PHOTOSENSITIZED CROSS-LINKING OF IF-3 TO *ESCHERICHIA COLI* 30 S SUBUNITS

B. S. COOPERMAN,[+] J. DONDON, J. FINELLI, M. GRUNBERG-MANAGO and A. M. MICHELSON
Institut de Biologie Physico-Chimique, 13, Rue Pierre et Marie Curie, 75005 Paris, France

Received 4 February 1977

1. Introduction

Initiation factor IF-3 is currently believed to have a dual function in protein synthesis. Initiation proceeds via dissociation of 70 S ribosomes into 30 S and 50 S subunits, and by binding tightly to the 30 S subunit, IF-3 favors this process. In addition, IF-3 appears to direct binding of 30 S subunits to start signals in messenger RNA [1]. A structurally detailed mechanism accounting for this dual functionality has been proposed [2], an important test for which would be the localization of the IF-3 binding site on the 30 S particle. Dye-photosensitized cross-linking of nucleic acids to proteins has been reported previously [3,4]. We here present results describing the successful application of a photosensitization procedure for inducing cross-links between IF-3 and both 30 S proteins and 16 S RNA.

2. Materials and methods

Purified IF-3 was prepared as described previously [5]. IF-3 was labeled via reductive methylation, using [^{14}C]formaldehyde [New England Nuclear, 44 Ci/mol] and sodium borohydride [6]. Typically, labeling of 3000–5000 cpm/μg was achieved. Microprotein determinations were accomplished using the method of Schaffner and Weissmann [7]. Labeled protein was freed from excess formaldehyde by Sephadex G-75 filtration. In accord with results previously reported [8], methylation led to no loss in IF-3 activity as measured by stimulation of fMet–tRNA^{fMet} binding to 70 S ribosomes [5]. 30 S Subunits were prepared as described previously [5], stored frozen, and preheated at 37°C for 20 min prior to use.

Reaction mixtures for photolysis were prepared using two different procedures. In the first, IF-3 (final concentration 0.8–4.0 μM) and 30 S subunits (final concentration 0.6–1.5 μM) were added to standard buffer (Tris 50 mM (pH 7.4), Mg^{2+} 8 mM, NH_4^+ or K^+ 100 mM) and incubated at 4°C for 15 min. Then the photosensitizing components flavin mononucleotide, riboflavin, $FeCl_3$ and NADH were added to final concentrations of 0.1 mM, 0.1 mM, 0.5 μM, and 0.125 mM, respectively. The complete reaction mixture was photolyzed for 5–10 min in narrow path-length optical cells (2 mm thickness) in volumes of 0.1–0.5 ml at 1–5°C, using a Blak-Ray B 100 A lamp (Ultraviolet Products), which has virtually all of its output energy at 364–366 nm. In the second procedure the reaction mixture, prepared as above but lacking the photosensitizing components, was ultracentrifuged through a 10% sucrose cushion made up in standard buffer, thus removing excess IF-3 from the IF-3·30 S complex. The complex, isolated as the resulting pellet and the immediate volume of sucrose cushion in contact with the pellet, was dialyzed against standard buffer, the photosensitizing components were added, and photolysis proceeded as described above.

Reaction mixtures containing IF-3 and 30 S subunits were analyzed on linear sucrose density-gradients (5–20%) containing standard buffer (low-salt) or standard buffer plus NaCl to a final concentration of 0.7 M (high-salt). Centrifugation was in an SW 56 rotor for 150 min at 50 000 rev./min, 4°C. The IF-3/30 S ratio was determined by measuring the radioactivity in pooled fractions shown by ultraviolet monitoring

[+]Department of Chemistry University of Pennsylvania Philadelphia, Pa. 19104, USA

to contain the 30 S subunit. Low-salt gradients were used to analyze non-photolyzed reaction mixtures for non-covalent binding or photolyzed reaction mixtures for the sum of non-covalent binding and incorporation. High-salt gradients were used to measure IF-3 incorporation. An alternative method for measuring IF-3 incorporation, which gave similar results, was to pass a photolyzed reaction mixture over a Sephadex G-75 column which was equilibrated with high-salt buffer. For preparative work, 30 S subunits containing incorporated IF-3 were separated from non-incorporated IF-3 by ultracentrifugation of the photolyzed reaction mixture through a 10% sucrose cushion made up in high-salt buffer. Protein was extracted from 30 S particles using 67% acetic acid [9], lyophilized or precipitated with acetone and taken up in gel buffer, prior to being electrophoresed. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli [10], using 15% acrylamide.

3. Results and discussion

3.1. *Incorporation into 30 S subunits*

The results of high- and low-salt gradient analysis of photolyzed and non-photolyzed reaction mixtures are presented in table 1. High salt concentrations have been shown to block IF-3·30 S complex formation. Thus, as expected, for a non-photolyzed reaction mixture virtually all of the co-migration seen in a low-salt gradient is abolished in a high-salt gradient (table 1, No. 4). By contrast, for a photolyzed reaction mixture, the IF-3/30 S ratio found in a high-salt gradient in 50% (No. 2) that found in a low-salt gradient (and 50–70% (Nos 1, 2, 4, 5) found in a low-salt gradient for a non-photolyzed reaction mixture) and we define this high-salt resistant co-migration as IF-3 incorporation into 30 S. Such incorporation is specific, at least in the sense that reaction mixture components (NaCl, aurin tricarboxylic acid (ATA), spermine) known to block non-covalent binding [8,12] also block incorporation (Nos 2, 3, 5–8). When the second photolysis procedure (Materials and methods) was used, in which the 30 S·IF-3 complex is isolated prior to photolysis, only 15–20% of non-covalently bound IF-3 was found to incorporate. The incorporation yield obtained in this manner is thus considerably lower than that obtained when 30 S particles are irradiated in the presence of excess IF-3 (table 1). However, the second procedure was utilized for the RNA and protein work described below, because it reduces the likelihood of non-specific incorporation arising from IF-3 in solution. IF-3 incorporates into both 30 S protein and 16 S RNA. Incorporation into RNA was 26 ± 7% of the total, as determined from the radioactivity remaining in the RNA pellet following acetic acid extraction of the 30 S subunit. No significant difference in the percentage of RNA labeling was found using either photolysis procedure.

Table 1
IF-3/30 S Ratios found by co-migration in sucrose density-gradients

No.	IF-3 (μM)	30 S (μM)	Addition (mM)	Photolysis	IF-3/30 S		
					Low-salt gradient	High-salt gradient	Relative incorporation
1	1.9	0.76	–	–	0.58	–	–
2	1.9	0.76	–	+	0.77	0.42	1.00
3	1.9	0.76	Spermine, 18	+	–	0.10	0.24
4	1.54	1.11	–	–	0.65	0.03	0.09
5	1.54	1.11	–	+	–	0.33	1.00
6	1.54	1.11	NaCl, 760	+	–	0.071	0.22
7	2.4	1.4	–	+	–	0.38	1.00
8	2.4	1.4	ATA, 0.04	+	–	0.19	0.50

Photolysis conducted via first procedure (Materials and methods). Calculations assume IF-3 mol. wt 22 500, $\epsilon^{1\text{ cm}}_{280\text{ nm}} = 1.4 \times 10^7$ for 30 S subunits [11].

3.2. *Incorporation into protein*

Figure 1 shows the results for a typical experiment in which proteins isolated from a 30 S sample containing incorporated IF-3 were analyzed by SDS–polyacrylamide gel electrophoresis. The presence of peaks I and II, corresponding approximately to mol. wt 52 000 and 40 000, respectively, confirms the cross-linking of IF-3 to 30 S proteins. Peak III corresponds to IF-3 and apparently represents protein that is somehow trapped into the 30 S structure on irradiation without becoming covalently cross-linked. If one assumes that peaks I and II reflect IF-3 cross-linking to single 30 S proteins, then peak I should arise from cross-linking to one or more of the group of proteins S2, S3, S4, whereas peak II should arise from cross-linking to one or more of the proteins S5–S12, it having been shown that cross-linked protein pairs migrate in an SDS–gel according to the molecular weight of the pair [13]. Here it should be noted that using bifunctional electrophilic reagents, IF-3 has been shown to cross-link to S7 [14], and to S1, S11, S12, S13, S19, and S21 [15,16]. At the present time we cannot exclude the possibility that the observed cross-links, especially peak I, arise from simultaneous cross-linking of IF-3 to two ribosomal proteins. Such a possibility would, however, be improbable statistically, since in the samples used for the protein cross-linking analysis most ($\geq$ 80–85%) of the non-covalently bound IF-3 does not even form a single cross-link. In a control experiment not shown, IF-3 was separately photolyzed and subjected to SDS–polyacrylamide gel electrophoresis. No significant radioactivity was seen in the region of the gel corresponding to mol. wt above 30 000, although a new peak was seen moving slightly slower than native IF-3 (mol. wt. $\sim$27 000), which presumably represents a photo-oxidized form of this protein.

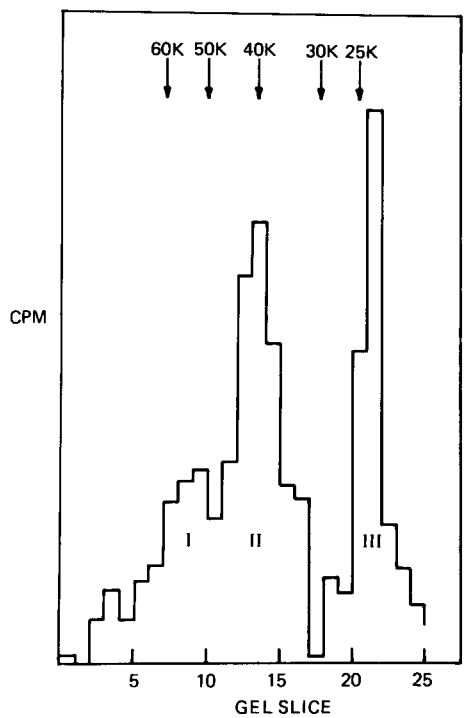

Fig.1. Radioactivity distribution in an SDS–polyacrylamide gel electrophoretogram of protein extracted from 30 S particles containing incorporated IF-3. The gel was calibrated using as mol. wt standards, bovine serum albumin (68 000), alcohol dehydrogenase (37 000), trypsinogen (25 000), cytochrome *c* (13 000) and insulin (6000). Radioactivity in gel slices was determined using an SDS extraction procedure [19]. Purified IF-3 used in these experiments is contaminated with small mol. wt proteins (8000–12 000) which bind tightly to 30 S subunits. Analysis of electrophoretograms obtained using IF-3 samples contaminated to differing extents led to the conclusion that peak I arises exclusively from cross-linking of IF-3, whereas peak II arises from cross-linking of both IF-3 and the smaller proteins.

3.3. *Incorporation into RNA*

30 S Particles containing incorporated IF-3 were treated with SDS and then subjected to sucrose density-gradient centrifugation. The results of one such experiment are shown in fig.2. The peak in $A_{260\ nm}$ at fraction 6 corresponds to 16 S RNA, and it is clear that labeled IF-3 co-migrates. This experiment was repeated using a series of pre-centrifugation SDS incubation conditions, and including 3 M urea in the gradient as summarized in table 2. From these data it appears highly probable that the value of 170 cpm/A_{260} represents genuine IF-3 cross-linking to RNA. The original 30 S sample used in this experiment had an IF-3 incorporation of 1020 cpm/A_{260}, so the fraction of IF-3 incorporated into 16 S RNA as measured by co-migration after extensive SDS treatment corresponds reasonably well with that determined measuring the radioactivity in the 67% acetic acid insoluble pellet (see above).

3.4. *Photochemistry of incorporation*

The photosensitizing system used in these experi-

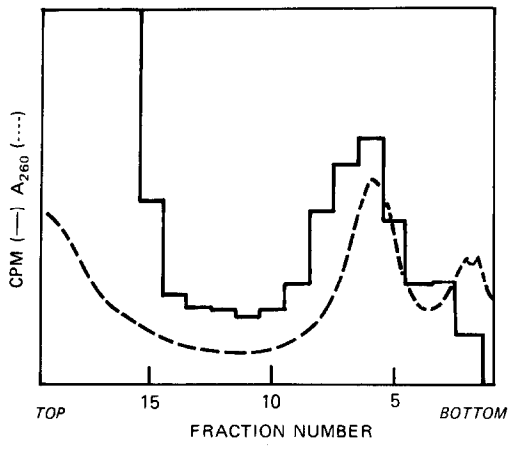

Fig.2. Comigration of radioactivity with 16 S RNA. 30 S Subunits containing incorporated IF-3 (1020 cpm/A_{260}) were suspended in a buffer (10 mM Tris, pH 7.4, 0.2 mM Mg^{2+}) containing 1% SDS. After being heated at 37°C for 2 min, they were layered on a 5–20% sucrose gradient containing 0.05 M sodium acetate (pH 5.8), 0.01 M EDTA, 0.5 M LiCl and 0.1% SDS and centrifuged at 4°C in an SW 56 rotor for 17 h at 42 000 rev/min.

ments was originally chosen because of its known ability [17] to generate singlet oxygen, superoxide and hydroxyl radicals, any of which could initiate free-radical processes leading to cross-linking. Added β-mercaptoethanol (7 mM) completely abolished incorporation, consistent with the notion of a free radical-dependent process. In preliminary experiments not presented we have shown that only the flavin-containing species are absolutely essential for incorporation. $FeCl_3$ has little or even a slight inhibitory effect, while the absence of NADH leads to a loss of incorporation of only about 30%. NADH acts essentially as hydrogen-donor for the photoreduction of flavin which on reoxidation gives rise to superoxide anion radicals. Free radicals such as $O_2^-\cdot$ and HO· can then react with protein or nucleic acid components to yield the corresponding active free radicals, particularly with aromatic residues. It may be noted (results not shown) that incorporation of [^{3}H]puromycin into 70 S ribosomes was strongly stimulated by our photosensitizing system, while under the same conditions, no incorporation was obtained for [^{3}H]dihydrostreptomycin.

Table 2
Co-migration of radioactivity and 16 S RNA in SDS-treated 30 S subunits containing incorporated IF-3

1% SDS pre-treatment	cpm/A_{260}	
2 min, 37°C	460	(340)
20 min, 37°C	280	–
20 min, 37°C + 10 min, 60.5°C	320	(170)

Experimental protocol as described in the legend to fig.2. Data are accurate to ±15%. Figures in parentheses refer to values found in gradients made up as described but containing, in addition, 3 M urea.

4. Concluding remarks

The experiments described in this communication demonstrate that it is possible to form protein–protein and protein–RNA cross-links in ribosomes via photosensitized production of active oxygen species. For protein cross-links this technique resembles cross-linking using bifunctional electrophilic reagents, with two important differences. Firstly, it is more likely to yield nearest neighbor cross-links, since no bridging molecule is involved. Secondly, several amino acid residues, presumably the aromatic- and sulfur-containing ones, are involved in the cross-link, as contrasted to the lysine cross-linking obtained with the electrophilic reagents. Thus use of both types of cross-linking techniques should yield complementary data defining a protein binding site. In this connection it will be interesting to compare our results (when the identities of the cross-linked proteins and RNA region(s) are determined) with those found earlier for IF-3 using electrophilic reagents [14–16].

Use of any non-specific cross-linking procedure raises the question of whether the covalent bonds formed reflect cross-linking from a native or denatured binding site. In our experiment, a response to this question depends on being able to extrapolate an observed cross-linking pattern back to zero (or short) irradiation time [18]. In experiments not shown, we have found the $t_{½}$ for photosensitized denaturation (standard photolysis conditions) of the 30 S particle, as measured by loss of poly(AUG) stimulated fMet-tRNA^{fMet} binding [5], to be two minutes, so that in future cross-linking experiments irradiation times shorter than this value will have to be used.

Acknowledgements

This work was performed during the tenure of an Alfred P. Sloan Fellowship (B.S.C.) and supported by grants from the following agencies: American Cancer Society (NP-176), Centre National de la Recherche Scientifique (Groupe de Recherche No. 18), Délégation Générale à la Recherche Scientifique et Technique (Convention No. 74.7.0356), Ligue Nationale Française contre le Cancer (Comité de la Seine), et le Commissariat à l'Energie Atomique.

References

[1] Grunberg-Manago, M. (1977) Prog. Nuc. Acid Res. 20, in press.
[2] Van Duin, J., Kurland, C. G., Dondon, J., Grunberg-Manago, M., Branlant, C. and Ebel, J. P. (1976) FEBS Lett. 62, 111–114.
[3] Smith, K. C. (1975) in: Photochemistry and Photobiology of Nucleic Acids (Wang, S. Y. ed) Academic Press, New York.
[4] Smith, K. C. and Hanawalt, P. C. (1969) in: Molecular Photobiology, p. 188, Academic Press, New York.
[5] Dondon, J., Godefroy-Colburn, T., Graffe, M. and Grunberg-Manago, M. (1974) FEBS Lett. 45, 82–87.
[6] Gualerzi, C., Wabl, M. R. and Pon, C. L. (1973) FEBS Lett. 35, 313–136.
[7] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
[8] Pon, C. L., Friedman, S. M. and Gualerzi, C. (1973) Mol. Gen. Genet. 116, 192–198.
[9] Hardy, S. J. S., Kurland, C. G., Voynow, P. and Mora, G. (1969) Biochemistry 8, 2897–2903.
[10] Laemmli, V. K. (1970) Nature (London) 227, 680–685.
[11] Godefroy-Colburn, T., Wolfe, A. D., Dondon, J., Grunberg-Manago, M., Dessen, P. and Pantaloni, D. (1975) J. Mol. Biol. 94, 461–478.
[12] Pon, C. L. and Gualerzi, C. (1976) Biochemistry 15, 804–811.
[13] Sommer, A. and Traut, R. R. (1975) J. Mol. Biol. 97, 471–481.
[14] Van Duin, J., Kurland, C. G., Dondon, J. and Grunberg-Manago, M. (1975) FEBS Lett. 59, 287–290.
[15] Sommer, A. and Traut, R. R. (1976) J. Mol. Biol. 106, 995–1015.
[16] Hawley, D. A., Slobin, L. I. and Wahba, A. J. (1974) Biochem. Biophys. Res. Commun. 61, 544–550.
[17] Michelson, A. M. and Durosay, P. (1977) Photochem. Photobiol. in press.
[18] Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R. and Cooperman, B. S., submitted for publication.
[19] Weber, K., Pringle, J. R. and Osborn, M. (1972) Meth. Enzymol. 26, 3.