Attachment of protein affinity-labeling reagents of variable length and amino acid specificity to *E. coli* tRNA^{fMet}

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ABSTRACT

Transamination with bifunctional amines in the presence of bisulfite has been used to attach side chains of variable length to the N⁴-position of single stranded cytidine residues in *E. coli* tRNA^{fMet}. Such side chains, terminating in reactive primary amino groups, have been coupled to a variety of N-hydroxysuccinimide esters. The resulting modified tRNAs carry protein affinity labeling groups capable of covalent reaction with a variety of amino acids.

INTRODUCTION

Cytidine and uridine residues in single-stranded regions of nucleic acids are readily modified by addition of sodium bisulfite to the 5,6-double bond of the pyrimidine base. Cytidine bisulfite adducts undergo deamination by reaction with water (1-3) and are converted to N⁴-substituted cytidine derivatives by transamination with an appropriate amine (4-6). Treatment of cytidine with bifunctional amines in the presence of bisulfite leads to formation of derivatives having side chains of variable length which terminate in reactive amino groups (Scheme I). Formation of such modified cytidine residues in polynucleotides allows attachment of a variety of amine-specific reagents to nucleic acids (7-9). In this paper, we describe the use of this reaction to attach protein affinity-labeling reagents to single stranded cytidine residues in *E. coli* tRNA^{fMet}.

MATERIALS AND METHODS

Nucleosides were obtained from Calbiochem. Bifunctional alkyl amines, carbonyldiimidazole, and fluorescein isothiocyanate were purchased from Aldrich Chemical Co. Sodium metabisulfite was Sigma grade I reagent, and sodium sulfite was obtained from Fisher Chemical Co. The N-hydroxysuccinimide esters of succinic acid and bromoacetic acid (IVa and IVf) were synthesized as described previously (8). Disuccinimidyl tartarate (IVb),

Scheme I

disuccinimidyl suberate (IVc), dithiobis (succinimidyl propionate) (IVd), ethylene glycol bis(succinimidyl succinate) (IVe), *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (IVg), *N*-hydroxysuccinimidyl 4-azidobenzoate (IVh), *N*-hydroxysuccinimidyl (4-azidophenyl)-1,3'-dithiopropionate (IVi), and *N*-hydroxysuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (IVj) were purchased from Pierce Chemical Co. Aminex A-25 and Aminex A-6 were purchased from Bio-Rad Laboratories. *E. coli* tRNA^{fMet} having a specific activity of 1720 pmol per A₂₆₀ unit was obtained from Boehringer Mannheim. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K₁₂ strain EM 20031 and assays for methionine acceptor activity were carried out as described before (10).

Preparation of N^4 -substituted cytosine and cytidine derivatives

Cytosine (100 mg) was dissolved in 8 ml of 0.1 M sodium acetate, pH 5.0 containing 1 M carbohydrazide. The solution was heated at 65° for 12 hrs, the volume reduced to 5 ml by boiling, and the solution allowed to cool to room temperature, yielding a white precipitate. The solid was collected by filtration, washed with 5 ml of water, and dried over P_2O_5 .

in a vacuum dessicator, yielding 110 mg (60%) of white powder. The product migrated as a single spot on thin layer chromatography and had the NMR spectrum, UV absorption spectrum, combustion analysis (C,H,N), and mass spectrum expected for N⁴-carbohydrazido-cytosine (Ib, R=H).

Cytidine (24.3 mg) was dissolved in 1 ml of 1 M carbohydrazide adjusted to pH 5.0 with acetic acid. The solution was heated at 65° for 17.5 hrs, cooled to room temperature and added to a 1 x 30 cm column of dihydroxyboryl cellulose equilibrated with 50 mM ammonium bicarbonate, pH 8.5. The column was eluted with the same buffer at a flow rate of 6 ml/hr and the major UV-absorbing peak was pooled and lyophilized. Analysis of this material showed that it was a single product having a UV absorption spectrum which closely corresponded to that of N⁴-semicarbazido-cytidine (11) (see Figure 2B).

Cytidine (9 mg) was dissolved in 1 ml of 1 M 1,3-diaminopropane, 2 M sodium bisulfite, pH 7.0 and incubated at 37° for 5 days. The solution was diluted 200-fold with 0.1 M Tris-HCl, pH 7.0 and allowed to stand at room temperature for 2 days. Chromatographic analysis showed that all of the cytidine had reacted, yielding 94% IIb (n=3) and 6% uridine. The product, N⁴-(3-aminopropyl)cytidine, was isolated by chromatography on a column of Dowex 50 equilibrated with 10 mM ammonium acetate, pH 5.0. After exhaustive washing of the column with the equilibration buffer, pure IIb was eluted with 1 M ammonium acetate, pH 5.0.

Rate of transamination of cytidine with bifunctional amines

Solutions of alkyl diamines and sodium bisulfite in water were adjusted to the appropriate pH by addition of HCl. Carbohydrazide-bisulfite solutions were prepared using mixtures of Na₂SO₃ and Na₂S₂O₅ to give the appropriate pH and concentration of bisulfite (7). Cytidine was dissolved in these solutions at a final concentration of 37 mM. An equal amount of adenosine, which is inert to reaction with bisulfite (3), was included in the reaction mixtures as an internal standard. Samples were incubated at 25° or 37°. Aliquots were withdrawn at various times, quenched by dilution with 100 volumes of 0.1 M Tris-HCl, pH 9, and incubated at 37° for 2 hrs to convert IIa → IIb and IIIa → IIIb. Samples containing Ib or IIb were analyzed by chromatography on a 0.6 x 30 cm column of Aminex A-25 at 50° by elution with 10 mM ammonium formate, pH 4.65 at a flow rate of 34 ml/hr. The column effluent was monitored at 254 nm using a Chromatronix Model 220 absorbance detector and a Sargent-Welch SRG recorder. Carbohydrazide-bisulfite reaction mixtures were analyzed by chromato-

graphy on Aminex A-6 at 50° by elution with 0.4 M ammonium formate, pH 4.7 (12).

Modification of tRNA^{fMet} with bifunctional amines

Carbohydrazide: Reaction mixtures contained 20 A₂₆₀/ml tRNA^{fMet} in 1 M carbohydrazide, 2 M sodium bisulfite, pH 6.0, 10 mM MgCl₂. Solutions were incubated at 25° for a given amount of time and reactions stopped by addition of 10 volumes of water. Samples were dialyzed vs. .15 M NaCl, 10 mM Tris, pH 7.0 followed by two dialyses vs. .05 M NaCl, 10 mM Tris pH 7.0, concentrated by evaporation to 20 A₂₆₀/ml and precipitated by addition of 2 volumes of 95% ethanol. The number of carbohydrazide side chains/mole was determined by quantitative attachment of fluorescein to the reactive amino groups as described elsewhere (7).

Bifunctional alkylamines: Reaction mixtures contained 16 A₂₆₀/ml tRNA^{fMet} in 1 M alkyl diamine, 2 M sodium bisulfite, pH 7.0, 10 mM MgCl₂. Solutions were incubated for a given amount of time, diluted with 2 volumes of water, and dialyzed as described above. One-tenth volume of 1 M Tris-HCl, pH 9.2 was added and the solutions incubated at 37° for 8 hrs, dialyzed vs. 10 mM Tris-HCl, pH 7, 5 mM MgCl₂, concentrated, and precipitated as above. The number of N⁴-substituted cytidine residues/mole tRNA was determined by attachment of fluorescein to the reactive amino groups. The precipitate of modified tRNA^{fMet} was dissolved in 1 M HEPES, pH 11.65 at a concentration of 27 A₂₆₀/ml and quickly mixed with an equal volume of DMSO containing freshly dissolved fluorescein isothiocyanate (10 mg/ml). The reaction mixture was incubated in the dark at 37° for 1 hr, neutralized with conc. HCl, adjusted to 0.24 M NaCl, and precipitated with 3 volumes of ethanol. Free dye was removed from the pellet by four reprecipitations from 0.1 M Tris-HCl, pH 7.0, 5 mM MgCl₂, 0.4 M NaCl with 3 volumes of ethanol. The tRNA was then dissolved in 0.1 M Tris-HCl, pH 7.0, 5 mM MgCl₂ and the amount of bound fluorescein calculated from the absorption at 495 nm as described elsewhere (7).

Coupling of N-hydroxysuccinimide esters to tRNA^{fMet} containing N⁴-substituted residues

N-Hydroxysuccinimide esters were dissolved in fresh DMSO just before use. Reactions with light-sensitive esters were carried out in the dark room. Modified tRNA^{fMet} was dissolved in 0.2 M HEPES, pH 7.8 at a concentration of 25 A₂₆₀/ml. The tRNA and ester solutions were mixed in a ratio of 1:0.75, incubated at 25° or 37° for various times and the re-

actions stopped by precipitation of the tRNA with 2 volumes of ethanol. The samples were chilled for 10 min at -20° and centrifuged. The tRNA was reprecipitated twice from 0.1 M sodium acetate, pH 6.0 to remove excess ester. The extent of reaction of N^4 -substituted cytidine residues in tRNA^{fMet} with the esters was determined by measuring the decrease in labeling of the modified tRNA with fluorescein isothiocyanate. The stability of products V(a)-(e) was determined as described before (8).

RESULTS

Transamination of cytidine with bifunctional amines

Treatment of cytidine with 1 M carbonylhydrazide, 2 M sodium bisulfite, pH 6.0 at 25° leads to rapid formation of the 4-carbonylhydrazido-cytidine-bisulfite adduct Ia (Figure 1). The product has a UV absorption spectrum which is essentially identical to that of the analogous semicarbazide-bisulfite adduct (13), with an absorption maximum at 245 nm and $\epsilon_{\text{max}}^7 = 17 \times 10^3$ (Figure 2A). Slow conversion of cytidine to the uridine-bisulfite adduct IIIa also occurs under these conditions, but at a rate which is insignificant in comparison to transamination with this

Figure 1. Rate of modification of cytidine in the presence of 1 M diamine, 2 M sodium bisulfite, pH 6.0 at 25° : ●-●, carbohydrazide; ○-○ 1,3-diaminopropane. Rate of conversion of cytidine to Ia (---) and IIIa (-.-.-) calculated by subtracting the amount of uridine formed in each reaction.

Figure 2. UV absorption spectra of transaminated cytidine derivatives in 0.1 M Tris-HCl, pH 7.0 (—), 0.1 N HCl (---), and 0.1 N KOH (-.-). A, bisulfite adduct of N^4 -carbohydrazido-cytidine (Ia); B, N^4 -carbohydrazido-cytidine (Ib); C, N^4 -(3-aminopropyl) cytidine (IIb, $n=3$).

highly reactive amine. In contrast, reaction with bifunctional alkyl amines at pH 6 results in little transamination, and the major product formed (75% of reacted cytidine) is the uridine-bisulfite adduct.

The deamination reaction is strongly suppressed at pH 7 and above (14,15). Transamination of cytidine with bifunctional alkyl amines in the presence of bisulfite at pH 7 leads to good yields of adducts IIa, although the reactions are much slower than transamination with carbohydrazide. The rate of modification of cytidine and the relative amounts of transamination vs. deamination vary with the chain length of the amine (Figure 3). 1,3-Diaminopropane reacts significantly more rapidly than diamines having longer alkyl chains and leads to higher yields of transamination. Essentially quantitative conversion to adduct IIa ($n=3$) can be achieved, with less than 5% deamination. The rate of transamination doubles on increasing the bisulfite concentration from 1 M to 2 M in the presence of 1 M amine at pH 7.0 (Figure 3). Transamination with 1,6-diaminohexane and 1,8-diaminooctane occurs at about half the rate observed with 1,3-diaminopropane and competes less favorably with deamination, the relative rates of formation of IIa and IIIa being 5:1 in the presence of 1 M amine, 2 M sodium bisulfite, pH 7.0.

Adducts IIa are unstable at pH 7, losing bisulfite to give the

Figure 3. A, Rate of modification of cytidine in the presence of bifunctional amines at 37°: 0-0, 1 M 1,3-diaminopropane, 2 M sodium bisulfite, pH 7.0; X-X, 1 M 1,6-diaminohexane, 2 M sodium bisulfite, pH 7.0; Δ - Δ , 1 M 1,8-diaminooctane, 2 M sodium bisulfite, pH 7.0; $\bullet$ - $\bullet$, 1 M 1,3-diaminopropane, 1 M sodium bisulfite, pH 7.0; $\square$ - $\square$, 1 M carbonylhydrazide, pH 6.0. B, Rate of transamination of cytidine in the presence of bifunctional amines. Symbols are the same as shown in A.

corresponding N⁴-substituted cytidine derivatives, IIb, after removal of excess reagent. The spectral properties of derivatives IIb are similar to those of the parent nucleoside (Figure 2C), however the transaminated products are readily separated from cytidine by chromatography on Aminex A-25 (Figure 4).

Bisulfite adduct Ia is stable at pH 7 in Tris-HCl buffer and eliminates bisulfite only slowly at pH 9. Similar properties have been reported for the analogous semicarbazide-bisulfite adduct of cytidine, however that adduct is quantitatively converted to 4-semicarbazido-cytidine by incubation at 37° for 2 hrs in 1 M sodium phosphate, pH 7 (13). Treatment of adduct Ia under these conditions results in only partial conversion to Ib, with formation of other unidentified products. This is presumably due to intra and/or intermolecular rearrangements involving reaction of the carbonylhydrazide side chain with the cytosine base, since these products no longer contain the reactive primary amino group of the side chain. Carbonylhydrazide is a sufficiently strong nucleophile that it transaminates cytidine directly in the absence of bisulfite at acidic pH

Figure 4. Chromatographic separation of N^4 -substituted cytidine derivatives from unmodified nucleosides on Aminex A-25, as described in Materials and Methods. All IIb derivatives elute in the same position.

(Figure 3), however this reaction is several orders of magnitude slower than the corresponding bisulfite-catalyzed transamination reaction. At pH 5, the rate of transamination with carbonylhydrazide alone is comparable to that seen with bifunctional alkyl amines in the presence of 2 M bisulfite at pH 7. The product, Ib, has spectral properties (Figure 2B) similar to those of the analogous semicarbazide derivative (11).

Reaction of *E. coli* tRNA^{fMet} with bifunctional amines

Treatment of *E. coli* tRNA^{fMet} with 1 M 1,3-diaminopropane in the presence of 2 M sodium bisulfite at pH 7.0 results in attachment of an average of one reactive side chain per mole of tRNA in 20 hrs at 37° or 66 hrs at 25° and leads to a partial loss of methionine acceptor activity. Single stranded uridine residues in tRNA^{fMet} form uridine-bisulfite adducts under these conditions, which also affects the ability of the tRNA to be aminoacylated (16). When the modified tRNA is incubated at pH 9 to reverse the uridine-bisulfite adducts prior to assay, linear pseudo first

order plots of methionine acceptor activity vs. time are obtained (Figure 5). Longer reaction times are required to attach an equivalent number of side chains to tRNA^{fMet} by reaction with other bifunctional amines and a somewhat greater loss of biological activity is observed due to the adverse effect of the accompanying deamination reaction on the ability of tRNA^{fMet} to be aminoacylated (17). Attachment of the bulky fluorescein moiety to the reactive amino group of cytidine derivatives IIB in tRNA^{fMet} has little or no further effect on methionine acceptance (Figure 5).

Modification of tRNA^{fMet} with 1 M carbohydrazide in the presence of 2 M sodium bisulfite at pH 6.0 results in rapid reaction, with formation of an average of one mole of Ia per mole of tRNA in 7 min at 25°. Transamination with this amine is significantly faster than either deamination or addition of bisulfite to single stranded uridine residues, and a linear first order loss of methionine acceptance is observed in the absence of any treatment with base prior to assay (Figure 5). Incubation of the modified tRNA in 0.1 M Tris-HCl buffer at pH 9 or in 1 M sodium phosphate, pH 7 results in a partial recovery of biological activity and

Figure 5. Loss of methionine acceptor activity following treatment of *E. coli* tRNA^{fMet} with bifunctional amines in the presence of bisulfite: (---), 1 M carbohydrazide, 2 M sodium bisulfite, pH 6.0 in 10 mM MgCl₂ at 25°; (○-○), 1 M 1,3-diaminopropane, 2 M sodium bisulfite, pH 7.0 in 10 mM MgCl₂ at 37°; (X-X), 1 M 1,3-diaminopropane, 2 M sodium bisulfite, pH 7.0 in 10 mM MgCl₂ at 37°, followed by labeling of the reactive side chains with fluorescein; (●-●), 1 M 1,3-diaminopropane, 2 M sodium bisulfite, pH 7.0 in 10 mM MgCl₂ at 25°.

produces a biphasic inactivation curve, however these conditions also lead to a 40% loss of reactive side chains. This is presumably due to rearrangements of the bisulfite adduct of 4-carbohydrazido-cytidine at the polynucleotide level similar to those observed with modified cytidine during attempts to convert the nucleoside adduct Ia $\rightarrow$ Ib.

A plot of the number of side chains attached to tRNA^{fMet} vs. methionine acceptor activity shows that the tRNA containing derivatives of type IIb can be aminoacylated to a greater extent than those containing adducts Ia (Figure 6).

The sites of the modified cytidine residues in tRNA^{fMet} are the same as those previously observed to be deaminated by treatment with sodium bisulfite alone at pH 6 (18) and to undergo transamination with methylamine in the presence of bisulfite at pH 7 (19). These include the unpaired cytidine at the 5' terminus, two cytidine residues in the dihydrouridine loop, the cytidine in the wobble position of the anticodon, and the two cytidine residues in the 3' terminal CCA sequence.

Attachment of protein affinity-labeling reagents to N⁴-substituted cytidine residues in tRNA^{fMet}

We have used a series of N-hydroxysuccinimide ester derivatives to attach protein affinity-labeling reagents to N⁴-substituted cytidines in tRNA^{fMet} (Scheme II). The kinetics of coupling esters IVa and IVd to tRNA^{fMet} containing an average of one residue of IIb (n=3) at 25° in 0.11 M

Figure 6. Effect of transamination of cytidine residues in *E. coli* tRNA^{fMet} on methionine acceptor activity: (●-●), Ia; (X-X), IIb (n=3).

Scheme II

Figure 7. Rate of coupling N-hydroxysuccinimide esters to $\text{tRNA}^{\text{fMet}}$ containing an average of one N^4 -substituted cytidine residue per mole of tRNA at 25° in 0.11 M HEPES, pH 8, 43% DMSO: (X-X) reaction of $\text{tRNA}^{\text{fMet}}$ containing I Ib ($n=3$) with a 100-fold excess of ester IVa; (●-●) reaction of $\text{tRNA}^{\text{fMet}}$ containing I Ib ($n=3$) with a 200-fold excess of ester IVd; (Δ - Δ) reaction of $\text{tRNA}^{\text{fMet}}$ containing Ia with a 200-fold excess of ester IVd.

HEPES, pH 8, 43% DMSO is shown in Figure 7 and the extent of reaction in 15 min with a variety of esters is summarized in Table I. Coupling reactions with tRNA^{fMet} containing other IIb derivatives are similar. In contrast, the reaction of N-hydroxysuccinimide esters with tRNA^{fMet} containing cytidine derivative Ia occurs much more slowly. This appears to be due to protection of the primary amino group of the carbohydrazide side chain from reaction by interaction with the tRNA structure, since adduct Ia in poly C has previously been observed to react at a rate comparable to that seen here with IIb derivatives in tRNA^{fMet} (8). The multiphasic kinetics of the coupling reaction with Ia in tRNA^{fMet} further suggests that esters react with carbohydrazide side chains in different environments of the tRNA structure at significantly different rates (Figure 7). Table II summarizes the extent of reaction of tRNA^{fMet} con-

Table I. Extent of reaction of tRNA^{fMet} containing an average of one mole of IIb (n=3) per mole of tRNA with various N-hydroxysuccinimide esters in 15 min at 25°.

N-hydroxysuccinimide Ester (IV)	$[\text{Ester}]_0^+ / [\text{IIb}]_0$	% Reaction
a	100	96
b	100	55
	200	75
c	100	53
	200	78
d	100	80
	200	97
e	100	77
	200	90
g	100	6
	200	68 [†]
h	200	14
i	200	57
j	200	21

[†] Note that the effective concentration of esters IV(a)-(e) is twice that of esters IV(g)-(j)

[‡] % reaction in 5 hrs at 25°

Table II. Extent of reaction of tRNA^{fMet} containing an average of one mole of Ia per mole of tRNA with a 200-fold molar excess of various N-hydroxy-succinimide esters

N-hydroxysuccinimide Ester (IV)	Reaction Time (min)	Temp. (°C)	% Reaction
a	60	25	78
d	15	25	33
	60	25	51
f	60	25	98
g	120	37	29
h	120	37	22
i	30	37	34
	60	37	52
j	120	37	22

taining an average of one residue of Ia per molecule in the presence of a 200-fold excess of various esters in 0.11 M HEPES, pH 8, 43% DMSO. Similar results have been obtained with tRNA^{fMet} containing cytidine derivative Ib.

The slow reaction of Ia and Ib has a particular disadvantage in coupling esters IV(a)-(e) to tRNA^{fMet} since the N-hydroxysuccinimide ester group of the product is partially hydrolyzed during the incubation, reducing the yield of lysine-reactive side chains in the tRNA. Approximately half of such side chains are still amine-reactive after a 2 hr incubation at 25° in 0.11 M HEPES, pH 8, 43% DMSO. The incubation period required to achieve adequate coupling to Ia or Ib can be reduced by increasing the initial concentration of ester or by repeated additions of fresh ester at 15 min intervals. Use of modified tRNA containing IIb derivatives is preferable for preparation of products V (a)-(e), however, since essentially quantitative coupling can readily be achieved under conditions which result in little hydrolysis of the lysine-reactive groups attached to the tRNA.

DISCUSSION

The procedures described here provide a simple method for the at-

tachment of a variety of protein affinity labeling reagents to single stranded cytidine residues in polynucleotides. Derivatives such as V (a)-(e) can undergo rapid reaction with lysine ϵ -amino groups, derivatives such as V (d), (f), (g) and (i) can react with cysteine sulfhydryl groups, and photoactivatable derivatives such as V (h)-(j) can form non-specific crosslinks with a variety of amino acids on exposure to visible light. Use of reversible crosslinking derivatives such as V (b), (d), (e) and (i) also permits the covalent couple formed between proteins and nucleic acids to be subsequently cleaved under mild reaction conditions (20-22).

Most naturally occurring nucleic acids are unreactive with N-hydroxy-succinimide esters, with the exception of a few species of tRNA that contain unusual nucleotide bases, such as 3-(3-amino-3-carboxypropyl) uridine (23). Transamination with bifunctional amines in the presence of bisulfite is used to introduce ester-reactive primary amino groups into nucleic acids at the sites of exposed cytidine residues. Carbohydrazide (pKa 4.3) reacts to form N⁴-substituted cytidine derivatives much more rapidly than bifunctional alkyl amines (pKa ~11). On the other hand, modified tRNA^{fMet} containing Ia or Ib reacts more slowly with N-hydroxy-succinimide esters than tRNA containing IIb, leading to lower yields of protein-reactive tRNA derivatives with certain esters. The greater stability of the alkyl side chain of IIb to extremes of pH and temperature may also be an advantage in certain protein-nucleic acid crosslinking studies.

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