tractable from trabecular as opposed to cortical bone, when expressed as units per milligram of hydroxyproline. The difference is still 2-fold when expressed in terms of units per milligram of calcium, but the hydroxyproline content is probably a more appropriate index of bone matrix in these samples than is calcium. Although we need to directly monitor the recovery of hSGF in our demineralization/extraction method to verify these findings, the observation that more hSGF activity is extractable from trabecular than cortical bone could be significant in terms of regulation, particularly since we know that trabecular bone responds differently than cortical bone in human subjects with osteoporosis in response to fluoride therapy (Bang et al., 1978). We should note that these yields, about 400 units/g of bone, are in reasonable agreement with our previous estimate (Farley & Baylink, 1982) and correspond to a concentration of about 100 μg/g of bone. Because in adult humans the amount of bone resorbed per day is about 1.7 g (Harris & Heany, 1969; Wergedal & Baylink, 1974), we can estimate that about 170 μg of hSGF would be released per day. This would represent a very low local concentration at a specific resorption site, relative to the dose/response values given in Figure 4.

In summary, we have shown the effect of hSGF on bone cell proliferation in vitro is inductive and prolonged and may be mediated by some secondary mechanism. This mitogenic effect does not depend on the synthesis of prostaglandin or cAMP. The magnitude of the mitogenic effect depends on initial cell density as well as on hSGF concentration, and it can be observed in confluent cultures.

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Specific Activation of Particulate Leucyl-tRNA Synthetase Complexes†

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ABSTRACT: We have identified a specific activation of the high molecular weight form of leucyl-tRNA synthetase. The form at 30 S is activated 3–5-fold by a 2.5S cytoplasmic factor, which has no effect in the activity of the smaller 8S form of leucyl-tRNA synthetase. This 2.5S activator appears to have pyrophosphatase activity.

The key role of aminoacyl-tRNA synthetases in the translational process suggests these enzymes could be critical targets for regulatory factors. The existence of such factors has indeed been claimed by a number of laboratories [see Dignam & Deutscher (1979) for a review]. Dignam & Deutscher (1979) very nicely showed that most described aminoacyl-tRNA synthetase activators in the literature may be inorganic pyrophosphatase, whose mode of activation is simply to remove the very powerful aminoacyl-tRNA synthetase inhibitor inorganic pyrophosphate. The alanyl-tRNA synthetase activator described by Hilderman (1977), however, appears to be a real

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regulator of the alanyl-tRNA synthetase activity in mammalian cells.

The aminoacyl-tRNA synthetases in mammals are often found in large complexes. These were first identified by Hird et al. (1964) and were later described in greater detail (Bandyopadhyay & Deutscher, 1971). They have since been described in a large number of laboratories with some variations noted between systems [see Denney (1977) for a review]. We have extensively described the complexes in Chinese hamster ovary (CHO)[1] cells (Hampel et al., 1979; Ritter et al., 1979).

While the existence of these complexes has been confirmed, the identification of functional roles has been more difficult. The complexes may contain RNA methylation enzymes (Agris et al., 1976), tRNA sulfur transferase (Harris et al., 1977), and other translational factors as well (Smulson et al., 1975). Thus the complexes appear to have a number of enzymatic activities involved in tRNA maturation, in tRNA activation, and in the translational process itself. Our laboratory has correlated the existence of these complexes with the physiological state of the cell. A decrease in the amounts of the large 30S aminoacyl-tRNA synthetase complex relative to the 20S complex is seen for four enzyme activities specific for Glu, Pro, Ile, and Leu in G-1-arrested CHO cells (Enger et al., 1978). Thus variations in the forms of these enzymes can occur under different cellular conditions.

Leucyl-tRNA synthetase (LeuRS) has three forms of the enzyme at 8 S, 20 S, and 30 S (Hampel et al., 1978), and this report describes a specific activation of the 30S particulate form of LeuRS with some effect on the 20S form and no effect on the initial velocity of the 8S LeuRS enzymatic activity. The CHO activator is about 2.5 S, has a M_w of 62000, and appears to have inorganic pyrophosphatase activity. Yeast inorganic pyrophosphatase has a similar activating effect on 30S LeuRS but is different since it also stimulates the 8S form of LeuRS to a lesser but real extent. The phenomenon of preferential activation of the LeuRS complexes presents a mechanism by which a simple cellular regulator can differentially control particulate enzymatic activity and could have in vivo significance.

Materials and Methods

Cell Culture. Cells used were Chinese hamster ovary (CHO) wild type (WT) (Puck et al., 1958) and the leucyl-tRNA synthetase mutant *ts*H1 (Thompson et al., 1973). Cells were grown on α-MEM (modified) medium (Flow Laboratories) with 10% calf serum (Microbiological Associates) and 50 μg/mL gentamicin sulfate (Schering Corp.). Filtered L-glutamine was periodically added to prepared media to replenish that broken down at a rate according to Tritsch & Moore (1962). Mycoplasma contamination was checked on live cells according to the Hoechst 33258 dye binding technique in Lab-Tek chambers (Chen, 1977).

Cells were grown at 34.5 °C in suspension culture to a density of 5×10^5 cells/mL (cell growth was logarithmic to 8×10^5 cells/mL) and collected by placing spinners in ice and simultaneously adding 250 mL of frozen (−20 °C) 0.25 M sucrose ice cubes/L of culture. Cooled cells were collected by centrifugation (1000$\bar{g}$, 10 min, 4 °C), resuspended with ice-cold 0.25 M sucrose, collected by centrifugation (1000$\bar{g}$, 12 min, 4 °C), resuspended (2×10^8 cells/1.8 mL of buffer) in buffer A (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5 at 25 °C), and stored frozen at −90 °C.

Sucrose Gradient Centrifugation. All operations were performed at 4 °C with nuclease-free solutions and glassware. Cells, 2×10^8, in 1.8 mL of buffer A were slowly thawed and lysed for 30 min by the addition of 0.2 mL of a 10% (w/v) Nonidet-P40 solution to give a 1% final concentration. Nuclei and cell debris were removed by centrifugation (1000$\bar{g}$, 30 min). The resulting cell lysates (2 mL) were carefully layered on 36 mL of 10–30% (w/v) linear sucrose gradients (Schwarz/Mann ultrapure sucrose) in buffer B (10 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10 mM Tris, pH 7.5 at 25 °C). The gradients were centrifuged in a Beckman SW 27 rotor at 27000 rpm for 7 or 18 h and fractionated into 1-mL fractions with an ISCO Model D gradient fractionator. $s_{20,w}$ values were calculated from a computer program as previously described (Moline et al., 1974).

Subcellular Fraction. Lysed cells (10 mL) with nuclei removed as above were centrifuged in a Beckman Type 65 rotor at 180000$\bar{g}$ for 2 h at 4 °C through a 0.75-mL 34% (w/v) sucrose pad. The pellet was labeled microsomes and the supernatant postribosomal supernatant (Hampel & Enger, 1973).

Relative amounts of the fractions as in Table I were standardized by taking the postribosomal supernatant and microsomal fractions to the same volume. Then during the assay the same volume of each fraction was used for combinations of fractions. Protein was determined by the method of Lowry et al. (1951) to monitor the protein concentration in the fractions, which was as we previously published (Hampel & Enger, 1973).

Aminoacyl-tRNA Synthetase Assay. Aminoacyl-tRNA synthetase assays were performed as previously described (Hampel et al., 1979). Leucyl-tRNA synthetase (LeuRS) activity was determined by using optimal assay conditions containing 10 mM Tris, pH 8.6 at 25 °C, 0.5 mM K_2EDTA, 0.1 mM dithiothreitol, 8 mM $MgCl_2$, 5 mM K_2ATP, 0.35 mM Na_2CTP, 0.75 mg/mL rat liver tRNA, 10 μM L-[^{3}H]leucine (10000 Ci/mol), and 10 μM each of the other 19 nonradioactive amino acids. Assays for enzyme activity in sucrose gradient fractions were done at 34 °C in a total volume of 50 μL (each contained 5 μL of the sucrose gradient fraction) and terminated after 6 min on Cl_3CCOOH-soaked disks; nonesterified amino acids were washed out, and the remaining esterified radioactivity was detected by a Mark III scintillation detector (Searle Analytic) at 60% efficiency.

Dose response with partially purified 2.5S component and 30S LeuRS was done by using peak tubes from higher resolution sucrose gradients that had been centrifuged for 18 h. Mixing of 2.5S and 30S forms was done by keeping the volume of one form constant and increasing the volume of the other in the assay mixture. A dose response both of varying 2.5S form to a constant amount of 30S form and varying 30S form to a constant 2.5S form was done. Background radioactivity and enzyme activity attributable to 30S LeuRS were subtracted from the total cpm.

Kinetic analysis was done on the post-sucrose-gradient 2.5S, 20S, and 30S fractions. The K_m and V_{max} for leucine were determined for the 20S and 30S LeuRS forms with and without the presence of the 2.5S form. Kinetic parameters for leucine were determined by using concentrations of 2, 4, 8, 12, 16, and 20 μM leucine in the assay mixture. Enzyme activity was compared for 0.5-, 1.0-, and 1.5-min assays at each leucine concentration. Double-reciprocal plots were used to calculate kinetic data.

[1] Abbreviations: CHO, Chinese hamster ovary; LeuRS, leucyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; PP_i, inorganic pyrophosphate; DEAE, diethylaminoethyl.

Table I: LeuRS Activity with Various Combinations of *ts*H1 and Wild-Type Subcellular Fractions[a]

	% of wild-type supernatant
wild-type supernatant	100
wild-type microsomes	51
wild-type supernatant and wild-type microsomes	286
*ts*H1 supernatant	13
*ts*H1 supernatant and wild-type microsomes	202
*ts*H1 microsomes	1
*ts*H1 supernatant and wild-type supernatant	134
*ts*H1 supernatant and *ts*H1 microsomes	16
wild-type supernatant and *ts*H1 microsomes	98

[a] All enzyme activities were determined at 0, 3, and 6 min and then normalized to wild-type supernatant (9.9×10^3 cpm/3-min assay = 100%).

Trypsin inactivation of the 2.5S form was done by dilution of Puck's trypsin solution 10× (final concentration 200 μg/mL trypsin). Ten microliters of diluted trypsin was mixed with 90 μL of post-sucrose-gradient 2.5S LeuRS and incubated for 10 min at 34 °C. This mixture was then immediately heated to 55 °C for 10 min to inactivate the trypsin. An aliquot of the 2.5S form was then mixed with the 30S form and assayed for activation for 6 min at 34 °C.

Inorganic Pyrophosphatase Assay. Inorganic pyrophosphatase activity was assayed as previously described (Dignam & Deutscher, 1979). Pyrophosphatase assays were performed by incubating aliquots of column fractions in 50 mM Tris, pH 7.5, 3 mM sodium pyrophosphate, and 8 mM $MgCl_2$. The total assay volume was 0.1 mL. Fractions were assayed for 15 min at 34 °C, and the assay was terminated by addition of 0.2 mL of 10% Cl_3CCOOH. Phosphorus determinations were done by adding 0.7 mL of a mixture of 1 part of 10% ascorbic acid to 6 parts of 0.42% ammonium molybdate in 1 N H_2SO_4 to the above assay mixture; the mixture was incubated for 5–10 min at 45 °C, and the absorbance at 820 nm was read (Chen et al., 1956). Yeast inorganic pyrophosphatase (Sigma, 500–600 units/mg) at activity equivalent to the pyrophosphatase activity of the 2.5S fractions was used to activate LeuRS. One unit of pyrophosphatase activity catalyzes the hyrolysis of 1 μmol of pyrophosphate in 15 min.

Molecular Weight. The molecular weight of the 2.5S LeuRS activator was determined by using Sephadex G-75 column chromatography (0.86 cm × 52 cm) at a flow rate of 12 mL h^{-1} cm^{-2}. The column was calibrated with blue dextran (V_0), K_2CrO_4 (V_i), lysozyme (14 300 daltons), ovalbumin (43 000 daltons), and yeast inorganic pyrophosphatase (63 000 daltons). The post-sucrose-gradient 2.5S form was run, the K averages for all proteins were calculated, and an estimated M_w for 2.5S activator was determined.

Results

Activation of Microsomal LeuRS. A 2–4-fold activation of LeuRS activity was observed when postribosomal supernatant was combined with the microsomal fraction of CHO cells. Table I shows LeuRS activity in various subcellular fractions and when these various subcellular fractions are combined. Wild-type microsomes had approximately 51% of the supernatant activity, but when combined with supernatant, activation was seen to a level 286% of that of wild-type supernatant. A large activation was also seen when *ts*H1 supernatant was mixed with wild-type microsomes. Thus wild-type or mutant supernatant gave a 2–4-fold increase of microsomal LeuRS activity. This enhancement of activity was not seen when microsomes from mutant *ts*H1 cells were used in place of wild-type microsomes or when combinations of supernatant were used. Furthermore, bovine serum albumin had no activation effect on any fractions. The dose response of increasing microsomal concentration with constant supernatant concentration on LeuRS activity showed the activated LeuRS activity is linear with a constant slope. A level of activation of about 2–4-fold at all relative intracellular ratios of microsomes/supernatant (1:1 to 1:13) assayed was observed.

We showed earlier that large amounts of LeuRS activity are found in normal wild-type CHO microsomes (Hampel & Enger, 1973), but none is found in the microsome fraction of the LeuRS mutant *ts*H1 (Hampel et al., 1978). A likely explanation for the results in Table I is therefore that a specific activation of the LeuRS in the microsomal fraction but not the LeuRS in the supernatant fraction is occurring.

Specific Activation of 30S LeuRS Form. The specificity of the activating activity was determined by sucrose gradients of whole cell lysate (minus nuclei and cell debris). The three primary LeuRS enzyme forms at 8 S, 20 S, and 30 S are seen in Figure 1A. To determine which forms of LeuRS were undergoing activation, we added whole cell supernatant to each gradient fraction at a physiologically appropriate intracellular ratio of about 1:1; the combination was assayed for LeuRS enzyme activity, and the LeuRS individual contribution of each supernatant and gradient fraction was subtracted from the total activity. The remaining activated activity (Figure 1B) was specifically in the LeuRS high molecular weight complex. It can be seen that this activation is primarily of the 30S form with only a small amount of the 20S form. No activation of the 8S LeuRS form occurred.

The size and location on the sucrose gradient of the supernatant activating component were identified by adding the 30S peak tube (tube 12) to each tube of the same gradient and assaying for LeuRS enzyme activity. Again the LeuRS activity of tube 12 and each gradient tube assayed was subtracted from the activity of the combination. The resulting activated activity occurred at 2.5 S and identified the LeuRS activator (Figure 1C). When this 2.5S activator alone was added to each gradient tube and activated LeuRS activity searched for, the same result as in Figure 1B was obtained.

The LeuRS mutant cell strain *ts*H1 has only an 8S form of LeuRS (Hampel et al., 1978). When supernatant was added to each fraction of the *ts*H1 whole cell lysate sucrose gradient and activation searched for, none occurred. The activator itself was located on the gradient at 2.5 S by adding 30S LeuRS (tube 12) from Figure 1A to each fraction of the *ts*H1 sucrose gradient in Figure 2 and measuring activated activity.

Identity of Activator. The activating activity was found near 2.5 S on all gradients run—some of which were run for longer times to more accurately determine the sedimentation coefficient. The absolute value of 2.5 S is accurate to 20%, but more importantly 2.5S activator serves as a working description for this activation activity.

When sucrose gradient column fractions were assayed for inorganic pyrophosphatase activity, this activity was found to be coincident with the 2.5S activating activity. After further purification of the activating activity in the 2.5S fraction by DEAE-Sephacel ion-exchange and Sephadex gel filtration chromatography, the activation could be coincident with inorganic pyrophosphatase activity. However, purification steps by DEAE column chromatography were complicated by

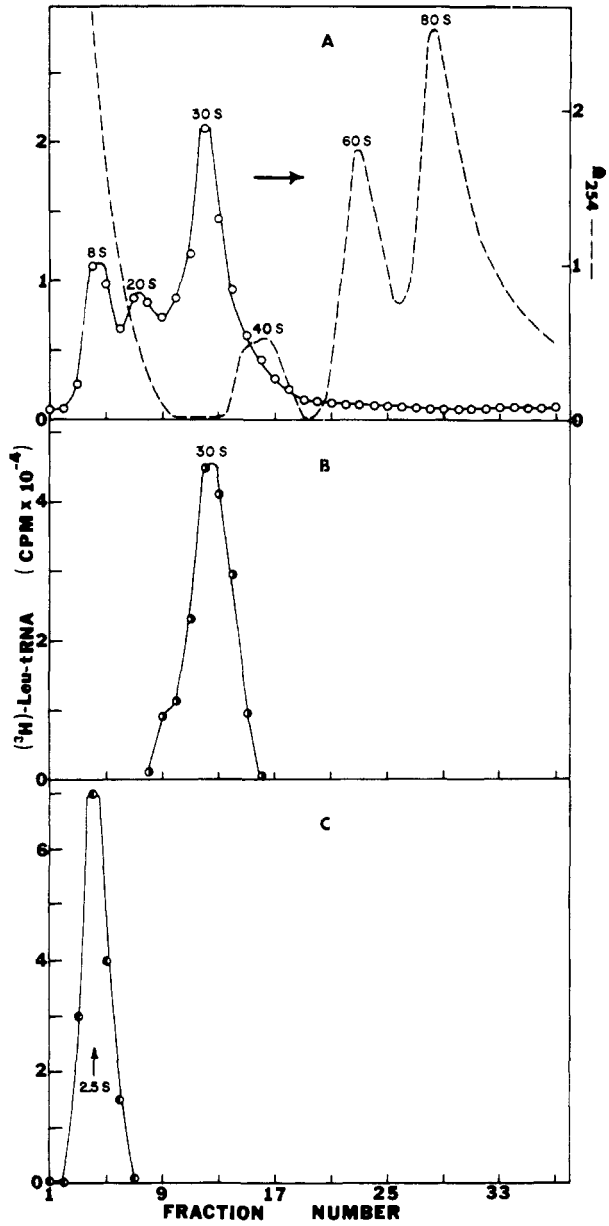

FIGURE 1: Sucrose gradient fractionation of LeuRS and LeuRS activation in CHO cells. Sucrose gradients (10–30%) of whole cell lysate less nuclei were run for 7 h at 27 000 rpm, fractionated, and assayed for (A) LeuRS activity, (B) activated LeuRS activity when supernatant was added to each gradient fraction, and (C) activated LeuRS activity when the 30S peak tube was added to each gradient fraction.

nonlinear activation effects of the 2.5S activating component after column chromatography. It was found that this activation activity was a nonlinear function of the amount of 30S LeuRS present and of the 30S/2.5S ratio. The function displayed a sharp activation minimum at a 30S/2.5S intracellular ratio of about 5. The reasons for this activation behavior of the purified 2.5S activator are intriguing but not yet understood by our laboratory. The molecular mass of the 2.5S activator as determined on a calibrated Sephadex G-75 column was 62 000 g/mol (data not shown), which is approximately the same as that for known inorganic pyrophosphatase (Schachman, 1952), and the activating activity likely has inorganic pyrophosphatase activity.

This then presents the question of what if any differential effects inorganic pyrophosphatase activity itself has on the various LeuRS enzyme forms. Does it activate the 30S form preferentially? Does it activate the 8S form at all?

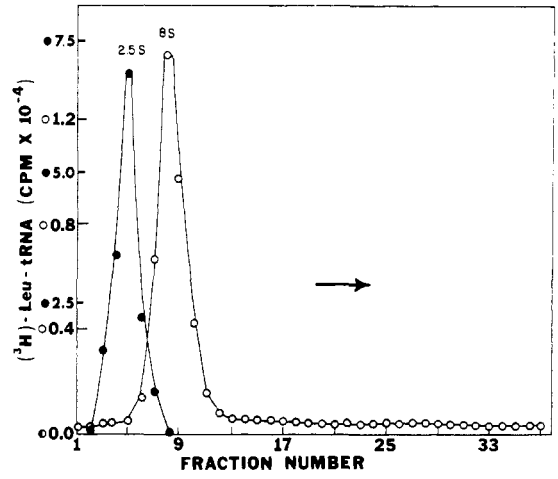

FIGURE 2: Sucrose gradient fractionation of LeuRS and activation components in mutant *ts*H1 CHO cells. Sucrose gradients as in Figure 1 were run for 18 h, fractionated, and assayed for LeuRS activity (○) and activated LeuRS activity when the 30S peak tube from the gradient in Figure 1C was added to each *ts*H1 gradient tube (●).

Table II: Percent Activation of 8S, 20S, and 30S LeuRS Forms by CHO 2.5S Activator (2.5S) and Yeast Inorganic Pyrophosphatase (YIP) [a]

	% activation	
	2.5 S	YIP
8 S	111	162
20 S	116	178
30 S	396	402

[a] Results are expressed with the LeuRS activity of nonactivated 8S, 20S, and 30S forms being 100%, respectively, and are the average of two experiments. An equivalent amount of inorganic pyrophosphatase activity (0.1 unit) was used in each activation assay.

Sucrose gradient peaks representing each of the LeuRS forms 8 S, 20 S, and 30 S were combined with either CHO 2.5S activator or yeast inorganic pyrophosphatase containing the same level of inorganic pyrophosphatase activity (0.1 unit/mL). The results in Table II show that the CHO 2.5S activator increases 30S LeuRS activity 4-fold while the change in 8S form activity (11%) is nearly within experimental error. Yeast inorganic pyrophosphatase also activates 30S LeuRS 4-fold but in addition enhances the 8S form activity 1.6-fold. Thus inorganic pyrophosphatase activity does activate the 8S LeuRS form. However, the activation is significantly less than that of the 30S LeuRS, indicating that indeed a greater activation of 30S LeuRS than 8S LeuRS occurs. The 8S form activation does not occur with the CHO 2.5S activator as is further shown in Figure 3. This latter point is made clear when the *ts*H1 8S LeuRS is activated by either CHO 2.5S activator or yeast inorganic pyrophosphatase, since *ts*H1 has only an 8S LeuRS form, avoiding complications from high molecular weight forms. Figure 3 shows the result where an activation of 8S LeuRS occurs in the presence of yeast inorganic pyrophosphatase, but no activation of the 8S LeuRS form occurs in the presence of the CHO 2.5S activator. In this experiment the CHO 2.5S activator was identified by pyrophosphatase activity.

Preferential enhancement of particulate enzyme activity by 2.5S inorganic pyrophosphatase activity was not limited to LeuRS but was observed by us for other synthetase activities as well. Extensive activation of aminoacyl-tRNA synthetase activities specific for Lys and Met was observed, which as we have previously shown both contain enzyme complexes (Ritter et al., 1976). An effect of activation on the leucine K_m was

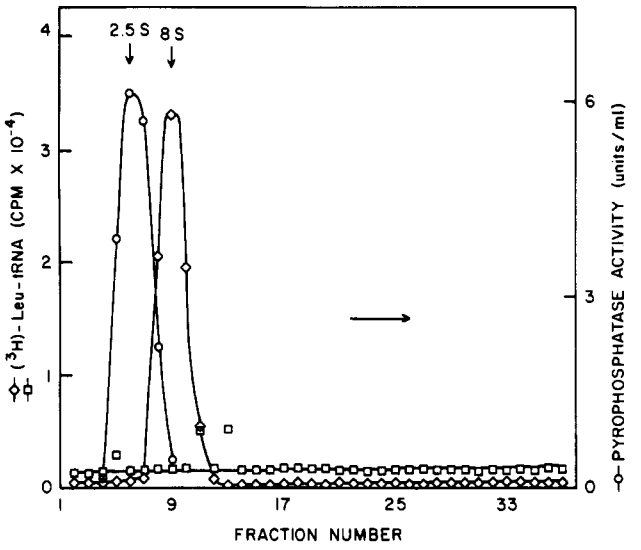

FIGURE 3: Activation of 8S LeuRS by inorganic pyrophosphatase but not by 2.5S CHO activating activity. Sucrose gradients of *ts*H1 cells as in Figure 2 were run, and pyrophosphatase activity was determined (O). This activity is coincident with the 2.5S activation activity of the 30S peak tube in Figure 2. Activated activity of *ts*H1 LeuRS by the 2.5S peak tube (□) and activated activity of *ts*H1 LeuRS by yeast inorganic pyrophosphatase (◇) are shown. All activation assays used 0.1 unit/mL inorganic pyrophosphatase per assay point.

seen with K_m values obtained as follows: 30 S, K_m = 9 μM; 30 S plus 2.5 S, K_m = 18 μM; 20 S, K_m = 8 μM; 20 S plus 2.5 S, K_m = 10 μM. These effects are not large and likely have little significance.

Discussion

The specific activation of the large particulate LeuRS complexes thus appears to be due to the removal of the inhibitor PP_i. The dose response of an increasing 30S/2.5S ratio (sucrose gradient fractions) on activation shows a linear effect with a constant activation level of about 4–5-fold, indicating that the inhibitory PP_i is generated during the aminoacylation reaction itself. The levels generated during the reaction are less inhibitory to the low molecular weight 8S form since little activation effect is seen here. The mechanism of PP_i inhibition is most likely pyrophosphorolysis of the E·aminoacyladenylate complex, which is independent of the concentration of tRNA (Lui et al., 1978). The extent of inhibition of PP_i, however, is dependent on the conformation of the tRNA since aminoacylation of heterologous tRNA is inhibited to a greater extent than that of homologous tRNA (Kull et al., 1969). It has also been shown that the PP_i exchange reaction is not detectable when the reaction is carried out in the presence of polyamines (Igarashi et al., 1971). Polyamines give the tRNA a different structure when they replace Mg^{2+} in binding tRNA (Teeter et al., 1980). A tRNA structure containing polyamines is very likely more correct physiologically since it accelerates the aminoacyl-tRNA synthetase reaction (Igarashi et al., 1978) and is less likely to be misacylated by the wrong amino acid (Loftfield et al., 1981). It would appear then that the greater sensitivity of the complexes to PP_i inhibition is due to differing extents of interaction of the enzyme with tRNA itself. The exact nature of these differences will require extensive further studies to elucidate this; however, the present studies do show a fundamental functional difference between the enzyme complexes and the low molecular weight form of LeuRS. This represents one more basic fact in the continuing quest to elucidate the basic functional roles of these high molecular weight complexes.

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