Trp^{221} Is Involved in the Protective Effect of Elongation Factor eEF-2 on the Ricin/α-Sarcin Site of the Ribosome*

(Received for publication, September 8, 1983)

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Elongation factor eEF-2 treated by N-bromosuccinimide under conditions which oxidize 2 Trp residues (Trp^{221} and Trp^{231}) is inactivated in ribosome-dependent GTP hydrolysis and polyphenylalanine synthesis, and inactivation correlates with the specific oxidation of Trp^{221} (Guillot, D., Penin, E., Di Pietro, A., Sontag, B., Lavergne, J. P., and Reboud, J. P. (1993) J. Biol. Chem. 268, 20911–20916). It is shown here that this oxidation prevents neither GTP binding to eEF-2 nor the formation of the ribosome-eEF-2-GPP(NH)P complex, but that oxidized eEF-2 is no longer able to protect ribosomes against ricin inactivation. These observations suggest that Trp^{221} or an amino acid sequence containing this residue interacts with the 28 S rRNA loop including the GAGA sequence, which is the target of ricin. Such a hypothesis is discussed in relation with data on RNA recognition motifs described in different proteins.

The eukaryotic elongation factor 2 (eEF-2) catalyzes the translocation of peptidyl-tRNA from the ribosomal A site to the P site in the protein elongation cycle. eEF-2 belongs to the GTP-binding superfamily of proteins, which is characterized by conserved structural motifs and by the ability to alternate between specific conformations by binding either GTP or GDP and, therefore, to function as "molecular switches" in the specific activation of other cellular components (Bourne et al., 1991). GTP binding changes the conformation of eEF-2 (Sontag et al., 1993; Guillot et al., 1993), and the resulting binary complex is able to bind to the ribosome. Consequently, translocation takes place, followed by hydrolysis of GTP into GDP and inorganic phosphate. The molecular mechanisms that trigger the movement of peptidyl-tRNA from the A site to the P site of the ribosome remain unknown. It has been recently proposed that the conformation of the 28 S RNA loop containing the GAGA sequence, which is the ribosomal site sensitive to the toxins ricin (Endo and Tsurugi, 1987) and α-sarcin (Endo and Wool, 1982), changes during each turn of the elongation cycle as a result of eEF-2 binding (Wool et al., 1992). In prokaryotes, elongation factor G (the counterpart of eEF-2) footprints in a homologous α-sarcin/ricin domain of 23 S RNA (Moazed et al., 1988).

We have recently shown that Trp^{221}, which is conserved and belongs to a well preserved domain of eEF-2 in eukaryotic cells and archaeabacteria, should be involved in the catalytic activity since its oxidation by N-bromosuccinimide (NBS) completely inactivates the factor. Moreover, our results strongly suggest that GTP induces a conformational change in the protein that unmask Trp^{221}, whereas GDP binding leads to a shielding of this residue (Guillot et al., 1993). Here we demonstrate that the eEF-2 with oxidized Trp^{221} could still bind GTP and form a complex with ribosome and an uncleavable analog of GTP but was no longer able to protect the 28 S RNA loop containing the GAGA sequence against modification by ricin A chain. A hypothesis is proposed concerning the probable interaction of an eEF-2 sequence containing Trp^{221} with this loop.

EXPERIMENTAL PROCEDURES

Materials—GTP was purchased from Pharmacia, NBS was from Sigma, and Micro-BCA protein assay reagent was from Pierce.

Procedure—Rat liver eEF-2 (>95% pure) was purified as previously described (Conquet et al., 1987) in the presence of 5 pmol of eEF-2 and 80 pmol of EF-1α. These conditions correspond to an excess of eEF-2, which was saturating. Formation of the ribosome-eEF-2-GTP(NH)P complex was measured as previously described (Guillot et al., 1993). The modified enzyme was equilibrated in the appropriate buffer for GTP binding, tryptophan residue titration, and measurement of activity, and protection of ribosomes against ricin inactivation. Tryptophan Titration—Titration of unmodified tryptophan residues was performed by measuring the fluorescence emission at 350 nm of eEF-2, which had been incubated overnight at 25 °C in the presence of 90 pmol of ribosomes, 30 pmol of eEF-2, and 20 μM [8-3H]GPP(NH)P in a final volume of 100 μl. Protein concentration was determined with the Micro-BCA protein assay (Smith et al., 1985).

Chemical Modification by NBS—eEF-2 (1 μM, 100 μl) was treated with increasing concentrations of NBS as previously described (Guillot et al., 1993). The modified enzyme was equilibrated in the appropriate buffer for GTP binding, tryptophan residue titration, measurements of activity, and protection of ribosomes against ricin inactivation. Tryptophan Titration—Titration of unmodified tryptophan residues was performed by measuring the fluorescence emission at 350 nm of eEF-2, which had been incubated overnight at 25 °C in the presence of 90 pmol of ribosomes, 30 pmol of eEF-2, and 20 μM [8-3H]GPP(NH)P in a final volume of 100 μl. Protein concentration was determined with the Micro-BCA protein assay (Smith et al., 1985).

Binding of GTP—Nucleotide binding to eEF-2 was measured by the method of Hummel and Dreyer (1962) with a gel filtration SW200 column (30 cm × 0.8 cm) from Millipore equilibrated with 10 μM GTP. A constant volume (100 μl, 20 μM GTP) containing 0.9 nmol of NBS-modified or unmodified eEF-2, was injected onto the column. Nucleotide concentration of the eluent was recorded by absorption spectrophotometry at 260 nm, and the area of the negative peaks was determined.

Treatment of Ribosomes with Ricin A Chain—Ribosomes were treated with ricin A chain as described by Brigotti et al. (1989). Briefly, rat liver ribosomes (10 pmol) were preincubated 15 min at 25 °C in 45 μl of buffer A (80 mM-Tris/HCl (pH 7.4), 120 mM KCl, 7 mM MgCl₂, 2 mM dithiothreitol) containing 45 nmol GTP and 51.5 pmol of native or NBS-treated eEF-2. Ricin A chain was then added to 5 μl of buffer A for a final concentration of 0.15 μM, and ribosomes were incubated for an additional 5 min at 25 °C. Finally, 10 μl were withdrawn from the reaction mixture and protein synthesis was assayed as described above.
RESULTS AND DISCUSSION

We recently found that, after oxidation of Trp221 residue by NBS, eEF-2 was no longer active in GTP hydrolysis and polyphenylalanine synthesis and, in addition, that eEF-2 intrinsic fluorescence could no longer be quenched by GTP (Guillot et al., 1993). To explain these observations, we proposed two different hypotheses. First, GTP did not bind to the modified eEF-2 any more, thus this could not be activated. Second, GTP still bound to the factor but did not quench its fluorescence, because the fluorescence of Trp221 in unmodified eEF-2 is specifically quenched by GTP. In this latter case, inactivation would be due to an impaired interaction of eEF-2 with ribosome.

In the present study, we used the chromatographic method of Hummel and Dreyer (1982) to measure the binding of GTP to NBS-treated eEF-2. The advantage of this method is that the complex does not dissociate during chromatography, even if it lasts a long time and the affinity constant is low, since the complex is always in equilibrium with the free ligand. Fig. 1 shows the stoichiometry of GTP binding to eEF-2 after treatment of the factor with increasing concentrations of NBS. The relationship between NBS concentration and the number of oxidized tryptophans, as well as the identification of the 2 tryptophans that are oxidized first, have been already described (Guillot et al., 1993). Oxidation of the most sensitive tryptophan (namely Trp343) had no effect on GTP binding to the factor, as could be expected since modification of this residue did not affect eEF-2 activity. Oxidation of the second tryptophan (namely TrpzZ1), which has been shown to inactivate eEF-2, still allowed the binding of 0.8 mol of GTP/mol of eEF-2. The successive oxidation of 2 more tryptophan residues (which have not been identified yet) induced 50 and 90% decreases in GTP binding, respectively. These results showed that the inactivation of eEF-2 following Trp221 oxidation was not due to the inhibition of GTP binding to the factor. Such a result is in agreement with the observation that Trp221 belongs neither to the consensuses GTP/GDP-binding sequences (Kohno et al., 1986) nor to the fragment that has been cross-linked with oxidized GTP (Nilsson and Nygård, 1988).

As Trp221 oxidation had no significant effect on GTP binding to eEF-2, we tested the hypothesis that this modification prevented the formation of the high affinity complex in the presence of an uncleavable GTP analog and ribosomes. As can be seen in Fig. 1, oxidation of Trp221 and Trp211 did not prevent either the formation of the ribosome-eEF-2-GPP(NH)P complex. This ternary complex did not form any more after oxidation of 2 additional tryptophan residues, as it was shown for GTP binding to eEF-2. Thus, the inactivation of eEF-2 consecutive to Trp221 oxidation could not be explained by the inability of eEF-2 to interact with the ribosome in the presence of GTP.

Interaction of modified eEF-2 with ribosome might be impaired in such a way that the factor could no more interact with a specific part of the ribosome triggering translocation. We tested this hypothesis by performing protection experiments against ribosome inactivation by ricin A chain. This toxin is an N-glycosidase that specifically catalyzes the depurination of the A4324 in the C4323AGA4326 sequence of 28 S rRNA (Endo and Tsurugi, 1987) leading to ribosome inactivation. Prior binding of eEF-2 to the ribosome in the presence of GTP or a non-hydrolyzable analog of GTP protects the ribosome from inactivation (Fernandez-Puentes et al., 1976; Brigotti et al., 1989). It has been suggested that the GAGA-containing loop has alternate conformers and that the switch between the two conformations, possibly initiated by the binding of eEF-2, could trigger the translocation process (Wool et al., 1992). Ribosome protection from ricin A chain inactivation and polyphenylalanine synthesis were measured using eEF-2 treated with increasing concentrations of NBS. As can be seen in Fig. 2, the protective effect of NBS-treated eEF-2 against ribosome inactivation decreased as a function of the number of oxidized tryptophans and is closely related to the decrease of eEF-2 activity in polyphenylalanine synthesis. Oxidation of the most sensitive Trp residue (Trp343) induced a decline of only 10% of the protective effect of eEF-2, whereas oxidation of Trp221 completely abolished this protection.

To explain this result, one could assume that an amino acid sequence containing Trp221 is involved in the interaction of eEF-2 with nucleotides located in the ricin-sensitive loop of the 28 S rRNA. A potential candidate would be the A4324 nucleotide, which would be exposed at the top of the loop (Wool et al., 1992).

**TABLE I**

| RNPI | RNPI2 |
|------|-------|
| Consensus | AAA A  | B A  | AP |
| LRL L | G A/GF | F  |
| GFLNLK | HGN A  | FLT |
Such an interaction would explain both the protective effect of eEF-2 against the depurination of the A\(^{3234}\) in 28 S RNA by ricin and the absence of protection when Trp\(^{221}\) is oxidized. Our observation from fluorescence measurements, that eEF-2 can interact not only with guanylic nucleotides but also with adenylic nucleotides (but not with cytidylic ones) (Sontag et al., 1993), may also be related to this interaction. We have noted already that Trp\(^{221}\) and the amino acid sequence surrounding this residue are remarkably conserved in EF-2 of eukaryotic cells and archaeabacteria (Guillot et al., 1993). A more detailed analysis of eEF-2 sequence reveals the presence of two ribonucleoprotein (RNP) consensus sequences, a hexamer termed RNP\(_2\) and an octamer termed RNP\(_1\) enclosing Trp\(^{221}\) (Table I).

These two sequences are characteristic of RNA-associated proteins such as heterogeneous nuclear RNP proteins, small nuclear RNP proteins, splicing factors, or transcription factors (reviewed by Keene and Query (1991)). In eEF-2, RNP\(_2\) is present in its entirety, whereas the C-terminal part of RNP\(_1\) contains an inversion between the aliphatic and the polar residue, and phenylalanine is replaced by lysine. Such replacements in structural residues of the RNP motifs are not unusual (Hoffman et al., 1991). More importantly, the three aromatic residues of the RNP motifs that are assumed to be involved in RNA recognition are found in eEF-2: Phe\(^{221}\) in RNP\(_2\) and Trp\(^{221}\) and Phe\(^{223}\) in RNP\(_1\).

Acknowledgment—We are grateful to Dr. Louise Rosenbaum for critical reading of our manuscript.

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