

Partial Purification by Affinity Chromatography of Tyrosine Aminotransferase-Synthesizing Ribosomes from Hepatoma Tissue Culture Cells

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ABSTRACT This report describes a cell-free protein-synthesizing system derived from hepatoma tissue culture cells in which the synthesis of tyrosine aminotransferase is enhanced in relation to total protein synthesis. The enhancement was achieved by using affinity chromatography to select a specific fraction of ribosomes in which the proportion of tyrosine aminotransferase synthesis is higher than it is in unfractionated ribosomes.

Gluconeogenic steroids induce a 10- to 15-fold increase in the rate of synthesis of tyrosine aminotransferase [L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5 (TAT)] in hepatoma tissue culture (HTC) cells (1). Experiments in whole cells suggest that this is due to an action of steroid hormones at the level of translation (2). With the hope of eventually testing this possibility more directly, we have devised a method for preparing from HTC cells a cell-free protein-synthesizing system that is capable of making TAT. Cell-free synthesis of specific proteins (3, 4), including TAT (5), by rat liver preparations has been reported, but the small amounts of specific protein synthesized have made quantitation difficult. Because HTC cells have the same disadvantage, we have sought to increase the specificity of the cell-free system by partially purifying those ribosomes engaged in TAT synthesis.

The purification of proteins by affinity chromatography is based on their specific, reversible binding to ligands (6-8). A crude extract containing the enzyme to be purified is passed through a column of agarose to which a specific ligand is covalently attached. The enzyme is preferentially retained and sometimes can be removed by appropriate buffers. TAT binds (9) to pyridoxal-*P* (K_m 10^{-8} M) and pyridoxamine-*P* (K_m 10^{-7} M). Agarose columns with pyridoxamine-*P* covalently bound can selectively remove TAT from HTC cell homogenates, with a resultant 100-fold purification upon elution (data to be published). Because TAT is thought to consist of four identical subunits, each binding one molecule of pyridoxal-*P* (10), at least one TAT subunit being synthesized on a polysome might be sufficiently complete to bind to the agarose-pyridoxamine-*P* column. If the entire complex of peptidyl-tRNA, mRNA, and ribosome remained intact and could be eluted, synthesis of TAT might proceed to comple-

Abbreviations: TAT, tyrosine aminotransferase; TCA, trichloroacetic acid; PALP, pyridoxal phosphate; HTC, hepatoma tissue culture cells.

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tion in a cell-free system. This paper presents evidence that this method can be used for partial purification of ribosomes that synthesize TAT.

MATERIALS AND METHODS

Agarose-pyridoxamine phosphate derivatives

(a) Pyridoxamine-*P* was coupled directly to Sepharose 4B (Fig. 1A), activated by 250 mg of cyanogen bromide per ml of packed agarose. The concentration of pyridoxamine-*P* in the coupling step was 20 mM in 0.2 M NaHCO₃ buffer, pH 9.8. Approximately 10 μmol of pyridoxamine-*P* was coupled per ml of packed agarose.

(b) Alternatively, pyridoxamine-*P* was linked to the agarose backbone through a hydrocarbon "arm" (Fig. 1, adsorbent B). Aminoethylimino-Sepharose was prepared by adding 0.3 M ethylenediamine in 0.2 M NaHCO₃, pH 9.8, to CNBr-activated Sepharose 4B. About 14 μmol of amino groups was present per ml of the Sepharose derivative. The ω-amino group was then succinylated with succinic anhydride (8). Pyridoxamine-*P* was coupled to the free carboxyl group with a water-soluble carbodiimide (8). 100 mg of pyridoxamine-*P* was added to a suspension containing 20 ml of derivatized Sepha-

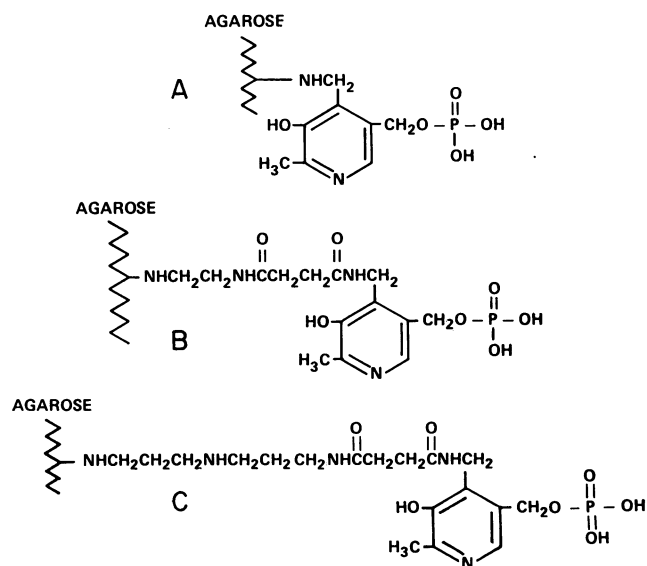


FIG. 1. Affinity chromatographic adsorbents A, B, and C prepared by attaching pyridoxamine-*P* to Sepharose 4B through side arms of increasing length (see *Methods*).

rose suspended in 20 ml of distilled water. The pH was adjusted to 4.7, and 1 g of 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide, dissolved in 3 ml of water, was added. The reaction was monitored for 2 hr while the pH was kept at 4.7, and the suspension was then stirred for 3 hr. The final adsorbent was washed with 8 liters of 0.1 M NaCl. Approximately 9 μ mol of pyridoxamine-*P* was coupled per ml of packed Sepharose.

(c) By similar methods, pyridoxamine-*P* was also linked to Sepharose by a longer arm consisting of succinyl 3,3'-diimino-dipropylamine (8) (Fig. 1, adsorbent C). The final, washed derivative contained 3 μ mol of pyridoxamine-*P* per ml of packed Sepharose.

Cells and ribosomes

HTC cells were grown in suspension culture (11, 1) in Swim's 77 medium. Crude ribosomes were prepared from cells induced for maximum TAT synthesis by growing them for 16 hr in 2 μ M dexamethasone phosphate, collecting them by low-speed centrifugation, and washing them twice in TKM buffer (0.05 M Tris·HCl (pH 7.6)–0.025 M KCl–0.005 M MgCl₂). The washed cells were allowed to swell for 20 min at 4°C in a volume of 5 mM MgCl₂ equal to half the packed cell volume. The cell suspension was homogenized in a Potter-Elvehjem homogenizer and then centrifuged at 20,000 $\times g$ for 10 min. The supernatant fraction was centrifuged at 100,000 $\times g$ for 2 hr, and the resulting ribosome pellets were stored frozen at –90°C.

PALP (pyridoxal-*P*) ribosomes

This term is used to designate the ribosomes obtained as follows: 10–15 mg of crude ribosomes suspended in 3 ml of TKM were fractionated on a 5-ml column of adsorbent B (Fig. 1) at 4°C. The column was washed with 40 ml of TKM buffer (pH 7.6) and eluted with 6 ml of 10 mM pyridoxal phosphate–50 mM Tris·HCl–25 mM KCl–5 mM MgCl₂–0.5 M NaCl–0.1% bovine serum albumin (all at pH 4.0). The yellow fraction (10–12 ml with a final pH between 5.5 and 6) was collected and centrifuged at 100,000 $\times g$ for 2 hr. The final yield of "PALP ribosomes" was 50–100 μ g protein.

Cell-free protein synthesis

Supernatant factors for protein synthesis were prepared by passing the 100,000 $\times g$ supernatant fraction through a Sephadex G-25 column with TKM buffer and collecting the void volume.

Cell-free protein synthesis was assayed by incubating, in a volume of 0.5 ml: 10–100 μ g of ribosomes [estimated as protein (12) or RNA (13)]; 2–3 mg (as protein) of supernatant factors; 1 mM ATP; 0.05 mM GTP; 1 mM phosphoenolpyruvate; 10 μ g of pyruvate kinase; 7.5 mM Mg acetate; 100 mM KCl; 50 mM Tris·HCl, pH 7.6; 2.5 mM β -mercaptoethanol; 4 μ M pyridoxal phosphate; 19 amino acids (not leucine), 40 μ M each; and 10 μ Ci of [³H]leucine (57 Ci/mmol) or 1 μ Ci of [¹⁴C]leucine (250 Ci/mol). After 30 min at 37°C, a 0.05-ml aliquot was removed, added to 1 mg of bovine serum albumin in 0.1 ml of 0.05 M leucine, and immediately precipitated with 10% trichloroacetic acid (TCA). The precipitate was heated at 90°C for 20 min in 5% TCA, collected by centrifugation, and twice dissolved in 0.1 M NaOH and reprecipitated with 5% TCA. The final precipitate was taken up in 0.5 ml of NCS solubilizer (Nuclear-Chicago), added to 10 ml of Liquifluor diluted with toluene, and counted in a Nu-

clear-Chicago liquid scintillation counter. When doubly labeled compounds were counted, the efficiency for tritium was 25% and for ¹⁴C 37%, with a 0.003% tritium spill and a 50% ¹⁴C spill. Quenching was uniform in all samples.

Immunoassay for TAT

The immunoprecipitation method for determining TAT synthesis has been described elsewhere (1, 14). In brief, unlabeled, partially purified TAT and nonspecific antiserum to TAT in slight excess were added to the incubation mixture to be tested, along with a 1000-fold excess of unlabeled amino acid. The precipitate was allowed to form at 37°C for 2 hr and then at 3°C for 16 hr. The precipitate was collected and the supernatant was reprecipitated in the same fashion. Both precipitates were washed five times with 0.5 ml of cold TKM buffer and counted as described above. The radioactivity in the first precipitate represents labeled TAT plus contaminants trapped in the precipitate, while that in the second represents only contaminants trapped in the precipitate. The difference between the two, therefore, is taken to represent labeled TAT (1).

TAT enzyme assay

The enzymatic activity of TAT was determined by the modified (9) method of Diamondstone (15) carried out at room temperature for 5 hr in the presence of 0.1% bovine serum albumin. Under these conditions, the assay is enzyme-dependent and linear for at least 5 hr.

RESULTS

Columns with pyridoxamine-*P* linked directly to agarose did not bind the TAT in homogenates of HTC cells. In contrast, adsorbents B and C (Fig. 1) gave substantial binding. The enzyme could be eluted from adsorbent B with 80% recovery at pH 4, and from adsorbent C only at pH 9 or above. In either case, the enzyme was purified at least 100-fold (data to be published).

When ribosomes prepared from induced HTC cells were passed through adsorbent B, less than 1% were retained. These could be eluted in the same way as TAT at pH 4. After concentration by high-speed centrifugation, the eluted ribosomes (PALP ribosomes) were as effective as unfractionated ribosomes in incorporating amino acids into TCA-precipitable protein. They were, however, enriched for the ability to synthesize immunologically detectable TAT when compared to unfractionated ribosomes (Table 1). The results given in Table 1 are taken from three separate double-label experiments. In each experiment PALP and unfractionated ribosomes were incubated separately, one with [³H]leucine and the other with [¹⁴C]leucine, in otherwise identical amino acid-incorporating systems. After 30 min at 37°C the paired mixtures were combined, and an excess of unlabeled leucine was added. Aliquots were then processed for determination of immunoprecipitable and TCA-precipitable counts. The first column of data shows the TAT-specific plus the nonspecific radioactivity in the first immunoprecipitation; the second column the nonspecific radioactivity in the second immunoprecipitation. The difference between the first and second represents the radioactivity in TAT (the third column). The fourth column shows the radioactivity incorporated into TCA-precipitable peptides and the last column the percentage of TCA-precipitable peptides that is immunoprecipitable TAT (col 3/col 4 \times 100).

TABLE 1. *Immunoassay of TAT synthesized by PALP and unfractionated ribosomes*

Expt.	Label	Ribosomes	Immunoprecipitation (cpm)			Total protein (cpm)	% TAT synthesized
			1	2	net		
1	¹⁴ C	Unfractionated	143	131	12	1,300	0.92
	³ H	PALP	5,070	1,500	3,570	28,350	12.6
2	¹⁴ C	Unfractionated	278	193	85	2,260	3.7
	³ H	PALP	12,800	1,150	11,600	25,800	45
	³ H	Unfractionated	12,500	9,190	3,310	120,000	2.8
	¹⁴ C	PALP	679	55	624	864	71
3	¹⁴ C	Unfractionated	375	160	215	6,000	3.6
	³ H	PALP	4,290	1,130	3,160	25,200	12.5
	¹⁴ C	Unfractionated + RNase	62	40	22	325	(6.5)
	³ H	PALP + RNase	1,800	670	1,130	9,250	(12.2)

Ribosomes were incubated independently in the complete amino acid-incorporating system. After 30 min at 37°C, the paired incubation mixtures were combined and 0.1 ml was precipitated with 10% TCA to determine incorporation into total protein. Another 0.3-ml aliquot of the combined incubation mixtures was immunoprecipitated as described in *Methods*. The total protein counts given above have been normalized to a volume of 0.3 ml.

In the third experiment the ribosomes were first incubated for 5 min at 37°C with 25 µg of pancreatic RNase and were then used as described above.

%TAT is calculated by dividing total protein cpm into net immunoprecipitable cpm. The amount of ribosomes, expressed as RNA, used in each incubation is as follows: Expt. 1, unfractionated 70 µg, PALP 20 µg; Expt. 2, unfractionated 55 µg, PALP 11 µg; Expt. 3, unfractionated 55 µg, PALP 20 µg.

In the first experiment of Table 1, PALP ribosomes incorporated 12.5% of the total peptide radioactivity into immunoprecipitable material, while the unfractionated ribosomes incorporated 0.9%. A qualitatively similar result was obtained in the second and third experiments. The second experiment also demonstrates that the percentage of immunoprecipitable counts incorporated is isotope-independent. The third experiment shows that the counts in the immunoprecipitate represent amino acids incorporated into protein and not adventitious binding of labeled amino acid. Half of the PALP and unfractionated ribosomes were incubated for 5 min with RNase prior to incubation in the amino acid-incorporating system. Such treatment substantially reduced the ability of both classes of ribosomes to incorporate amino acids and concomitantly reduced the counts in the immunoprecipitate. Other experiments not shown here proved that the incorpora-

tion of amino acids into TCA-precipitable and immunoprecipitable counts is time dependent.

The experiments in Table 1 were performed using a double-label technique to eliminate sample variations in the precipitated counts and to minimize possible artifacts in the immunologic method. Despite this it could be argued that ribosomes that are adsorbed to and then eluted from the column are altered in some nonspecific way, making the radioactivity they incorporate more readily precipitated by the antiserum than that of controls. The experiment in Table 2 was designed to study this possibility. If TAT is passed through adsorbent C (Fig. 1) it is retained, but only 1-2% of that retained can be eluted at pH 5. If ribosomes are passed through such a column, some of those that bind are eluted at pH 5, but one might not expect any enrichment for the ability to make TAT since their elution was not obtained by conditions that release TAT. For the experiment in Table 2 such ribosomes were compared with unfractionated ribosomes. There was no enrichment for synthesis of immunoprecipitable material in the pH 5 PALP ribosomes. Therefore, merely adsorbing and nonspecifically eluting ribosomes did not seem to enrich their ability to synthesize immunoprecipitable TAT. In other experiments, the binding (and consequent purification) of the TAT-specific ribosomes on adsorbent B was blocked by amounts of free TAT just sufficient to saturate the column. When ribosomes from the same preparation were passed through the same column in the absence of free TAT, the usual purification resulted.

TABLE 2. *Immunoassay of TAT produced by ribosomes isolated from adsorbent C*

Label	Ribosomes	Immuno-precipitation (cpm)			Total protein (cpm)	% TAT synthesized
		1	2	net		
¹⁴ C	Unfractionated	134	82	52	1,840	2.8
³ H	PALP (pH 5)	1,165	745	420	14,320	2.9

PALP (pH 5) ribosomes were prepared by passing 10 mg of resuspended crude ribosomes through a 5-ml column of adsorbent C and eluting at pH 5. The ribosomes in the eluate were concentrated by centrifugation at 100,000 × *g* for 2 hr. Immunoprecipitable and total protein cpm were assayed as described in Table 1 and *Methods*.

50 µg of unfractionated ribosomes and 15 µg of PALP ribosomes were used in the respective incubation mixtures.

Evidence that the PALP ribosome fraction is enriched for the ability to synthesize enzymatically active TAT is presented in Table 3. The components of the amino acid-incorporating system used were the same as those used above, except that the supernatant factors were passed through an agarose column to which TAT antibody was covalently bound. This removed more than 99% of the TAT activity. The ex-

periments were done by incubating in the amino acid incorporating system ribosomes alone, supernatant factors alone, or the two combined, with and without cycloheximide. The first column of data in Table 3 shows the counts incorporated into TCA-precipitable peptide. The second column represents TAT activity, derived by subtracting the sum of the TAT activity of the supernatant factors alone plus that of ribosomes alone from the activity found when the two were incubated together. The contribution to A_{331} of the unfractionated ribosome preparation alone was due to TAT trapped in the ribosome pellet and in other experiments was substantially reduced by resuspending and repelleting the ribosomes.

When unfractionated ribosomes were incubated with supernatant factors in the complete system, there was no net increase in TAT activity. PALP ribosomes, when incubated in the complete system with supernatant factors, appeared to synthesize enzymatically active TAT. This net increase in enzyme activity was blocked by cycloheximide in proportion to total protein synthesis. This same result has been obtained with anisomycin or EDTA as the blocking agent. Thus, enzyme activity appears concomitantly with and is proportional to protein synthesis in the presence of PALP ribosomes. The possibility of some other effect, such as enzyme activation dependent upon protein synthesis or reduction in enzyme decay cannot be eliminated, but the data are consistent with the hypothesis that new enzyme molecules are completed.

DISCUSSION

Two lines of evidence have been advanced to support the contention that agarose-pyridoxamine-*P* columns are capable of partially purifying ribosomes synthesizing TAT: the apparent synthesis of enzyme activity, and the immunoprecipitation data. The latter show that fractionated ribosomes incorporate more radioactive amino acid into immunoprecipitable material than did unfractionated ribosomes and that this incorporation is isotope-independent and blocked by RNase.

One nonspecific mechanism that might produce such results would be that TAT is adsorbed randomly to ribosomes, causing them to bind to the columns. This TAT might then cause immunoprecipitation of ribosomes not at all engaged in TAT synthesis. Four lines of evidence are against this possibility. First, TAT is added (in the supernatant factors) to each cell-free incorporation experiment and in large amounts as part of the immunoprecipitation procedure. If ribosomal binding of TAT occurred to any appreciable extent, one would expect no difference in the immunoprecipitable radioactivity. Second, washing ribosomes by recentrifuging them through a sucrose step gradient readily removes any associated TAT activity, which implies that TAT is not tightly bound. Third, experiments cited in *Results* showed that ribosomes passed through adsorbent B were purified in the absence but not in the presence of free TAT. Apparently, free TAT, rather than assisting ribosome adsorption, competes for the specific binding sites. Fourth, random binding would not explain the experiments in which TAT activity appeared to be synthesized by the PALP ribosomes. The changes in absorbance, though small, were within the sensitivity of the assay and were reproducible in six consecutive experiments done over a period of 3 months.

If TAT-synthesizing ribosomes are preferentially retained by the pyridoxamine-*P* column, some interesting questions

TABLE 3. Enzymatic assay of TAT synthesized by PALP and unfractionated ribosomes

Components added to incorporation system	Total protein (cpm)	TAT, as net increase in A_{331}
Unfractionated ribosomes + supernatant factors	45,000	0.02
Unfractionated ribosomes + supernatant factors + cycloheximide*	4,000	0.01
PALP ribosomes + supernatant factors	2,800	0.105
PALP ribosomes + supernatant factors + cycloheximide*	1,600	0.055

The indicated components were added to the otherwise complete amino acid-incorporation system in a total volume of 0.25 ml and incubated for 30 min at 37°C. At the end of the incubation, 0.05 ml was removed for estimation of incorporation of [³H]leucine into total protein (col. 2). The remaining 0.2 ml was assayed for TAT activity in the presence of 0.1% bovine serum albumin. All readings were done against a blank containing 0.2 ml of the incorporating system minus both ribosomes and supernatant factors.

The values for net increase in A_{331} were calculated in the following manner: each ribosome preparation alone was incubated in the complete system, and the A_{331} produced was determined; the supernatant factor preparation alone was incubated in the complete system and the A_{331} produced by it was determined. The A_{331} from these single incubations was summed and subtracted from the A_{331} produced when these components were incubated together. That is, $A_{331}[r + SF] - A_{331}r - A_{331}SF =$ net increase. In the case of the unfractionated ribosomes, the actual numbers were $[0.335] - (0.25) - (0.115) = 0.02$ and $[0.375] - (0.25) - (0.115) = 0.01$. For the PALP ribosomes the values were $[0.220] - (0.00) - (0.115) = 0.105$ and $[0.17] - (0.00) - (0.115) = 0.055$. In this experiment, 63 μ g (RNA) of unfractionated ribosomes and 3.3 μ g of PALP ribosomes were used.

* 2 mM.

about the nature of the binding are raised. TAT is a tetramer, consisting of four identical subunits that bind one molecule of pyridoxal-*P* each (10). The mechanism conceived as the basis for this study was that a nearly complete TAT peptide subunit could fold sufficiently to bind to the cofactor. If this is so, it is evidence that the nascent peptides with their partially completed primary structure can fold properly to a considerable extent. Although no unequivocal proof of this exists, interactions of a specific sort have been suggested that involve the nascent peptides of β -galactosidase (16) and the heavy and light chains of immunoglobulins (17-19). In addition, nascent peptides of immunoglobulins have been shown to interact with antiserum against the appropriate chain or even whole IgG (20, 21). There is much evidence, however, that does not support this idea (22). For example, staphylococcal nuclease has been shown to require at least 85% of, and probably the entire, primary sequence for accurate folding (23).

Another possibility is that fully synthesized peptide subunits provide the necessary link to the cofactor column by adhering to polysomes engaged in TAT synthesis. In several cases, presumably freshly completed enzymes have been described that adhere to ribosomes (16, 24). Studies of TAT synthesis in HTC cells have shown no evidence for a subunit pool (1, 25), although a small, rapidly turning-over pool cannot be excluded by the existing data.

Finally, it may be that the enrichment for TAT-producing polysomes occurs by virtue of some unsuspected mechanism. Nonetheless, the system described in this paper may facilitate studies on the regulation of the synthesis of TAT. It also may be a method that can be generalized to permit the examination of the synthesis of other proteins.

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