Variations in Attachment of a Cysteine Conjugate to Soluble Ribonucleic Acid*

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SUMMARY

A cell-free system was prepared from rat liver which catalyzed the incorporation of cysteine and the analogue of cysteine, S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine, into ribosomal protein. Fractionation of this system with (NH₄)₂SO₄ resulted in the separation of the cysteinyl soluble ribonucleic acid synthetase (cysteinyl-sRNA synthetase) from the aminocetyl-sRNA synthetases, presumably those for valine and isoleucine, that catalyzed the transfer of the S-substituted cysteine analogue to sRNA. Furthermore, in incubation media that contained the synthetases for cysteine and analogue transfer, an excess of unlabeled cysteine did not inhibit aminoacylation of sRNA by the H- or S-labeled analogue. However, the addition of either valine or isoleucine produced a marked inhibition of the transfer of radioactive tetrahydrohydroxynaphthylcysteine to sRNA. These observations were consistent with the concept that valyl- and isoleucyl-sRNA synthetases and their respective transfer RNAs are operational for the cysteine analogue. In contrast, the reaction of 1,2-epoxy-1,2,3,4-tetrahydronaphthalene with cysteinyl-sRNA resulted in the formation of the analogue attached to the cysteine transfer RNA. The incorporation of (1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine into protein should occur in a position determined by the species of sRNA that is acting as the transfer agent.

The conjugation of polycyclic aromatic hydrocarbons with cellular proteins has been implicated in carcinogenesis (2). Previous evidence (3, 4) has indicated also that a hydrocarbon-protein conjugate can be a precursor for mercapturic acid formation. S-Arylcysteines or certain of the dihydrohydroxy derivatives have been found in tissues from the original protein conjugate (5) and from S-dihydrohydroxyarylglutathione (6, 7). The activation of these S-substituted cysteine analogues apparently involved the pathway for the incorporation of amino acids into protein. These findings, plus observations that S-substituted arylcysteines can be formed in tissue (5-7), offered a mechanism for the attachment of aromatic hydrocarbons to cellular proteins alternative to direct binding.

Previously we reported (1, 8) that stored preparations of rat liver enzyme lost catalytic activity within a few days for the activation of tetrahydrohydroxynaphthylcysteine and chlorophenylcysteine, whereas the cysteine-activating enzyme remained active for several days. One possibility is that the cysteinyl-sRNA synthetase is not involved in the activation and transfer of either analogue into sRNA. Furthermore, it appears reasonable that if the cysteine-activating enzyme were not functioning in this capacity, the cysteine transfer RNA would not accept either analogue. Tetrahydrohydroxynaphthylcysteine was used to study the proposed hypothesis, with the use of components of a cell-free system from rat liver and yeast sRNAs. The results are described in this report.

EXPERIMENTAL PROCEDURE

Preparation of Enzymes and Microsomes—The liver from a male Holtzman rat weighing approximately 150 g was placed in 25 ml of ice-cold 0.25 M KCl in a cold stainless steel cup of a Waring Blender and homogenized for 40 sec. The homogenate was centrifuged at 18,000 x g for 30 min. The supernatant fluid was centrifuged at 105,000 x g for 2 hours in a Spinco model L centrifuge with a No. 40 analytical rotor. The supernatant fluid was decanted and stored at -20°C. The sedimented microsomal pellets were rinsed twice with 1 ml of 0.01 M Tris-HCl, pH 7.5. The microsomal preparation was then resuspended in 0.1 M Tris-HCl and kept cold to await further treatment.

Preparation of Deoxycholate Ribosomes—The deoxycholate-treated ribosomes were prepared as described by Robinson and Novelli (9). The ribosomes were collected by centrifugation, washed once, dissolved in 0.01 M Tris-HCl, pH 7.5, and stored at -20°C.

Preparation of Yeast sRNA—The yeast sRNA was prepared from baker's yeast as described by Hoagland et al. (10) for sRNA from rat liver. The sRNA was passed through Sephadex G-25 to remove low molecular weight contaminants (8).

Preparation of S-Substituted Cysteine—S-(1,2,3,4-Tetrahydro-

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that free cysteine and S-substituted cysteine migrated from the origin after incubation with alkali, but no migration of free dilute alkali. The radioactively labeled S-substituted cysteine alkali at pH 9.5. Radioautograms exposed for 50 days indicated Sephadex G-25, and was isolated from free cysteine or S-substituted cysteine by an adaptation of the method of Bucovaz and Davis (12). The recovered S-substituted cysteinyl-sRNA was was doubled (Fig. 1, Reaction 1).

Purification of S-Substituted Cysteinyl-sRNA—The S-substituted cysteinyl-sRNA (Fig. 1, Reaction 2), which was prepared as described in the legend to Table II, was dialyzed for 15 hours against three changes of H2O. The dialyzed, S-substituted cysteinyl-sRNA was transferred to a column which contained Sephadex G-25, and was isolated from free cysteine or S-substituted cysteine by an adaptation of the method of Bucovaz and Davis (12). The recovered S-substituted cysteinyl-sRNA was chromatographed in a butanol-acetic acid-H2O (2 : 1: 1, v/v) system before and after incubation at 37° for 30 min in dilute alkali at pH 9.5. Radioautograms exposed for 50 days indicated that free cysteine and S-substituted cysteine migrated from the origin after incubation with alkali, but no migration of free cysteine or analogue could be detected prior to incubation in dilute alkali. The radioactively labeled S-substituted cysteine analogue was prepared either with tritiated naphthalene or 35S-labeled cysteine, or by dual labeling with both isotopes.

Assay for Incorporation into Ribosomes—After incubation, the reaction mixture was diluted to 10 ml with 0.1 M Tris-HCl, pH 7.5, and centrifuged at 105,000 × g for 60 min, which resulted in sedimentation of the ribosomes. The ribosomal particles were washed according to the method of Schneider (13) with 5% trichloroacetic acid followed by extraction with 5% trichloroacetic acid at 90° for 15 min and with ether-ethanol (1:1, v/v). The precipitates were dissolved in 3 ml of 90% formic acid. An aliquot from each of these solutions was assayed for protein content and radioactivity.

Analytical Methods—Total protein was determined by measuring the turbidity developed on the addition of trichloroacetic acid (14). The ornithin method of Hendler (15) was used to measure sRNA. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

RESULTS

Table I shows results of a study in which cysteine and the cysteine analogue, tetrahydrodihydroxynaphthyleysteine, were transferred to sRNA by various enzyme fractions of rat liver. These experiments differ only in the pretreatment of the enzyme fractions. Whenever the 50% (NH4)2SO4 precipitate was used, cysteine transfer to sRNA decreased markedly and tetrahydrodihydroxydihydroxynaphthyleysteine transfer increased several fold over that observed with the 50% supernatant fraction. In some experiments, all of the enzyme activity which catalyzed the transfer of tetrahydrodihydroxynaphthyleysteine was lost in the 50% supernatant fraction.

Most of the enzyme which catalyzed transfer of the S-substituted cysteine was precipitated by 50% (NH4)2SO4, and the major portion of cysteine activity remained in the 50% supernatant fraction.

Evidence for the reaction of 1,2-epoxy-1,2,3,4-tetrahydrodihydroxynaphthalene with cysteinyl-sRNA is shown in Table II. Soluble RNA which had been labeled with 35S-cysteine was allowed to react with 3H-1,2-epoxy-1,2,3,4-tetrahydronaphthalene. The proposed reaction product, S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinyl-sRNA, was doubly labeled with 3H and 35S. As indicated by the data in Table II, the tritiated epoxide appeared to be bound to the cysteinyl-sRNA recovered from the reaction mixtures. Experiment 3 shows that a portion of the epoxide was bound in some manner to the sRNA structure, but a significantly greater amount appeared to be bound to sRNA which had previously been charged with cysteine (Experiments 1 and 2). Based on radioactivity measurements, 40% of the sulfhydryl groups of cysteinyl-sRNA were conjugated with the epoxide.

Evidence to support the data recorded in Table II was obtained by charging sRNA with 35S-cysteine (16 μCi per pmole). The (NH4)2SO4 supernatant fraction of Table II was used as the

![Fig. 1](image-url)
source of enzyme. One micromole of ^35S-cysteinyl-sRNA (containing 400,000 cpm per pmole of sRNA) was incubated with approximately 7 pmoles of unlabeled 1,2-epoxy-1,2,3,4-tetrahydroxynaphthalene and was recovered from the reaction mixture by alkaline hydrolysis to remove bound amino acids.

TABLE II

Reaction of 1,2-epoxy-1,2,3,4-tetrahydroxynaphthalene with cysteinyl-sRNA

The left-hand column indicates the condition of the sRNA. The ^35S-cysteinyl-sRNA and the unlabeled cysteinyl-sRNA were prepared as described in Table I, with the 50% (NH₄)₂SO₄ supernatant fraction as the source of enzyme. The reaction mixture contained 1 pmole of sRNA charged with 3 pmoles of ^35S-cysteine containing 510 cpm; 23.4 pmoles of ^3H-1,2-epoxy-1,2,3,4-tetrahydroxynaphthalene containing 1286 cpm; and 60 mM Tris-HCl, pH 7.5, in a total volume of 10 ml. Experiments 2 and 3 contained unlabeled cysteinyl-sRNA and stripped sRNA, respectively. Other components were identical with those in Experiment 1. The reaction mixtures were incubated with ^3H-epoxide for 60 min at 37°. After incubation, each sample was extracted twice with ether to remove unbound epoxide, and the sRNA was isolated after precipitation with ethanol. The product from each reaction mixture was dialyzed for 3 hours against water and eluted from a column containing Sephadex G-25 with 0.01 M Tris-HCl, pH 7.5, in a total volume of 10 ml. Experiments 2 and 3 contained unlabeled sRNA charged with 3 mmoles of a%-cysteine (flasks 5 to 10) and 40 pmole of a%-THN-cysteine (flasks 11 to 16), with the expected tetrahydroxynaphthylcysteine and cysteine, respectively. The products were identified by paper chromatography in a butanol-acetic acid-water (2:1:1, v/v) system, followed by radioautography. When chromatographed, the portion of sRNA which was not exposed to alkali treatment showed radioactivity only at the origin of the chromatogram.

Since the cysteinyl-sRNA used in the experiment just described as well as in the experiments outlined in Table II was produced by the action of cysteinyl-sRNA synthetase and cysteine acceptor RNA, all available evidence indicates that the arylecysteine moiety produced by this reaction should transfer the amino acid derivative to a site in protein normally occupied by cysteine, by virtue of its attachment to cysteine acceptor RNA. When ^3H- or ^35S-labeled S-tetrahydroxynaphthylcysteine was incubated with a partly purified mixture of synthetases and sRNA, an enzyme system other than the cysteinyl-sRNA synthetase was involved (Table I). Consequently, it might also be anticipated that a different species of sRNA would be involved. Table II presents evidence supporting this hypothesis. When the reaction of ^3H- or ^35S-labeled S-tetrahydroxynaphthylcysteine was observed in the presence of unlabeled diluents in the incubation mixture (Reactions 3 to 4 and 7 to 10), it was found that an excess of unlabeled cysteine (Reaction 3) or a mixture of 17 other naturally occurring amino acids (Reaction 10) did not appreciably inhibit the extent of transfer of the analogue. In contrast, however, when the unlabeled analogue (Reactions 4 and 7), valine (Reaction 9), or isoleucine (Reaction 9) was used as a diluent, a significant decrease in the specific activity of the transferred radioactive analogue was observed. Reaction 2 indicates that the tritiated S-substituted aromatic hydrocarbon is not hydroyzed from the expected tetrahydroxynaphthylcysteine and cysteine, respectively.

TABLE III

Effect of unlabeled amino acids upon tetrahydroxynaphthylcysteine transfer to sRNA

Each incubation mixture contained 0.5 pmole of sRNA; 6.6 mM MgCl₂; 5 pmoles of disodium ATP (where indicated); 66 mM Tris-HCl, pH 7.5; 0.374 pmole of ^3H-tetrahydroxynaphthylcysteine (containing 43,700 cpm) (flasks 1 to 4) or 4 pmole of ^3S-labeled analogue (containing 21,000 cpm) (flasks 5 to 10); and the 50% (NH₄)₂SO₄-precipitated enzyme fraction containing 1.8 mg of protein in a total volume of 3 ml. In addition, 40 pmoles of unlabeled amino acids were added where indicated. Assay procedures are described in the text.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Components</th>
<th>Additions</th>
<th>Specific activity of sRNA (cpm/pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ^3H-THN-cysteine + sRNA</td>
<td>Cysteine</td>
<td>230.0</td>
<td></td>
</tr>
<tr>
<td>2. ^3H-THN-cysteine + sRNA + ATP</td>
<td>THN-cysteine</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td>3. ^3H-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. ^3S-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. ^3H-THN-cysteine + sRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. ^3S-THN-cysteine + sRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. ^3S-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. ^3S-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. ^3S-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. ^3S-THN-cysteine + sRNA + ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The choice of valine and isoleucine used as diluents in this experiment was predetermined by AT^4P→P^4P exchange studies. The analogue and other amino acids were used as diluent controls.

* The abbreviation used is: THN-cysteine, S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine.

* The amino acid mixture contained 17 natural amino acids other than cysteine, valine, and isoleucine.
cysteine during the course of the transfer reaction. As a whole, the data support the concept that different systems function for the transfer of the S-substituted cysteine analogue and cysteine to sRNA. It may be concluded from the data of Table III that the transfer of the S-substituted cysteine analogue and cysteine is catalyzed by different systems, each of which has sufficient intrinsic reactivity to form covalent bonds with amino acids or proteins. Furthermore, the study confirms that the cysteine transfer RNA does not function as an acceptor of tetrahydrohydroxynaphthylcysteine.

Table IV shows a comparison of cysteine and tetrahydrohydroxynaphthylecysteine incorporation into deoxycholate ribosomal protein of rat liver. A stimulation of the incorporation of either cysteine or the cysteine analogue was observed when all necessary components were present in the incubation medium. The omission of necessary components caused a reduced incorporation of both cysteine and analogue. As indicated in Table IV, the incorporation of either tetrahydrohydroxynaphthylcysteine or cysteine was strongly inhibited by 0.2 mM puromycin.

**DISCUSSION**

These studies provide evidence for the binding in *vitro* of activated aromatic hydrocarbons at different stages in the pathway of protein biosynthesis, and suggest the possibility of comparable bindings in *vivo*. It is unlikely that polycyclic hydrocarbons have sufficient intrinsic reactivity to form covalent bonds with amino acids or proteins. Thus, binding of hydrocarbons to protein observed by Miller (2), Mills and Wood (3), and Heidelberger and Moldenhauer (16) must include a preliminary activation of the hydrocarbon to a reactive form. Booth, Boyland, and Sims (7) have suggested that this activated form is an epoxide, and they have demonstrated the reactivity of arylepoxide with cysteine, or the protein-bound hydrocarbon may be formed by normal degradation of the glutathione conjugate which results from detoxication (17). This investigation shows that the activated hydrocarbon may be incorporated into protein by reaction with the cysteinyl-sRNA. The present concepts of protein biosynthesis suggest that this type of reaction would result in binding of the hydrocarbon to protein via the cysteine residue at the positions occupied by cysteine (18). This would coincide with the positions expected for attachment of the hydrocarbon by direct reaction with the sulfhydryl groups of the complete protein. On the other hand, if the activated hydrocarbon reacts with cysteine to form the analogue prior to its attachment to sRNA, the aminoacyl-sRNA synthetases and the transfer RNAs for valine and isoleucine would be involved (Table III).

The final step is not documented, since evidence has not been obtained to show that the cysteine analogue which is transferred into deoxycholate ribosomal protein (Table IV) is incorporated at a site which is normally occupied by valine and isoleucine. It has been shown, however, that the specificity for transfer from sRNA to microsomal protein is determined by the structure of the sRNA and not by the amino acid attached (18). The premise that tetrahydrohydroxynaphthylcysteine and, possibly, other arylecysteine analogues must be incorporated into protein at a site which is aberrant in terms of cysteine incorporation is diagrammed in Fig. 2.

At present no definitive relationship of these reactions with carcinogenesis by hydrocarbons has been demonstrated. A priori, this reaction would be intracellular, nonpredominant, and indirect. This appears evident because the principal sites of detoxication, the liver and kidney cells, are resistant to carino-

**TABLE IV**

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>Omissions</th>
<th>Radioactivity incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactive tracer</td>
<td>Puromycin</td>
<td>cfpm</td>
</tr>
<tr>
<td><strong>25S-Cysteine</strong></td>
<td>None</td>
<td>3275</td>
</tr>
<tr>
<td></td>
<td>- ATP, GTP, phosphoenolpyruvate, pyruvate, kinase</td>
<td>2085</td>
</tr>
<tr>
<td></td>
<td>+ None</td>
<td>265</td>
</tr>
<tr>
<td><strong>25S-Labeled S-tetrahydrohydroxynaphthylecysteine</strong></td>
<td>None</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>- ATP, GTP, phosphoenolpyruvate, pyruvate, kinase</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>+ None</td>
<td>70</td>
</tr>
</tbody>
</table>

**FIG. 2**

Diagrammatic summary of the proposed method by which tetrahydrohydroxynaphthylecysteine could occupy uniquely different positions in protein. *EPOXIDE*, 1,2-epoxy-1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl-1-cysteine; *THN-CYSTEINE*, S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-1-cysteine.
gencis, and daughter cells of susceptible tissues maintain the cancerous imprint, but contain no bound hydrocarbon. In the detoxication reactions, most activated hydrocarbon molecules combine with glutathione or water. The former reaction product would be metabolized to a mercapturic acid, and the latter, to a phenolic derivative. However, it is probable that an occasional reaction of the activated hydrocarbon with cysteine, cysteinyll-sRNA, or protein sulfhydryl groups can occur (5). Whether an aberrant reaction of this nature can influence the synthesis and coding of DNA remains to be seen.

REFERENCES
Variations in Attachment of a Cysteine Conjugate to Soluble Ribonucleic Acid
Edsel T. Bucovaz, John C. Morrison and John L. Wood


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