In effect the *restriction point* block in progression, which is less evident in virally transformed and tumor cells (Pardee, 1974) is located at the *metabolic crossover point* cited in the quotation from Bloxham and Lardy (loc. cit.).

The liver in tryptophan deficiency

In 1968 Munro (1968) and Sidransky et al. (1968), reported that tryptophan deficiency in rats and mice is rapidly followed by disaggregation of liver polyribosomes. Residual liver tryptophan is quickly metabolized and becomes limiting (Biggio et al., 1974) causing a decrease in charging level of its tRNA (Allen et al., 1969). Jefferson and Korner (1969) with the use of a perfused rat liver system soon demonstrated that depletion of several other amino acids also caused such breakdown of polyribosomes. That this could be due to the inhibition of phosphofructokinase may be inferred from evidence presented by Oravec and Sourkes (1970) who showed that α-methyltryptophan, which *decreased* hepatic protein synthesis by stimulating pyrrolase activity and tryptophan degradation, *promoted* gluconeogenesis. Such promotion of gluconeogenesis through inhibition of phosphofructokinase has been reported for glucagon (Claus et al., 1980). By inhibiting glycolysis at this step, the action of the opposing enzyme of the phosphofructokinase-fructose diphosphatase couple, fructose diphosphatase, is accentuated, thus enhancing the pathway for reversal of glycolysis (Clark et al., 1974).

Uncharged tRNA as the signal reporting amino acid deprivation

Several reports in the literature suggest that uncharged tRNA is the cellular signal reporting amino acid deprivation. Thus Vaughan and Hansen (1973) found with both diploid human embryonic fibroblasts and HeLa cells that amino acid analogs which inhibit charging of the tRNA corresponding to their natural metabolites rapidly cause the disaggregation of the cellular polyribosomes. They used histidinol to block charging of histidine-tRNA and O-methylthreonine to inhibit charging of isoleucine-tRNA. Of particular interest was their observation that when the analogs were added together the degree of protein synthesis inhibition was additive. *A priori*, one would expect that the extent of inhibition would be limited to that obtained by the more stringent deprivation, since a co-existing lesser deficiency should supply suffi-

cient product to support the rate determined by the more stringent deficiency. However, if the signal were uncharged tRNA, its combined accumulation could result in an additive inhibition.

Further support for a role for uncharged tRNA comes from studies with mutant cells which have a temperature sensitive aminoacyl tRNA synthetase that cannot charge its tRNA at the non-permissive temperature (Clemens et al., 1987). In such cases the effects of amino acid deprivation can be observed without nutritional manipulation. The possibility that the signal for events following the appearance of uncharged tRNA could be mediated by a small molecular metabolite, such as guanosine tetraphosphate (ppGpp), as observed in bacteria, has been considered and discounted in a review by Silverman and Atherly (1979). However in experiments where uncharged tRNA was added to lysates of Ehrlich ascites cells, Austin et al. (1972) conclude that uncharged tRNA is not *directly* involved in inhibiting peptide chain initiation. This is correct, since it has been shown that the mechanism (Fig. 1) involves inhibition of phosphofructokinase by uncharged tRNA (Rabinovitz, 1991, 1992) an *indirect* action which limits the availability of the product of the enzyme's activity, fructose-1,6-diphosphate, the proximate effector (vide infra). The model suggests that no specificity is accorded the uncharged species, but that, as reported by Negrutskii and Deutscher (1991, 1992), the charged form is completely sequestered within the protein synthetic apparatus and is therefore unavailable for interaction with phosphofructokinase.

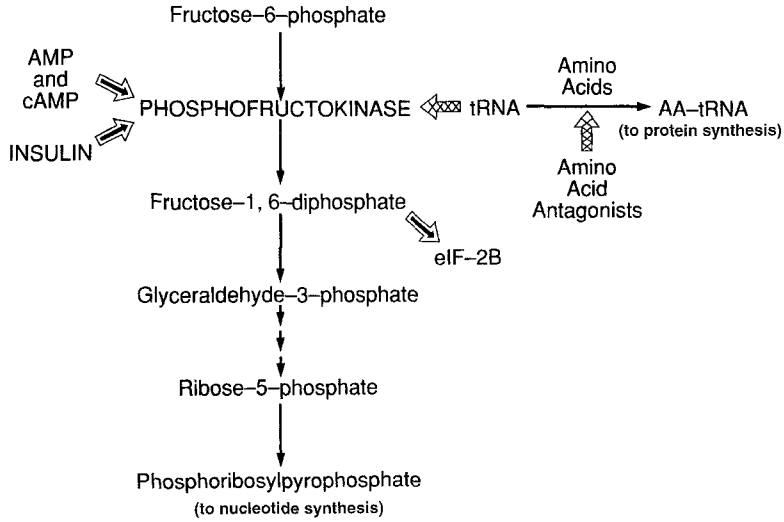

Fig. 1. Diagrammatic outline of interacting effectors. Broad arrows with internal arrows, stimulation. Broad arrows with XX, inhibition. The interaction between yeast phenylalanine tRNA and rabbit muscle phosphofructokinase is described (Rabinovitz, 1992). It involved interaction of the tRNA with the inactive dissociated forms of the enzyme thereby blocking their equilibrium with the active tetramer. This type of control has been generally accepted for modulation of phosphofructokinase interactions (Lehotzky et al., 1993 and references cited therein). All other interactions are described in the indicated literature

Cyclic AMP, only at high unphysiological concentrations stimulates both phosphofructokinase and protein chain initiation

Cyclic AMP, in the millimolar range, far above its normal concentration as a co-factor, stimulates lysates of energy depleted reticulocytes (Giloh-Freudenberg and Mager, 1975) and gel filtered lysates of these cells (Legon et al., 1974; Wu et al., 1978) at the initiation stage of translation. Also Pinilla and Luque (1977) reported that such high concentrations were required to activate phosphofructokinase of rat erythrocyte lysates, mimicking linear AMP, a well recognized allosteric activator of the enzyme. With respect to its role in peptide chain initiation Legon et al. state (1974) "The high concentration required would seem to rule out any direct involvement in the normal regulatory process but one might speculate that it is acting as an analogue of some small molecule which is directly involved". With respect to its role as phosphofructokinase activator Sols explains (Sols, 1981) "The complexity regarding the number of different sites for adenylnucleotides is increased by the report of cAMP as an activator of animal phosphofructokinase; this report was first made by Mansour and Mansour as early as 1962. Nevertheless, cAMP, at least in higher animals, is merely an analog of AMP, acceptable in vitro but never able to act *in vivo*, because *in vitro* cAMP operates only in the physiological concentration range for AMP, a concentration which cAMP never approaches *in vivo* because of thermodynamic limitations. For this reason there never has been selective evolutionary pressure to have an AMP site discriminating against cAMP. Exactly the opposite should be expected for a cAMP regulatory site, which needs both high affinity for cAMP and sharp discrimination with respect to the much more abundant AMP".

This coincidence, the requirement for an extraordinary high concentration of cyclic AMP to stimulate both the initiation of peptide chain translation and the activity of phosphofructokinase suggests that the nucleotide may be stimulating this enzyme in the lysate engaged in protein synthesis. Further support for this view is the report that this nucleotide acts synergistically with fructose-1,6-diphosphate in a gel filtered lysate of reticulocytes which is engaged in peptide chain initiation (Wu et al., 1978).

Fructose-1,6-diphosphate, the proximate effector

The indications of a role for phosphofructokinase in metabolic control is supported by evidence that the product of its activity, fructose-1,6-diphosphate is a participant in peptide chain initiation. Its role had been somewhat obscured by evidence that another sugar phosphate, glucose-6-phosphate was also an activator of protein synthesis in gel-filtered reticulocyte lysates. However, in a series of three papers (Jackson et al., 1983a,b; Hunt et al., 1983). Jackson, Hunt and coworkers demonstrated that the glucose phosphate acted solely as a substrate for glucose-6-phosphate dehydrogenase, generating NADPH to maintain a reductive environment. Other methods for maintaining a reductive environment were also effective, such as with a functional thioredoxin/thioredoxin reductase system, isocitrate, or dithiothreitol.

Fructose-1,6-diphosphate did not provide for a reductive environment but acted synergistically with dithiothreitol to promote peptide chain initiation. The diphosphate has been shown to be the best sugar phosphate stimulant (Gross et al., 1991a) and bound allosteric effector (Singh et al., 1994) of eIF-2B (also known as GEF) the guanine nucleotide exchange factor. eIF-2B permits the recycling of the initiation factor eIF-2 (Konieczny and Safer, 1983; Aroor et al., 1994), which is a component of the ternary complex, GTP:eIF-2:met-tRNA$_I$ and becomes associated with the small ribosomal subunit in the process of initiation. Upon formation of the 80S initiation complex GTP is hydrolyzed to GDP, which is released along with eIF-2 (Gross et al., 1991b). Only through the action of eIF-2B can eIF-2 be reconstituted with GTP. When the former factor is limiting, there is a reduction in the level of the small ribosomal subunit containing the ternary complex(43S subunit). This is seen under all conditions of deprivation: that of glucose and amino acids in cellular preparations and fructose phosphates in lysate preparations (loc. cit.). Added purified eIF-2B to gel filtered reticulocyte extracts can satisfy the requirement of sugar phosphates (Jackson and Hardon, 1985) and when added to extracts of Chinese hamster ovary cells can repair the defect of amino acid deficiency (Clemens et al., 1985).

The phosphofructokinase crossover point is related to the G1 amino acid restriction point

Pardee (1974) reported the presence of a restriction point in the cell cycle of "normal" cells under conditions of amino acid deprivation. Such cells became arrested in the G1 phase of the cell cycle and remained viable for extended times (Allen and Moskowitz, 1978 and references cited therein). Several transformed or cancer cells however were not turned off, and by continued proliferation died of the deficiency and were susceptible to the cytotoxicity of S phase chemotherapeutic agents. This observation has received several confirmations (Newman et al., 1983 and references cited therein). Using the above approach Warrington (1978) was able to eliminate HeLa cells cocultivated with normal human fibroblasts. However, with the same cell types in a study specifically concerned with the control of initiation of protein synthesis, Vaughan and Hansen (1973) found that no essential difference existed between them. Indeed, as described previously, others have used Ehrlich ascites cells to study the disaggregation of polyribosomes under conditions of amino acid deprivation and found a rapid response to this deficiency. A resolution to what appears to be this anomaly was found by Newman et al. (1983). With the use of BALB/3T3 cells and their virally transformed counterparts SV-T2 cells, they found that BALB/3T3 cells could be reversibly inhibited in cell cycle traverse by 1.5 mM histidinol but that SV-T2 cells required 4.0 mM inhibitor. Thus, there is a window of opportunity to bring about a selective reversible inhibition of proliferation in the normal cell. In view of the earlier discussion in this paper, it is concluded that the selectivity is based on the level of phosphofructokinase activity among the different

cell types, a more stringent deficiency being required where a higher phosphofructokinase activity exists. Such extranuclear regulation of the cell cycle has been suggested by Pardee (1994).

Most tumor or transformed cells have higher phosphofructokinase activities and fructose-1,6-diphosphate concentrations than their normal counterparts. Thus Vora et al. (1985) report on the isozyme profile and increased activity of phosphofructokinase in various human neoplasms. Boscá and Corredor (1984) review the reports that ascites tumor cells have 65 times the fructose-1,6-diphosphate concentration as liver or muscle and 300 times the concentration of erythrocytes and platelets. Higher phosphofructokinase activities in neoplasms compared to their tissue of origin has also been reported for rat hepatoma (Dunaway et al., 1972) rat glioma (Dastidar and Sharma, 1989) human thyroid (Van Der Heijden et al., 1986) and in virus transformed chick embryo cells (Singh et al., 1974). Although there was only a 70% increase in phosphofructokinase activity, an analysis of glycolytic intermediates indicated a three fold increase in fructose-1,6-diphosphate (Singh et al., 1974). Indeed, estimation of enzyme activity in cell lysates may not be indicative of cellular activity, which can be elevated by the potent stimulant, fructose-2,6-diphosphate. This phenomenon is discussed by Kole et al. (1991 and references cited therein) for *ras*-transformed rat fibroblasts, and has been recently reviewed (Hue and Rousseau, 1993).

Summary and projected studies

The central role of phosphofructokinase modulation in metabolic control and that of the product of its activity, fructose-1,6-diphosphate has received attention in several quarters (Ovádi, 1988; Sols et al., 1981). The mechanism described here emphasizes this activity in yet another respect, that relating to amino acid insufficiency. Although a direct effect of tRNA on phosphofructokinase activity in a purified enzyme system has been found and several correlations between this enzyme and its product at the initiation stage of protein synthesis is described in cell lysates, the cellular correlations noted varied across a broader biological spectrum. Although this supports the global nature of this control mechanism, a more restricted verification would be desirable. For example, Elson et al. (1992) have transfected PC 12 cells with human liver type phosphofructokinase which then became overexpressed in these cells. One might test to see whether such cells would be less sensitive to amino acid depletion, requiring a more stringent deficiency to show the enzyme's crossover point and thus would lose the restriction point of their normal counterpart.

Another area for investigation has been indicated by Sols et al. (1981) as the "in situ approach", utilizing permeabilized cells to bridge the gap between cell free and cellular conditions. A similar approach has been applied by Negrutskii and Deutscher (1991, 1992) in their evaluation of the sequestration of aminoacyl-tRNA within protein synthetic machinery. Thus, the addition of phosphofructokinase to cells by permeabilization and sealing or by continu-

ous exposure to fructose-1,6-diphosphate may convert them to a metabolic state resembling the neoplastic phenotype. One might expect such cells to lose their G1 restriction point if they could still progress through the cell cycle.

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Author's address: M. Rabinovitz, Ph.D., 4504 Traymore Street, Bethesda, MD 20814-3965, U.S.A.

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