ON PHOTOAFFINITY LABELING OF *ESCHERICHIA COLI* RIBOSOMES USING AN AZIDOCHLORAMPHENICOL ANALOGUE

Peter E. NIELSEN, Vagn LEICK* and Ole BUCHARDT

Department of General and Organic Chemistry, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen and **Biochemical Institute B, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark*

Received 11 August 1978

1. Introduction

The chloramphenicol binding site on 70 S *Escherichia coli* ribosomes has been studied by thermal affinity labeling with ambiguous results [1,2]. Consequently, it is of interest to attempt to clarify this problem using photoaffinity labeling, a technique which has frequently been applied in various other studies of *E. coli* ribosomes [3,4].

We describe here a photoaffinity labeling experiment on *E. coli* ribosomes with azidochloramphenicol (N_3-CAP) and a photochemical study of this compound.

2. Materials and methods

Escherichia coli ribosomes, 70 S, and purified subunits were prepared as in [5]. N_3-CAP was synthesized as in [6] and was ^{3}H-labeled by a heterogeneously-catalyzed ^{3}H-exchange reaction at room temperature, performed by New England Nuclear, Boston, MA. The crude product was purified to constant specific activity by thin-layer chromatography, giving final spec. act. ~15 mCi/mmol.

Irradiation (affinity labeling) experiments were performed in quartz tubes at room temperature in a buffer mixture consisting of 0.06 M Tris–HCl, pH 7.4, 0.4 M KCl, 0.02 M Mg-acetate, 0.012 M β-mercaptoethanol[+], using a Bausch and Lomb SP 200 lamp equipped with Pyrex or WG 320 nm cut-off filter. The concentration of ribosomes or ribosomal subunits was 1 mg/ml and 10^6 cpm of [^{3}H] N_3-CAP in 100 μl methanol was used/ml mixture.

Photolysis of N_3-CAP at 77 K was performed with the SP 200 lamp (Bausch and Lomb) at 260 nm using a B and L monochromator.

Ultraviolet spectra were recorded on a Cary 14 spectrophotometer.

Flash experiments were undertaken in 10 cm quartz or Pyrex cells using two flash lamps (0.5 μF, 15 kV) as in [8]. Monochromatic light was used in the analyzing beam.

3. Results and discussion

We have described [6] the synthesis of N_3-CAP, which was found to exhibit significant antibiotic activity (10% activity in the 'fragment reaction' [6], and 50% growth inhibition in vitro**, compared to that of chloramphenicol (CAP), and which is highly photoactive at wavelengths ($\lambda > 300$ nm), which do not damage the ribosomes (table 1).

Upon irradiation, [^{3}H]N_3-CAP was covalently attached to 50 S subunits in a time-dependent reac-

Abbreviation: N_3-CAP, (1R,2R)-1-(4-azidophenyl)-2-dichloroacetamido-1,3-propanediol

[+] It was checked by ultraviolet spectroscopy that N_3-CAP was thermally stable under these conditions [7]

** Hansen, J. B., Buchardt, O., Leick, V. and Nielsen, P. E. (1978) unpublished results

Table 1
Photolysis of CAP and N_3-CAP

Compound	Half-life ($\tau_{1/2}$ min)		
	No filter	Pyrex filter	WG '320 nm' filter
CAP	> 30	–	–
N_3-CAP	0.5	3	5

Half-life measurements upon irradiation with ultraviolet-light (Bausch and Lomb SP 200 light source) were carried out in quartz cuvettes (methanol, 0.03 mg/ml) and the reaction followed by recording the ultraviolet spectra

tion (fig.1). However, a similar attachment to 30 S subunits was discovered, and when 70 S subunits were labeled, analysis on a sucrose gradient (fig.2) showed equal labeling of the 50 S and 30 S subunits. The ribosomal proteins were labeled to a higher extent than the RNA (table 2), but this label was rather non-specific as examined by two-dimensional gel-electrophoresis [9].

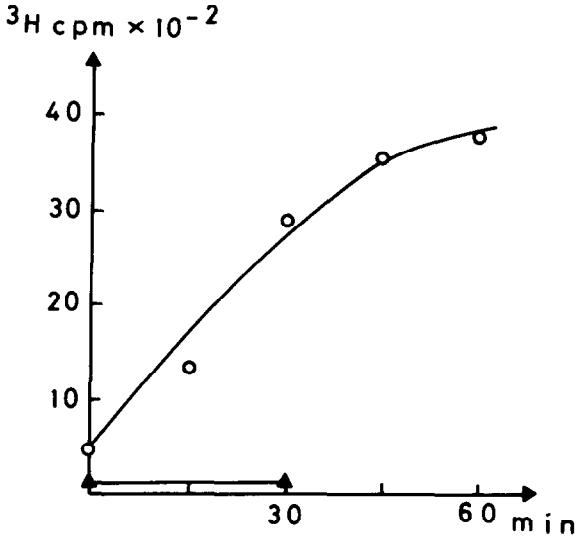

Fig.1. Affinity labeling of *E. coli* 50 S subunits: 20 A_{260} subunits were labeled for the periods indicated, precipitated in 66% ice-cold EtOH, and washed twice by resuspension in 200 μl H_2O followed by precipitation in 66% EtOH (0°C). This was followed by filtration on Whatmann GF/C filters and washing with 3 × 5 ml ice-cold EtOH, ^{3}H cpm were determined using toluene-based scintillation liquid (–○–○–), in the absence of light (–Δ–Δ–). If [^{3}H]N_3-CAP was first irradiated and then incubated with ribosomes no labeling was observed.

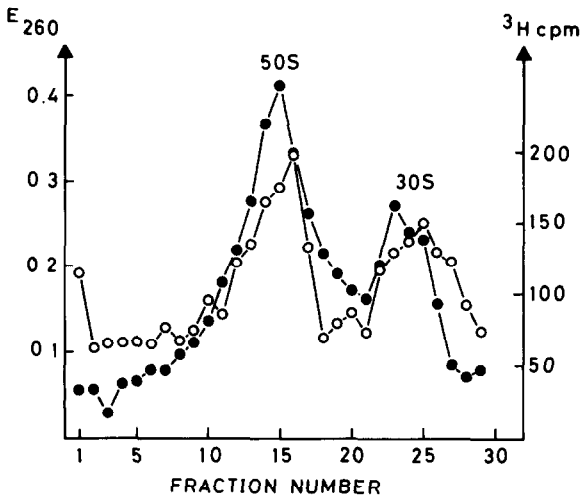

Fig.2. Affinity labeling of 70 S ribosomes. Ribosomes, 70 S, were affinity labeled as described, and after precipitation with 66% ice-cold EtOH and resuspension in a low Mg^{2+} (0.3 mM) buffer, layered on a 5–20% (w/w) sucrose gradient and centrifuged for 8.5 h at 21 000 rpm (4°C) in a Beckmann SW 27 rotor. (–●–●–) A_{260}; (–○–○–) ^{3}H cpm of EtOH-precipitated material.

In order to understand this non-specific labeling better a photochemical study of N_3-CAP was undertaken. Irradiation of N_3-CAP (λ = 260 nm) at 77 K in a glass consisting of ether–isopentane–

Table 2
Distribution of labeling between ribosomal protein and rRNA

	^{3}H cpm	%
Total	846 769	100
rRNA	138 31	11
Ribosomal protein	652 631	80

50 A_{260} 50 S subunits were affinity labeled for 30 min, using the WG 320 filter. Samples, 300 μl, were taken and covalently attached. ^{3}H cpm were determined as in fig.1. Ribosomal RNA was extracted from the samples with an equal volume phenol and precipitated once with 90% ice-cold alcohol. Ribosomal protein was extracted by resuspending the ethanol ribosome pellet in 50 μl H_2O + 50 μl 8 M urea. The mixture was incubated for 20 min with 2 μg pancreatic RNase and the proteins precipitated in 90% EtOH

Table 3
Properties of the '415' nm transients

	Rate of formation (s^{-1})	Half-life ($\tau_{1/2}$ s)
N_3-CAP[a]	1.8×10^3	0.4
N_3-CAP (3 mM methylamine)[a]	–	8×10^{-3}
N_3-CAP (1 mM diethylamine)[a]	–	2×10^{-2}
N_3-CAP in dioxane	2.5×10^2	1.0
N_3-CAP in H_2O pH 7.4[b]	2×10^3	0.1

[a] In 96% ethanol
[b] 10 mM Tris–HCl

Solutions, 10^{-4} M, were flash-photolyzed and the rates of formation and half-lives of the transients calculated by analysis of the ΔA_{415} by time monitored on an oscilloscope

ethanol (EPA) (2:5:5) under simultaneous examination by ultraviolet-spectroscopy permitted the observation of a new maximum at 415 nm which was thermostable at 77 K. A similar transient absorption (λ = 415 nm) was observed upon flash photolysis. The transient was generated from a precursor at a rate of $1.8 \times 10^3 s^{-1}$, and it had a $\tau_{1/2}$ of 0.4 s in ethanol. The transient was scavenged by amines (table 3) and reacted with 70 S ribosomes (fig.3) but

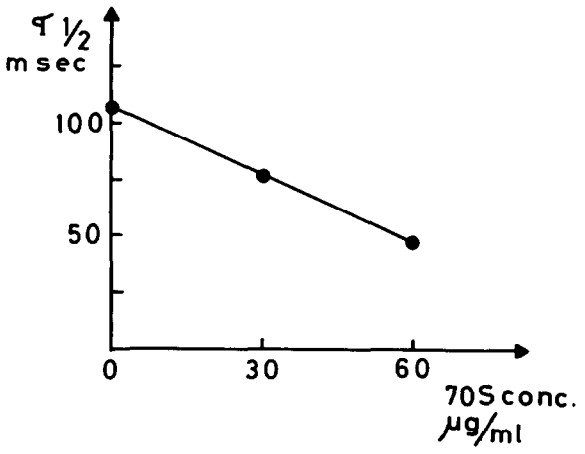

Fig.3. Reaction of the '415 nm' species with 70 S ribosomes. N_3-CAP was flash-photolyzed in 10 mM Tris–HCl (pH 7.4) and $\tau_{1/2}$ from the transient was measured at various ribosome concentrations.

it was not affected by the presence of piperylene.

It has been assumed that azidoaryl photoaffinity labeling reagents react with the binding site via triplet nitrene insertion [3,10,11]. Simple phenyl nitrenes are stable in EPA at 77 K and have λ_{max} at ~350–380 nm [12], whereas their $\tau_{1/2}$ in ethanol at room temperature are 10^{-3}–10^{-4} s [13]. This shows that the transient from N_3-CAP is not a triplet nitrene. More probably it is a ring-expanded product (a 1,2,4,6-azacycloheptatetraene)+ formed directly from the excited N_3-CAP or from its corresponding singlet nitrene [14]; rearrangement products from singlet nitrenes are known to react with amines [15] and thiols [16], whereas insertion reactions by singlet nitrenes have only been observed intramolecularly [17].

Thus the low labeling specificity observed may in part be due to rapid formation of the 415 nm species under the photoaffinity labeling experiments. A species with such a half-life would have time to leave the active site before reacting. Conversely, if generated in solution it would be able to react with the ribosomes (amino and sulfhydryl groups of the proteins) in a non-specific manner after diffusion.

It is noteworthy that we could not detect any species corresponding to the triplet nitrene and thus the 415 nm transient seems to be an important reaction intermediate in the photoaffinity labeling experiment. Since the species is more stable in an aprotic environment (dioxane versus H_2O) and reacts selectively with, e.g., amines, it is considered a less desired intermediate than a nitrene in a photoaffinity labeling experiment. Since preliminary experiments indicate that other *para*-substituted aryl azides behave analogously, we are presently investigating this reaction pathway in more detail.

Acknowledgements

We wish to thank Dr N. Harrit for his help with the flash experiments. This work was supported by the Danish Medical Science Research Council and the Danish Natural Science Research Council which is gratefully acknowledged.

+ On photolysis in polyvinyl chloride film at 77 K an IR absorption at 1885 cm^{-1} was detected [14]

References

[1] Sonenberg, N., Wilchek, M. and Zamir, A. (1973) Proc. Natl. Acad. Sci. US 70, 1423–1426.
[2] Pongs, G., Bald, R. and Erdmann, V. A. (1973) Proc. Natl. Acad. Sci. US 70, 2229–2233.
[3] Bayley, H. and Knowles, J. R. (1977) Methods Enzymol. 46, 69–114.
[4] Cooperman, B. S. and Nicholson, A. W. (1978) FEBS Lett. 90, 203–208.
[5] Anderson, S., Bretscher, M., Clark, B. and Marcher, K. (1967) Nature 215, 490–492.
[6] Nielsen, P. E., Leick, V. and Buchardt, O. (1975) Acta Chem. Scand. B29, 662–666.
[7] Staros, J. V., Bayley, H., Standring, D. N. and Knowles, J. R. (1978) Biochem. Biophys. Res. Commun. 80, 568–572.
[8] Tomer, K. B., Harrit, N., Rosenthal, I., Buchardt, O., Kumler, P. G. and Creed, D. (1973) J. Am. Chem. Soc. 95, 7402–7406.
[9] Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412.
[10] Hall, J. H., Hill, J. W. and Fargher, J. M. (1968) J. Am. Chem. Soc. 90, 5313–5314.
[11] Splitter, J. and Calvin, M. (1967) Tetrahedron Lett. 1445–1448.
[12] Reiser, A., Bowes, G. and Horne, R. J. (1966) Trans. Faraday Soc. 62, 3162–3169.
[13] Reiser, A., Willets, F. W., Terry, G. C., Williams, V. and Marley, R. (1968) Trans. Faraday Soc. 64, 3265–3275.
[14] Chapmann, O. L. and Le Roux, J.-P. (1978) J. Am. Chem. Soc. 100, 282–285.
[15] DeGraff, B. A., Gillespie, D. W. and Sundberg, R. J. (1974) J. Am. Chem. Soc. 95, 7491–7496.
[16] Carroll, S. E., Nay, B., Scriven, E. F. V., Suschitzky, H. and Thomas, R. D. (1977) Tetrahedron Lett. 3175–3178.
[17] McRobbie, I. M., Meth-Cohn, O. and Suschitzky, H. (1976) Tetrahedron Lett. 929–932.