The Interaction of Aminoacyl Transferase II and Ribosomes*

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SUMMARY

The kinetics of aminoacyl transfer from aminoacyl soluble ribonucleic acid to ribosomal protein has been examined with rat liver preparations. A lag in the initial rate of incorporation with resolved aminoacyl-transfering enzymes is observed with low (4 mM) concentrations of reduced glutathione; this lag is abolished when transferase II is incubated with a sulfhydryl compound, such as glutathione or 2-mercaptoethanol, prior to the addition of the other reaction components. The sulfhydryl activation is dependent on time and on glutathione concentration, and is inhibited by oxidized glutathione. The total extent of amino acid incorporation is also dependent on sulfhydryl concentration. A sulfhydryl requirement in aminoacyl transfer, other than for the activation of transferase II, was not detected. The initial rate of amino acid incorporation is markedly stimulated when transferase II is incubated with GTP and a sulfhydryl compound, such as glutathione or 2-mercaptoethanol, prior to the addition of the other reaction components. The sulfhydryl-activated enzyme and ribosomes, prior to the participation of aminoacyl transferring (or aminoacyl-polymerizing) factors and GTP. However, studies with reticulocyte preparations (7) indicate that aminoacyl-sRNA binding requires one of the two transferring enzymes and GTP, while peptide bond synthesis requires the other enzyme. Some recent studies with rat liver preparations appear to be consistent with the former requirement (14) and others with the latter (15, 16).

The observations presented here suggest the occurrence of additional interactions, between aminoacyl-transferase II and sulfhydryl compound and between the sulfhydryl-activated enzyme and ribosomes, prior to the participation of aminoacyl-sRNA. It should be emphasized that the sulfhydryl requirement is satisfied by a variety of compounds and is not specific for glutathione (1, 2). Although glutathione has been used in the experiments described here, similar results have been obtained with 2-mercaptoethanol.

EXPERIMENTAL PROCEDURE

Isolation of Ribosomes and Transfer Factors—After removal of mitochondria, ribosomes were prepared from the supernatant fraction of rat liver homogenates by sedimentation of the microsomes at 100,000 × g for 90 min, extraction of crude ribosomes from microsomes with 0.23% deoxycholate (17), and purification of the ribosomes by repeated centrifugation in high (0.01 M) magnesium ion solutions (3). After removal of the microsomes the supernatant fraction was adjusted to pH 5.2 and the pH 5-insoluble amino acid-activating enzyme and sRNA complex ("pH 5 enzymes" fraction) were removed by centrifugation (18, 19). The protein in the pH 5 supernatant solution was partially purified by chromatography on calcium phosphate gel and ammonium sulfate fractionation, and the two aminoacyl transferases (I and II) were then resolved from this partially purified soluble protein fraction by gel filtration on columns of Sephadex G 200 (3).

14C-Labeled Aminoacyl-sRNA1 Preparation—Aminoacyl-sRNA, labeled with 14C-leucine or with a 14C-amino acid hydrolysate, was extracted from incubations of the "pH 5 enzymes" fraction, ATP, and the isotopically labeled amino acid or acids by the phenol extraction procedure (18, 20, 21). The 14C-leucyl-sRNA preparations also contained endogenous, unlabeled, esterified amino acids. Small amounts of contaminating, high molecular weight RNA were removed from aminoacyl-sRNA by molecular sieve chromatography on columns of Sephadex G 200 in 2% potassium acetate, pH 4.9 (Fig. 1). The specific radioactivities

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$ The abbreviation used is: sRNA, soluble ribonucleic acid.
of the purified aminoacyl-sRNA preparations used were approximately 125,000 cpm per mg of RNA.

Incubation Procedures—Incubations of the complete system, accomplished at 37° in a total volume of 2 ml, contained the following components: approximately 1 mg of purified ribosomes (3), 0.2 mM GTP, 6 mM MgCl₂, 80 mM NH₄Cl, 60 mM Tris buffer, pH 7.4, and 30 to 60 μg of transferase I. Varying concentrations of ¹⁴C-aminoacyl-sRNA, glutathione, and transferase II were added as noted below. At the end of the incubation period the hot (90°) 5% trichloracetic acid-insoluble fraction was prepared and collected on Millipore filters for radioactivity determinations (3).

In experiments in which transferase II was preincubated, the first incubation contained transferase II, 6 mM MgCl₂, 60 mM Tris buffer, pH 7.4, and varying concentrations of glutathione in a total volume of 0.25 ml. In some experiments, ribosomes, GTP, and NH₄Cl were included in this incubation mixture. Incubation was performed at 37° for 15 min. Transferase I, ¹⁴C-aminoacyl-sRNA, and ribosomes, GTP, or NH₄Cl, if absent from the first part of the incubation, were added as noted above. The concentration of glutathione in this incubation was approximately 2 mM, and some incubations received 22 mM oxidized glutathione. The second incubation was carried out at 37° for 5 min in a total volume of 1.85 ml. For the third incubation approximately 0.1 mg of transferase I and 52 μg of ¹⁴C-aminoacyl-sRNA (7000 cpm, labeled with a ¹⁴C-amino acid hydrolysate) were added to the contents of the second incubation. GTP, MgCl₂, NH₄Cl, and Tris buffer were added to maintain the same concentrations, and any component omitted during the first two steps was added. The complete system was then incubated at 37° in a total volume of 2 ml for varying periods of time. At the end of the third incubation period the hot 5% trichloracetic acid-insoluble fraction was prepared and collected on Millipore filters for radioactivity determinations.

RESULTS

The resolution of high molecular weight RNA and aminoacyl-sRNA (obtained from ¹⁴C-amino acid-labeled “pH 5 enzymes” fractions) by gel filtration on Sephadex G-200 is shown in Fig. 1. Radioactive amino acids were associated only with the sRNA peak, which was retarded by the gel (Fractions 40 to 70). Rechromatography or sucrose density gradient analysis of the sRNA revealed only one component, while the material emerging with the void volume (Fractions 15 to 30) revealed a pattern similar to that of ribosomal RNA. Aminoacyl-sRNA purified on Sephadex G-200 was used in all of the experiments described below. The purification of sRNA by similar gel filtration procedures has been reported (15, 22).

Preliminary studies (4) indicated that the initial lag in amino acid incorporation, observed in incubations with the resolved transferases, was not abolished by increasing the concentration of either transferase; however, preincubation of transferase II and glutathione, prior to the addition of the other reaction components, eliminated the initial lag. Fig. 2 shows that the early lag is markedly reduced and the total extent of incorporation is increased by increasing the concentration of glutathione in the incubation ( — O— O). When transferase II is first incubated in 4, 8, or 20 mM glutathione, prior to its addition to the incuba-
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TIME OF INCUBATION (MINUTES)

Fig. 3. The effect of glutathione on the initial rate of aminoacyl transfer. Incubations were carried out for 5 min at 37°C, as described in Fig. 2. One series was incubated directly in varying concentrations of glutathione (○—○); others contained transferase II preincubated for 15 min with similar concentrations of glutathione.

Fig. 4. The effect of glutathione on the preincubation of transferase II. A, varying amounts of transferase II were incubated for 15 min at 37°C in 60 mM Tris buffer and 0 (Line 1), 4 (Line 3), 8 (Line 4), 12 (Line 5), 20 (Line 6), and 32 (Line 7) mM glutathione; the reaction mixtures were then diluted to a final concentration of 4 mM glutathione and 54 µg of transferase II per sample; transferase I, ribosomes, GTP, NH₄Cl, MgCl₂, Tris buffer, and 23 µg of W-leucyl-sRNA (3060 cpm), as described in the text, were added and incubations at 37°C, in a total volume of 2 ml, were continued for varying periods of time. Line 2 (○—○) represents a non-preincubated control series in 4 mM glutathione. B, transferase II was incubated for 15 min at 37°C in 60 mM Tris buffer and 20 mM glutathione. One series was then maintained at 20 mM glutathione (Line 4, □—□); others were diluted to 4 mM (Curve 3, □—□) or 0.5 mM (Curve 2, △—△) glutathione. Transferase I, ribosomes, GTP, NH₄Cl, MgCl₂, Tris buffer, as described in the text, and 55 µg of ¹⁴C-leucyl-sRNA (7000 cpm) were then added. All samples contained 54 µg of transferase II in a final volume of 2 ml. Incubations were continued at 37°C for varying periods of time. ○—○ (Line 1), non-preincubated control in 0.5 mM glutathione.

Fig. 5. Dependence of aminoacyl transfer on the concentration of transferase II. A, incubations, as described in the text and Fig. 2, in 4 mM glutathione with 11 µg of ¹⁴C-leucyl-sRNA (1500 cpm) and varying concentrations of transferase II (○—○) or of transferase II preincubated for 15 min at 37°C in 5 mM glutathione (●—●); incubation time at 37°C, 25 min. B, incubations, in 0.5 mM glutathione, contained 52 µg of ¹⁴C-leucyl-sRNA (7000 cpm) and varying concentrations of transferase II preincubated for 10 min at 37°C in 20 mM glutathione; incubation time at 37°C, 60 min.
higher concentrations of transferase II were required in control incubations (— — — ) as compared to incubations with transferase II preincubated for 15 min in 8 mM glutathione. Fig. 5B shows the effect of transferase II concentration in experiments in which the first incubations contained the enzyme and 20 mM glutathione, and the second incubation was carried out in 0.5 mM glutathione. As described in Fig. 4B (Line 1) and in Fig. 6 (△—△), glutathione with a concentration of 0.5 mM or lower does not support aminocyl transfer in control incubations with non-preincubated enzymes. It is interesting to note, however, that the addition of transferase II to incubations in 0.3 mM glutathione with sulfhydryl-activated transferase II (Fig. 6) did not inhibit the activity of the preincubated enzyme; indeed, incorporation in the presence of transferase II plus glutathione-activated transferase II (○—△) was slightly but significantly greater than the sum of the individual activities of activated transferase II (○—○) or non-preincubated transferase II (△—△).

Experiments in which transferase II and glutathione were preincubated in the presence of other reaction components but in the absence of transferase I and 14C-aminoacyl-sRNA indicated that markedly higher initial rates of incorporation were obtained when transferase II, glutathione, ribosomes, GTP, MgCl₂, and NH₄Cl were present in the first incubation (Fig. 7, Line 1). Preincubation of these components in the absence of GTP (Line 5) or ribosomes (Line 4) led to lower reaction rates at early times, which rates were not too different from those for experiments in which only transferase II and glutathione were present in the first incubation (Line 2). Similar data were obtained when ammonium chloride was omitted from the first incubation. Line 3 of this figure represents a series in which transferase II and glutathione were preincubated in one flask, and ribosomes, GTP, MgCl₂, and NH₄Cl were preincubated in another; the two flasks were then combined and transferase I and 14C-aminoacyl-sRNA were added prior to the second incubation. Thus, although the initial lag in aminocyl transfer (Line 1) is overcome when transferase II is preincubated with glutathione, the presence of ribosomes, GTP, MgCl₂, and NH₄Cl results in a further increase in the initial rate.

Fig. 8 summarizes an experiment in which GTP was allowed to react with ribosomes, transferase II, and glutathione, as described above, and the preincubation mixture was then diluted to lower the GTP concentration before the addition of transferase I and 14C-aminoacyl-sRNA. Incubation of the complete system, in 2.5 × 10⁻⁸ M GTP, did not catalyze aminocyl transfer (△—△). Preincubation of transferase II, glutathione, ribosome, MgCl₂, NH₄Cl, and GTP at 10⁻⁶ M, followed by a second incubation in the presence of 10⁻⁶ M GTP, led to aminocyl transfer (○—○) similar to that described in Fig. 7, Line 6; however, when the GTP concentration was diluted from 10⁻⁶ M to 2.5 × 10⁻⁸ M, but glutathione, MgCl₂, and NH₄Cl were maintained at the same concentration, the addition of transferase I and 14C-aminoacyl-sRNA did not result in amino acid incorporation (△—△). Thus, although GTP appears to be essential for the initial interaction observed above between activated transferase II and ribosomes (in the presence of MgCl₂ and GTP, 80 mM NH₄Cl, and 6 mM MgCl₂ (Line 6). Some series did not receive GTP (Line 6) or ribosomes (Line 4). In one series, transferase II and glutathione were preincubated separately from ribosomes, GTP, NH₄Cl, and MgCl₂, and these were combined prior to the next step (Line 3). Line 2 represents transferase II preincubated with glutathione. All series then received transferase I and 40 μg of 14C-leucyl-sRNA (5200 cpm) and the concentration of the following components adjusted, in a final volume of 2 ml, as follows: glutathione, 4 mM; GTP, 0.3 mM; NH₄Cl, 80 mM; MgCl₂, 6 mM; and Tris buffer, 60 mM. Incubations, at 37°, were then continued for varying periods of time. Line 1 represents a non-preincubated control series.
inhibited by oxidized glutathione, and that the sulfhydryl-activated transferase II. When transferase II was preincubated with oxidized glutathione (20 mM), and the oxidized sulfhydryl-activated transferase II interacted with ribosomes in the reaction involving GTP. This relationship was further examined as a function of ribosomal concentration in a series of three-step incubations (Fig. 10). The presence of GTP and the other components (Line 5) protected the system to a certain extent against inhibition by oxidized glutathione.

It has been observed that the addition of oxidized glutathione to incubations of the complete system completely inhibits the aminoacyl transfer reaction. This inhibition also occurs with sulfhydryl-activated transferase II. When transferase II was preincubated with oxidized glutathione (20 mM), and the oxidized glutathione was then extensively diluted (to 2 mM) prior to the aminoacyl transfer reaction. This inhibition also occurs with sulfhydryl-activated transferase II. When transferase II was preincubated with oxidized glutathione (20 mM), and the oxidized sulfhydryl-activated transferase II interacted with ribosomes in the reaction involving GTP. This relationship was further examined with oxidized glutathione as shown in Fig. 9. A series of three-step incubations was carried out as follows. The first incubation contained transferase II, reduced glutathione (16 mM), ribosomes, MgCl₂, NH₄Cl, and Tris buffer; two sets of tubes also received GTP. The second incubation contained the components described above but the reduced glutathione was diluted to 2 mM; one series of tubes with GTP and one series without GTP received oxidized glutathione (22 mM). For the third incubation, all tubes received transferase I, ¹⁴C-aminoacyl-sRNA, and GTP if previously omitted. The rates of incorporation when all the precubination components were present (●●●●) was markedly lower when GTP was omitted from the first and second incubations (□□□□). When oxidized glutathione was added in the presence of GTP (○○○○), the reaction did not proceed beyond 5 to 10 min and the total extent of incorporation was considerably less than in its absence. However, when oxidized glutathione was added to the series of flasks preincubated in the absence of GTP (□□□□), aminoacyl transfer did not occur.

The activated transferase II-ribosome-GTP interaction was also examined as a function of ribosomal concentration in a series of three-step incubations (Fig. 10). The first incubation contained transferase II, reduced glutathione (16 mM), NH₄Cl, MgCl₂, and varying amounts of ribosomes as indicated. Some incubations received GTP, one series received GDP, and another ATP; MgCl₂ was omitted from one series containing GTP. The second incubation contained the components present in the first step but the reduced glutathione was diluted to 2 mM, and 22 mM oxidized glutathione was added. The third incubations received transferase I, ¹⁴C-aminoacyl-sRNA, and GTP or MgCl₂, if absent from the previous incubations; ribosomes were added to all tubes to an equivalent final concentration. When GTP was absent from the first two phases of these incubations, oxidized glutathione completely inhibited aminoacyl transfer (Line 5). The presence of GTP and the other components (Line 5) protected the system to a certain extent against inhibition by oxidized glutathione.
were continued for 20 min at MgCl₂ or GTP omitted from first and second steps of incubation; all samples then received transferase I, 52 pg of 1%-

...dized glutathione, and this protection also appeared to be dependent on the concentration of ribosomes in the preincubation period. Other nucleotides such as GDP (Line 4) and ATP (Line 3) were not as effective in this respect as GTP, and may reflect the presence of some nucleoside diphosphokinase activity. The results with GTP cannot be interpreted unequivocally because, as in the E. coli system (23), GDP appears to be a competitive inhibitor of GTP in the reticulocyte system. As mentioned above, binding of sRNA to ribosomes appears to occur nonenzymatically in the E. coli system, but requires one of the transferring factor and GTP in the reticulocyte system. Studies in this laboratory with rat liver preparations indicate that neither transferase I nor transferase II quantitatively in-

...the presence of some nucleoside diphosphokinase activity. The results with GTP cannot be interpreted unequivocally because, as in the E. coli system (23), GDP appears to be a competitive inhibitor of GTP in the reticulocyte system. When MgCl₂ was absent during the preincubation period, incorporation was not subsequently observed. In one experiment, not presented here, NH₄Cl was omitted from the preincubation but approximately 2.5 mM potassium ions were present in the buffered salts used to resuspend ribosomes, transferase II, and other reaction components; incorporation in this medium, which was low in monovalent cations, was about 50% of that observed when 50 mM NH₄Cl was present.

**DISCUSSION**

The kinetics of the aminoacyl transfer reaction has been examined in experiments in which combinations of various reaction components were incubated prior to the synthesis of peptide bonds, which was initiated by the addition of ¹⁴C-aminoacyl-sRNA and transferase I. The results presented above indicate that the sulfhydryl requirement in aminoacyl transfer is related to the sulfhydryl function of transferase II. The initial lag in amino acid incorporation with resolved enzymes is decreased by increasing glutathione concentrations. The rate and the total extent of the reaction are proportional to the concentration of sulfhydryl compound in the incubation. The early lag is completely eliminated by preincubation of transferase II with glutathione. Aminoacyl transfer with sulfhydryl-preincubated transferase II is also influenced by the concentration of glutathione in the incubation; however, significant incorporation is obtained even when the glutathione concentration in the first incubation step is extensively diluted prior to the addition of the activated transferase II to the second incubation in the complete system. The amount of this enzyme required for incorporation is markedly lower in the presence of relatively high levels of glutathione (20 mM) or when glutathione-preincubated enzyme is used. These observations suggest that sulfhydryl compound is only required for the activation of transferase II.

A marked stimulation of the initial rate of aminoacyl transfer, 3 to 4 times greater than that obtained when transferase II and glutathione were preincubated, was observed when the first incubation also included ribosomes, GTP, and NH₄Cl. The requirement for all of these components, for this very rapid initial rate, might reflect an interaction between sulfhydryl-activated transferase II and ribosomes which is dependent on the presence of GTP and a monovalent cation. The finding that GTP could not be extensively diluted, after its participation in the transferase II-ribosome interaction, suggests that the phenomenon responsible for the high initial activity observed requires GTP in both the preincubation and the incubation phases. The possibility exists that GTP may participate in the reversible formation of a complex between ribosomes and the enzyme, but is dissociated from it at low GTP concentrations. These results with GTP are in contrast to those in which incorporating activity was demonstrated when glutathione was extensively diluted after being allowed to react with transferase II during the preincubation period.

Experiments with oxidized glutathione which inhibits the aminoacyl transfer reaction suggest that its effect is due to the reoxidation of transferase II, which is inactive, or to the formation of a mixed disulfide between the enzyme and glutathione. The finding that the GTP-dependent interaction of transferase II and ribosomes is less sensitive to inhibition by oxidized glutathione is of particular interest. Thus, when sulfhydryl-activated transferase II is preincubated with ribosomes and GTP, the resulting preparation catalyzes a very rapid initial rate of amino acid incorporation and is partially protected from inhibition by oxidized glutathione. A monovalent cation and MgCl₂ may also be required, but other nucleotides such as ATP or GDP do not substitute for GTP.

As mentioned above, binding of sRNA to ribosomes appears to occur nonenzymatically in the E. coli system, but requires one of the transferring factor and GTP in the reticulocyte system. Studies in this laboratory with rat liver preparations indicate that neither transferase I nor transferase II quantitatively influences or modifies the binding of aminoacyl-sRNA to ribosomes. Incubations were carried out with ribosomes, MgCl₂, NH₄Cl, glutathione, GTP, and ¹⁴C-aminoacyl-sRNA in the presence...
of transferase I or II, individually. The ribosomes were sedimented from the incubation mixture through a discontinuous sucrose gradient (24) and analyzed for total radioactivity and their ability to transfer amino acids to protein. The amount of $^{14}C$ bound noncovalently to the ribosomes was the same as in control incubations in the absence of the transferase; protein-bound radioactivity was negligible in these experiments with the individual transferases until the complementary transferring factor was added. When the transferase omitted from such incubations was then added, together with a large pool of nonisotopic aminocetyl-sRNA, the kinetics and the total extent of incorporation were the same as in experiments in which $^{14}C$-aminocetyl-sRNA was not present in the preincubation period but was added at the same time as the pool of unlabeled aminocetyl-sRNA. Nonenzymatic binding of aminocetyl-sRNA to rat liver ribosomes has been obtained in the presence of MgCl₂; KCl and NH₄Cl inhibit this binding and release ribosome-bound aminocetyl-sRNA, although aminocetyl transfer to protein requires a monovalent cation. It has not yet been possible to determine whether aminocetyl-sRNA binding to ribosomes is specific with respect to the coding sequence on the template or the site on the ribosome where peptide synthesis is catalyzed.

Studies with E. coli ribosomes and synthetic polynucleotides, however, have shown that the attachment of sRNA is specific with respect to the amino acid acceptor sRNA chains and the synthetic oligonucleotides used as artificial templates (25-27). Attempts are in progress to determine whether aminocetyl-sRNA bound nonenzymatically to ribosomes in this rat liver system, which contains endogenous messenger RNA, is a direct precursor of polypeptides or whether incorporation occurs only after it is released, perhaps from none specific adsorption sites on ribosomes. The studies presented here represent efforts to elucidate some of the intermediary steps involved in polypeptide biosynthesis prior to the synthesis of the peptide bond. Although the binding of aminocetyl-sRNA does not appear to be influenced by either of the aminocetyl-transfering enzymes, the binding which does occur has not been shown to be an obligatory step. Evidence is presented here, however, for an additional interaction, among activated transferase II, ribosomes, GTP, and NH₄, which occurs prior to peptide bond synthesis and which must be considered in terms of the mechanism of the reaction. Evidence to be published subsequently, for an interaction between aminocetyl-sRNA and transferase I prior to the reaction of this factor with ribosomes and transferase II, must also be considered. It is possible that a polyribosomal event occurs prior to the participation of aminocetyl-sRNA and transferase I, and that a sequence of reactions is repeated as single amino acids are incorporated into nascent peptide chains. The stimulatory effect on the initial kinetics described above may reflect a ribosome-messenger RNA event prior to the participation of transferase I and aminocetyl-sRNA and prior to peptide bond synthesis resulting in the priming of active ribosomes for the addition of the next aminocetyl-sRNA to the growing ends of peptidyl-sRNA chains.

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