# Role of Sulfhydryl Groups in Activating Enzymes. Properties of *Escherichia coli* Lysine–Transfer Ribonucleic Acid Synthetase*

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ABSTRACT: The role of the SH groups in the lysine–transfer ribonucleic acid (t-RNA) synthetase from *E. coli* has been investigated. The native enzyme contains two reactive SH groups per mole, and one additional SH group is exposed in 6 M urea.

Sulfhydryl reagents do not inhibit the pyrophosphate–adenosine triphosphate exchange activity of the enzyme. This enzyme is shown to be the only amino acid activating enzyme from *E. coli* that is not inhibited by *p*-mercuribenzoate (PMB). PMB and $HgCl_2$ inhibit the transfer of lysine from the enzyme to the RNA. $HgCl_2$ causes a marked shift in the absorption maxima of the RNA, which apparently reflects disruption of structure.

The mechanism of activation of amino acids, the first step in protein synthesis, can be written

$$\text{RC(=O)—O}^- + \text{E} + \text{ATP} \overset{Mg^{2+}}{\rightleftarrows} \text{E·RC(=O)–AMP} + \text{PP}_i$$

in which the adenylic acid group of adenosine triphosphate (ATP)[1] is transferred to the carboxyl group of the amino acid. This amino acyl–adenylate forming reaction is analogous to a large class of reactions including the activation of acetate in the formation of acetyl CoA, pantoic acid in the synthesis of pantothenic acid, and the activation of luciferin that precedes the light-emitting reaction catalyzed by firefly luciferase, all of which also proceed through an acyl–adenylate enzyme intermediate.

The sensitivity of these enzymes to low concentrations of *p*-mercuribenzoate (PMB) has been reported by several investigators (Moustafa, 1963; Davie *et al.*, 1956; Allen *et al.*, 1960; DeLuca *et al.*, 1964; Webster and Campagnari, 1962). In the tryptophan-activating enzyme from beef pancreas, it has been shown that the presence of substrates results in the apparent loss of four SH groups out of a total of eight per mole (DeLuca and McElroy, 1965). In addition the substrates protect the enzyme from inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and PMB. An analogous situation has been demonstrated for firefly luciferase, in which two sulfhydryl groups out of seven will no longer react with PMB in the presence of dehydroluciferyl adenylate. The two protected SH groups appear to be essential for catalytic activity since the substrates also protect the enzyme from inhibition by sulfhydryl reagents (DeLuca *et al.*, 1964).

The possibility that SH groups are involved in the function of these enzymes is also suggested by the observation that loss in activity of stored enzyme preparations can be partly restored by the addition of low molecular weight thiol compounds such as glutathione (Allen *et al.*, 1960) or β-mercaptoethanol (Bergmann *et al.*, 1961).

These observations suggest that, as a class, the amino acid t-RNA synthetases and the other activating enzymes that catalyze the formation of enzyme-bound acyl–adenylates contain one or more sulfhydryl groups that are essential for activity. In contrast to these observations the present report demonstrates that the lysine-activating enzyme from *E. coli* is insensitive to mercurials and other sulfhydryl reagents. Furthermore, the SH groups that react with DTNB in the native enzyme are not essential for enzyme activity, measured either as the ATP–pyrophosphate exchange reaction or the t-RNA acylation reaction. It is shown further that lysyl-t-RNA synthetase is the only amino acid activating enzyme from *E. coli* in which the pyrophosphate–ATP exchange reaction is not inhibited by PMB. However, mercurials do inhibit the transfer of lysine to t-RNA, apparently due to an effect of the inhibitor on the RNA.

## Materials

Inorganic [$^{32}$P]pyrophosphate, [$^{14}$C]ATP, and L-[$^{14}$C]-lysine were purchased from New England Nuclear Corp. All unlabeled amino acids were from the Cali-

* From the National Institutes of Dental Research, National Institutes of Health, U. S. Department of Health, Education and Welfare, Bethesda, Maryland, and the McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland. *Received August 4, 1965; revised September 29, 1965.* This work was supported in part by grants from the National Institutes of Health, the National Science Foundation, and the Atomic Energy Commission.

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[1] Abbreviations used in this work: PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; ATP, adenosine triphosphate.

fornia Corp. for Biochemical Research except for L-lysine, which was obtained from Mann Research Laboratories. DEAE-Sephadex, A-50 medium anion-exchange resin, was the product of Pharmacia, Uppsala, Sweden. Unlabeled ATP, as the crystalline sodium salt, was obtained from Sigma Chemical Co. DTNB was obtained from Aldrich Chemical Co. Millipore filters (HA, 25 mm, 0.45 $\mu$) were obtained from Millipore Filter Corp., Bedford, Mass. *E. coli* strain B t-RNA was the product of General Biochemicals. Kilogram quantities of frozen *E. coli* strain B (M) were purchased from Grain Processing Corporation, Muscatine, Iowa. The cells were frozen immediately after harvesting and stored at −15° until used. Cells stored for several months in this way did not lose significant amounts of enzyme activity.

## Methods

Sulfhydryl (SH) determinations were carried out using DTNB (Ellman, 1959) at pH 7.9. Absorbancy changes were followed at 412 m$\mu$, and total SH content in any sample was calculated assuming an $\epsilon_m$ of 13,600 for the reagent. Stability of the color is dependent on metal-free reagents; therefore, $10^{-4}$ M EDTA was routinely included in the cuvet.

Protein was determined spectrophotometrically by absorption at 280 m$\mu$. A molecular weight of 100,000 was used for the calculations (R. Stern, unpublished observations). Preparations of enzyme is the same as that described in an earlier publication (Stern and Mehler, 1965) with some modifications. The DEAE-cellulose and hydroxylapatite columns were replaced by two successive DEAE-Sephadex A-50 columns.

The first column (2.2 × 25 cm) was eluted with a linear gradient between 0.15 M potassium phosphate buffer, pH 7.0, and 0.35 M potassium phosphate buffer, pH 6.5. A total gradient volume of 2000 ml was used. One hundred fractions were collected, and fractions 40–50 were combined, diluted, and applied to a second column (2.2 × 10 cm). Potassium phosphate buffer, pH 6.0 (0.01 M), and a linear gradient between 0.05 and 0.40 M NaCl, 600-ml total gradient volume, was used. Fractions (140) were collected and the peak fractions 80–110 were combined. The specific activity of protein from the combined fractions from this second column was the same as the final product from the original procedure. Assays of the ATP–pyrophosphate exchange reaction and the t-RNA acylation reaction were described earlier (Stern and Mehler, 1965).

## Results

The SH content of the lysine–t-RNA synthetase was determined as described under experimental methods. Table I gives values of the SH content from two preparations of the enzyme. The maximal number of groups reacting in the "native" enzyme is two/mole. One additional SH/mole is found in the presence of either 6 M urea or 5 M guanidine.

The reaction in buffer requires 40 min for completion at room temperature. In urea it is somewhat faster, while in guanidine it is complete in less than 5 min. Variability among samples determined in buffer may be due to the low levels of enzyme used or a result of spontaneous oxidation of sulfhydryl groups.

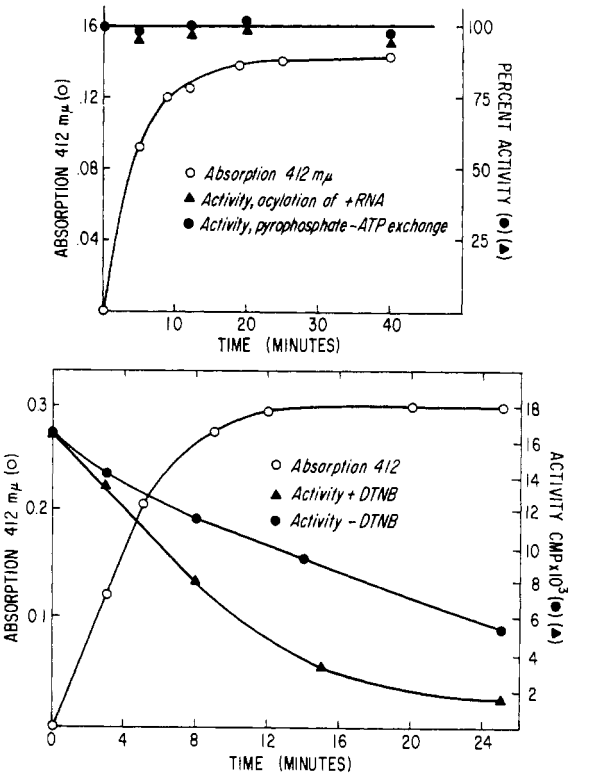


FIGURE 1: The reaction of DTNB with purified lysyl-s-RNA synthetase. Enzymatic activity was measured by the ATP–pyrophosphate exchange reaction and transfer of lysine to t-RNA. Results are expressed as per cent of a control sample containing everything except DTNB. Titration of sulfhydryl groups with DTNB was followed by absorption at 412 m$\mu$. The reaction was carried out in 0.1 M Tris buffer, pH 7.9 (a), and 0.1 M Tris buffer, pH 7.9, plus 6 M urea (b). A 1-ml cuvet contained 0.8 ml of buffer and urea, 0.1 ml of concentrated purified enzyme (7.1 m$\mu$moles), and 250 m$\mu$moles of DTNB. At indicated intervals a 10-$\mu$l aliquot of the sample was removed directly from the cuvet, diluted into 0.4 ml of 0.1 M Tris buffer, pH 7.9, and assayed for enzymatic activity.

In one of the two enzyme preparations (Table I, expt I) 0.5–1 sulfhydryl group/mole was lost after concentration and storage for several days. The total number of reactive sulfhydryl groups in this sample in urea or guanidine was two/mole, rather than three.

Additional determinations were performed in the presence and absence of lysine, ATP, and $Mg^{2+}$. The greatest difference in sulfhydryls measured in the presence and absence of substrates was 0.3 of an SH group/mole of enzyme, which is not significant.

TABLE I: Sulfhydryl Content of Lysine-t-RNA Synthetase.[a]

| Sample | SH/mole |
|---|---|
| I. 0.1 M phosphate, pH 7.9 | 1.8 |
| | 1.7 |
| | 1.4 |
| Enzyme after storage. | |
| 0.1 M phosphate, pH 7.9 | 1.2 |
| 6 M urea–0.1 M phosphate, pH 7.9 | 1.8 |
| 5 M guanidine–0.1 M phosphate | 2.2 |
| II. 6 M urea–0.1 M phosphate, pH 7.9 | 2.9 |
| | 3.1 |
| | 3.0 |

[a] Samples contained from 5 to 10 mμmoles of purified enzyme, 250 mμmoles of DTNB, and the buffer indicated. Absorbancy at 412 mμ was followed until no further increase was observed.

Inhibition Studies

The enzyme was incubated with DTNB in order to determine whether the reactive SH groups were essential for enzymatic activity. The DTNB was followed spectrophotometrically, and aliquots of the enzyme were removed from the cuvet at various intervals and assayed for the ability to catalyze the ATP–pyrophosphate exchange reaction and the transfer reaction to t-RNA. Figure 1a shows the results of such an experiment. No effect on either of the two catalytic activities of the enzyme can be seen after all the accessible sulfhydryls have reacted.

The effect of DTNB on the substrate-binding capacity of the enzyme, *i.e.*, the ability to form an amino acid–AMP–enzyme complex, was also investigated (Allende *et al.*, 1964; Lagerkvist and Waldenstrom, 1965; Norris and Berg, 1964). Identical samples of enzyme were treated with buffer or excess DTNB for 30 min at room temperature. [$^{14}$C]ATP, lysine, and $Mg^{2+}$ were then added and after 5 min both samples were placed on 1 × 25 cm Sephadex G-25 columns and eluted with 0.1 M Tris, pH 7. Fractions were collected and the radioactivity associated with enzyme-bound acyl–adenylate was determined by plating and counting. The two samples contained equal amounts of protein-bound radioactivity, demonstrating that DTNB does not affect the formation or stability of the complex. The reactive sulfhydryl groups are not involved in the formation of the lysyl–adenylate complex.

It seemed possible that the "buried" sulfhydryl, reactive only in the presence of urea, might be essential for enzymatic activity. The enzyme was incubated in 6 M urea, 0.1 M potassium phosphate buffer, pH 7.9, in the presence and absence of DTNB. Aliquots were removed and diluted into buffer at various time intervals. The results (Figure 1b) indicate there is a time-dependent irreversible loss of enzymatic activity in urea alone; however, DTNB treatment results in a greater loss of activity. It is impossible from this experiment to decide whether the additional loss due to DTNB is the result of a participation of the SH in catalytic activity or occurs because the incorporation of DTNB molecule interferes with reversal of the enzyme to the "native" conformation.

In an attempt to decrease the irreversible loss of activity after 6 M urea treatment experiments were carried out in 2 M urea. There was no loss of activity after 40 min in the control sample or in the presence of DTNB, and the buried SH was not detected. Ap-

TABLE II: Effect of PMB on 20 Amino Acyl-t-RNA Synthetases of *E. coli*.[a]

| L-Amino Acid | Enzyme Activity (units/mg) | Activity in PMB[b] |
|---|---|---|
| Alanine | 1.2 | 0.4 |
| Arginine | 0.6 | 1.8 |
| Aspartic acid | 0.7 | 6.7 |
| Asparagine | 1.5 | 0.0 |
| Cysteine | 3.8 | 13.0 |
| Glutamic acid | 0.2 | 0.0 |
| Glutamine | 0.2 | 0.0 |
| Glycine | 0.2 | 0.0 |
| Histidine | 1.5 | 11.5 |
| Isoleucine | 6.1 | 4.9 |
| Leucine | 8.2 | 0.0 |
| Lysine | 3.2 | 150.0 |
| Methionine | 8.6 | 0.0 |
| Phenylalanine | 0.9 | 0.0 |
| Proline | 0.3 | 0.0 |
| Serine | 0.8 | 0.0 |
| Threonine | 0.8 | 0.0 |
| Tyrosine | 4.6 | 0.0 |
| Tryptophan | 4.0 | 13.0 |
| Valine | 8.8 | 10.0 |

[a] Effect of PMB on amino acyl synthetase reactions as measured by the ATP–pyrophosphate exchange. The reaction mixture contained in 1.0 ml: 100 μmoles of Tris buffer, pH 8.0, 10 μmoles of NaF, 8 μmoles of $MgCl_2$, 2 μmoles of ATP, and 2 μmoles of $Na_4P_2O_7$. Sufficient [$^{32}$P]$Na_4P_2O_7$ was added to yield a specific activity of $10^5$ cpm/μmole. The enzymes were added as 100 μl of the streptomycin supernate as previously described (Stern and Mehler, 1965). A 10-min preincubation at room temperature with and without $1 \times 10^{-3}$ M PMB was followed by the addition of 20 μmoles of each of the amino acids. No addition was made to the control tube. The samples were then incubated at 37° for 15 min. [b] Per cent of total activity remaining after 10-min preincubation with $10^{-3}$ M PMB at room temperature.

parently this concentration of urea does not disrupt structure sufficiently to expose the additional thiol group. The loss of activity in 6 M urea after 40 min is not as great if the dilution is made into buffer containing substrates and $1 \times 10^{-3}$ M $\beta$-mercaptoethanol.

To determine whether the lysine-t-RNA synthetase is the only activating enzyme from *E. coli* that is not inhibited by sulfhydryl reagents, the crude extract from a streptomycin supernatant was prepared (Stern and Mehler, 1965). ATP–pyrophosphate exchange activity was assayed with each of the 20 amino acids in the presence and absence of $1 \times 10^{-3}$ M PMB. Table II strikingly emphasizes the complete resistance of only the lysine-t-RNA synthetase. Although the other enzymes are inhibited to varying extents, the lysine enzyme appears to be stimulated by PMB. This effect has not been observed with the purified enzyme.

*Effect of SH Reagents on Acylation of t-RNA.* An unusual effect is observed in the transfer of amino acid to t-RNA if PMB is included in the incubation. At $1 \times 10^{-3}$ M PMB there is a marked decrease of [$^{14}$C]-lysine incorporated into RNA (Table III). Experiment II shows this inhibition is completely reversible by addition of excess $\beta$-mercaptoethanol. Experiment III demonstrates that a comparable concentration of PMB has no effect on the PP–ATP exchange activity of the enzyme.

Table IV shows that two other sulfhydryl reagents, DTNB and NEM, do not inhibit lysine incorporation, while mercuric chloride behaves similarly to PMB. The inhibition of the transfer reaction at increasing concentrations of PMB is shown in Figure 2. At $1.5 \times 10^{-3}$ M PMB the incorporation of [$^{14}$C]lysine into RNA is completely prevented.

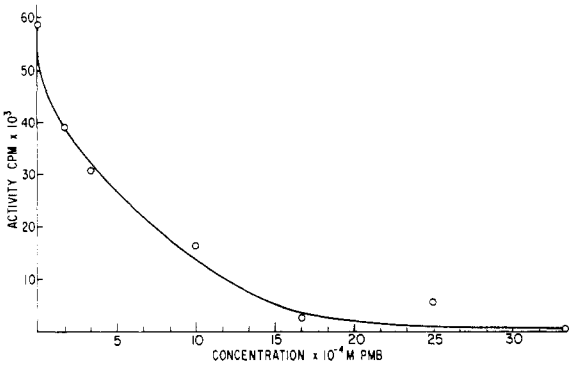


FIGURE 2: Effect of DTNB on transfer reaction. Each tube contained in 0.2 ml: 25 m$\mu$moles of Tris buffer, pH 8.0, 1 $\mu$mole of $MgCl_2$, 0.2 $\mu$mole of ATP, 0.5 mg of t-RNA, 10 m$\mu$moles of L-[$^{14}$C]lysine, and PMB at the concentration shown. The samples were allowed to stand for 10 min at room temperature followed by addition of enzyme and incubation at 37° for 10 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. This suspension was chilled, filtered through a millipore filter, washed, and counted as described previously (Stern and Mehler, 1965). All assays were done in duplicate.

TABLE III: Effect of PMB on Acylation of t-RNA and PP–ATP Exchange.[a]

| | Additions | Cpm in t-RNA | Inhibition (%) |
|---|---|---|---|
| I. | None | 60,991 | |
| | $1.2 \times 10^{-3}$ M PMB | 4,361 | 93 |
| | $3 \times 10^{-4}$ M PMB | 32,531 | 47 |
| II. | None | 56,487 | |
| | $1.2 \times 10^{-3}$ M PMB, and after 10 min $9 \times 10^{-3}$ M $\beta$-mercaptoethanol | 57,662 | |
| | $1.2 \times 10^{-3}$ M PMB | 3,569 | 95 |

| | Additions | Cpm Exchanged into ATP | Inhibition (%) |
|---|---|---|---|
| III. | None | 3,820 | 0 |
| | $1 \times 10^{-3}$ M PMB | 3,500 | 0 |
| | None | 6,320 | 0 |
| | $1 \times 10^{-3}$ M PMB | 6,850 | 0 |

[a] The inhibitors were incubated with the t-RNA at room temperature, pH 7.9, for 30 min. The enzyme was then added followed by incubation at 37° for 10 min. Assays were done as described previously (Stern and Mehler, 1965).

TABLE IV: Effect of Sulfhydryl Reagents on Acylation of t-RNA.[a]

| Additions | Cpm in t-RNA | Inhibition (%) |
|---|---|---|
| | 26,000 | |
| $1 \times 10^{-3}$ M DTNB | 38,000 | 0 |
| | 48,000 | |
| $1 \times 10^{-3}$ M $HgCl_2$ | 500 | 99 |
| | 60,000 | |
| $1 \times 10^{-3}$ M NEM | 70,000 | 0 |

[a] Conditions were the same as in Table III except the NEM sample was allowed to react with the RNA at pH 7.5 for 30 min. The pH was then brought to 7.9 before addition of the enzyme.

## Discussion

In view of recent reports of inactivation of t-RNA by mild iodine oxidation (Carbon *et al.*, 1965) and the isolation of a thiopyrimidine from *E. coli* t-RNA (Lipsett, 1965), it seemed possible the PMB was inhibiting acylation by reaction with a thiol group in the t-RNA. However, the lack of inhibition by either NEM or DTNB does not support this hypothesis. For the same reason it is also unlikely that the inhibition is caused by reaction with an enzyme sulfhydryl that is required for binding the t-RNA. It is possible that only PMB and $HgCl_2$ react with the third SH of the enzyme when RNA is present and inhibit the transfer of the lysine to the RNA. It seems more reasonable that the reaction of the mercurials is with some other group in the RNA. This is substantiated by the observation that at pH 7.9 in the presence of mercuric chloride the absorption maxima of the RNA is shifted from 260 m$\mu$ to about 276 m$\mu$. Due to the high absorbance of PMB at 260 m$\mu$, it was impossible to demonstrate an effect of PMB on the absorption of the RNA. Similar spectral changes have been observed with nucleosides in the presence of methylmercuric and mercuric ions (Simpson, 1964). These result from an association of the mercuric compounds with ring nitrogens and amino groups of the bases.

The present results do not support the idea that an SH group in t-RNA is essential for acceptor activity. It is possible, of course, that an SH in the RNA could be susceptible to iodine oxidation but will not react with either NEM or DTNB. We have demonstrated, however, that both NEM and DTNB will react with thiouracil. Perhaps the iodine inactivation is due to oxidation of some group in the t-RNA other than a thiol.

A report of inhibition of lysyl-t-RNA synthetase from wheat germ (Moustafa, 1964) by PMB as evaluated by the transfer reaction alone may not be due to inhibition of the enzyme but rather an effect on the RNA. In assessing the effect of sulfhydryl reagents on the amino acid activating enzymes it is necessary to assay the activation and transfer reactions independently.

The lysyl-t-RNA synthetase is different from the other 19 amino acid-t-RNA synthetases from *E. coli*, all of which require a free SH for activity. It has been reported that the lysine-t-RNA synthetases from wheat germ (Moustafa, 1964) and rat liver (Hele, 1961) are sensitive to PMB. We have assayed the PP–ATP exchange activity of the lysyl-t-RNA synthetase in crude yeast and ascites tumor extracts and also found both enzymes to be completely inhibited by $1 \times 10^{-3}$ M PMB.

If the catalytic mechanism for acyl-adenylate formation is the same for all activating enzymes, the results with this lysine-activating enzyme indicate that sulfhydryls do not participate directly in the reaction. However, free sulfhydryls need not be invoked as a part of the "active site" of these enzymes but perhaps as groups required to maintain the proper secondary and tertiary structure. It seems most reasonable to postulate that the lysine enzyme of *E. coli* differs from other amino acyl-t-RNA synthetases in being able to maintain an active conformation with modified sulfhydryl groups but that it resembles the other enzymes with respect to catalytic mechanism.

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