AFFINITY LABELING OF ISOLEUCYL-tRNA SYNTHETASE WITH N-BROMOACETYLISOLEUCYL-tRNA

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SUMMARY: Isoleucyl-tRNA synthetase from *Escherichia coli* B forms an irreversible complex with N-bromoacetyl-[^{3}H]Ile-tRNA which is not disrupted after equilibration with excess $tRNA^{Ile}$. The presence of $tRNA^{Ile}$ in the incubation protects the enzyme from alkylation by this reagent, whereas $tRNA^{Phe}$ has no effect. Under comparable conditions, bromoacetate does not form a covalent link with the enzyme. The results demonstrate that N-bromoacetyl-Ile-tRNA binds reversibly to the enzyme by non-covalent interactions with the tRNA moiety, and then forms a covalent bond between the alkylating group and an amino acid residue of the protein.

In conjunction with ongoing studies of the aminoacyl-tRNA synthetase, we have been attempting to develop affinity labeling techniques which would be of general applicability to this group of enzymes. Recently, acylation of Met-tRNA synthetase from *E. coli* has been reported to occur upon treatment with *p*-nitrophenyl-carbamyl-Met-tRNA (1). We sought to prepare a chemically reactive derivative of aminoacyl-tRNA which is capable of reacting with a wider variety of nucleophiles than the aforementioned reagent, and which would yield a modified amino acid readily amenable to isolation and identification. Affinity labeling reagents of this type should have general utility in characterization of the active sites of these enzymes and might facilitate co-crystallization of enzyme-tRNA complexes.

Bromoacetamides are capable of alkylating histidine, cysteine, methionine and lysine residues of proteins (2,3), and N-bromoacetyl-Phe-tRNA has recently been reported to be a labeling reagent for the 50S particle of *E. coli* ribo-

somes (4). We describe here the preparation of N-bromoacetyl-Ile-tRNA, and provide evidence that this analog serves as an affinity labeling reagent of Ile-tRNA synthetase (IRS).

Materials and Methods:

Purified IRS from *Escherichia coli* B was prepared as described by Baldwin and Berg (5). The N-hydroxysuccinimide esters of bromoacetic and acetic acids were prepared by the method of Anderson *et al.* (6) and stored at -15° over $CaCl_2$. The former compound had mp 116-118° after recrystallization from chloroform-petroleum ether and gave correct C,H,N combustion analysis. L-[^{3}H]isoleucine (1-5 Ci/mmole) was purchased from New England Nuclear. [^{3}H]-Bromoacetic acid (116 mCi/mmole) and L-[^{3}H]phenylalanine (500-1000 mCi/mmole) were products of Amersham/Searle. Unfractionated *E. coli* tRNA was purchased from Schwarz/Mann and would accept 42 pmoles of Ile per A_{260}. $tRNA^{Phe}$ was a gift from Oak Ridge National Laboratory, and was *ca.* 80% pure (997 pmoles $tRNA^{Phe}$ per A_{260}). Ile-tRNA was prepared with purified IRS as described by Yarus and Berg (7). N-bromoacetyl- and N-acetyl-[^{3}H]Ile-tRNA (1 Ci/mmole) were prepared by the following procedure: [^{3}H]Ile-tRNA (340 pmoles) in 100 µl of 0.1*M* triethanolamine hydrochloride (pH 7.8) was treated with 0.6 µmole of the N-hydroxysuccinimide ester of the appropriate acid in 30 µl of 1,4-dioxane. After 75 minutes at room temperature, three volumes of ice-cold ethanol were added. The cloudy suspension was chilled at -15° for 15-30 minutes and the precipitated tRNA was collected by centrifugation. The extent of N-acylation was determined as the amount of radioactivity remaining after treatment with Cu^{2+}/Tris-HCl (8). Yields equivalent to acylation of 95-100% of the Ile-tRNA were typical. The nitrocellulose membrane filter assay for detection of the complex was that described by Yarus and Berg (7,9). Nitrocellulose membrane filters were obtained from Schleicher and Schuell (type B-6, 2.4 cm). Filters were dissolved in 10 ml of Bray's solution (10) and counted in a liquid scintillation counter with 45% efficiency.

A typical alkylation experiment consisted of IRS (40 pmoles), N-bromoacetyl-[^{3}H]Ile-tRNA (90 pmoles), sodium cacodylate (0.2 µmole; pH 7.5), and water in a final volume of 260 µl. Incubations were conducted at room temperature. In the competition experiment, unfractionated tRNA containing 1150 pmoles $tRNA^{Ile}$ was included in the reaction mixture. Aliquots (50 µl) were withdrawn from the reaction mixture at various times and added to a solution containing an excess (*ca.* 13-fold) of $tRNA^{Ile}$ and 0.2 m*M* unlabelled L-isoleucine in the standard filter assay buffer. After 5 minutes at room temperature the solutions were filtered and washed in the manner described by Yarus and Berg (7). These conditions were shown in control experiments to result in exchange of greater than 95% of reversibly-bound [^{3}H]Ile-tRNA, and the radioactivity remaining represents irreversibly bound complex. At the beginning of each experiment one point was taken which was not subjected to the exchange process. The binding observed for this point constitutes an approximation of the maximum binding (*vis.* reversible and irreversible) expected for any given experiment.

Results and Discussion

The data in Table I and Figure 1 show that incubation of IRS with N-bromoacetyl-[^{3}H]Ile-tRNA results in a time dependent incorporation of radioactivity into the protein which may not be removed upon equilibration with $tRNA^{Ile}$. The conditions used for the equilibration were sufficient to remove all reversibly bound [^{3}H]Ile-tRNA and N-acetyl-[^{3}H]Ile-tRNA from IRS. When a control experiment was performed in which N-acetyl-[^{3}H]Ile-tRNA (an analog which does not possess an alkylating group) was incubated with IRS under identical conditions (Table I), the protein bound radioactivity was negligible. These results are in accord with irreversible alkylation of IRS by the bromoacetyl analog to give a derivative in which IRS is covalently attached to $tRNA^{Ile}$ (*vis.* IRS-CH_2CO-Ile-tRNA.

Several additional lines of evidence suggest that the irreversible alkylation proceeds *via* unimolecular reaction within a reversible complex

Table I

Affinity Labeling of IRS with N-bromoacetyl-[^{3}H]Ile-tRNA[a]

Source of Label	Time of Incubation	dpm not Exchanged	Apparent % of IRS labelled
N-bromoacetyl-[^{3}H]Ile-tRNA[b]	5 min	2,000	11
	24 hr.	16,750	93
[^{3}H]bromoacetic acid + tRNA[c]	5 min	0	0
	24 hr.	0	0
N-acetyl-[^{3}H]Ile-tRNA[b]	1 hr.	651	3.7
	23 hr.	927	5.2

[a]Incubations and assays were performed as described in Materials and Methods. [b]The specific activity was 2200 dpm/pmole and 50 µl aliquots of the reaction mixture were treated as described in Materials and Methods. [c][^{3}H]Bromoacetic acid (86 pmoles, 116 mCi/mmole) and unfractionated tRNA^{Ile} (89 pmoles) were used instead of N-bromoacetyl-[^{3}H]Ile-tRNA.

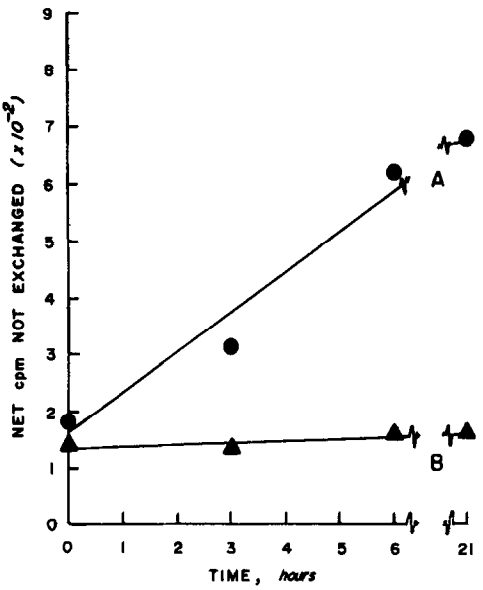

Figure 1: Incorporation of non-exchangeable radiolabel of N-bromoacetyl-[^{3}H]Ile-tRNA into IRS as a function of time. (A) Reaction mixture (260 µl) was as described in Materials and Methods; total initial binding of the 50 µl assayed aliquot (without exchange by $tRNA^{Ile}$) was 835 cpm. (B) In addition to the above, the assay mixture (260 µl) contained 1150 pmoles of unfractionated $tRNA^{Ile}$.

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rather than non-specific bimolecular reaction between the reactive α-bromoacetamide and IRS. First, the reported second-order rate constant for alkylation of IRS by iodoacetamide (0.79×10^{-1} $M^{-1}min^{-1}$) (11) suggests that, at the concentrations used here, bimolecular reaction should not be significant over the time of incubation. In fact, when IRS was incubated for 24 hr with [^{3}H]bromoacetic acid and $tRNA^{Ile}$ (Table I), the incorporation of radioactivity into protein was negligible. In this case the alkylating agent is of comparable reactivity to that of N-bromoacetyl-[^{3}H]Ile-tRNA, but will not form a reversible complex. Second, if the incubation is performed in the presence of a 14-fold excess of $tRNA^{Ile}$ as a competitor, the incorporation of radioactivity into IRS is greatly diminished (Figure 1). A similar experiment using $tRNA^{Phe}$ as the competitor had no effect on the amount of protein bound radioactivity.

From the above, it may be concluded that N-bromoacetyl-[^{3}H]Ile-tRNA is an effective affinity labeling agent for IRS. Competition experiments with

$tRNA^{Ile}$ and sham experiments using an analogous reactive chemical group demonstrate that the specificity of the irreversible reaction resides in the nucleic acid moiety. Further studies on the isolation and characterization of the covalently bound IRS-CH_2CO-Ile-tRNA complex are in progress.

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