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Amino Acid Transfer Factors from Yeast. II. Interaction of Three Partially Purified Protein Fractions with Guanosine Triphosphate*

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ABSTRACT: Two protein fractions (1-P and 2-P) isolated from yeast supernatant fluid exhibited two kinds of enzymatic activity when combined: they catalyzed (1) peptide chain elongation and (2) ribosome-dependent guanosine triphosphate hydrolysis. 1-P was able to form a complex with guanosine triphosphate. Further purification of 1-P and 2-P by adsorption chromatography and gel filtration yielded T_1 and T_2 still complementary in guanosine triphosphate splitting but without any transfer effect. Purified T_1 had lost most of the guanosine triphosphate binding activity.

The T factors showed great heat lability. By contrast, a further ribosome-dependent guanosine triphosphatase was obtained from the supernatant fluid, which was more heat stable and had a higher molecular size than T_1 and T_2. This stable guanosine triphosphatase did not complete the T factors in amino acid polymerization; however, the transfer effect of an additionally isolated protein fraction was enhanced twofold by the combined T factors.

For some years it has been known that peptide chain elongation with purified ribosomes needs more than one component from the soluble fraction of the cell. Three enzyme factors isolated from bacterial sources (Lucas-Lenard and Lipmann, 1966) possessed amino acid incorporation activity only when combined, hence their functions in the elongation process must be different. One factor (G) was shown to contain a ribosome-dependent GTPase activity (Nishizuka and Lipmann, 1966) and a second (T_u) was found to bind GTP (Gordon, 1967; Allende *et al.*, 1967; Ravel *et al.*, 1967; Lucas-Lenard and Haenni, 1968). In mammalian systems, however, only two complementary transfer fractions could be separated up to now (Arlinghaus *et al.*, 1963; Gasior and Moldave, 1965; Klink *et al.*, 1967a). One of these (Klink *et al.*, 1966; Arlinghaus *et al.*, 1964) or both (Rao and Moldave, 1967; Ibuki and Moldave, 1968) were reported to interact with GTP.

In contrast to bacterial enzymes, transfer factors from

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yeast resembled those from liver in as much as they were quite active in liver incorporation systems (Klink and Richter, 1966). From yeast supernatant three different protein fractions could be obtained (Richter and Klink, 1967). In chain elongation tests with polysomes, however, one fraction was found complementary to each of the other fractions. Ayuso and Heredia (1967) also described two yeast enzymes which were complementary in polymerization. Therefore the question remained whether there exist more than two amino acid transfer enzymes with different functions in nonbacterial organisms. In the present report, we want to show that two of the yeast fractions (1-P and 2-P) described in the preceding paper are complementary not only in polymerization but also in ribosome-dependent GTP hydrolysis and that 1-P has a GTP-binding activity. Evidence will be given that 1-P and 2-P contain additional components involved in amino acid transfer. A combination of two factors derived from 1-P and 2-P by further purification steps was active in GTP hydrolysis but not in polymerization. In order to avoid further confusion by introducing new symbols, we propose to call the purified enzymes the T factors, following the nomenclature of Nishizuka and Lipmann (1966). In addition we want to demonstrate that a ribosome-dependent yeast GTPase somewhat resembling the G factor from *Escherichia coli* did not complete our T factors in amino acid transfer.

Materials and Methods

Preparation of the Cell-Free Extracts. Saccharomyces cerevisiae strain Kaneka was grown in a peptone–glucose–salt solution, harvested, and disrupted as described (Richter and Klink, 1967).

Isolation of the Ribosomes. Yeast polysomes (standard ribosomes) were prepared as recently reported (Richter and Klink, 1967). Poly U dependent yeast ribosomes were isolated by centrifugation of the supernatant fluid S-1 (78,000*g* for 2 hr). The upper four-fifths of the supernatant was removed, and the ribosome pellets were dissolved in the remaining supernatant and diluted with 2.5 volumes of buffer (0.02 M Tris-HCl (pH 7.6), 0.6 M KCl, 0.008 M $Mg(CH_3COO)_2$, and 0.25 M sucrose). This solution was clarified by centrifugation (10 min at 10,000*g*) and spun down (2 hr at 78,000*g*). The pellets were redissolved in buffer (0.02 M Tris-HCl (pH 7.6), 0.004 M $Mg(CH_3COO)_2$, 0.1 M KCl, and 0.25 M sucrose). After centrifugation (30 min at 78,000*g*) the supernatant was dialyzed against buffer (0.02 M Tris-HCl (pH 7.6), 0.1 M KCl, 0.25 M sucrose, and 0.008 M $Mg(CH_3COO)_2$). This solution (29 ml) containing 25 *A* units (260 mμ, 1%, 1 cm) was layered over sucrose (3 ml of 1.7 M sucrose in 0.02 M Tris-HCl (pH 7.6), 0.004 M $Mg(CH_3COO)_2$, and 0.1 M KCl) and centrifuged for 2 hr at 78,000*g*. The upper 20 ml of the supernatant was discarded. The remaining solution was gently homogenized and centrifuged at 78,000*g* for 30 min (twice) to remove large aggregates. The supernatant was carefully collected, diluted with the same volume of buffer (0.02 M Tris-HCl (pH 7.6), 0.1 M KCl, 0.004 M $Mg(CH_3COO)_2$, and 0.05 M sucrose), and centrifuged at 78,000*g* for 2 hr. After removing 20 ml of the supernatant the ribosome pellet was dissolved in the remaining supernatant, and dialyzed against buffer (0.02 M Tris-HCl (pH 7.6), 0.008 M $Mg(CH_3COO)_2$, 0.1 M KCl, and 0.25 M sucrose). The concentration of this washed-ribosome preparation was adjusted to 80 *A* units (260 mμ, 1%, 1 cm).

Isolation of the Polymerization Factors by Anion-Exchange Chromatography. Fractions 1-P, 2-P, and 3-P were prepared from the ammonium sulfate fraction S-2 as described (Richter and Klink, 1967). Chromatography of the S-2 fraction was simplified. After the first elution step with KCl-free buffer (Richter and Klink, 1967), the KCl gradient which followed was replaced by a stepwise elution with 0.2 M KCl (for 2-P) and 0.35 M KCl (for 3-P), respectively. The buffer used for elution contained 0.02 M Tris-HCl (pH 7.4), 0.001 M $Mg(CH_3COO)_2$, 0.001 M DTT,[1] and KCl as indicated. In some cases the elution of the enzymes was started with 0.2 M KCl; the eluate obtained was called crude fraction. The isolated fractions were fractionated with solid ammonium sulfate as previously described (Richter and Klink, 1967).

Adsorption Chromatography with Alumina Cγ Gel. An ammonium sulfate fraction of 1-P or 2-P, containing 20–30 mg/ml of protein, was equilibrated with 0.005 M phosphate buffer (pH 6.6), and stirred with 1 ml of alumina Cγ gel/ml of protein (Bio-Rad). After 10 min the gel was centrifuged. The supernatant was discarded, and the gel was washed once more with the same volume of phosphate buffer (0.005 M). The bulk of the GTPase activity was eluted from 2-P between 0.05 and 0.15 M phosphate (pH 7.4), and from 1-P in the range of 0.1 M phosphate (pH 7.4). All phosphate buffers contained 0.001 M DTT. The eluate was dialyzed against buffer (0.02 M Tris-HCl (pH 7.4), 0.008 M $Mg(CH_3COO)_2$, 0.1 M KCl, and 0.001 M DTT).

Gel Filtration. Separation of the fractions by gel filtration on the dextran gels Sephadex G-100 and G-200 was carried out using a descendent technique as described (Richter and Klink, 1967).

Preparation of [γ-^{32}P]*GTP and* [γ-^{32}P]*ATP.* [γ-^{32}P]GTP and [γ-^{32}P]ATP were prepared by the method of Klink *et al.* (1967b). The specific activities of these triphosphates were in the range of 50–80 mCi/mmole.

Assay for GTPase Activity. GTP hydrolysis by the polymerization factors was carried out at 37° for 10 min. The total volume of 0.5 ml contained 25 μmoles of Tris-HCl (pH 7.6), 4 μmoles of $Mg(CH_3COO)_2$, 50 μmoles of KCl, 15 μmoles of cysteamine, 3–8 mμmoles of [γ-^{32}P]GTP (sp act. 5–10 μCi/μmole), 50 μl of washed-ribosome solution (*A* (260 mμ, 1%, 1 cm) 2.0–4.0), and polymerization factors as indicated. In ATPase tests 20–25 mμmoles of [γ-^{32}P]ATP (sp act. 20 mCi/mmole) were used. Before adding the ribosomes to the reaction mixture, they were preincubated at 55° for 5 min. This preincubation caused an inactivation of an unspecific GTPase present in the ribosome solution.

The reaction was stopped by adding 2 ml of an ice-

[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: DTT, dithiothreitol.

cold 2% trichloroacetic acid solution containing 0.6 μmole of KH_2PO_4, 60 mg of charcoal (Norit A), and 1.3 μmoles of $K(CH_3COO)$. After centrifugation ^{32}P liberation was determined by counting aliquots of the supernatant in a Tri-Carb liquid scintillation spectrometer. The scintillator was a dioxane solution of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Assay for GTP Binding. The binding reaction was investigated with [γ-^{32}P]GTP or [^{3}H]GTP using Millipore filters (HA 0.45-μ pore size) or Sephadex G-25 columns (0.8 × 1.3 cm). Under standard conditions the binding was tested in a total volume of 0.25 ml: 12.5 μmoles of Tris-HCl (pH 7.6), 25 μmoles of KCl, 2 μmoles of $Mg(CH_3COO)_2$, 7.5 μmoles of cysteamine, 100–300 μg of 1-P, and 2–3 mμmoles of [γ-^{32}P]GTP (sp act. 70 mCi/mmole), or 2.5 mμmoles of [^{3}H]GTP (sp act. 1.47 mCi/mmole). The solution was incubated at 0° for 2 min, diluted with 1 ml of ice-cold buffer (0.02 M Tris-HCl (pH 7.4), 0.008 M $Mg(CH_3COO)_2$, and 0.1 M KCl), and passed through a Millipore filter. The filter was washed with the same buffer, dried, and counted in a liquid scintillation spectrometer.

When using Sephadex G-25 columns we incubated a total volume of 0.5 ml containing 25 μmoles of Tris-HCl (pH 7.6), 50 μmoles of KCl, 4 μmoles of $Mg(CH_3COO)_2$, 15 μmoles of cysteamine, 2–4 mg of 1-P, and 4–6 mμmoles of [γ-^{32}P]GTP, 4–6 mμmoles of [γ-^{32}P]ATP, or 5 mμmoles of [^{3}H]GTP. The mixture was incubated at 0° for 2 min and then passed through a Sephadex G-25 column. The elution buffer was the same as used for Millipore filter technique. The eluate was collected dropwise and counted in the Tri-Carb spectrometer.

Preparation of [^{14}C]Aminoacyl-tRNA and [^{14}C]Phenylalanyl-tRNA. These preparations were carried out by the methods recently reported (Klink *et al.*, 1967a).

Assay for Amino Acid Incorporation into Ribosomal Protein. The standard conditions for polypeptide formation were modified. The total volume of the reaction mixture was reduced to 0.5 ml containing 50 μl of ribosome solution (*A* (260 mμ, 1%, 1 cm) 3.0), 20–30 μg of [^{14}C]phenylalanyl-tRNA (2500–3000 cpm), 0.30 μmole of ATP, 0.16 μmole of GTP, 2.0 μmoles of phosphoenolpyruvate, 12 μg of pyruvate kinase, 15 μmoles of cysteamine, 7.5 μmoles of $Mg(CH_3COO)_2$, 50 μmoles of KCl, 25 μmoles of Tris-HCl (pH 7.6), and 80 μg of poly U. The reaction was stopped as described (Richter and Klink, 1967). The protein was washed two times with hot 2% trichloroacetic acid and passed through a glass paper filter (GF/C, 2.5-cm o.d.). The filter was washed thoroughly with ethanol, dried, and counted in a Tri-Carb liquid scintillation spectrometer. Protein was determined by the method of Lowry (Lowry *et al.*, 1951).

Materials. Poly U was obtained by Boehringer (Mannheim, Germany). DTT was purchased from Calbiochem Corp. (Lucerne, Switzerland). [^{14}C]Phenylalanine (sp act. 400–700 μCi/μmole), [^{14}C]yeast protein hydrolysate (sp act. 800 μCi/μmole), and carrier-free [^{32}P]phosphoric acid were supplied by Philips-Duphar (The Netherlands). [^{3}H]GTP (sp act. 1.47 Ci/μmole) was purchased from New England Nuclear Corp. (Boston). Glass paper filter was a product of Whatman (England). Alumina Cγ gel (aged) was purchased from Bio-Rad Laboratories (Richmond, Va.).

Results

Identification of Two Fractions Complementary in Splitting GTP. Two of three transfer-active fractions (1-P and 2-P) isolated from yeast supernatant by TEAE-cellulose chromatography catalyzed amino acid polymerization with polyribosomes from yeast or liver only when combined. The third fraction (3-P) represented an association of at least two transfer factors, one of them being complementary to 2-P (Richter and Klink, 1967).

In search of a ribosome-dependent GTPase factor like the enzymes G (*E. coli*) or FI (calf liver) we examined the yeast fractions separately and in various combinations. Only the combination of 1-P and 2-P hydrolyzed GTP in the presence of ribosomes (see Table I), whereas the separate fractions and other combinations showed little ribosome-dependent activity. Some preparations of 2-P had greater effects which were not enhanced by ribosomes, however. In the experiments described we used poly U dependent washed ribosomes (see Methods) because yeast polysomes by themselves were considerably active in splitting GTP.

TABLE I: Splitting of GTP by Two Complementary Fractions in the Presence of Washed Ribosomes.[a]

	[γ-^{32}P]GTP Hydrolyzed (mμmoles)	
	Fractions Alone	Fractions Combined with Washed Ribosomes[c]
1-P	0.35	0.44
2-P	0.65	0.70
3-P[b]	0.40	0.65
1-P + 2-P	0.85	3.85
1-P + 3-P	0.65	1.25
2-P + 3-P	1.05	1.75

[a] The fractions 1-P, 2-P, and 3-P were isolated as described in Methods. [b] 3-P was filtered on Sephadex G-200 as reported (Richter and Klink, 1967). [c] The blank of washed ribosomes was 0.05 mμmole of GTP.

Purification of the Complementary GTPase Fractions. It was of interest if further purification of the two complementary fractions would influence both functions, GTP hydrolysis and amino acid polymerization, to a different extent. Therefore the ammonium sulfate fractions of 1-P (45–60% saturation) and of 2-P (50–70% saturation) were treated with alumina Cγ gel. GTPase ac-

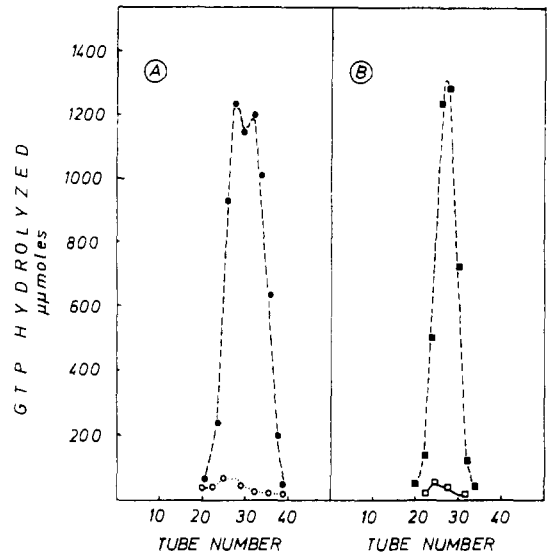

FIGURE 1: Gel filtration on Sephadex G-100 of fractions 1-P and 2-P. The fractions were isolated as described in the Results section and dialyzed against buffer (0.02 M Tris-HCl (pH 7.4), 0.008 M $Mg(CH_3COO)_2$, 0.1 M KCl, and 0.005 M cysteamine). A solution of 0.8 ml containing 20–30 mg of protein was passed through the columns (2.7 × 40 cm). The elution buffer was the same as used for dialysis (cysteamine was replaced by 0.001 M DTT). Fraction volume, 2.0 ml. For assay of GTPase activity, see Methods. (A) Gel filtration of 1-P. Hydrolysis of GTP by: (○—○) eluate fractions alone; (●- - -●) eluate fractions combined with washed ribosomes and T_2 (40 μg). A blank of washed ribosomes and T_2 was already substracted. No additional GTP splitting was observed by the combination of washed ribosomes and fraction 26 or by the combination of T_2 and fraction 26. (B) Gel filtration of 2-P. Hydrolysis of GTP by: (□—□) eluate fractions alone; (■- - -■) eluate fractions combined with washed ribosomes and T_1 (30 μg). A blank of washed ribosomes and T_1 has been substracted. Fraction 28 combined with washed ribosomes or with T_1 had no effect on GTP hydrolysis.

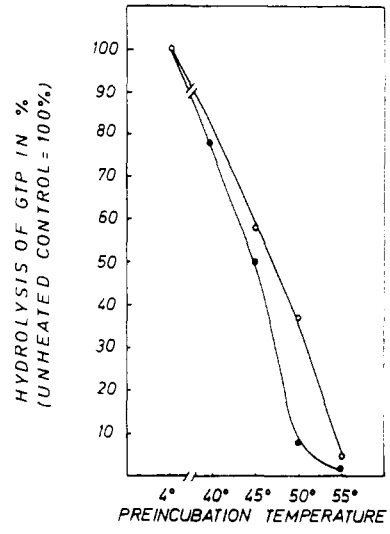

FIGURE 2: Inactivation of the GTPase activities of T_1 and T_2 by heating for 5 min at the temperatures indicated. GTPase activity of the heated fractions was investigated at 32° for 10 min. For other conditions of GTP hydrolysis, see Methods. GTPase activity in per cent of the unheated control: (○——○) T_1; (●——●) T_2.

tivity from 1-P was eluted from the gel with 0.1 M phosphate buffer (pH 7.4). Fraction 2-P was purified in a similar manner using phosphate concentrations in the range of 0.05–0.15 M (pH 7.4). Subsequently these alumina Cγ gel fractions were passed through Sephadex G-100 columns (Table II). The purified factors obtained in this way from 1-P and 2-P will be called T_1 and T_2, respectively. Obviously the polymerization effect of the enzyme combination has disappeared but their GTP-splitting activity with ribosomes is almost unchanged. The ribosome-independent GTPase activity is minimized. From dextran gels T_1 and T_2 were eluted in single peaks (see Figure 1); from their positions one may assume molecular weights below 100,000 for both T_1 and T_2.

Characteristics of GTP Hydrolysis by the T Factors. T_1 and T_2 together had very little GTPase activity which was stimulated about 15-fold by addition of washed yeast ribosomes (see Table II). The interchangeability of the yeast ribosomes with polysomes or recombined ribosomal subunits from liver is demonstrated in Table III. The ribosomal subunits were fully dependent upon polyuridylic acid for polyphenylalanine synthesis. No poly U was added in the GTPase tests; hence, mRNA seems to be dispensable for the ribosomal effect on GTP hydrolysis. In experiments with washed yeast ribosomes, being not fully but largely messenger free, addition of poly V slightly stimulated the GTPase activity (see Table IV). Aminoacyl-tRNA influenced more strongly GTP splitting. The GTPase effect of a mixture of T_1, T_2, ribosomes, and poly U was increased more than twofold by adding phenylalanyl-tRNA. It is of particular interest that ribosomes could be substituted by aminoacyl-tRNA for GTPase stimulation to a considerable extent; in the absence of ribosomes, phenylalanyl-tRNA

TABLE II: Activity of Fractions 1-P and 2-P and the Factors T_1 and T_2 in Phenylalanine Polymerization and GTP Hydrolysis.[a]

	[^{14}C]Phenylalanine Polymerization (cpm) with Washed Ribosomes and Poly U	[$^{32}\gamma$-P]GTP Hydrolysis (mμmoles)	
		Without Washed Ribosomes[b]	With Washed Ribosomes[b]
1-P	30	0.48	0.60
2-P	70	1.15	1.85
1-P + 2-P	280	1.50	4.65
T_1	0	0.08	0.15
T_2	1	0.10	0.40
$T_1 + T_2$	3	0.19	3.12

[a] For isolation of T_1 and T_2, see Methods. [b] The blank of washed ribosomes was 50 μμmoles of GTP.

TABLE III: Interchangeability of Yeast Ribosomes, Liver Polysomes, and Ribosomal Subunits from Liver in GTPase Stimulation Effect.

	[γ-^{32}P]GTP Hydrolyzed (μμmoles)			
		Fractions with		
	Fractions[a] Alone	Washed Ribosomes from Yeast	Polysomes from Liver	Recombined Ribosomal Subunits[b] from Liver
T_1	47	96	75	79
T_2	150	332	245	204
$T_1 + T_2$	342	1490	1250	1155
Ribosomes		90	34	17

[a] The T factors were prepared from 1-P or 2-P by molecular sieve chromatography omitting the adsorption step with alumina Cγ gel. [b] Recombined subunits from liver were prepared as described (Klink *et al.*, 1967a,b).

enhanced the GTPase effect of ($T_1 + T_2$) to 54% of the value produced by the combination of T_1, T_2, and ribosomes. A more detailed analysis of tRNA effects on the T factors will be given in a subsequent paper. As indicated in Figure 2, there were some differences between T_1 and T_2 in heat sensitivity. Heating at 50° for 5 min destroyed about 60% of the GTPase effect of T_1, whereas T_2 was inactivated almost completely by this treatment. T_2 lost nearly all activity when stored frozen at −20° for a few days. Free SH groups were needed for GTP hydrolysis by the T factors. Gel filtration of T_2 in the absence of SH substances inactivated the enzyme; the activity was restored only partially by readdition of cysteamine.

Complex Formation between Yeast Fractions and GTP. At first, GTP-binding experiments were performed with the three crude fractions 1-P, 2-P, and 3-P using [γ-^{32}P]-GTP and the Millipore filter technique. Table V (expt 1) indicates that 1-P was able to combine with GTP to a considerable degree while 2-P and 3-P showed smaller binding effects. GTP binding was dependent upon the concentration of 1-P and GTP. As reported

TABLE IV: Influence of Washed Ribosomes, Poly U, and [^{14}C]Phenylalanyl-tRNA on the GTPase Activity of T_1 and T_2.[a]

	[γ-^{32}P]GTP Hydrolyzed (mμmoles)	
	Fractions Alone	Fractions Combined with Washed Ribosomes
$T_1 + T_2$	0.065	2.005
$T_1 + T_2$ + poly U	0.235	2.35
$T_1 + T_2$ + [^{14}C] phenylalanyl-tRNA	1.11	4.80
$T_1 + T_2$ + poly U + [^{14}C]phenyl-alanyl-tRNA	1.20	5.67

[a] T_1 and T_2 were prepared from 1-P and 2-P as described in Results. The incubation mixtures contained 20 μg of T_1 and 30 μg of T_2 protein, and, as indicated, 10 μg of poly U and 30 μg of phenylalanyl-tRNA.

TABLE V: GTP Binding Activity of the Yeast Fractions.[a]

Fractions	Incubation Time at 0°	Binding of [γ-^{32}P]GTP (μμmoles)
	Experiment I	
1-P	2 min	9.37
2-P	2 min	1.34
3-P	2 min	0.71
1-P heated[b]	2 min	0.54
	Experiment II	
1-P	30 sec	14.0
1-P	2 min	15.8
1-P	5 min	15.1
1-P	10 min	15.7
Washed ribosomes[c]	2 min	0.27

[a] Formation of GTP complex was determined by Millipore filter (see Methods). Fraction 1-P contained 300 μg, 2-P 800 μg, and 3-P 400 μg of protein. [b] Fraction 1-P was heated at 55° for 5 min. [c] GTP binding activity of ribosomes was tested with 0.1 ml of ribosome solution (A (260 nm, 1%, 1 cm) 4.0).

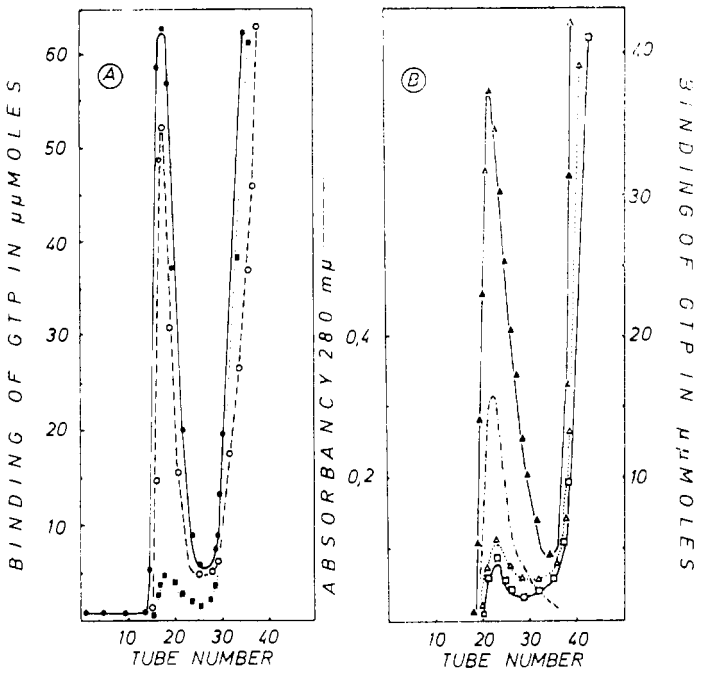

FIGURE 3: Gel filtration of the 1-P–GTP complex on Sephadex G-25 columns. The reaction mixtures contained in part A 10 mμmoles and in part B 6 mμmoles of [γ-^{32}P]GTP. For other conditions for the GTP-binding reaction, see Methods. (– – – –) Void volume estimated with dextran blue. (A) Complex formation of 1-P with [γ-^{32}P]GTP. (●—●) In the presence of DTT; (○–○) in the absence af DTT; (■· · · · · ■) inhibition of GTP binding by heating 1-P at 55° for 5 min. (B) Complex formation of 1-P with: (▲——▲) [γ-^{32}P]GTP; (△· · · · · △) [γ-^{32}P]ATP. (□—□) Influence of GDP on the formation of the 1-P–GTP complex; in the reaction mixture GTP was replaced by 70 mμmoles of GDP. After incubation of the solution (at 0° for 2 min), 6 mμmoles of [γ-^{32}P]GTP was added. The solution was passed through the column as described in Methods.

TABLE VI: $^{32}P_i$ Liberation from the Complex of 1-P with [γ-^{32}P]GTP.[a]

		$^{32}P_i$ Liberation (μμmoles) from the	
Temp (°C)	Time (min)	Complex	Complex boiled[b]
		Experiment I	
0	0	4.96	11.7
0	10	5.25	12.8
30	5	13.2	
30	10	15.8	12.9

		$^{32}P_i$ Net Liberation (μμmoles) from the Complex by		
		T_2	Ribosomes[c]	T_2 + Ribosomes
		Experiment II		
30	10	7.0	1.6	26.1
				Complex boiled
30	10			1.5
			T_2 heated at 50° for 5 min	
30	10	0.9	1.0	2.7

[a] Formation and isolation of the complex: Fraction 1-P (17 mg) (ammonium sulfate fraction of 50–60% saturation) was incubated at 0° for 2 min in the presence of 100 mμmoles of [γ-^{32}P]GTP (sp act. 60 μCi/μmole). The total volume of 3.0 ml was passed through a Sephadex G-25 column (1.8 × 40 cm) and eluted with buffer as indicated in Methods. The void volume of the column was estimated with a dextran blue solution. Fractions (0.8 ml) were collected. The fractions containing the complex were pooled and tested for [$^{32}P_i$]phosphate liberation using the adsorption technique with charcoal (see Methods). The total volume of the reaction mixture was 0.5 ml containing 60 μμmoles of bound GTP. The concentrations of T_2, washed ribosomes, and other components are the same used for measuring GTP hydrolysis. [b] The complex was boiled for 5 min. [c] Washed ribosomes (*A* (260 nm, 1%, 1 cm) 4.0) were used.

TABLE VII: Decrease of the Binding Capacity by Purification of 1-P.[a]

Fraction	Binding of [^{3}H]GTP by 100 μg of Protein ($\mu\mu$moles)
1-P	74.5
1-P-Alu Cγ[b]	9.3
T_1[c]	4.0

[a] Binding tests were carried out by filter technique. [b] Fraction 1-P was adsorbed on alumina Cγ and eluted as described in Methods. [c] Fraction T_1 was isolated from the 1-P-Alu Cγ fraction by gel filtration.

with *E. coli* fractions (Ravel *et al.*, 1967; Gordon, 1967; Allende *et al.*, 1967), interaction of 1-P with GTP occurred immediately in the cold (see Table V, expt 2).

The nucleotide specificity of the complex formation was tested with [γ-^{32}P]ATP and [γ-^{32}P]GTP. Figure 3B indicates an about sevenfold higher affinity of 1-P to GTP than to ATP. The formation of the complex was inhibited by GDP and by heating 1-P at 55° for 5 min (see Figure 3A,B). The interaction of GTP with 1-P was only slightly decreased by removing free SH groups (see Figure 3A). Aminoacyl-tRNA did not influence the binding reaction with 1-P. The complex of 1-P and GTP, isolated by filtration through Sephadex G-25, was relatively unstable. Storage at 0° for 3 hr caused a 50% dissociation of the complex. Under these conditions hardly any P_i release was detectable. Only 10% of the Sephadex complex was retained when passing through a Millipore filter. These findings resembled those reported recently by Gordon (1968). Incubation of the gel-filtered 1-P-[γ-^{32}P]GTP complex with T_2 and washed ribosomes at 37° for 10 min resulted in a significant release of P_i (see Table VI). The complex was completely destroyed by boiling it for 5 min.

Our findings detailed above suggested that 1-P by itself was able to form a protein–GTP complex. However, each purification step lowered the GTP-binding capacity

TABLE VIII: Heat Sensitivity of Peaks II and III (from Figure 4a).

Enzymes	GTP Hydrolysis (mμmoles) Without Ribosomes	With Ribosomes
Peak II	1.1	2.5
Peak II, heated[a]	0.08	1.3
Peak III	0.42	1.05
Peak III, heated[a]	0.14	0.22

[a] 5 min at 50°.

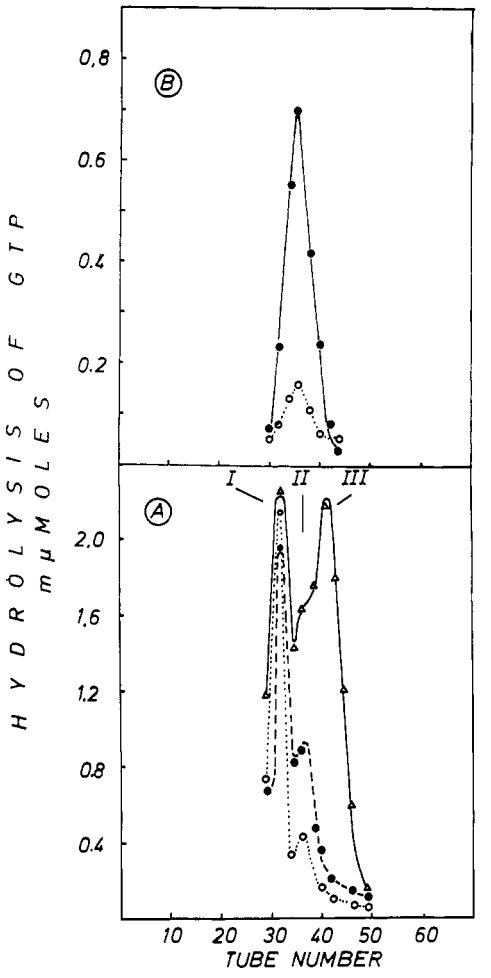

FIGURE 4: Gel filtration of the crude fraction (A) and rechromatography of the stable GTPase (B) on Sephadex G-100. GTP hydrolysis in the presence of washed ribosomes. GTP splitting by: (○· · · · ·○) 100 μl of eluate fractions alone; (●—●, ●– –●) 100 μl of eluate fractions combined with ribosomes (a blank obtained by the ribosomes alone was already subtracted); (△——△) 100 μl of eluate fractions combined with ribosomes and 25 μg of T_1 protein.

(see Table VII). At present a protein fraction different from T_2 is under study which restores the binding capacity of T_1.

Occurrence of a Stable GTPase Fraction. When using 1-P or 2-P as starting material we failed to isolate a fraction like G (*E. coli*) or FI (liver) (Nishizuka and Lipmann, 1966; Klink *et al.*, 1966) which did not require another complement for GTP splitting except ribosomes. However, we detected such a fraction by modifying the initial chromatography step of the enzyme preparation. Yeast supernatant was filtrated in 0.2 M KCl buffer through DEAE-cellulose, yielding the crude fraction containing all the material of 1-P and 2-P. If this crude fraction was chromatographed on Sephadex G-100, a shoulder at the back of the main peak showed a ribosome-dependent GTPase activity which was more heat stable than the T factors and therefore was called stable GTPase (see Figure 4A, peak II, dotted lines). Following this fraction an additional peak (III, see Figure 4A) appeared in the elution diagram if GTPase tests

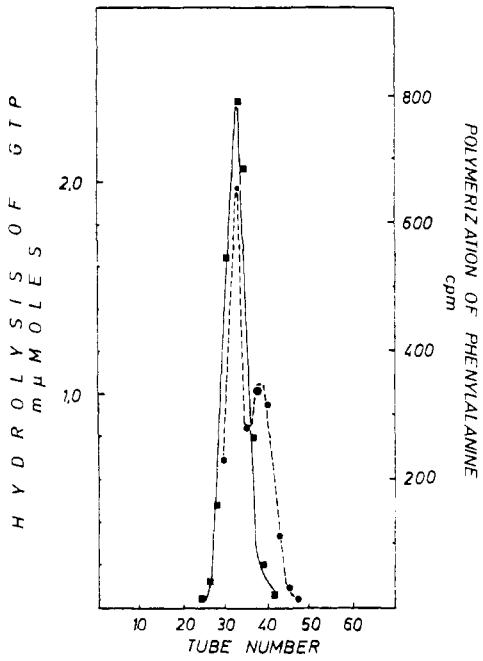

FIGURE 5: Comparison between GTP splitting and polyphenylalanine synthesis of the crude fraction filtrated through Sephadex G-100. The conditions for chromatography and GTP splitting are the same as indicated in Figure 4A. GTP hydrolysis by: (●- - -●) 100 μl of the eluate fractions in combination with washed ribosomes; for GTPase activity of the fractions alone, see Figure 4A. (■—■) Polymerization of phenylalanine by the eluate fractions and washed ribosomes. For assay of peptide synthesis, see Methods.

were performed in the presence of T_1. Peak II could be separated from I and III by rechromatography at Sephadex G-100 (see Figure 4B). Table VIII gives a comparison between the heat labilities of peaks II and III (Figure 4A), suggesting that the GTP-splitting activity of peak III is identical with that of T_2. It should be noted that the ribosome-independent effect of the stable GTPase can very easily be reduced by heat. The rechromatographed enzyme was not stimulated by (T_1 + T_2) in polymerization (see Table IX). Stable GTPase did not bind GTP.

TABLE IX: Comparison between GTPase and Polymerization Effects of T Factors and Stable GTPase.[a]

Enzymes	GTP Hydrolysis (mμmoles)	[^{14}C]Phenylalanine Polymerization (cpm)
T_1	0.10	12
T_2	0.25	35
Stable GTPase	0.25	30
T_1 + T_2	1.95	51
T_1 + T_2 + stable GTPase	2.05	85

[a] T_1 and T_2 were isolated by adsorption chromatography (alumina) and gel filtration (Sephadex G-100). Stable GTPase was refiltered on Sephadex G-100. GTP hydrolysis and polymerization were carried out as described in Methods.

TABLE X: Influence of the T Factors on Polyphenylalanine Synthesis with Washed Ribosomes.

Fraction	[^{14}C]Phenylalanine Polymerization (cpm)
Pool[a]	475
Heated pool[b]	104
T_1 + T_2	3
T_1 + heated pool	107
T_2 + heated pool	121
Heated pool + T_1 + T_2	206

[a] The pool was obtained using the fractions 28–35 of Figure 5; 100 μl of the pool was used in each case. [b] The pool was heated at 50° for 5 min. For assay of peptide synthesis, see Methods. The stable GTPase was isolated as described in Results.

Effects of the T Factors on Amino Acid Polymerization. The crude fraction (see above) was transfer active in itself; the bulk of this activity was recovered from Sephadex G-100 in a single peak distinct from the stable GTPase (see Figure 5). We looked for the missing polymerization complement to T in this peak: The eluate fractions 28–35 (Figure 5) were pooled. The transfer activity of this pool decreased to 20% when heated at 50° for 5 min and was partially recovered only by addition of T_1 and T_2 (see Table X). In order to find out which component of the pool was inactivated by heat we performed GTPase tests. Neither the heated pool nor combinations from the heated pool and T or from the heated pool and T_2 showed ribosome-dependent GTPase activity.

Discussion

In a previous paper the separation of three different amino acid transfer fractions (1-P, 2-P, and 3-P) from yeast supernatant has been reported (Richter and Klink, 1967). Fractions 1-P and 2-P had a transfer effect when combined. As shown above a combination of the purified factors T_1 and T_2 preserved ribosome-dependent GTPase activity but no longer catalyzed peptide synthesis. The GTP-binding effect of 1-P largely disappears in the change of 1-P into T_1:T_1 and obviously needs a second factor for complex formation with GTP. New results to be presented in a later paper give evidence that a second GTP-binding factor different from T_2 exists. Ertel *et al.* (1968) demonstrated that also in *E. coli* GTP binding depends upon two factors: T_s and T_u. Ravel *et al.* (1967) observed simultaneous binding and splitting of GTP by their fraction FII from *E. coli*, but this only occurred in the presence of aminoacyl-tRNA. As shown in Table IV aminoacyl-tRNA had a strong stimulating

effect on the GTP splitting by the yeast factors T_1 and T_2. It remains to be further investigated, however, to what extent ribosomes and tRNA can substitute each other in stimulating the T factors and what significance a tRNA-induced ribosome-independent GTP splitting has in the peptide chain elongation process. A discussion of these problems will also be left to a later paper where we want to treat the relation of the T factors with the GTP-binding factors from liver and *E. coli* (Rao and Moldave, 1967; Ibuki and Moldave, 1968; Gordon, 1967; Lucas-Lenard and Haenni, 1968).

In *E. coli*, the G factor is shown to complete the T factors for polymerization. Yeast-stable GTPase, though similar to G in several points, failed to stimulate the transfer reaction in combination with any other yeast fraction. Hence it remains open if the stable GTPase is an additional enzyme or a somehow stabilized form of one or two T factors; in the latter case, however, one should ask for a GTP-binding effect of this fraction.

On the other hand, the results listed in Table X suggest that there are more than two complementary polymerization factors in yeast supernatant. However, the third fraction besides T_1 and T_2 was a partially inactivated, crude fraction without detectable GTP-hydrolyzing activity. At present we do not know which part of this fraction has been inactivated by the heating procedure that drastically decreases its incorporation activity. Though the heat sensitivity of factor T_2 only seemed to be high enough, both, T_2 and T_1, had to be added in order to restore incorporation activity. A more detailed analysis of the crude fraction and of 3-P (see above) will be presented later in a following publication.

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