Lack of Inhibition by L-1-Chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one (L-1-Tosylamido-2-phenylethyl Chloromethyl Ketone) of the Formation of Bacterial Polypeptide Chain Initiation Complex

By J. JONÁK* and B. F. C. CLARK
Medical Research Council Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, U.K.

(Received 18 June 1973)

The chymotrypsin inhibitor L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one does not inhibit the function of the initiation factor during the formation of the polypeptide chain initiation complex *in vitro*. Since the inhibitor has been shown previously to inhibit polypeptide chain elongation by reacting with elongation factor EF-Tu, the inhibitor can be used to investigate the initiation and elongation steps of protein biosynthesis separately.

Bacterial initiator tRNA, i.e. fMet-tRNA_f, is bound to ribosomes by mRNA in the formation of a polypeptide chain initiation complex, which also involves the participation of the three types of initiation factor, IF-1, IF-2 and IF-3 (Lucas-Lenard & Lipmann, 1971). Definition of the role of factor IF-2 in chain initiation has suggested a role for factor IF-2 analogous to that of elongation factor EF-Tu. Factor IF-2 is thought to form an intermediate complex with GTP and fMet-tRNA_f (Rudland *et al.*, 1971; Lockwood *et al.*, 1971, 1972; Dubnoff & Maitra, 1972), just as factor EF-Tu forms a complex with any other aminoacyl-tRNA and GTP (Ravel *et al.*, 1967; Gordon, 1968; Ono *et al.*, 1968); factors IF-2 and EF-Tu also have associated guanosine triphosphate activities on the ribosome (Kolakofsky *et al.*, 1969; Lelong *et al.*, 1970), and both contain thiol groups that have been reported to be essential for activity (Mazumder *et al.*, 1969). The specific chymotrypsin inhibitor L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one (TPCK) (Schoellmann & Shaw, 1963) has been shown to inhibit protein chain elongation through inactivation of the factor EF-Tu (Jonák *et al.*, 1973; Richman & Bodley, 1973). Further, the thiol groups of factor EF-Tu from *Bacillus stearothermophilus* have been shown to be the site of action of the inhibitor (Jonák *et al.*, 1973). We have therefore tested the action of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one on the formation of the initiation complex and in particular on the activity of factor IF-2, in order to obtain further characterization of its properties in comparison with those of factor EF-Tu.

Experimental

For the assays, crude ribosomes (65 mg/ml) and NH₄Cl-washed ribosomes from *Escherichia coli* M.R.E. 600 were prepared essentially as described by Haseltine *et al.* (1972), except that the ribosomes were stored at 4°C in their buffer B (10 mM-Tris-acetate buffer, pH 7.8, containing 14 mM-magnesium acetate, 60 mM-potassium acetate and 1 mM-dithiothreitol) until use. Additional washes each with 2 M- and 1 M-NH₄Cl solutions in buffer B were included at the beginning of the preparation of NH₄Cl-washed ribosomes. The washed ribosomes (130 mg/ml) were then stored under liquid N₂ in buffer B but containing 0.2 mM-dithiothreitol. Initiation factor IF-2 was prepared by the method of Remold-O'Donnell & Thach (1970), except that small phosphocellulose columns were used for the concentration of the factor preparations. Factor IF-2 (1.55 mg/ml) was stored under liquid N₂ in 50 mM-Tris-HCl buffer, pH 7.4, containing 0.4 M-NH₄Cl and 0.2 M-dithiothreitol. For the preparation of initiation factor IF-1 from the 45–75% saturation-(NH₄)₂SO₄ cut of the 2 M-NH₄Cl ribosomal wash, the method of Hershey *et al.* (1969) was adopted, with the omission of the Sephadex G-50 step. The resulting preparation contained both factor IF-1 and factor IF-3 activities, and on electrophoresis in the presence of sodium dodecyl sulphate two bands corresponding to factors IF-1 and IF-3 were detected. This factor IF-1-IF-3 complex (2.25 mg/ml) was stored, under liquid N₂, in 20 mM-Tris-HCl buffer, pH 7.5, containing 750 mM-NH₄Cl, 1 mM-EDTA, 0.2 mM-dithiothreitol and 10% (v/v) of glycerol.

The assay for the binding of fMet-tRNA_f used a 50 µl reaction mixture composed of 50 mM-Tris-HCl buffer, pH 7.4, containing 5 mM-MgCl₂, 50 mM-NH₄Cl, A-U-G (0.125 E₂₆₀ unit) or bacteriophage

* Present address: Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo n. 2, Prague 6, Czechoslovakia.

R17 RNA (0.1 mg) as indicated, 0.4 M-GTP, crude ribosomes (130 µg) or initiation factors and NH₄Cl-washed ribosomes (130 µg), and [¹⁴C]Met-tRNA_f as shown. Just before addition to the reaction mixture the R17 RNA was preincubated with 0.1 mM-EDTA for 2 min at 37°C. Reaction mixtures were incubated for 15 min at 25°C (experiments with A-U-G) or for 10 min at 37°C (experiments with R17 RNA), then cooled down to 4°C and 2 ml of buffer A (50 mM-Tris-HCl buffer, pH 7.4, containing 50 mM-NH₄Cl and 5 mM-MgCl₂) was added. The mixture was filtered through Millipore filters (HAWP; diameter 25 mm, pore size 0.45 µm). The filters were washed three times with 3 ml of buffer A and dried, and the radioactivity retained by the filters was counted in a Packard Tri-Carb scintillation spectrometer with an efficiency of about 75%.

Before exposure to L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one, crude ribosomes (0.2 ml) were dialysed against buffer B (100 ml) without dithiothreitol for 3 h at 4°C. Dialysed ribosomes were preincubated with 1 mM-L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one and 5% (v/v) of methanol, and 0.02 mM-GDP where indicated, for 6½ h at 4°C before their activity was tested. Factor IF-2 or IF-1-IF-3 complex was first diluted with 3 vol. of 50 mM-Tris-HCl buffer, pH 7.4, and then preincubated with 0.5 mM-L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one and 5% (v/v) of methanol for 6 h at 4°C and with 0.02 mM-GDP where indicated. Control samples were preincubated with 5% (v/v) of methanol alone or with 5% (v/v) of methanol and 0.02 mM-GDP where indicated. Untreated samples were not preincubated with any of the reagents.

[¹⁴C]Methionine (250 µCi/µmol) came from NEN Chemicals G.m.b.H. (Frankfurt, Germany); L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one and GTP (lithium salt) were from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.); *N*-ethylmaleimide was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.); GDP (lithium salt) and

A-U-G were from the Boehringer Corporation (Mannheim, Germany). R17 RNA was prepared by Dr. M. Yoshida; tRNA_f was purified by Mr. R. Coulson from source material isolated at the Microbiological Research Establishment (Porton, Wilts., U.K.) and was charged with [¹⁴C]methionine and formylated by the procedure of Clark & Marcker (1966).

Results

The effect of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one on initiation was tested with the crude ribosomes. As shown in Table 1, crude ribosomes [sedimented through 40% (w/v) sucrose only] were found to be very active in the binding of fMet-tRNA_f in response to the triplet A-U-G. Exposure of ribosomes to 1 mM-L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one under the conditions that led to the complete inactivation of factor EF-Tu, however, did not influence their binding capacity for fMet-tRNA_f (Table 1), and no significant effect of GDP, which increases the rate of inhibition of factor EF-Tu by L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one (Jonák *et al.*, 1973; J. Jonák & I. Rychlík, unpublished work), was observed. It is not demonstrated here, but it was also found that the preincubation with 10 mM-*N*-ethylmaleimide did not affect the binding activity of crude ribosomes.

Table 2(a) (Expt. 1) shows the effect of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one on the activity of purified initiation factor IF-2 in the stimulation of the binding of initiator tRNA to washed ribosomes directed by A-U-G triplet or by natural mRNA. After 6 h of preincubation with 0.5 mM-L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one, essentially no inhibition of activity of factor IF-2 was observed in the binding reaction mediated by the triplet A-U-G. Under the assay conditions with R17 RNA only a slight (about 15%) inhibition of the binding of fMet-tRNA_f binding to ribosomes occurred. GDP was again found

Table 1. *Effect of L-1-chloro-4-phenyl-3-toluene-p-sulphonamidobutan-2-one on activity of crude ribosomes in the binding of fMet-tRNA_f*

The experiment was performed under the triplet (A-U-G)-binding assay conditions as described in the text. Each reaction mixture contained 9 pmol of fMet-tRNA_f.

Preincubation treatment of ribosomes	fMet-tRNA _f bound (pmol)
None, minus A-U-G	0.18
None	4.75
5% methanol	4.50
1 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol	4.65
5% methanol + 0.02 mM-GDP	.25
1 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol + 0.02 mM-GDP	4.31

Table 2. *Effect of L-1-chloro-4-phenyl-3-toluene-p-sulphonamidobutan-2-one on initiation factors*

(a) Effect of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one and *N*-ethylmaleimide on the activity of factor IF-2 in the binding reaction. Expt. 1: each reaction mixture contained 4.65 µg of treated or untreated factor IF-2 as indicated, 6.7 µg of untreated IF-1-IF-3 complex, 8.3 pmol of fMet-tRNA_f and other components as described in the text. Expt. 2: factor IF-2 was first diluted with 3 vol. of 50 mM-Tris-HCl buffer, pH 7.4, and incubated with 10 mM-*N*-ethylmaleimide for 30 min at 25°C (factor IF-2, *N*-ethylmaleimide-treated) before the addition to the reaction mixture, which contained otherwise the same components as described in Expt. 1. Control factor IF-2 was preincubated with water only. (b) Effect of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one on the activity of the IF-1-IF-3 complex in the binding reaction. Each reaction mixture contained 6.7 µg of treated or untreated IF-1-IF-3 complex as shown, 4.65 µg of untreated factor IF-2 and the remaining components as described in (a). The experiment was performed under the R17-RNA assay conditions.

	fMet-tRNA _f bound (pmol)	
	A-U-G assay	R17-RNA assay
(a) Treatment of factor IF-2		
Expt. 1		
None, minus messenger	0.07	0.06
None, minus IF-1-IF-3 complex	0.29	0.24
None	2.98	2.81
5% methanol	3.03	3.01
0.5 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol	2.70	2.51
5% methanol + 0.02 mM-GDP	2.93	3.03
0.5 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol + 0.02 mM-GDP	2.59	2.60
Expt. 2		
None	2.89	2.91
10 mM- <i>N</i> -Ethylmaleimide	1.06	0.32
(b) Treatment of IF-1-IF-3 complex		
	fMet-tRNA _f bound (pmol)	
None, minus R17 RNA	0.09	
None, minus factor IF-2	0.12	
None	2.82	
5% methanol	2.69	
0.5 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol	2.62	
5% methanol + 0.02 mM-GDP	2.62	
0.5 mM-L-1-Chloro-4-phenyl-3-toluene- <i>p</i> -sulphonamidobutan-2-one + 5% methanol + 0.02 mM-GDP	2.52	

to have no effect on the action of L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one. The activity of factor IF-2 was also not affected by preincubation with 5% (v/v) methanol. In additional control experiments, preincubation of factor IF-2 with 10 mM-*N*-ethylmaleimide under the conditions described by Mazumder *et al.* (1969) resulted in decreases of 63.5 and 89% in the binding of fMet-tRNA_f to ribosomes in response to A-U-G and R17 RNA respectively, thus confirming that thiol group(s) are essential for the activity of initiation factor IF-2 [Table 2(a), Expt. 2].

As shown in Table 2(b), the stimulatory activity of the IF-1-IF-3 complex in the binding reaction under the assay conditions with R17 RNA was also found to be unaffected by preincubation with L-1-

chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one or with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one + GDP.

Discussion

Our results thus indicate that neither the formation of the initiation complex as a whole nor an individual initiation factor was affected by L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one. This suggests that L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one may be used *in vitro* as a specific inhibitor of the elongation step of protein synthesis, acting by inhibiting the formation of the elongation factor EF-Tu ternary complex (J. Sedláček & J. Jonák, unpublished work;

Richman & Bodley, 1973). L-1-Chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one therefore differentiates the factor-EF-Tu-binding activity of aminoacyl-tRNA from the factor-IF-2-binding activity of the initiator tRNA.

Finally we wish to mention that the initiation factor IF-2 used in our experiments, prepared by the method of Remold-O'Donnell & Thach (1970), which contained both species IF-2 α and IF-2 β (as detected on electrophoresis in the presence of sodium dodecyl sulphate) (Miller & Wahba, 1973), was found to possess very low activity, if assayed alone by the A-U-G- or R17-RNA-dependent ribosomal binding of fMet-tRNA_f [Table 2(a)], although in the simultaneous presence of the IF-1-IF-3 complex strong stimulation of fMet-tRNA_f binding occurred [Tables 2(a) and 2(b)]. Similar results have also been reported by Miller & Wahba (1973). Therefore it appears that, for the effective binding of initiator tRNA on to the ribosome with certain factor preparations, the simultaneous presence of all three initiation factors is essential.

We are grateful to the European Molecular Biology Organization for financial support for J. J. during this work and to Dr. C. J. Bruton and Dr. A. A. Travers for critical reading of the manuscript.

- Clark, B. F. C. & Marcker, K. A. (1966) *J. Mol. Biol.* **17**, 394-406
- Dubnoff, J. S. & Maitra, U. (1972) *J. Biol. Chem.* **247**, 2876-2883
- Gordon, J. (1968) *Proc. Nat. Acad. Sci. U.S.* **59**, 179-183
- Haseltine, W. A., Block, R., Gilbert, W. & Weber, K. (1972) *Nature (London)* **238**, 381-384
- Hershey, J. W. B., Dewey, K. F. & Thach, R. E. (1969) *Nature (London)* **222**, 944-947
- Jonák, J., Sedláček, J. & Rychlík, I. (1973) *Biochim. Biophys. Acta* **294**, 322-328
- Kolakofsky, D., Dewey, K. F. & Thach, R. E. (1969) *Nature (London)* **223**, 694-697
- Lelong, J. C., Grunberg-Manago, M., Dondon, J., Gros, D. & Gros, F. (1970) *Nature (London)* **226**, 505-510
- Lockwood, A. H., Chakraborty, P. R. & Maitra, U. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 3122-3126
- Lockwood, A. H., Sarkar, P. & Maitra, U. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 3602-3605
- Lucas-Lenard, J. & Lipmann, F. (1971) *Annu. Rev. Biochem.* **40**, 409-448
- Mazumder, R., Chae, Y. B. & Ochoa, S. (1969) *Proc. Nat. Acad. Sci. U.S.* **63**, 98-103
- Miller, M. J. & Wahba, A. J. (1973) *J. Biol. Chem.* **248**, 1084-1090
- Ono, Y., Skoultchi, A., Klein, A. & Lengyel, P. (1968) *Nature (London)* **220**, 1304-1307
- Ravel, J. M., Shorey, R. L. & Shive, W. (1967) *Biochem. Biophys. Res. Commun.* **29**, 68-73
- Remold-O'Donnell, E. & Thach, R. E. (1970) *J. Biol. Chem.* **245**, 5737-5742
- Richman, N. & Bodley, J. W. (1973) *J. Biol. Chem.* **248**, 381-383
- Rudland, P. S., Whybrow, W. A. & Clark, B. F. C. (1971) *Nature (London) New Biol.* **231**, 76-78
- Schoellmann, G. & Shaw, E. (1963) *Biochemistry* **2**, 252-255