Structure-function Relationship in *Escherichia coli* Initiation Factors

IDENTIFICATION OF A LYSINE RESIDUE IN THE RIBOSOMAL BINDING SITE OF INITIATION FACTOR BY SITE-SPECIFIC CHEMICAL MODIFICATION WITH PYRIDOXAL PHOSPHATE

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Incubation of *Escherichia coli* initiation factor 3 (IF3) with pyridoxal phosphate (PLP) followed by reduction with sodium borohydride resulted in the selective modification and inactivation of this protein. The ribosomal-binding site (RNA-binding site) of IF3 is the target of PLP modification, since (a) the phosphate residue of PLP is required for inactivation; (b) RNA as well as synthetic polynucleotides (especially guanine-containing ones) protect IF3 from inactivation; and (c) 30 S, but not 50 S ribosomal subunits, protect IF3 from PLP modification and from inactivation.

The incorporation of PLP into IF3 occurred exclusively at lysine residues by reduction of the Schiff bases yielding ε-(5'-phosphopyridoxyl)lysine. The PLP-modified lysines were identified by amino acid analysis and sequencing of the PLP-modified peptides. Out of the 20 lysines of the factor, only Lys 2, Lys 5, Lys 99, Lys 112, Lys 168, and an unidentified Lys of the central cluster of the molecule (Lys 86, 87, 90, 91, 96) were found to be modified to varying degrees. The incorporation of 3 to 4 mol of PLP/mol of IF3 is accompanied by a substantial (≥80%) inactivation of this protein; the loss of activity follows apparent first-order kinetics, and the inactivation results from the modification of just 1 Lys residue. This essential Lys residue was identified by various criteria to be Lys 112. The identification of an "active region" in the IF3 molecule is emerging from this as well as from other chemical modification studies.

Site-specific chemical modification of proteins with pyridoxal phosphate has been widely used to inactivate phosphate-binding enzymes. In many instances, this reaction proved to be selective for those Lys residues displaying affinity for negatively charged phosphate groups (1–6). Thus, this reaction is potentially useful in the study of structure-function relationships in the protein synthetic machinery where many nucleic acid (and nucleotide)-protein interactions occur. Furthermore, the fluorescent properties of the reaction product can be exploited in spectrofluorimetric investigations.

Recently, we made use of the PLP1 reaction in the study of the active sites of both *Escherichia coli* ribosomal subunits (7–10) as well as of initiation and elongation factors. Among the initiation factors, IF3 interacts with nucleic acids (11–16) and binds to the 30 S ribosomal subunits via the 16 S rRNA (12, 16). In the present paper, we present data on the identification of an active site of IF3 responsible for the interaction of the molecule with the ribosome.

**EXPERIMENTAL PROCEDURES**

**General Preparations**—*E. coli* MRE600 30 S and 50 S ribosomal subunits (17), 16 S and 23 S rRNAs (18), and initiation factors (19) were prepared as described previously. In *vitro* labeling of initiation factors by reductive methylation with [14C]formaldehyde was performed as described (20).

**Assay of IF3 Activity**—Unless otherwise specified, IF3 activity was determined by the 30 S poly(U)–N-acetylphenylalanyl-tRNA ternary complex equilibrium perturbation method as described previously (21). Binding of radioactive IF3 to ribosomes was studied by sucrose density gradient centrifugation as described elsewhere (16).

**Reaction with Pyridoxal Phosphate**—IF3 was incubated with PLP (Serva, Heidelberg) either in 20 mM triethanolamine–HCl, pH 7.8, and 30 mM KCl (TEA buffer) or in 12.5 mM triethanolamine–HCl, pH 7.8, 20 mM KC1, 6 mM Mg acetate, and 4 mM β-mercaptoethanol (TEA-Mg buffer) as specified in each figure legend. This reaction was stopped by the addition of either unlabeled NaBH₄ or NaBH₄, (Amersham Buchler) as specified in each figure legend. The reaction mixture was left for at least 10 min at 0 ºC to ensure complete reduction by NaBH₄ before further processing. To determine the number of PLP molecules incorporated into IF3, bovine serum albumin (1 mg/ml) was added as a carrier to the reaction mixtures containing known amounts of the factor. Acetic acid (0.1 N) was added to decompose excess NaBH₄, and after 30 min at 0 ºC, the mixture was neutralized by the addition of 0.1 N NaOH. Aliquots of the mixture were then placed on Whatman 3MM filter paper discs and the hot TCA-insoluble radioactivity was determined (22). The number of PLP residues incorporated by each IF3 molecule was calculated from the specific activity of the radioactive borohydride used, assuming 1 tritium atom/PLP incorporated. The stoichiometry of PLP incorporation determined in this way was found to be in good agreement with that determined spectrophotometrically (3).

**Protein Chemical Analyses**—Protein hydrolysis with *Staphylococcus aureus* protease (Miles), two-dimensional peptide fingerprinting, amino acid analysis, and manual amino acid sequencing were carried out as described previously (23, 24).

Radioactive fingerprints were subjected to fluorography as described (25). Identification of Arg, His, and Tyr residues on thin layer plates was performed by means of the colored reactions described by Yamada and Itano (26), Sanger and Tuppy (27), and Acher and Gere (28), respectively.

**Preparation of N¹-Pyridoxyl-lysine—N¹-Pyridoxyl-lysine was prepared essentially according to the method of Schnackez and Noltmann (29). Poly(lysine hydrobromide) (Miles) (2.5 mg) was treated with PLP (0.1 mmol) in 10 mM Na phosphate buffer, pH 7.5, for 15 min at 37 ºC. The reaction mixture was reduced for 30 min at 0 ºC by the addition of 1 mmol NaBH₄, followed by the addition of 1/50

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1 The abbreviations used are: PLP, pyridoxal 5'-phosphate; SP, *Staphylococcus aureus* protease; poly(A, U, G), polyadenylate, uridylylguanylate; RNase A, ribonuclease A (EC 3.1.27.5); RNase T₁, ribonuclease T₁ (EC 3.1.27.3); AUG, adenylyluridylylguanosine; IF, initiation factor.

2 A. Giovane, H. Oshawa, C. L. Pon, and C. Gualerzi, manuscript in preparation.
volume CH₃COOH and extensive dialysis against 2% CH₃COOH. The product showed a major absorption band at 325 nm, characteristic for the reduced Schiff base (29). After lyophilization, this was dissolved in a small amount of 0.1 N HCl and dried over NaOH. Hydrolysis was performed in 5.7 N HCl for 20 h at 110 °C in vacuo. The hydrolysate was dried over NaOH, dissolved in H₂O and applied as a streak onto a Cel 300 thin layer plate (Macherey-Nagel, Düren).

Electrophoresis was run for 2.5 h at 400 V in the same buffer used for fingerprinting. A single, fluorescent, ninhydrin-positive band, in addition to the band corresponding to unreacted lysine, was detected. The fluorescent band was scraped off and extracted twice with 50% CH₃COOH. The extract was lyophilized and dissolved in H₂O. The preparation thus obtained gave only one ninhydrin-positive and fluorescent spot after two-dimensional electrophoresis and chromatography.

RESULTS

Mechanism and Specificity of the Reaction—The scheme of the reaction of a protein with PLP is shown in Fig. 1. The ε-NH₂ group of lysines forms a Schiff base with the aldehyde group of pyridoxal phosphate; the Schiff base is then reduced by NaBH₄ to form ε-(5'-phosphopyridoxyl)lysine. This compound can be identified chromatographically from its fluorescence or from its radioactivity if tritiated NaBH₄ is used to reduce the Schiff base. In this study, we have primarily used the latter method to identify and quantify the reaction products of IF3 with PLP.

When E. coli initiation factor IF3 is reacted with PLP, the only product found following acid hydrolysis of the protein, electrophoresis, chromatography, and autoradiography is N⁵ pyridoxyl lysine (Fig. 2). When pyridoxal phosphate is substituted by other pyridoxal derivatives, negligible amounts of radioactivity are incorporated by IF3 (not shown), and, unlike with PLP, little or no inactivation of the factor takes place (Table I). It is clear from the table that both aldehyde and phosphate groups are necessary for the reaction and for the inactivation of IF3.

Identification of the Residues Modified by PLP—To determine which Lys residues of IF3 are modified by the reaction, purified factor was incubated with pyridoxal phosphate, reduced with tritiated NaBH₄, dialyzed, hydrolyzed with S. aureus protease, and subjected to two-dimensional peptide mapping. A fluorography of such a fingerprint showing four

![Fig. 2. Identification of the reaction product following PLP reaction of IF3. IF3 was modified by PLP following reduction by NaB\[^{14}H\]₄, as described under "Experimental Procedures." The reaction mixture was extensively dialyzed against 2% acetic acid and lyophilized. The sample (5 × 10⁶ cpm) was hydrolyzed with 5.7 N HCl for 20 h at 110 °C in vacuo, dried, and dissolved in a small amount of H₂O. The hydrolysate was applied onto a cellulose thin layer sheet (Cel 300) together with a N⁵ pyridoxyl-lysine standard prepared as described under "Experimental Procedures." Electrophoresis was for 2.5 h at 400 V, followed by ascending chromatography in 1-butanol: pyridine:acetic acid-water (5:5:1:4). The position of the standard N⁵ pyridoxyl-lysine was identified by its fluorescence. The plate was then subjected to fluorography as described (25). Complete overlap between pyridoxyl lysine fluorescence and radioactivity was obtained.

### Table I

| Compound | Structure | Activity lost |
|----------|-----------|--------------|
| 1. Pyridoxal 5'-phosphate | R₅ = CHO | R₅ = PO₂H₂ | 100 |
| 2. Pyridoxamine 5'-phosphate | R₅ = CH₂NH₂ | R₅ = PO₂H₂ | <1 |
| 3. Pyridoxol 5'-phosphate | R₅ = CH₂OH | R₅ = PO₂H₂ | <1 |
| 4. Pyridoxal | R₅ = CHO | R₅ = H | 13 |
| 5. Pyridoxamine | R₅ = CH₂NH₂ | R₅ = H | 3 |
| 6. Pyridoxol | R₅ = CH₂OH | R₅ = H | <1 |

![Fig. 1. Scheme of reaction of pyridoxal phosphate with lysine residues in proteins. In the absence of reduction with NaBH₄, the formation of the Schiff base is reversible (1). If NaB\[^{14}H\]₄ is used for reduction, the resulting ε-(5'-phosphopyridoxyl)lysines will be radioactive. Alternatively, identification of the reaction product can be based on fluorescence properties of pyridoxyl lysine (3). Since trypsin fails to hydrolyze next to pyridoxyl lysine (2), in the present paper peptide mapping for the identification of the modified peptides was performed following enzymatic hydrolysis of IF3 with S. aureus protease.](http://www.jbc.org/)
radioactive peptides is presented in Fig. 3A. The identification of these peptides was aided by the use of colored reactions to locate on the plate peptides containing specific amino acids (i.e., Arg, Tyr, His). The unequivocal identification of the peptides, however, was obtained, following their elution from the thin layer plates, by comparison of their amino acid composition and sequence with the known IF3 sequence (30). These results are summarized in Table II.

The identified peptides (SP1, SP11, SP7, SP16) correspond to a total of 5 lysine residues (Lys 2, Lys 5, Lys 99, Lys 112, and Lys 166) out of the 20 lysines present in the IF3 molecule (30). Of the above lysines, Lys 166 is modified to a lesser extent, which also varied somewhat from experiment to experiment. The picture shown in Fig. 3A can be regarded as a typical example of this kind of analysis, and the above-mentioned peptides are always among the main, and often the only targets of PLP modification. However, depending upon the particular batch of IF3 used, and perhaps upon slight, uncontrollable variations in the reaction conditions, another peptide was sometimes found modified. This peptide was identified to be SP9, which contains 5 lysines (Lys 86, 87, 90, 91, 96) of the central cluster of the IF3 molecule. Although no attempt was made to identify which of the above lysines are modified, it can be deduced from the stoichiometry of modification that, on average, no more than 1 lysine is modified per SP9 peptide. A fluorogram of a two-dimensional fingerprint in which SP9 was modified is shown in Fig. 3B.

**Inactivation of IF3 by PLP Reaction**—As mentioned above, the PLP modification of IF3 results in the loss of its biological activity. In the following experiment (Fig. 4A), the rate of inactivation of IF3 was measured at various PLP concentrations. It can be seen from the figure that the loss of IF3 activity proceeds more or less rapidly, depending upon the PLP concentration, and that the inactivation follows apparent first order kinetics at any given concentration of PLP, at least during the initial period of incubation. In Fig. 4B we present a log-log plot of the half-times of the inactivation (which are related to the apparent first order rate constants) versus the respective PLP concentrations. This plot yields a straight line with a slope of 0.9, thus indicating that the reaction is first order with respect to the PLP concentra-

**FIG. 3.** Fluorogram of a staphylococcal protease peptide map of PLP-modified IF3. In A, IF3 (350 μg) was incubated in 1 ml of TEA buffer with 0.6 mM PLP for 10 min at 37 °C and reduced at 0 °C by 5.5 mM NaBH₄. The reaction mixture was exhaustively dialyzed against 2% acetic acid, lyophilized, dissolved in 50 mM CH₂COONH₄, pH 4, containing 0.1 mM diithiothreitol, and digested with staphylococcal protease as described under "Experimental Procedures." The digest was lyophilized, dissolved in a small volume of H₂O, and subjected to two-dimensional peptide mapping as described under "Experimental Procedures." Fluorography of the fingerprint was performed as described (25). B, fluorogram of a hydrolysate of a different preparation of IF3 modified by PLP as described for A. In this case, SP9 was also among the modified peptides as described in the text.

**Table II**

| SP peptide identified | Position | Amino acid composition* | Sequence (from NH₂ terminus) |
|-----------------------|----------|-------------------------|-----------------------------|
| SP7                   | 107-121  | Asx, 1.3 (1); Ser, 1.1 (1); Glx, 2.8 (2); Gly, 1.4 (1); Val, 1.2 (1); Ile, 1.4 (1); Leu, 2.8 (3); Tyr, 0.64 (1); Phe, 0.97 (1); Lys, 0.28 (1); Arg, 2.00 (2) | Gly-Asp ... |
| SP16                  | 162-168  | Asx, 0.85 (0); Thr, 1.00 (1); Ser, 0.96 (1); Glx, 2.6 (1); Pro, 1.1 (1); Gly, 1.0 (0); Val, 0.48 (0); Ile, 1.8 (1); Leu, 1.6 (0); Tyr, 0.16 (0); Phe, 1.0 (1); Lys, 0.35 (1); Arg, 0.87 (0) | Gly-Phe ... (Ser) |
| SP11                  | 98-106   | Asx, 1.2 (1); Thr, 0.87 (1); Glx, 1.5 (1); Pro, 0.90 (1); Gly, 1.1 (1); Ala, 0.26 (0); Val, 0.28 (0); Ile, 0.83 (1); Leu, 0.49 (1); Phe, 0.73 (1); Lys, 0.29 (1); Arg, 1.00 (1) | He-Lys-Phe ... (Leu) |
| SP1                   | 1-18     | Asx, 1.3 (2); Thr, 0.59 (1); Glx, 3.2 (2); Pro, 0.62 (1); Gly, 2.0 (3); Ala, 1.0 (1); Val, 0.93 (1); Ile, 1.6 (1); Lys, 1.1 (2); Arg, 2.3 (3) | Lys-Gly-Gly-b |

*SP16 was contaminated with SP7, so that some amino acids of the latter (including the NH₂ terminus) were detected in SP16.

- According to Chang (34), when the NH₂-terminal amino acid is blocked (in this case, N-methylmethionine), the 2nd residue (Lys) appears as the 1st due to the splitting off of the blocked amino acid before the first cycle.

**FIG. 4.** PLP concentration dependence of the inactivation of IF3. In A, IF3 was incubated at 10 °C in TEA buffer with the concentration of PLP indicated above each line. At various times, aliquots containing 3.5 μg of IF3 were taken, mixed with 1/15 volume of 100 mM NaBH₄, and chilled at 0 °C. These aliquots were assayed for their biological activity as described under "Experimental Procedures." B, determination of the reaction order. The rate constants obtained as described for A were plotted versus the PLP concentration in a log-log plot. The reaction order was obtained from this plot as the slope, following the procedure of Levy et al. (35).
nucleic acids (both synthetic and natural). Fig. 8 summarizes these data. As seen from the figure, there is a substantial protection of IF3 activity in the presence of all nucleic acids used. However, some nucleic acids are more effective than others in protecting IF3. In particular, all guanosine-containing polynucleotides appear to be more effective than comparable amounts (by weight) of polynucleotides containing no guanosine. Among the natural nucleic acids, 16 S rRNA seems to protect IF3 slightly better than does double-stranded DNA.

It should be noted, however, that no differences in the capacity to protect IF3 from PLP inactivation were seen between 16 S and 23 S rRNA (see under "Discussion"). It is also noteworthy that, unlike poly(AUG), even high concentrations (0.34 mM) of the initiation triplet, ApUpG, were without effect on the PLP inactivation of IF3; GMP was also without effect (Table II). These results indicate that the protected site is not merely a phosphate or a nucleotide-binding site, but rather a nucleic acid-binding site.

The lack of specificity between 16 S and 23 S rRNA in protecting IF3 against PLP modification and inactivation is in contrast to the specific protection provided by 30 S ribosomal subunits but not by 50 S subunits. The following experiment presents a comparison of the inactivation of IF3 activity in the presence of either 30 S or 50 S ribosomal subunits as a function of PLP concentration (Fig. 9A) and as a function of the concentration of either ribosomal subunit (Fig. 9B). It can be seen from these figures that 30 S ribosomal subunits are able to provide a substantial protection of IF3 against inacti-

**Fig. 5.** Loss of ribosomal-binding capacity of PLP-modified IF3. [14C]methyl-IF3 (27 μg; 63,000 cpm/μg) prepared according to Gualerzi and Pon (20) were incubated for 10 min at 37 °C in 75 μl of TEA buffer containing 2.5 mM PLP and reduced in the presence of 6.25 mM NaBH₄. After dialysis against TEA-Mg buffer and concentration by "dialysis" against dry Sephadex G-150, the PLP-[14C]-methyl-IF3 was incubated with 2 A₂₆₀ units of 30 S ribosomal subunits in 200 μl of the same buffer containing 50 μg of bovine serum albumin. After 30 min of incubation at 37 °C, the sample was centrifuged for 2.5 h at 48,000 rpm in the SW 60 Ti rotor through a 10 to 30% (w/v) sucrose gradient containing the same buffer. A. control with [14C]-methyl-IF3 not modified with PLP; B. PLP-[14C]-methyl-IF3. The gradients were fractionated and the radioactivity (•) as well as the absorbance at 260 nm (○) of each fraction was determined.

**Protection Experiments**—To find out which of the modified lysine residues is responsible for the inactivation, we performed a number of "substrate" protection experiments which are presented below.

The first of these experiments (Fig. 6A) shows that, in the presence of the random polynucleotide poly(AUG), IF3 is strongly protected from PLP reaction. Thus, when IF3 is incubated with 1 mM PLP in the presence of poly(AUG), a negligible amount of PLP is incorporated per IF3 molecule and only a small fraction of the biological activity is lost. In the control experiment, in which IF3 was modified under the same conditions but in the absence of poly(AUG), approximately 3 molecules of PLP were incorporated per IF3 molecule with roughly 80% loss of the biological activity (Fig. 6B).

In addition to reacting with IF3, PLP could theoretically react with poly(AUG). Indeed, some evidence for PLP reaction with RNA has been reported (31). In light of this fact, an experiment was performed to check whether the protection of IF3 by poly(AUG) could stem from the reaction of PLP with the polynucleotide (Fig. 7). In this experiment, IF3 was incubated with PLP and poly(AUG) and reduced with NaBH₄, under conditions in which partial protection is obtained. The sample was then divided into two aliquots, one of which was treated with RNases A and T1. Both samples were then subjected to gel filtration on Sephadex G-25. As seen in the figure, in the sample not treated with RNases, both absorbance at 260 nm and radioactivity appear together in the void volume of the Sephadex column. After enzyme digestion, however, the radioactivity and the absorbance peaks became separated; the radioactive material was still eluted in the void volume of the column, while the A₂₆₀ absorbing material was retarded. The lack of radioactivity in the latter fractions clearly indicates that the PLP reaction occurred only with IF3 and not with poly(AUG) under experimental conditions where IF3 protection is observed.

To find out whether the protection of IF3 depends upon the type of nucleic acid used, the extent of protection was measured in the presence of various concentrations of different
vation. Thus, when equimolar amounts of 30 S ribosomal subunits and IF3 are present in the PLP reaction mixture (indicated by the arrow in Fig. 9B), over 60% protection of IF3 activity is obtained, and virtually complete protection is obtained when a 3-fold molar excess of 30 S over IF3 is used. That the protection depends upon the binding of IF3 to 30 S ribosomal subunits can be deduced from the fact that comparable amounts of 50 S ribosomal subunits produce only a minor protection of the IF3 activity and that the capacity of the 30 S subunits to protect is lost in the presence of 0.5 M NaCl, which prevents the ribosomal binding of IF3 (16).

Identification of the Protected Peptides—A comparison of the peptides modified by PLP in the presence or absence of IF3 binding to 30 S ribosomal subunits is presented in Fig. 10 as fluorograms of two staphylococcal protease fingerprints. Both samples, containing the same amount of IF3, were modified with PLP in the presence of 30 S ribosomal subunits, but only the IF3 of Fig. 10B was actually bound to the ribosomes, while the reaction mixtures of Fig. 10A contained 0.5 M NaCl, which prevented most of the interaction between the factor and the ribosomes. It is obvious from the figure that when IF3 is ribosome-bound, a much-reduced amount of PLP

![Diagram A](image1)

**FIG. 7. Lack of PLP reaction with poly(AUG).** IF3 (200 μg) was incubated for 10 min at 37 °C in 1.5 ml of TEA buffer containing 500 μg of poly(AUG) and 0.625 mM PLP. The sample was then reduced at 0 °C by the addition of 6.25 mM (final concentration) NaB[35]H4, dialyzed against 20 mM Tris-HCl, pH 7.7, containing 8 mM urea, and then dialyzed against the same buffer without urea. The bulk of this material was used for peptide fingerprinting (not shown), while two 60-μl aliquots were incubated for 2 h at 37 °C with or without 2 μg of RNase A and 0.4 μg of RNase T1 (Boehringer Mannheim). Both samples were then loaded on a Sephadex G-25 fine column (1 x 30 cm) equilibrated with 20 mM Tris-HCl, pH 7.7, containing 8 mM urea. Fractions of 1 ml each were collected. After the determination of the absorbance at 260 nm (.), 0.1 ml of each fraction was removed for [3H] radiolabel counting according to the addition of 1 ml of H2O and 10 ml of Bray's solution (○).

**Fig. 8. Specificity of polynucleotide protection of IF3 activity.** IF3 (4.3 μg) was incubated for 10 min at 37 °C in 30 μl of TEA buffer containing PLP (0.625 mM) and 2 μg of the various kinds of the polynucleotides or nucleic acids as indicated. After reduction with NaBH4 (6.25 mM), 5-μl aliquots of each reaction mixture were assayed for IF3 activity. All synthetic polynucleotides used, as well as calf thymus double-stranded DNA, were purchased from Boehringer Mannheim.

| Residual activity (%) | None | poly (A,U,G) | poly (U,G) | poly (C,G) | poly (G) | poly (A) | poly (U) | poly (C) | poly (A,U) | DNA | 16S RNA |
|-----------------------|------|--------------|------------|------------|----------|----------|----------|----------|------------|-----|---------|
| 0                     |      |              |            |            |          |          |          |          |            |     |         |
| 50                    |      |              |            |            |          |          |          |          |            |     |         |
| 100                   |      |              |            |            |          |          |          |          |            |     |         |

**TABLE III**
Comparative protection of IF3 activity from PLP modification by poly(AUG), AUG, and GMP

| Reaction conditions | Addition | Residual activity (AUG) | Residual activity (AUG) | Residual activity (%) |
|---------------------|----------|-------------------------|-------------------------|-----------------------|
| Complete PLP modification reaction | None | ≤1 |
| As above poly(AUG) (0.89 mM) | 96.6 |
| As above AUG (0.67 mM) | 2.5 |
| As above GMP (0.90 mM) | 8.5 |
| Omit PLP | None | 100 |

*Expressed as PI concentration.

**Fig. 9. Protection of IF3 from PLP inactivation by 30 S and 50 S ribosomal subunits as a function of PLP concentration (A) and as a function of subunit concentration (B).** In A, IF3 was incubated for 5 min at 37 °C with 4.5 A260 units of either 30 S ribosomal subunits (○), 50 S ribosomal subunits (●), or without ribosomes (△); PLP was added to the indicated final concentrations and incubation was then continued for 10 min at 37 °C, followed by reduction with NaBH4 (6.25 mM) at 0 °C. The final volume of each reaction mixture was 32 μl. To these, 96 μl of 8 M urea and 4 M LiCl were added. After 15 min incubation at 50 °C, 50 μl of each sample were assayed for IF3 activity. In B, the PLP reaction was essentially identical with that described for A, with the exceptions that the amounts of 30 S (○) or 50 S (●) in the preincubation were varied as indicated, and the PLP concentration in the reaction was 2.5 mM. In addition to the 30 S ribosomal subunit, one sample also contained 0.5 M NaCl (△). After reduction with NaBH4 (see above), the samples were treated with urea/LiCl, incubated, and tested for activity (see above). One hundred per cent activity was calculated by comparison with samples in which comparable amounts of an untreated IF3 were tested in the presence of the equivalent amount of ribosomal subunit.
Fig. 10. Identification of SP peptides of IF3 protected by 30 S ribosomal subunits from PLP modification. In A, IF3 (75 μg) was preincubated for 5 min at 37 °C with 73 A260 units of ribosomal subunit in a TEA-Mg buffer containing 0.5 M NaCl, then treated for 10 min at 37 °C with PLP (1.25 mM) followed by reduction with NaBH4 (1.25 mM). The total volume of the reaction mixture was 400 μl. In B, all conditions were identical with those described above, with the only exceptions being the absence of NaCl and a 2-fold scaling up of the entire reaction mixture. After reduction, the excess NaBH4 was consumed by adding PLP to a final concentration of 2.5 mM, and the samples were loaded onto sucrose gradients (10 to 30%, w/v) in TEA-Mg buffer and spun for 3 h at 48,000 rpm at 4 °C in an SW 60 Ti rotor. The combined top fractions of the gradients of sample A were lyophilized after extensive dialysis against 2% acetic acid. The 30 S peak fractions from the gradients of sample B were collected by centrifugation after precipitation by 0.7 volumes of ethanol and suspended in 1 ml of TEA-Mg buffer containing 0.4 M NH4Cl to dissociate IF3 from 30 S. These samples were then loaded onto a second gradient (10 to 30% w/v) in a TEA-Mg buffer containing 0.4 M NH4Cl and centrifuged as above. The top fractions from these gradients containing IF3 dissociated from 30 S were combined and lyophilized after extensive dialysis against 2% acetic acid. The IF3 sample thus obtained was further purified from some contaminating ribosomal proteins by gel filtration on a Sephadex G-150 superfine column equilibrated with 2% acetic acid containing 6 M urea. Purified IF3 was finally dialyzed against 2% acetic acid and lyophilized. *Staphylococcus* protease peptide fingerprinting and fluorography of these samples were performed as described under "Experimental Procedures."

is incorporated. In addition, not all peptides are equally protected from PLP modification; thus, while SP1 + SP9, SP7, and SP16 were strongly protected, the modification of SP11 was little affected by the binding of the factor to the ribosomes. To quantitate the protection of the individual peptides by 30 S ribosomal subunits, the radioactivity associated with the various peptides was determined. The results of this experiment are presented in Table IV.

An experiment similar to that described in the legend to Fig. 10 and Table IV was also carried out to quantitate the protection of individual SP peptides in the presence of poly(AUG). In this experiment, IF3 activity was protected about 50% (from 85 to 35% inactivation) when the modification was carried out in the presence of the polynucleotide. In this case, however, all peptides were found to be substantially protected. The highest protection was found with SP7 (64%) and SP11 (68%), although the latter was not affected by the presence of 30 S ribosomal subunits. SP1 and SP9 were also protected by poly(AUG) (38 and 36%, respectively), although proportionately less than by 30 S ribosomal subunits. SP16 was not heavily modified, even in the absence of poly(AUG), but its protection by the polynucleotide was 47%.

Taken together, one can conclude that the modification of SP11 does not affect the binding site of IF3, since the modification of this peptide, although prevented by the presence of poly(AUG), is hardly affected by the 30 S ribosomal subunits, and IF3 molecules with strongly modified SP11 still retain substantial activity. Furthermore, although we cannot exclude the possibility that the modification of peptides SP9 and SP16 leads to the inactivation of the factor, it seems unlikely that their modification is the major cause of the inactivation. In fact, although these peptides are protected by both 30 S and poly(AUG), SP16 is always found to be modified to a much lower and variable degree compared to the other peptides without any obvious correlation with the loss of activity, and in many, if not most of the cases, inactivation of IF3 occurs without any modification of SP9.

Thus, one can deduce from the protection experiments that the modification of SP7 or SP1 is the most likely reason for IF3 inactivation; both peptides are always heavily modified in free IF3 and strongly protected by poly(AUG) as well as by 30 S subunits to an extent roughly proportional to the activity protected.

**Table IV**

Quantitative determination of the extent of modification of individual SP peptides of IF3 bound and unbound to 30 S ribosomal subunits

The radioactive spots were cut out and the radioactivity was determined in a scintillation counter. The radioactivity remaining at or near the origin represented 46 and 33% of the total radioactivity applied for the unbound and bound IF3, respectively.

| Peptide | IF3 | Protection |
|---------|-----|------------|
| SP1 (Lys 2, 5) + SP 9 | 113,162 (cpm) 50,134 (cpm) | 56 |
| SP11 (Lys 99) | 33,368 (cpm) 27,322 (cpm) | 18 |
| SP7 (Lys 112) | 29,741 (cpm) 13,437 (cpm) | 55 |
| SP16 (Lys 166) | 13,783 (cpm) 8,062 (cpm) | 42 |
| SP? | 4,715 (cpm) 1,194 (cpm) | 75 |
| Residual activity of IF3 | 43.9 (%) 69.3 (%) | 45 |

Fig. 11. Comparison of the rate of the inactivation of "long" and "short" IF3 by PLP reaction. A short form of IF3 missing the first 6 amino acids was prepared and purified as will be described elsewhere. A, SDS-polyacrylamide (15%) gel electrophoresis of (1) native, (2) mixture of native and short, and (3) purified short IF3. In B, 10 μg of either native (○) or short (○) IF3 were incubated at 10 °C with 2.5 mM PLP in a final volume of 0.15 ml of the TEA buffer. At the indicated times, the samples were reduced with 6.25 mM NaBH4, and the activity of each sample was determined as described under "Experimental Procedures."
Modification of SP7 Is Responsible for IF3 Inactivation—
The PLP protection experiments indicated peptides SP1 (Lys 2 and 5) or SP7 (Lys 112) as the most likely candidates to play a role in IF3 inactivation. However, a "short" form of IF3 is known in which the first 6 amino acid residues from the NH₂ terminus are missing (32, 33). Since this shorter form of IF3 is somewhat active, it seems unlikely that Lys 2 and Lys 5 play an essential role in IF3 function, although their modification with the negatively charged PLP could indirectly lead to the inactivation of the factor. Thus, the interpretation of the PLP modification experiments, not only in terms of the location of the active site but, more importantly, in terms of the mechanism leading to the inactivation, would greatly differ depending upon which peptide (SP1 or SP7) was responsible for the inactivation. To solve this problem, a short form of IF3 lacking the first 6 amino acids was produced by mild digestion with proteolytic enzyme according to a procedure which will be described elsewhere. This short form was then purified (see Fig. 11A) and its PLP-dependent inactivation was determined and compared to that of the native (long) IF3. It can be seen from Fig. 11B that the short form of IF3 not only is inactivated by the reaction with PLP, but also that the rate of PLP inactivation of the short IF3 is nearly identical with the rate of inactivation of the native factor.

Thus, even in the absence of Lys 2 and 5, IF3 can be inactivated by PLP reaction. Since fingerprint analysis of the PLP-modified short form of IF3 revealed a pattern of radioactive PLP peptides essentially similar to that of native IF3 (except for the obvious absence of SP1 in the short IF3), we can conclude from the experiment of Fig. 11 that the modification of SP7 (Lys 112) is the major cause of the inactivation of IF3, although we cannot rule out that also the modification of SP1, when present in the intact IF3 molecule, may contribute to the inactivation.

DISCUSSION

In this paper, we have investigated the reactivity of initiation factor IF3 toward the site-specific lysine reagent pyridoxal phosphate. From the primary structure, it is known that this protein contains 20 lysine residues (30). Among these residues, Lys 2, 5, 99, 112, and 166 are found to be modified by PLP under conditions leading to the nearly complete loss of biological activity. The position of these lysines in the primary sequence of IF3 is schematically presented in Fig. 12, which also shows the position of all other Lys residues. Of the reactive lysines, Lys 166 is normally found to be modified to a lesser and more variable extent than the others. In addition, depending upon the reaction conditions and, more so, upon the IF3 preparation used, another unidentified lysine belonging to the central cluster of the molecule may be modified.

As a result of the PLP reaction, the IF3 activity is lost. The inactivation reaction is first order with respect to PLP. That 1 or more of the lysines modified by PLP are part of the IF3 active site involved in ribosomal binding can be deduced from various lines of evidence presented under "Results". It was thus shown that the binding of IF3 to the 30 S ribosomal subunits is impaired by PLP reaction, and that IF3 is protected from PLP modification and inactivation in the presence of various nucleic acids, both synthetic and natural, as well as by binding to 30 S ribosomal subunits. On the other hand, no protection was observed in the presence of 50 S ribosomal subunits with which IF3 interacts weakly and nonspecifically, or in the presence of an AUG triplet or GMP. The lack of protection by 50 S ribosomal subunits in contrast to the protection obtained with 23 S RNA and the equal protection obtained with 16 S and 23 S rRNAs are not surprising, since it has been shown that IF3 can bind almost equally well to both 16 S and 23 S rRNAs and that substantial binding to 50 S ribosomal subunits and spurious binding to 30 S ribosomal subunits can be obtained after stripping some proteins from these particles (12).

As seen under "Results," the phosphate group of PLP is necessary for IF3 inactivation, but the lack of protection by either AUG triplet or GMP, while providing evidence that the protection obtained with nucleic acids is not merely due to the presence of phosphate and/or guanines, suggests that the site protected is a rather complex nucleic acid-binding site. It should be noted, in this connection, that recent data could be interpreted to indicate in average a stretch of 14 nucleotides bound/IF3 molecule (36). In addition, the definite preference of IF3 for guanine-containing nucleotides seen in the polynucleotide protection experiments is consistent with the finding that the binding site of IF3 on the 30 S ribosomal subunit shows some properties of single-stranded, guanosine-containing RNA in being affected by either RNase T1 digestion or kethoxal modification (12, 16).

By comparison of the extent of IF3 inactivation and protection with the extent to which the individual lysine residues are modified by PLP and protected in the presence of 30 S ribosomal subunits or poly(AUG), it was concluded that modification of Lys 112 in peptide SP7 is the major cause of IF3 inactivation and that this residue is localized in the ribosomal-binding site of the factor. Concerning the other lysines, it seems unlikely that a role in binding to 30 S ribosomes is played by Lys 99, since this residue is hardly protected by the 30 S ribosomal subunits and its extensive modification does not interfere with IF3 activity. On the other hand, the participation of Lys 2 and 5 in the ribosomal-binding site of IF3 could be compatible with the extent to which these residues are modified by PLP and protected by 30 S and nucleic acids. However, if these 2 lysines play any role in the ribosomal binding of IF3, this must be a marginal one, since a short form of IF3 missing these two residues can still bind, albeit with lesser affinity, to the 30 S ribosomes (32, 33). In addition, the fact that the rate of PLP inactivation is nearly identical for the short and the native form of IF3 strongly suggests that the modification of Lys 2 and 5 is not the major cause of inactivation.

Finally, Lys 166 and one of the lysines in the central cluster of the molecule are also modified by PLP—the former, always, but to a variable extent; the latter, only with some IF3 preparations. Due to this irregularity in their behavior, their modifications cannot be regarded as the major cause of the observed IF3 inactivation. This does not exclude, however,
there is a tyrosine residue (Tyr 109) which displays essentially similar properties. Thus, a previous study showed that iodination of Tyr 109 inhibits the binding of IF3 to 30 S ribosomal subunits and that rRNAs and 30 S but not 50 S ribosomal subunits protect this tyrosine from enzymatic iodination (37). Also suggestive is the fact that the rate of chemical modification of Tyr 109 is affected by the presence of IF1 which, in turn, affects the ribosomal binding of IF3. Further evidence for the involvement of at least 1 tyrosine in IF3 binding comes from spectrofluorimetric studies. In addition to Tyr 109, other residues in the neighborhood of Lys 112 are likely to be important components of the IF3 active site. Among these, Arg 114 probably constitutes the primary recognition site for the phosphate of PLP (PLP is thought to bridge 2 amino acid residues by interacting via phosphate with one and forming a Schiff base with the amino group of the other). In addition, other possible important residues are Asp 105 and Glu 106 for their potential capacity to recognize guanines in single-stranded regions (38). Finally, since model oligopeptides of the type Lys-Tyr-Lys and Lys-X-Tyr-X-Lys were shown to bind preferentially to single-stranded nucleic acids (39), one can see how the region around peptide SP7 of the IF3 molecule has the potential information to select single-stranded RNA and to recognize guanine residues, two of the established properties of the ribosomal-binding site of IF3.

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