Structure–Function Relationship in *Escherichia coli* Initiation Factors: Role of Tyrosine Residues in Ribosomal Binding and Functional Activity of IF-3†

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ABSTRACT: When *Escherichia coli* initiation factor IF-3 was labeled in vitro with ^{125}I by lactoperoxidase or chloramine T, three to six iodines were incorporated by each IF-3 molecule. Following enzymatic digestion of the modified IF-3, fingerprinting, and sequence determination of the resulting peptides, three tyrosine residues (positions 71, 76, and 109) as well as His-139 were found modified to varying degrees to both mono- and diiodotyrosine as well as iodohistidine. The three tyrosine residues were not equally reactive. With lactoperoxidase at pH 5.7 and 7.0, the rate of modification was found to be Tyr-76 > Tyr-71 > Tyr-109 ≫ His-139. Furthermore, the iodination of Tyr-76 seemed to increase the iodination of Tyr-71 and the iodination of Tyr-71 and/or Tyr-76 seemed to enhance the rate of iodination of Tyr-109. When the iodination is completed, the IF-3 activity is lost by 90%, as determined by its capacity to promote the dissociation of ternary complexes of poly(U)–N-AcPhe-tRNA–30S ribosomal subunits. The capacity of IF-3 to bind to the 30S ribosomal subunit is also lost, albeit to a lesser extent. All three tyrosines of native unbound IF-3 were accessible to lactoperoxidase, suggesting their "exposure" to the solvent. Upon binding of IF-3 to the 30S ribosomal subunits, however, all tyrosines became protected from iodination and the IF-3 activity was preserved. Unlike 30S subunits, 50S subunits did not protect IF-3. Protection of IF-3 from iodination and inactivation was also provided with equal efficiency by 16S and 23S rRNA. Analysis of the peptides modified in the presence or absence of the rRNAs and comparison of the rates of modification of the individual tyrosines with the rate of IF-3 inactivation led to the conclusions that modification of Tyr-76 does not result in loss of biological activity, that modification of Tyr-71 does not impair the 30S binding of IF-3 but prevents the functional interaction of the factor with the ribosomal subunit, and that modification of Tyr-109 is accompanied by the loss of the ribosomal binding capacity of IF-3.

Initiation factor IF-3 binds to the 30S ribosomal subunit by interacting with the 16S rRNA (Gualerzi & Pon, 1973; Pon & Gualerzi, 1976; Pon et al., 1977). Since the primary sequence of IF-3 is known (Brauer & Wittmann-Liebold, 1977) and the identification of the region of the rRNA to which IF-3 binds is in progress, it would be desirable to obtain some information concerning the nature and the number of active sites of this important component of the protein synthetic machinery. In this paper we present experiments of selective enzymatic modification of tyrosine residues and substrate protection, which allow the identification of possibly two active sites in the IF-3 molecule. From these experiments, information concerning the environment of the tyrosine residues in the native IF-3 molecule can also be deduced.

Materials and Methods

Buffers. Buffer A, 10 mM Tris-HCl (pH 7.7), 10 mM $Mg(OAc)_2$, and 100 mM NH_4Cl; buffer B, 40 mM Tris–HOAc (pH 7.0) and 7 mM $Mg(OAc)_2$; buffer C, 200 mM *N*-methylmorpholine hydrochloride (pH 8.0); buffer D, 40 mM Tris–HOAc (pH 7.0), 1 mM $Mg(OAc)_2$, and 40 mM NH_4Cl; buffer E, 30 mM Tris-HCl (pH 7.3), 30 mM NH_4Cl, 15 mM $Mg(OAc)_2$, and 2 mM 2-mercaptoethanol.

General Preparations. Initiation factor IF-3 was purified to electrophoretic homogeneity (>95%) from *Escherichia coli* MRE 600 cells (Microbiological Research Establishment, Porton Down) as will be described elsewhere (C. Gualerzi and C. L. Pon, unpublished procedure). Unless otherwise specified, preparations of ribosomes and ribosomal subunits and activity tests of IF-3 were performed as previously described (Pon & Gualerzi, 1979). Preparation of radioactively methylated IF-3 by reductive alkylation was performed as previously described (Gualerzi & Pon, 1979). rRNAs were prepared from ribosomes by either the phenol method (Folkhard et al., 1975) or the urea–acetic acid method of Hochkeppel et al. (1976), as specified later.

The 30S–IF-3 complexes were separated by centrifugation on sucrose gradients (10–30% w/v) containing buffer A with 5 mM β-mercaptoethanol in an SW 60 Ti rotor for 3 h at 4 °C and 42 krpm. Similar gradients were run with the only exception that the gradients contained 350 mM NH_4Cl to separate IF-3 from 30S ribosomal subunits.

Iodination. Iodination was performed by a lactoperoxidase-catalyzed reaction essentially as described (Morrison & Bayse, 1970; Litman & Cantor, 1974). Lactoperoxidase was purchased from Sigma and purified according to Morrison & Hultquist (1963). Carrier-free $Na^{125}I$ was purchased from New England Nuclear. Two reactions, which were identical but for the addition of a minute amount of $Na^{125}I$ (20–50 μCi) to one of them, were normally carried out simultaneously at 22–24 °C. A typical reaction mixture for the enzymatic iodination contained 20 μg of IF-3 and 3 pmol of lactoperoxidase [calculated assuming the extinction coefficient given by Morrison & Bayse (1970)] in 0.315 mL of buffer B containing 6×10^{-5} M NaI and 6×10^{-5} M freshly diluted H_2O_2 to start the reaction. At the desired times, appropriate amounts of the reaction mixture were withdrawn and the reaction was stopped by addition of β-mercaptoethanol (~40 mM final concentration). The samples derived from iodination with nonradioactive NaI were used for activity tests, while the radioactively labeled samples were used to determine the extent of modification as well as to identify the modified peptides.

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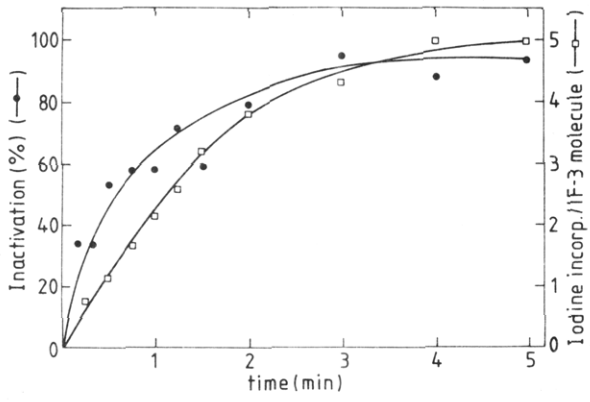

FIGURE 1: Time course of LPO-catalyzed iodination of IF-3. The iodination reaction was carried out as described under Materials and Methods and stopped at the indicated times. The extent of iodine incorporated per IF-3 molecule was determined after electrophoresis as described under Materials and Methods. The activity of the modified IF-3 was determined from its capacity to induce the dissociation of ternary complexes of 30S ribosomal subunits, poly(U), and radioactive N-AcPhe-tRNA as previously described (Pon & Gualerzi, 1979).

For determination of the extent of modification, 5 μg of carrier IF-3 was added to portions of the incubation mixtures, which were then subjected to polyacrylamide gel electrophoresis in $NaDodSO_4$[1] (Laemmli, 1970; Studier, 1973). The stained bands corresponding to IF-3 were cut out and solubilized with Soluene 350 (Packard), and their radioactivity was determined by scintillation counting. Alternatively, the radioactivity incorporated by a known amount of IF-3 was determined by cold Cl_3AcOH precipitation on Whatman 3MM filter paper disks (Kaji, 1968).

Protein Chemical Methods. Tryptic digestions and fingerprinting of the tryptic peptides on cellulose thin-layer sheets (Cel 400, Macherey-Nagel) were performed as described (Yaguchi et al., 1975). Extraction from TLC plates and manual sequencing of the peptides by the modified Edman degradation method were performed as previously described (Chang et al., 1978) but without the performic acid oxidation step of the peptides. Autoradiographies were performed by using Agfa Curix RP1 X-ray films.

Results

Chemical or Enzymatic Iodination of IF-3. Figure 1 presents the time course of LPO-catalyzed iodination of IF-3. It can be seen from the figure that, under the conditions of the experiment, the reaction is complete after 3–5 min of incubation. At this time approximately five iodine molecules have been incorporated into each molecule of IF-3 and ~90% of the biological activity of the factor has been lost. Control experiments showed that the simultaneous presence of both LPO and H_2O_2 in the reaction mixture was necessary for iodination and inactivation of the factor. Separate experiments carried out with denatured IF-3 (by 6 M urea or 2% $NaDodSO_4$) by using chloramine T as iodine-oxidizing agent (Hunter & Greenwood, 1962) yielded a similar amount of iodine incorporated per IF-3 molecule and similar radioactive peptide maps. These results indicated that all potential substrates are eventually reached by lactoperoxidase and must therefore occupy more or less "external" positions in the native IF-3 molecule.

[1] Abbreviations used: LPO, lactoperoxidase (EC 1.11.1.7); $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; TLC, thin-layer chromatography; poly(U), poly(uridylic acid); DABTH, 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiazolinone.

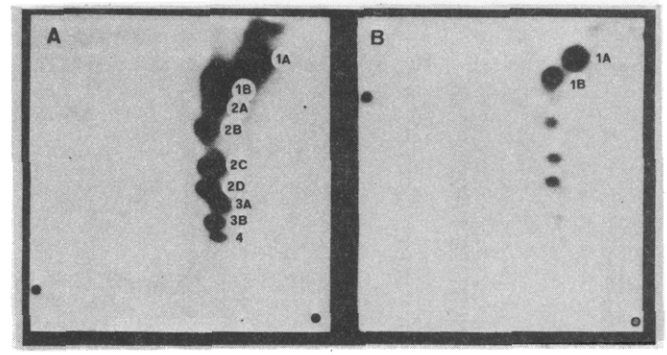

FIGURE 2: Autoradiography of two-dimensional peptide maps of iodinated IF-3. (A) 2 mg of purified IF-3 was iodinated in buffer A for 5 min at 24 °C, and the reaction was stopped as described under Materials and Methods. After extensive dialysis against 2% acetic acid and lyophilization, the sample was dissolved in 90 μL of 6 M urea and diluted with 800 μL of buffer C. Three portions of 10 μg of TPCK-trypsin (Worthington) were added at zero time, after 2 h, and after 7 h. Total incubation time with trypsin was 17 h at 37 °C. After trypsinization, the sample was lyophilized and dissolved in ~1 mL of 10% acetic acid. For analytical purposes a small aliquot of this solution (5–10 μL) was applied to a Cel 400 TLC plate and subjected to fingerprinting as described under Materials and Methods. The rest of the sample was processed as described in the legend to Table I. (B) In this experiment iodination was performed in buffer D essentially as described in (A), except for the addition of 16S rRNA in a 1.6:1 molar ratio to IF-3. After the reaction was stopped, the 16S rRNA was digested by incubation with 5 μg of RNase A (Boehringer, Mannheim) at 22 °C for 12 h. Trypsinization and peptide map analysis were performed as described above.

Table I: Identification of Iodinated Peptides of IF-3[a]

peptide	amino acid sequence determined	corresponding tryptic peptide	Tyr residue
1A	Phe-Leu-I_2Tyr...	T22	Tyr-76
1B	Phe-Leu-ITyr...	T22	Tyr-76
2A	Ile-Met(ox)-Asp-I_2Tyr...	T13	Tyr-71
2B	Ile-Met(ox)-Asp-ITyr...	T13	Tyr-71
2C	Ile-Met-Asp-I_2Tyr...	T13	Tyr-71
2D	Ile-Met-Asp-ITyr...	T13	Tyr-71
3A	Phe-Arg-Pro-Gly-Thr-Asp-Glu-Asn-I_2Tyr...	T17	Tyr-109
3B	Phe-Arg...	T17	Tyr-109
4	Glu-Met...	T4	

[a] The sample was prepared as described under the legend to Figure 2. Urea was removed from the tryptic hydrolysate by passage through a Sephadex G-10 column (1 × 15 cm) equilibrated with 10% acetic acid. After lyophilization and dissolving in deionized water, 10 μL of the hydrolysate corresponding to ~150 μg of the original protein was subjected to peptide mapping as described under Methods. Ninhydrin staining of the plates, autoradiography, extraction of the peptides, and peptide sequencing were performed as described (see Materials and Methods). DABTH derivatives of monoiodo- and diiodotyrosine were identified by comparison to DABTH derivatives prepared from standard mono- and diiodotyrosine (Sigma).

Identification of the Iodinated Amino Acid Residues. When ^{125}I-iodinated IF-3 is digested with trypsin and subjected to two-dimensional peptide mapping and autoradiography, the picture of Figure 2A showing nine discrete radioactive spots is obtained. For identification of the modified peptides, the material corresponding to each radioactive spot was extracted and subjected to manual amino acid sequencing, which also allowed the discrimination between monoiodo- and diiodotyrosine. The results of these analyses are shown in Table I. As seen in the table, eight out of the nine radioactive peptides correspond to the three tyrosine residues of IF-3, modified to either the monoiodo or diiodo form. Thus, Tyr-76 is found

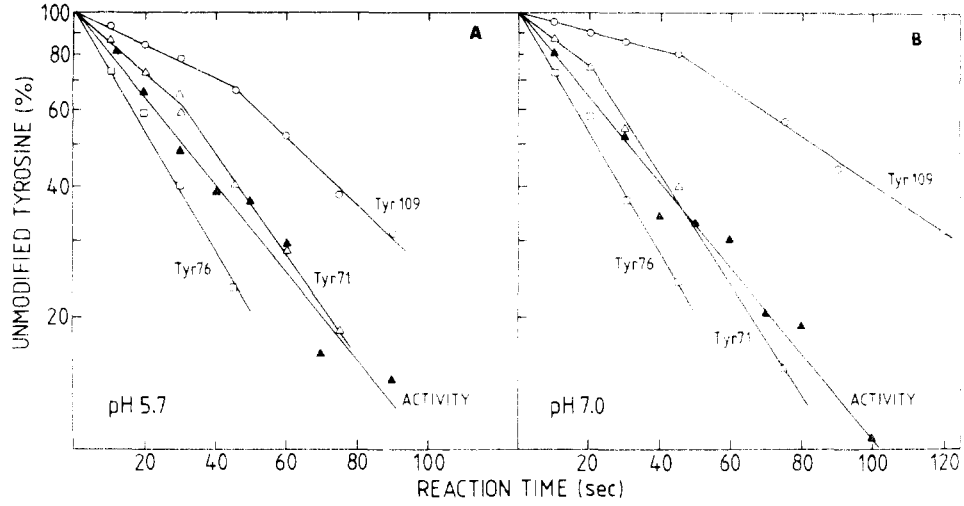

FIGURE 3: Rate of modification of individual Tyr residues by LPO-catalyzed iodination at pH 5.7 and 7.0. Iodination was carried out essentially as described under Materials and Methods at pH 5.7 (A) or pH 7.0 (B). The IF-3 samples modified with nonradioactive NaI were used to determine the residual biological activity (see Materials and Methods). The IF-3 samples modified with $Na^{125}I$ were divided into two portions. The first portion, corresponding to 0.1 of each sample, was used to determine in duplicate the total amount of iodine incorporated per IF-3 molecule by Cl_3AcOH precipitation (see Materials and Methods). 25 μg of carrier IF-3 and 150 μg of bovine serum albumin were added to the remaining 0.9 of each sample. After dialysis against 2% acetic acid, the samples were lyophilized, dissolved in buffer C, and subjected to trypsin digestion. The iodinated peptides were located by autoradiography and cut out of the TLC plates, and their radioactivity was determined by liquid scintillation counting. The counts of each peptide were normalized by reference to the total amount of counts recovered in each plate and to the total amount of iodine incorporated by IF-3 for each incubation time. The normalized counts corresponding to the same tyrosine residues were summed after making an appropriate correction for the twofold higher specific activity of the peptides containing diiodotyrosine. From these total counts the percent modification of each tyrosine was calculated by reference to samples in which the modification of each tyrosine residue was complete. The entire experiment was done in duplicate, and two to three plates were obtained for each modification time. The variations between the values determined for each tyrosine residue from different plates of the same sample were less than 15%.

in peptides 1A and 1B and Tyr-109 in peptides 3A and 3B. Tyr-71 is present in four different peptides since, in addition to being modified to monoiodo- (2B and 2D) and diiodotyrosine (2A and 2C), it is in a peptide containing either methionine (2C and 2D) or methionine sulfoxide (2A and 2B). The ninth peptide (4) was found to correspond to an iodinated form of His-139.

Rate of Modification of the Individual Tyrosine Residues. The kinetics of modification of the individual tyrosine residues of IF-3 by LPO-catalyzed iodination at pH 5.7 (Figure 3A) and pH 7.0 (Figure 3B) were determined in the following experiment. It can be seen from the figure that at both pH values the three tyrosine residues do not react at the same rate; Tyr-76 is the most reactive, and its modification seems to follow apparent first-order kinetics up to ~70% modification. The modifications of Tyr-71 and Tyr-109, on the other hand, do not yield straight lines in the semilog plots, but their modifications seem to proceed through an initial slow phase, followed by a second phase during which the rates of modification become considerably enhanced. Overall, Tyr-71 is modified faster than Tyr-109. The results of parts A and B of Figure 3 could be interpreted to mean that initially all three tyrosines of IF-3 can be reached by the enzyme, but their accessibility and/or reactivity as determined by their microenvironment or freedom of rotation are quite different. The iodination of Tyr-76 could then bring about a modification of these parameters (conformational change?) resulting in an increased rate of modification of Tyr-71. The modification of the latter and/or of Tyr-76 could in turn bring about changes resulting in the increased rate of Tyr-109 modification. In the experiment of Figure 3 the modification of His-139 was not taken into account, since its modification is quantitatively negligible and occurs much later compared to the modification of the tyrosine residues. Figure 3 also shows the loss of IF-3 activity during iodination. In spite of some scattering of the experimental points, it is clear from these data that the inactivation of IF-3 follows apparent first-order kinetics and that the rate at which Tyr-76 becomes modified is faster than the rate at which the factor becomes inactivated. On the other hand, since the inactivation proceeds faster than the rate of modification of either Tyr-71 or Tyr-109, one could hypothesize that the modification of either one of these two residues could lead to the inactivation of the factor.

Table II: Protection of Individual Tyr Residues of IF-3 by rRNAs

additions	% modification[b] of Tyr-71	Tyr-76	Tyr-109	residual act. (%)
none	67	95	27	35
16S or 23S RNA	30 (54)[a]	67 (28)	3 (89)	75 (62)

[a] The values in parentheses represent the percent protection from modification or inactivation. The experiment was carried out as described in the legend to Figure 2B with the exception that urea-acetic acid extracted rRNAs (see Materials and Methods) were used. The rRNA/IF-3 molar ratios were 2.3:1 for 16S rRNA and 1.5:1 for 23S rRNA. [b] The percent modification of each Tyr residue was calculated as described in the legend to Figure 3.

Protection of Tyrosine Residues by Ribosomal Subunits and rRNA. When the LPO-catalyzed iodination of IF-3 is carried out in the presence of either 16S rRNA or 23S rRNA, a substantial reduction of the total amount of iodine incorporated per IF-3 molecule is observed, at least within the first period of incubation. Peptide analysis and autoradiography of IF-3 radioactively iodinated under these conditions revealed that the protection concerned primarily Tyr-71 and Tyr-109 (Figure 2B). Since this experiment was done using rRNAs prepared by conventional phenol extraction (Folkhard et al., 1975), we considered the possibility that the protection was due to traces of phenol remaining in the rRNA preparations. The experiment was therefore repeated with rRNAs extracted with urea–acetic acid (Hochkeppel et al., 1976) and yielded basically similar results (Table II). As seen in the table, after 4 min of iodination in the presence of either 16S or 23S rRNA, the activity of IF-3 was lost by ~25% while the control sam-

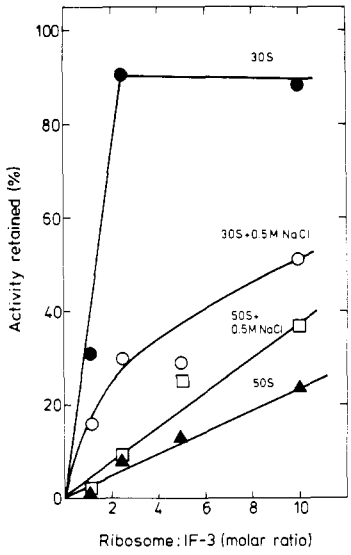

FIGURE 4: Protection of IF-3 from inactivation in the presence of 30S or 50S ribosomal subunits. Several reaction mixtures were prepared, each containing 3.5 μg of IF-3 and varying amounts of 30S or 50S ribosomal subunits, to yield the indicated ribosome/IF-3 ratios. The ribosome concentration in the reaction mixtures was kept constant at 2.4×10^{-6} and 1.2×10^{-6} M for the 30S and 50S ribosomal subunits, respectively. The iodination was carried out in buffer B with 0.25 mM NaI, 5×10^{-8} M LPO, 0.25 mM H_2O_2, and, wherever indicated, 0.5 M NaCl. After 1, 2, and 4 min of incubation at 24 °C, samples corresponding to 0.8 μg of IF-3 were removed from the reaction mixture and the reaction was stopped by addition of β-mercaptoethanol (final concentration 30 mM) and NH_4Cl (final concentration 0.5 M). The samples (400 μL each) were loaded onto sucrose gradients and centrifuged to separate IF-3 from the ribosomal subunits (see Materials and Methods). The upper 1.5 mL of each gradient was used for the activity test of IF-3.

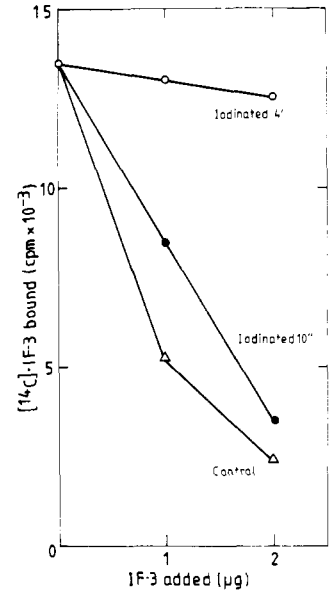

FIGURE 5: Competition between iodinated and native IF-3 for binding to 30S subunits. Iodination was performed as described under Materials and Methods. The incubation mixture for each competition experiment contained 0.8 μg of [^{14}C]methyl-IF-3 (see Materials and Methods) and the indicated amounts of either native (Δ), 10-s iodinated (●), or 4-min iodinated (○) IF-3 in 300 μL of buffer A. 30S ribosomal subunits (0.25 A_{260} unit) were then added to each tube, and the samples were incubated for 15 min at 37 °C. The amount of [^{14}C]methyl-IF-3 bound to 30S ribosomal subunits was determined following sucrose gradient centrifugation (see Materials and Methods).

ples incubated in the absence of rRNA had lost ~65% of their activity. This protection from inactivation was accompanied by a reduction of the modification of the tyrosine residues. However, while modification of Tyr-76 was found to be reduced by less than 30%, Tyr-71 was protected over 50%, and nearly complete protection of Tyr-109 was observed. These results indicate that, in the presence of rRNA, Tyr-71 and Tyr-109 become less accessible to the enzyme and/or less reactive, while Tyr-76 is hardly affected by the presence of the rRNA. Noteworthy is also the lack of specificity in the protection provided by 16S and 23S rRNA, since identical amounts (by weight) of either rRNA produce a substantially similar degree of protection from iodination and inactivation. Finally, the substantial protection of the biological activity without a corresponding protection of Tyr-76 further supports the conclusion that the iodination of this residue is not responsible for the IF-3 inactivation. Separate experiments showed that the extent of IF-3 protection by rRNAs varies with the ionic concentration of the incubation mixture; the protection is higher in the presence of 1 mM Mg^{2+} and 40 mM NH_4Cl than in the absence of these ions.

More selective protection of IF-3 against inactivation is observed by binding IF-3 to ribosomal subunits. Thus, in the presence of 30S ribosomal subunits, IF-3 activity is protected over 90% while little protection is observed in the presence of 50S ribosomal subunits (Figure 4). In the presence of 0.5 M NaCl, which is known to inhibit the ribosomal binding of IF-3 (Pon & Gualerzi, 1976), protection by 30S ribosomal subunits is substantially reduced while nonspecific protection by 50S subunits is somewhat increased (Figure 4).

A separate experiment showed that upon binding of the factor to the 30S ribosomal subunit all tyrosine residues of IF-3 are shielded from LPO-catalyzed iodination (not shown).

Relationship between IF-3 Iodination and IF-3 Activity. The expression of the biological activity of IF-3 presumably requires two different steps: the binding of the factor to its site on the 30S ribosomal subunit, and a specific molecular interaction between the bound factor and its neighboring molecules on the ribosome. These two steps can be separated following IF-3 iodination. Thus, it will be shown in the following experiments that (a) although the affinity of iodinated IF-3 for the 30S ribosomal subunit decreases as the iodination reaction proceeds, the ribosomal binding of the factor is still possible even after extensive iodination and (b) iodinated IF-3 can bind to the ribosomes without expression of biological activity. As seen in Figure 5, iodinated IF-3 displays a reduced capacity to compete with noniodinated IF-3 for ribosomal binding. This is much more pronounced in the case of IF-3 molecules iodinated for a longer time (4 min) than for a shorter time (10 s). The same phenomenon accounts for the result shown in Figure 6. In this experiment the ratio between the 30S-bound [^{125}I]IF-3 counts and the total [^{125}I]IF-3 counts is plotted against the time of the iodination reaction. It can be seen in the figure that this ratio is low at the beginning of the reaction, when a large proportion of yet unmodified IF-3 molecules compete with the labeled IF-3 in binding, but markedly increases between 10 and 30 s. The subsequent slow decrease in the ratio reflects a net loss of the 30S binding capacity of iodinated IF-3. The ability, though somewhat decreased, of iodinated IF-3 to compete with the native factor for the ribosomal binding site (Figure 5) and the ability of the native factor to chase iodinated IF-3 from its ribosomal binding site (Figure 6 and experiments not shown) indicate, though do not prove, that the ribosomal binding site of iodinated IF-3 is the same or at least partially overlaps the physiological one. Furthermore, when complexes between 30S ribosomal subunits and IF-3 iodinated for various times were isolated from sucrose gradients and their capacity to express IF-3 activity was de-

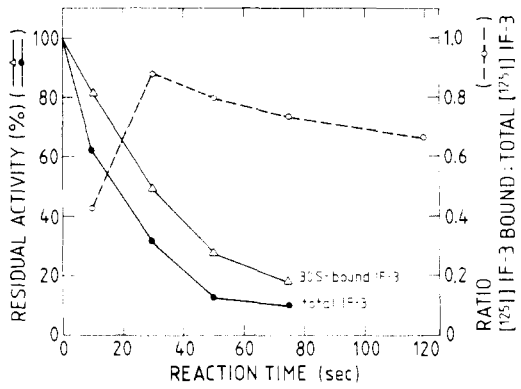

FIGURE 6: Differential effect of iodination on the capacity of IF-3 to bind to 30S ribosomal subunits and to express biological activity. Two 150-μg portions of IF-3 were iodinated as described under Materials and Methods by using either radioactive NaI (mixture A) or nonradioactive NaI (mixture B). At the indicated times aliquots of both incubation mixtures (A and B) corresponding to 22 μg of IF-3 were withdrawn and the reaction was stopped as described under Materials and Methods. Each sample was then divided into two portions containing 4 and 18 μg of IF-3, respectively. 100 μg of carrier IF-3 was added to the minor portions of the samples containing radioactively labeled IF-3 (A), and these samples were used for peptide map analysis (see Table III) as well as for the determination of radioactive iodine incorporated per IF-3 molecule (see Materials and Methods). The residual biological activity of IF-3 was determined in duplicate (●) by using the minor portion of the samples derived from mixture B (see Materials and Methods). 14 A_{260} units of 30S ribosomal subunits were added to the main portion of each sample, and after 15 min of incubation at 37 °C the 30S–IF-3 complexes were isolated on sucrose gradients (see Materials and Methods). After determination of the amount of radioactive IF-3 associated with the 30S ribosomal subunits, the bulk of 30S–[^{125}I]IF-3 complex isolated from the sucrose gradients was used to analyze the distribution of iodine among the various IF-3 peptides following trypsin digestion (see Table III). The figure shows the normalized ratio between the counts of IF-3 bound to 30S subunits and the total IF-3 radioactivity present in each binding experiment as a function of the iodination time (○). The capacity of ribosome-bound IF-3 to express its biological activity was assessed by an equilibrium perturbation experiment similar to the one described (Pon & Gualerzi, 1974). Poly(U) (110 μg) and N-Ac[^{3}H]Phe-tRNA (260 pmol) were added to portions of the pooled fractions from the sucrose gradient, each containing 4.5 A_{260} units of 30S–IF-3 complex. The mixtures were incubated for 20 min at 37 °C until equilibrium in the formation of the ternary complexes was attained, and portions of this mixture, each corresponding to 0.5 A_{260} unit of 30S–IF-3 complex were diluted 10- to 100-fold with buffer E. The ability of 30S-bound IF-3 to express its biological activity was then determined from the extent to which the complexes were able to attain a new equilibrium within 5 min of incubation after dilution (Pon & Gualerzi, 1974, 1979) (Δ).

termined, it was found that this capacity is rapidly lost as the extent of iodination is increased, in spite of the fact that the amount of IF-3 present in these complexes remains substantially constant. This proves that a large amount of iodinated IF-3 can be found in association with the 30S ribosomal subunit in an apparently inactive form.

The following experiment was designed to identify which modification of the IF-3 molecule is still compatible with the ribosomal binding of the factor but not with the expression of the biological activity. Thus, radioactively iodinated IF-3 was incubated with 30S ribosomal subunits and the 30S-bound iodinated IF-3 was separated from free IF-3 by sucrose gradient centrifugation. The distribution of the radioactivity among the various peptides in the 30S-bound factor was then determined by peptide analysis and compared to the distribution of radioactivity in the unfractionated iodinated IF-3. From this experiment one can deduce the extent to which the individual peptides are modified in the two types of IF-3. The results of this experiment (Table III) show that the distribution

Table III: Influence of Modification of Different Tyr Residues on the 30S Ribosome Binding Capacity of IF-3[a]

	extent (%) of Tyr modification after			
	10-s reaction		4-min reaction	
residue	unfractionated IF-3	30S-bound IF-3	unfractionated IF-3	30S-bound IF-3
Tyr-71	7.0	2.6	89.0	77.1
Tyr-76	12.2	11.8	87.5	96.0
Tyr-109	2.0	0.3	31.7	6.9

[a] This experiment is essentially described in the legend to Figure 6. Radioactively iodinated IF-3 (mixture A) was incubated with 30S ribosomal subunits, and the 30S-bound factor was separated from the unbound factor by sucrose gradient centrifugation. Peptide maps of the unfractionated, 30S-bound and unbound IF-3 were then obtained, and the extent of modification of Tyr residue was determined as described in the legend to Figure 3. The table shows the data concerning unfractionated and 30S-bound IF-3 for the shortest and the longest iodination times. The data concerning the extent of modification in the unbound IF-3 are not included in the table because, due to the low yields, they were not as reliable as the other data from the quantitative point of view. Qualitatively, however, iodinated IF-3 isolated from the top of the sucrose gradient (i.e., iodinated IF-3 with strongly reduced or no affinity for the 30S ribosomal subunits) showed a proportionally higher modification of Tyr-109, thus lending further support to the data shown in the table.

of the radioactivity among the three tyrosine residues is distinctly different between the unfractionated and the ribosome-bound IF-3. Thus, after 10 s of incubation in the presence of a substantial amount of yet unreacted IF-3, the 30S ribosomal subunits seem to have selected those IF-3 molecules with reduced Tyr-71 modification and with a negligible modification in Tyr-109 (compared to unfractionated IF-3). After 4 min of incubation, when nonmodified IF-3 molecules presumably no longer exist, the 30S ribosomal subunits can still bind (though in an inactive form, as seen in Figure 6) IF-3 molecules with substantially modified Tyr-76 and Tyr-71 but clearly discriminate against those molecules bearing a modified Tyr-109. From these data it can be concluded that the modification of Tyr-71 produces IF-3 molecules still able, though with decreased affinity, to bind to the 30S particles but unable to express their biological activity. The modification of Tyr-109, on the other hand, lowers even further the affinity of IF-3 for the 30S ribosomal subunits, thus producing molecules substantially unable to bind to ribosomes and to express biological activity. Finally, the above data provide additional evidence that modification of Tyr-76 has a negligible effect on the biological activity of the factor.

Discussion

The results presented in this paper allow some conclusions to be drawn concerning the environment of the three tyrosine residues in the native IF-3 molecule. When IF-3 is bound to the 30S ribosomal subunit, none of these tyrosines can be reached by the enzyme lactoperoxidase (M_r 78 000) and neither iodination of the tyrosines nor inactivation of the IF-3 function takes place. By contrast, all three tyrosines are accessible to the enzyme when IF-3 is free in solution, implying the exposure of these tyrosines to the aqueous phase. Initially, the rate at which the tyrosines react is distinctly different for the three residues (Tyr-76 > Tyr-71 > Tyr-109) at all pH values tested. This behavior possibly reflects small differences in the local pK values and/or in the rigidity of the steric orientation of the planar phenolate rings of the tyrosines. However, as the reaction proceeds, the rates of modification of both Tyr-71 and Tyr-109 increase to become similar to that

of Tyr-76. This is probably due to the change of one or both of the above-mentioned parameters brought about by the iodination of Tyr-76. That the microenvironment of the three tyrosine residues is indeed slightly different is shown by the fact that the transition from monoiodo- to diiodotyrosine, which is faster in a more hydrophobic environment (Mayberry & Hockert, 1970), occurs more readily for Tyr-109 than for Tyr-71 and -76 (not shown).

The results of this paper also demonstrate the participation of one of the tyrosines (Tyr-109) in the formation of the 30S binding site of IF-3. It is noteworthy that Tyr-109, in addition to being protected from iodination by the 30S ribosomal subunits, is also protected by rRNA (either 16S or 23S rRNA) as well as by synthetic polynucleotides (not shown).

Tyr-109 is located two residues away from a lysine residue (Lys-112), which shows affinity for negatively charged phosphate groups, is protected by both 30S ribosomal subunits and rRNA, and was found to be essential for the ribosomal binding of IF-3 (Ohsawa and Gualerzi, unpublished experiments). It is known that IF-3 is endowed with nucleic acid binding capacity (Sabol et al., 1970; Gualerzi & Pon, 1973), and several observations suggest that it can bind to both single-stranded and double-stranded RNA as well as to DNA, tRNA, and polynucleotides (Sabol et al., 1970; Gualerzi & Pon, 1973; Kaempfer & Kaufmann, 1973; Pon & Gualerzi, 1976). Among these nucleic acids, IF-3 shows a marked preference for guanine-containing single-stranded RNA or polyribonucleotides (Wickstrom, 1974; Jay et al., 1974), and the IF-3 binding site on the 30S ribosomal subunit shows some properties of single-stranded, guanine-containing RNA in being sensitive to RNase T1 and to kethoxal treatment (Gualerzi & Pon, 1973; Pon & Gualerzi, 1976).

Using model oligopeptides of the type Lys-Tyr-Lys, Helene and co-workers (Helene, 1977; Mayer et al., 1979) were able to demonstrate a selective recognition of single-stranded vs. double-stranded regions of nucleic acids by these model compounds. Furthermore, Helene and collaborators proposed the specific recognition of guanines in single-stranded nucleic acids by glutamic and aspartic acid residues and of guanines in double-stranded regions by asparagine and glutamine. Indeed, an aspartic acid and a glutamic acid residue (Asp-105[2] and Glu-106) are present in the IF-3 molecule very close to Lys-112 and Tyr-109 along with a number of other amino acid residues (i.e., Asn-108 and Arg-114) whose potential importance in establishing hydrogen-bonding interactions with nucleic acids has long been recognized (Seemann et al., 1976). In conclusion, the RNA binding region of IF-3, which in turn represents the ribosome binding site of IF-3, is probably localized in this region of the molecule and contains both Tyr-109 and Lys-112 and at least the potential information to select single-stranded RNA and to recognize guanine residues in both single- and double-stranded regions of the RNA. In addition, the RNA binding region of IF-3 probably extends toward the center of the molecule to include one or more of the lysine residues which are clustered between residues 78 and 91. An α-helical secondary structure is predicted for this region of the molecule (Ohsawa and Gualerzi, unpublished experiments).

Very little can be said at the moment concerning the interaction between Tyr-109 and the ribosome. Unpublished results show that not only iodination but also nitration of Tyr-109 prevents the binding of IF-3 to the ribosome. Nitration and iodination produce two types of effects, which could both account for the loss of the biological activity. These are (a) a drastic reduction of the pK of the phenolate residue and (b) a substantial thickening of the phenol ring. Preliminary experiments in which the normal pK of the phenolate was restored by reduction of nitrotyrosine to aminotyrosine indicated that the modification of the phenolate pK is not the cause for the lowering of the binding, leaving the second proposed explanation (i.e., the thickening of the phenolate ring) as the most likely reason for the inhibition.

The modification of Tyr-71 was shown to produce IF-3 molecules still able to bind to the 30S ribosomal subunit but unable to express the biological activity. Previous experiments have shown the possibility of forming 30S–IF-3 complexes with reduced biological activity below 4 °C (Gualerzi et al., 1979). It is impossible at present to decide whether any analogy exists at the molecular level between these two situations, but both findings seem to indicate that the expression of the biological activity of IF-3 is the result of two separate processes: the direct binding of IF-3 to the ribosome and a molecular interaction between the bound factor and one or more ribosomal components. In the absence of any knowledge concerning the tertiary structure of IF-3, it is not known whether Tyr-71 occupies a position close to Tyr-109. If this is not the case, the results concerning the effect of Tyr-71 modification imply that IF-3 contains two separate sites for the interaction with the ribosomal subunits: one directly responsible for binding and the other implicated in the functional interaction with the subunit.

Acknowledgments

We express our gratitude to Professor H. G. Wittmann for his constant interest and encouragement and to Dr. C. L. Pon for her decisive help in peptide sequencing, as well as for the many valuable discussions.

References

Brauer, D., & Wittmann-Liebold, B. (1977) *FEBS Lett. 79*, 269–275.

Chang, Y. J., Brauer, D., & Wittmann-Liebold, B. (1978) *FEBS Lett. 93*, 205–214.

Folkhard, W., Pilz, I., Kratky, O., Garrett, R., & Stöffler, G. (1975) *Eur. J. Biochem. 59*, 63–71.

Gualerzi, C., & Pon, C. L. (1973) *Biochem. Biophys. Res. Commun. 52*, 792–799.

Gualerzi, C., & Pon, C. L. (1979) *Methods Enzymol. 59*, 783–795.

Gualerzi, C., Risuleo, G., & Pon, C. L. (1979) *J. Biol. Chem. 254*, 44–49.

Helene, C. (1977) *FEBS Lett. 74*, 10–13.

Hochkeppel, H.-K., Spicer, E., & Craven, G. R. (1976) *J. Mol. Biol. 101*, 155–170.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Jay, G., Abrams, W. R., & Kaempfer, R. (1974) *Biochem. Biophys. Res. Commun. 60*, 1357–1364.

Kaempfer, R., & Kaufmann, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1222–1226.

Kaji, A. (1968) *Methods Enzymol. 12*, 692–699.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Litman, D. J., & Cantor, C. R. (1974) *Biochemistry 13*, 512–518.

Mayberry, W. E., & Hockert, T. J. (1970) *J. Biol. Chem. 245*, 697–700.

Mayer, R., Toulme, F., Montenay-Garestier, T., & Helene, C. (1979) *J. Biol. Chem. 254*, 75–82.

[2] Our sequence data of peptide T17 indicate an Asp residue in position 105 instead of Asn as reported by Brauer & Wittmann-Liebold (1977).

Morrison, M., & Hultquist, D. E. (1963) *J. Biol. Chem. 238*, 2847–2849.
Morrison, M., & Bayse, S. B. (1970) *Biochemistry 9*, 2995–3000.
Pon, C. L., & Gualerzi, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4950–4954.
Pon, C. L., & Gualerzi, C. (1976) *Biochemistry 15*, 804–811.
Pon, C. L., & Gualerzi, C. (1979) *Methods Enzymol. 60*, 230–239.
Pon, C. L., Brimacombe, R., & Gualerzi, C. (1977) *Biochemistry 16*, 5681–5686.
Pon, C. L., Wittmann-Liebold, B., & Gualerzi, C. (1979) *FEBS Lett. 101*, 157–160.
Sabol, S., Sillero, M. A. G., Iwasaki, K., & Ochoa, S. (1970) *Nature (London) 228*, 1269–1273.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804–808.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
Wickstrom, E. (1974) *Biochim. Biophys. Acta 349*, 125–130.
Yaguchi, M., Wittmann, H. G., Câbezon, T., De Wilde, M., Villarroel, R., Herzog, A., & Bollen, A. (1975) *Mol. Gen. Genet. 142*, 35–43.

A Novel Enzymatic Activity of Phenylalanyl Transfer Ribonucleic Acid Synthetase from Baker's Yeast: Zinc Ion Induced Transfer Ribonucleic Acid Independent Hydrolysis of Adenosine Triphosphate†

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ABSTRACT: Phenylalanyl-tRNA synthetase from baker's yeast in the presence of phenylalanine or other amino acids misactivated by the enzyme, ATP, and low concentrations of Zn^{2+} is able to hydrolyze ATP to AMP and PP_i very efficiently. After dialysis of the enzyme against ethylenediaminetetraacetic acid (EDTA), this amino acid dependent but $tRNA^{Phe}$-independent hydrolysis is suppressed to negligible levels. The ATP hydrolysis can be restored by the addition of Zn^{2+} to the EDTA-dialyzed enzyme. During aminoacylation of $tRNA^{Phe}$ the Zn^{2+}-induced ATP hydrolysis parallels the aminoacylation reaction, leading to nonstoichiometric production of AMP. Mechanistically, we conclude that Zn^{2+} can be bound to phenylalanyl-tRNA synthetase and can influence the stability of ATP if an activatable amino acid is present. The influence of Zn^{2+}, if any, on the aminoacylation of $tRNA^{Phe}$ is not known. In practice, this side reaction is of the utmost importance in all cases in which the fate of ATP during aminoacylation is followed, especially if the stoichiometry of ATP consumption in relation to Phe-$tRNA^{Phe}$ formation has to be determined.

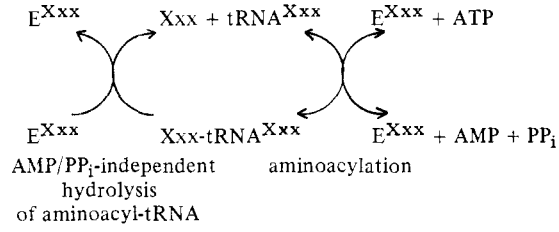
ATP is the essential energy source during the aminoacylation of tRNA by aminoacyl-tRNA synthetases. According to the generally accepted reaction sequence (eq 1; Xxx is the amino acid and E^{Xxx} is the corresponding aminoacyl-tRNA synthetase), one tRNA should be aminoacylated for each ATP consumed.

$$Xxx + ATP + E^{Xxx} \rightleftharpoons [E^{Xxx}\cdot AMP\text{-}Xxx] + PP_i \quad (1)$$

$$[E^{Xxx}\cdot AMP\text{-}Xxx] + tRNA \rightleftharpoons Xxx\text{-}tRNA + E^{Xxx} + AMP$$

The quantitation of this process and the possibility of editing or proofreading processes acting on the product (which would release free tRNA and thereby decrease the stoichiometry) have recently been the subject of some comment (Hopfield et al., 1976; Mulvey & Fersht, 1977). Whereas Hopfield et al. (1976) found a value of 0.65 Ile-$tRNA^{Ile}$/ATP, Mulvey & Fersht (1977) obtained a value of 0.99 for the same process. One adequate explanation of this discrepancy (involving the use of excess ATP over tRNA and thus including in the calculation the contribution of ATP involved in the synthesis of E·Ile-AMP after complete aminoacylation of tRNA) has been given by Mulvey & Fersht (1977). Nevertheless, the importance of a determination of ATP consumption during the overall aminoacylation process (Igloi et al., 1979) makes it imperative for one to be confident that the production of AMP does indeed stem from the aminoacylation and not from other "side" reactions.

The tRNA-dependent continuous ATP hydrolysis during the aminoacylation reaction has been established for several synthetases (von der Haar & Cramer, 1976) and has been called the "AMP/PP_i-independent hydrolysis of aminoacyl-tRNA" to emphasize its interpretation as a *cyclic reaction* (eq 2) and to distinguish it from the AMP/PP_i-dependent deacylation of aminoacylated tRNA.



We would now like to report a novel enzymatic activity of phenylalanyl-tRNA synthetase in which a different but readily interconvertible form of the enzyme brings about ATP hydrolysis in the absence of tRNA. This activity can be manipulated by the removal or addition of Zn^{2+} and leads to the conclusion that phenylalanyl-tRNA synthetase is a metalloprotein.

Materials and Methods

Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified to homogeneity from baker's yeast according to von der Haar

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0006-2960/80/0419-1676$01.00/0