A new simpler photoaffinity analogue of peptidyl tRNA

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ABSTRACT

The synthesis of the n-hydroxysuccinimide ester of N-(2-nitro-4-azidophenyl)glycine (NAG) is described. This reacts with E. coli phe-tRNA^{Phe} to yield the photoaffinity label NAG-Phe-tRNA^{Phe}. This peptidyl tRNA analogue binds correctly to the peptidyl site of the E. coli ribosome. The only significant covalent products found after irradiation of a peptidyl site bound NAG-Phe-tRNA^{Phe}-70S-poly(U) complex are 50S proteins L11 and L18. After irradiation the complex can still bind [^{3}H]Phe-tRNA to the amino acyl site and participate in peptide bond formation with the covalently attached NAG-Phe moiety. Alternatively, one can allow peptide bond formation to occur first, prior to photolysis. The reaction products are still L11 and L18. Hence, both of these two proteins appear to be centrally located at the peptidyl transferase center.

INTRODUCTION

Affinity labels have been quite successful at locating proteins in or near functional sites of the E. coli ribosome[1-6]. Recently we reported the preparation and properties of p-(2-nitro-4-azidophenoxyl)phenylacetyl-phenylalanyl-tRNA^{Phe} (NAP-Phe-RNA^{Phe}).[7] This photoaffinity peptidyl tRNA covalently reacted primarily with proteins L11 and L18 of the 50S subunit. Photoactivated affinity reagents have the advantage that the precise time of reaction can be controlled. They have a very broad spectrum of reactivity. The major shortcoming of NAP-Phe-tRNA^{Phe} and potentially other photolabels as well is the high risk of nonspecific reaction[8,9]. To circumvent this problem, a second photoaffinity reagent, N-[2-nitro-4-azido-phenyl]glycyl-phenylalanyl-tRNA^{Phe} (NAG-Phe-tRNA^{Phe}), has now been prepared and tested. The NAG moiety is far less hydrophobic than NAP. Therefore one might predict, and in fact we

find, its reaction pattern with ribosomal proteins is far more specific.

EXPERIMENTAL

Materials and Methods: Ribosomes were obtained from E. coli A19 cells (General Biochemicals). Ribosomes, ribosomal subunits, and modified [^{3}H]Phe-tRNA^{Phe} were prepared as described previously.[7] The analysis of ribosomal proteins was preformed by two-dimensional gel electrophoresis.[10] The tritium content of the proteins was determined as described previously.[7]

Synthesis of NAG-Phe-tRNA^{Phe}: All work with azides was carried out in the absence of direct room or sun light. 300 mg (1.65 mmole) of 4-fluoro-3-nitrophenylazide (Pierce Chemical Co.) was dissolved in 8.5 ml of ethanol. 111 mg of glycine (1.65 mmole) and 226 mg of K_2CO_3 (1.65 mmole) were dissolved in distilled water and added to the ethanol mixture. After refluxing for 4 1/2 hours at 90-95° C, the reaction mixture was cooled and acidified. Water was added to facilitate the precipitation of the acid and the mixture was allowed to stand overnight at 4° C. The reddish precipitate was filtered, washed with water and dried under vacuum.

Purification of the product was accomplished by biphasic extraction. The precipitate was dissolved in aqueous $NaHCO_3$ and extracted with ethyl acetate approximately 5 times. The $NaHCO_3$ layer was acidified and the product extracted into the ethyl acetate layer 3 times, dried over $MgSO_4$, and rotoevaporated. N-(2-nitro-4-azidophenyl)glycine (NAG) was recrystallized from a mixture of ethanol/water (75:25), mp 168°-169° C. TLC (90:10 ethyl acetate:acetic acid) showed a single spot. IR (KBr pellet) showed bands at 3.3μ (O-H), 4.7μ (N≡N), and 5.8μ (C=O). NMR (DMSO-d_6) showed a sharp singlet at δ6.8-7.9. The mass spectrum gave a parent peak at 209, indicating a rapid loss of N≡N (28) from the molecular weight 237.18 and also showed the absence of impurities in the compound. Analysis: Found (calculated: C, 40.72 (40.51);

H, 3.07 (2.98); N, 29.20 (29.52).

Equimoles of NAG and N,N'-dicyclohexylcarbodiimide (DCC) were dissolved in ethyl acetate. A slight excess of N-hydroxysuccinimide was added and the mixture allowed to react at 37° C for 1 1/2 hr. Dicyclohexylurea which precipitated was filtered away and the solution was roto-evaporated. TLC (ETOAc) indicated substantial reaction had occurred. The succinimide ester of N-(2-nitro-4-azidophenyl) glycine (SNAG) was used without further purification for preparation of NAG-Phe-tRNA^{Phe}. Using the Cu^{2+} hydrolysis method[11] of determining the amount of N-blocked aminoacyl-tRNA, an optimal yield (60-75%) of NAG-Phe-tRNA^{Phe} was observed after 1 1/2 hr of incubation of SNAG with [^{3}H]Phe-tRNA^{Phe} exactly as described for NAP-Phe-tRNA^{Phe}.[7]

Reactions of NAG-Phe-tRNA^{Phe} with ribosomes were carried analogously to NAP-Phe-tRNA^{Phe} reactions.[7] Photolysis of free SNAG above 310 nm in aqueous ethanol shifted the azide absorption from 445 nm to a spectrum with very weak broad peaks at 455 nm and 330 nm. The photolysis was complete in 45 min, so all reactions with ribosomes were carried out for a minimum of 1 hr at 4° C to insure complete reaction.

RESULTS

Binding of NAG-Phe-tRNA^{Phe} to Ribosomes: NAG-Phe-tRNA^{Phe} should behave similarly to NAP-Phe-tRNA^{Phe} and other peptidyl-tRNAs or analogues. The binding of NAG-Phe-tRNA^{Phe} to 70S ribosomes is stimulated by the addition of poly(U). This is seen in Table I. In three separate determinations, the presence of poly(U) enhanced NAG-Phe-tRNA^{Phe} binding to 70S ribosomes approximately 4-fold when measured by millipore binding assays (Table I) and sucrose centrifugation analysis (results not shown).

Covalent Binding of NAG-Phe-tRNA^{Phe} to Ribosomes: Complexes with tRNA analogs, poly(U) and ribosomes were prepared as described previously.[7] The appearance of [^{3}H] counts in the subdivided rRNA and protein fractions was a measure of covalent reaction products from NAG photolysis

TABLE I

Total Binding of [^{3}H]NAG-Phe-tRNA^{Phe} to Ribosomes

Sample*	tRNA Input (cpm)	Cpm Incorporated -poly(U)	Cpm Incorporated +poly(U)	poly(U) stimulation
1	9,780	1100	4680	4.2
2	17,520	3100	9700	3.1
3	23,200	2600	17,050	4.7

*This assay was performed according to Nirenberg (12).

TABLE II

Incorporation of Radioactivity into Ribosomal RNA and Protein Fraction

Sample	[^{3}H] cpm/mg of Ribosomal Fraction† #1	#2	#3
1. 30S subunit			
Protein	248	141	-
RNA	230	132	-
2. 50S subunit			
Protein	10,700	2010	4700
RNA	130	80	60

† RNA and protein fractions were obtained by adding an equivolume of 8M urea and 4M LiCl to either 30S or 50S ribosomal subunits and incubating for 48 hours at 4° C. Following incubation, the rRNA was removed by centrifugation.

(Table 2). Since 85-95% of the total tritium counts appears to be attached to the 50S ribosomal protein fraction, these were the only NAG-Phe-tRNA^{Phe} reaction products characterized further.

Two-dimensional gel electrophoresis was carried out on radioactive 50S proteins obtained from ribosomal complex containing poly(U) and NAG-[^{3}H]Phe-tRNA^{Phe}. The average results from four determinations are exhibited in Fig.1. The major modified proteins appear to be L11 and L18 with minor reactivities assigned to L1, L5, L27, and L33.

NAG-Phe-tRNA^{Phe} Binds to the Peptidyl Site: Reaction of the NAG-Phe-tRNA^{Phe}-poly(U)-ribosome complex with the antibiotic puromycin permits analysis of the binding site of this tRNA. These results are exhibited in Table 3. Puromycin releases both N-acetyl-[^{3}H]Phe-tRNA^{Phe} and NAG-Phe-tRNA^{Phe} to a comparable degree, suggesting that the positioning of NAG-[^{3}H]Phe-tRNA^{Phe} is mainly in the peptidyl (P) site. To rigorously prove the above results, nonradioactive NAG-Phe-tRNA^{Phe} was added to the complex either before or after photolysis. Consequently, only if at least one peptide bond formation has occurred will covalent incorporation of radioactivity be possible.

The appearance of substantial radioactivity in 70S ribosomal complexes containing either prephotolyzed (prior to [^{3}H]Phe-tRNA addition) or post-photolyzed (after [^{3}H]Phe-tRNA addition) NAG-Phe-tRNA^{Phe} indicates that peptide bond formation as well as covalent modification has indeed occurred. This strongly supports the evidence that NAG-Phe-tRNA^{Phe} is situated in the peptidyl site of the ribosome. Two-dimensional gel electrophoreses were performed on these reacted proteins (Fig.1). The major covalent products from pre-photolyzed NAG-Phe-tRNA^{Phe} samples are L11 and L18. Minor reactions occur with L2, L5, L19, L26, and L33. Because of the possibility of overlapping spots or changed mobilities after modification[2,13], L19 is most likely part of modified L18. We consistently extract the area around a protein to insure the maximum yield of modified protein.

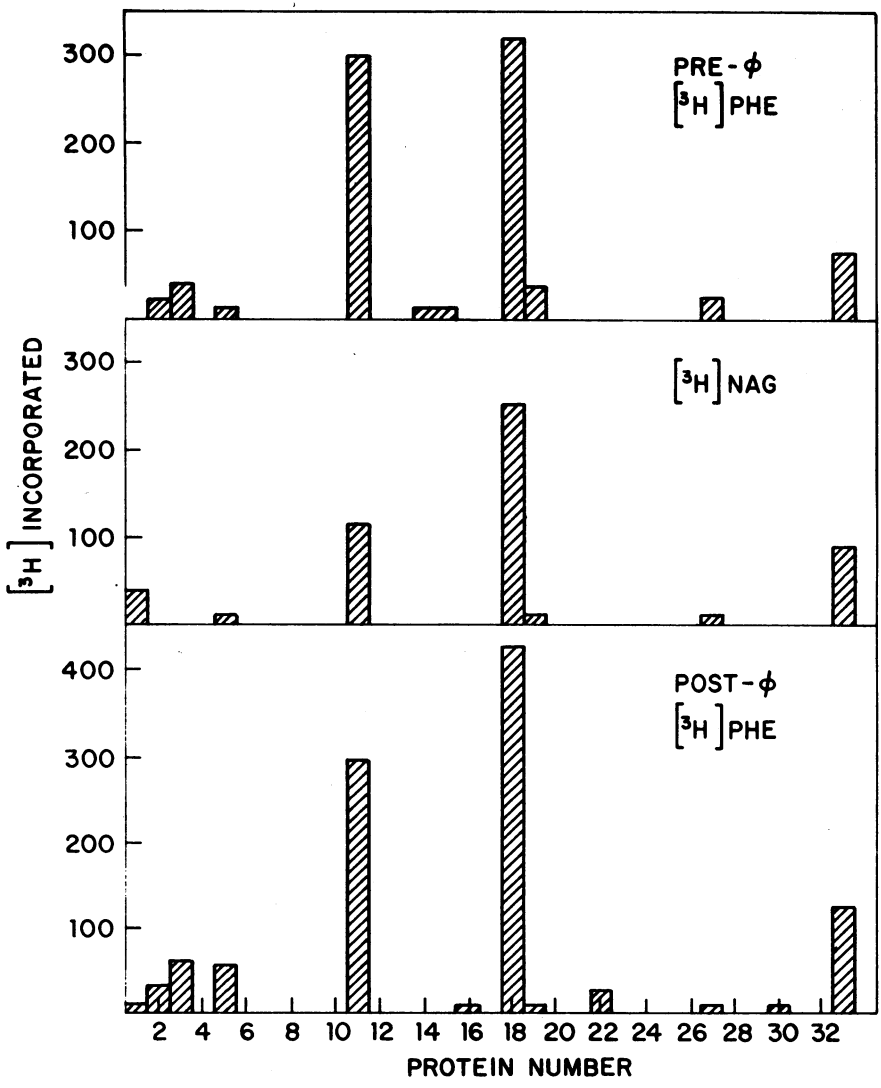

Fig.1. Identification of covalently modified 50S ribosomal proteins. The average distribution of radioactivity found on a two-dimensional gel.

Pre-φ[^{3}H]Phe: Ribosomes containing nonlabeled NAG-Phe-tRNA^{Phe}; [^{3}H]Phe-tRNA added after photolysis.

[^{3}H]NAG: Ribosomes containing NAG-[^{3}H]Phe-tRNA^{Phe}.

Post-φ[^{3}H]Phe: Ribosomes containing nonlabeled NAG-Phe-tRNA^{Phe}; [^{3}H]Phe-tRNA added before photolysis.

TABLE III

Puromycin Reactivity of Bound $tRNA^{Phe}$ †

Sample	Puromycin (1mM)	$tRNA^{Phe}$ Input (cpm)	$tRNA^{Phe}$ Bound cpm bound	% bound	Puromycin Release cpm released	cpm released by bound tRNA
1. [^{3}H]Ac-Phe-$tRNA^{Phe}$	+	52,000	37,600	53%	26,320	70%
	-	52,000	37,600	53%	6,400	17%
2. [^{3}H]NAG-Phe-$tRNA^{Phe}$	+	46,400	34,100	73%	26,370	78%
	-	46,400	34,100	73%	8,120	4%

† Assays were performed as described previously.[7]

Very similar covalent modification results were observed for proteins obtained from postphotolyzed NAG-Phe-tRNA^{Phe}-ribosome complexes (Fig.1). Again, the major reaction species were L11 and L18 with minor reactions occurring in proteins L2, L5, L27, and L33. The product of the postphotolyzed reaction mixture is presumably NAG-Phe-[^{3}H]Phe-tRNA^{Phe} bound in the amino acyl (A) site.

DISCUSSION

The synthesis of NAG-Phe-tRNA^{Phe} provides a particularly selective photoaffinity label for probing functional sites of the ribosomes. NAG-Phe-tRNA^{Phe} appears to resemble a functional peptidyl-tRNA. Poly(U) stimulates the binding of NAG-Phe-tRNA^{Phe} about 4-fold (Table 1). Furthermore, the high puromycin reactivity with noncovalently bound NAG-Phe-tRNA^{Phe} implies the location of the tRNA analogue is in the P site.

Further evidence that NAG-Phe-tRNA^{Phe} is functionally situated in the P site is shown in experiments where nonradioactive NAG-Phe-tRNA^{Phe} is bound and photolyzed, and then [^{3}H]Phe-tRNA^{Phe} is added to the ribosomal complex. The covalent incorporation of radioactivity in the ribosome can occur only as the result of peptide bond formation after photolysis to produce NAG-Phe-[^{3}H]Phe-tRNA^{Phe}. In order to react with [^{3}H]Phe-tRNA^{Phe} bound in the A site, the NAG-Phe-tRNA^{Phe} must have been covalently positioned in the P site.

The comparable protein reactivities of all three experiments (Fig.1) suggest that the 3' end of a tRNA with a peptide chain is fairly flexible. L11 and L18 are the major reaction products in all three photolyses. Similarly, both L11 and L18 are the major modified proteins with NAP-Phe-tRNA^{Phe}[7], which contains one more phenyl group and is approximately one amino acid longer than NAG-Phe-tRNA^{Phe}. Although we thought these differences in the inherent makeup of the tRNA analogues might produce different reaction patterns, not only were the major products identical, but also the minor products, L2, L5, L27, and L33. This suggests that either L11 and L18 are situated in the pathway of the growing peptide chain or the flexibility of the peptide chain is significant

and the N-terminus of the chain retains a specific conformation near L11 and L18.

One basic difference between the two photoaffinity labels lies in the greater reactivity of NAG-Phe-tRNA^{Phe}. The yield of reaction products with NAG-[^{3}H]Phe-tRNA^{Phe} appears to be twice as much as the yield upon photolysis of NAP-[^{3}H]Phe-tRNA^{Phe}. Furthermore, the extent of nonspecific reactions is greatly reduced with NAG-[^{3}H]Phe-tRNA^{Phe}. Of the total tritium counts eluted from the 2-D gel, 70% of the counts were found in L11 and L18 and the remaining counts were distributed among five proteins. In contrast, with NAP-[^{3}H]Phe-tRNA^{Phe}, only 30% of the total radioactivity on the 2-D gel was found in L11 and L18. The remaining radioactive counts were isolated from approximately 21 proteins.

NAG-Phe-tRNA^{Phe} has provided strong support for our previous conclusions.[7] Partial reconstitution experiments of Nierhaus and Montejo using protein deficient cores indicated L11 as a necessary component of peptidyl transferase activity.[14] Furthermore, L11 and neighboring proteins in the A site, L6 and L16[3,15,16], have been shown to influence each others' ribosomal functions.[17] L18 is one of two 50S proteins that specifically bind to the 5S RNA.[18] This complex exhibits GTP and ATP hydrolysis which are inhibited by the same antibiobiotics which inhibit EF-G dependent GTPase activity.[19] A photoaffinity label of GDP which complexed with EF-G and fusidic acid modified proteins L5, L11, L18, and L30.[8] Clearly, L11 and L18 appear to be intimately involved in the active center of the ribosome.

It is also worth mentioning that the N-hydroxy-succinimide ester of NAG used as a precursor of NAG-Phe-tRNA^{Phe} can itself easily be synthesized at a high specific activity from [^{3}H]-glycine. This precursor would appear to be an attractive general photocrosslinking agent.

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