Involvement of arginine residues in inhibition of protein synthesis by ricin A-chain

Keiichi Watanabe+ and Gunki Funatsu*

Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan

Received 29 April 1986

Modifications of arginine residues in ricin A-chain with phenylglyoxal (PGO) and 1,2-cyclohexanedione (CHD) caused a marked loss in its inhibitory activity on cell-free protein synthesis. The loss of activity caused by modification with PGO was much faster than the loss of total arginine residues. More than 90% activity was lost with PGO modification of about three arginine residues. Regeneration of arginine residues from the CHD-modified residues resulted in complete recovery of the activity. These results strongly suggest the involvement of definite arginine residue(s) in A-chain activity. Analysis of the peptides, produced by peptic digestion of the [^{14}C]PGO-modified A-chain, showed that some of the six arginine residues in the N-terminal region of A-chain react with PGO faster than other arginine residues.

Ricin A-chain *Ribosome inactivation* *Active site* *Arginine residue*

1. INTRODUCTION

Ricin, a toxic lectin present in castor bean (*Ricinus communis*) seeds, consists of two different polypeptide chains (A- and B-chains) linked by a single disulfide bond [1]. The B-chain binds galactose-containing receptors on the cell surface [2]. The A-chain strongly inhibits protein synthesis by enzymatically damaging the 60 S ribosomal subunit [3] and is thought to affect the elongation factor-dependent functions [4,5].

The primary structure of the A-chain has been determined in our laboratory [6] and it has become clear that the A-chain is a glycoprotein consisting of 265 amino acid residues. Knowledge about the essential amino acid residues for A-chain activity will be important for understanding the relationship between its structure and activity, and thus the molecular mechanism of action of the A-chain. Although chemical modifications of ricin with various reagents have been reported [7–9], the essential amino acid residues for A-chain activity have not been found. The ability of the isolated A-chain to inhibit protein synthesis in a cell-free system is much stronger than that of the intact ricin molecule [2]. It is therefore necessary to subject the isolated A-chain to chemical modifications for elucidation of the essential amino acid residues.

We report here the modification of arginine residues in the A-chain and the results demonstrating the involvement of definite arginine residue(s) in the inhibition of protein synthesis by A-chain.

2. MATERIALS AND METHODS

The A-chain of ricin (ricin D [10]) was prepared as described in [11]. [^{14}C]PGO hydrate

* To whom correspondence should be addressed
+ Present address: Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Ihnestrasse 63–73, D-1000 Berlin 33 (Dahlem), Germany

Abbreviations: PGO, phenylglyoxal; CHD, 1,2-cyclohexanedione; HPLC, high-performance liquid chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

(32600 dpm/μmol) was prepared from [1-^{14}C]acetophenone [12] which had been prepared from benzene and [1-^{14}C]acetic anhydride (Amersham) [13].

Modification of A-chain with PGO (Aldrich) was performed at 25°C in the dark at a protein concentration of 1 mg/ml and a reagent concentration of 10 mM (310-fold molar excess) in 0.125 M $NaHCO_3$, pH 8.3. Aliquots were withdrawn at intervals and immediately diluted into ice-cold 20 mM Tris-HCl buffer, pH 7.5, and subsequently dialyzed against the same buffer at 5°C. Modified arginine residues were determined by amino acid analysis after HCl hydrolysis.

Modification with CHD (Nakarai Chemicals) was carried out essentially according to [14]. A-chain (1 mg/ml) was incubated with 0.1 M CHD at 37°C for 2 h in 0.2 M sodium borate buffer, pH 8.3. After dialysis against 5 mM Tris-HCl buffer, pH 7.5, in the cold, the sample was divided into two portions. One portion was incubated at pH 7.5 with 0.2 M hydroxylamine at 37°C for 12 h under N_2 to regenerate arginine residues, then dialyzed again. Modified arginine residues were determined by amino acid analysis.

Peptic digestion of [^{14}C]PGO-modified A-chain was carried out with a 1 : 100 (w/w) amount of pepsin (Sigma) at pH 2.1 at 25°C for 16 h. The radioactive peptides were separated by reverse-phase HPLC (Jasco Fine SIL C_8 column) with a linear gradient of acetonitrile in 5 mM phosphate buffer at pH 6.0 and further purified by rechromatography with a propanol or acetonitrile gradient in aqueous 0.1% trifluoroacetic acid. The eluate was monitored at 250 nm because the PGO derivative of arginine has a maximum absorption at this wavelength [15]. Radioactivity was measured with a liquid scintillation counter (Aloka LSC-602). Amino acid compositions were analyzed as in [6]. N-terminal sequences of peptides were determined by the 4-*N,N*-dimethylamino-azobenzene-4′-isothiocyanate / phenylisothiocyanate double-coupling method [16].

The cell-free system of protein synthesis, consisting of unfractionated lysate from rabbit reticulocyte, was prepared as in [7,9]. The inhibitory activity of the A-chain on protein synthesis was determined based on the amount required to give 50% inhibition.

3. RESULTS AND DISCUSSION

When A-chain was incubated with a 310-fold molar excess of PGO at pH 8.3 and 25°C, the inhibitory activity on cell-free protein synthesis was lost very rapidly (fig.1). The time necessary for 90% inactivation under these conditions was about 4 min. Amino acid analyses of samples of modified A-chain showed that in the course of PGO modification, the content of arginine residues decreased (fig.1) without significant change in the composition of other amino acids. This result indicates that the loss of A-chain activity is due to the modification of arginine residues.

A molecule of A-chain contains 20 arginine residues [6]. The semilogarithmic plot for loss of arginine in A-chain gave an almost linear curve up to 180 min, when 75% of total arginine residues were modified (fig.1). This observation indicates that there are several classes of arginine residues which react with PGO at different rates. The fact

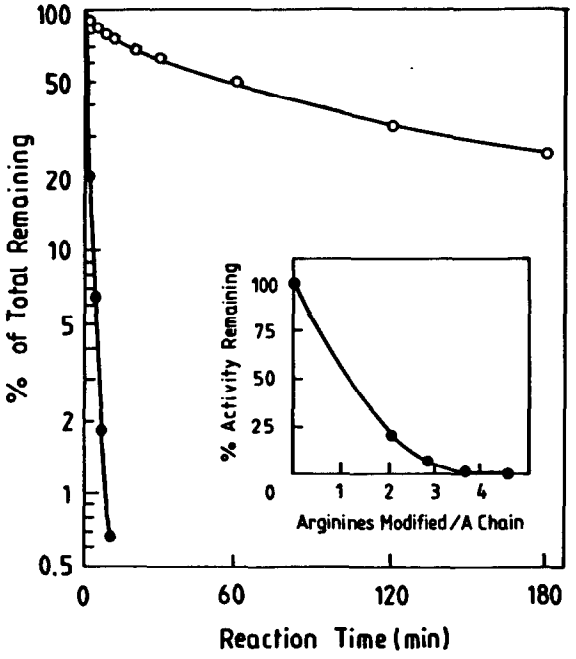

Fig.1. Loss of arginine residues (○) and loss of inhibitory activity on protein synthesis (●) in ricin A-chain by modification with PGO. Ricin A-chain was modified with PGO, and the arginine content and activity were determined as described in section 2. Inset: loss of activity as a function of the number of modified arginine residues per molecule of A-chain.

that the loss of A-chain activity is much faster than that of the total arginine residues demonstrates that the inactivation of A-chain results from the modification of definite arginine residue(s) which react with PGO faster than other arginine residues. As shown in the inset to fig.1, more than 90% of the A-chain activity was lost with modification of about three arginines per A-chain. Similar loss of activity as a function of modified arginines was observed when A-chain was modified with decreasing amounts of PGO (15–60-fold molar excess) for 60 min at the same pH and temperature.

To confirm the inactivation of the A-chain by the specific modification of arginine residue(s), A-chain was modified with a different α-dicarbonyl reagent, CHD. In this case, arginine can be readily regenerated from the modified residues by treatment with hydroxylamine [14]. As shown in table 1, modification with CHD also caused inactivation of the A-chain. Subsequent treatment with hydroxylamine resulted in the complete recovery of activity although some of the modified arginines still remained (table 1). The reactivation observed indicates that the loss of activity caused by treatment with CHD is due to the specific modification of arginine residue(s) and not to irreversible effects on the A-chain.

An attempt was made to identify the modified arginine residues by analyzing the peptides generated by proteolytic digestion of a sample of A-chain in which about two arginines had been modified with [^{14}C]PGO. Because the PGO derivative of arginine is particularly stable under acidic conditions [12], digestion by pepsin at pH 2.1 was chosen. The resulting peptic peptides were separated by reverse-phase HPLC using a linear gradient of acetonitrile at pH 6.0 followed by 2-propanol (fig.2). More than 60% of the injected radioactivity was present in the last three fractions (F-1, F-2, F-3). The longer retention times of the radioactive fractions may be due to increased hydrophobicity of PGO-modified peptides. Fraction F-3, which was eluted with 2-propanol, contained most of the radioactivity. Four radioactive peptides were isolated from this fraction by rechromatography with a linear gradient of 2-propranol in 0.1% trifluoroacetic acid. N-terminal sequencing of these peptides gave Ile-Arg-Ala- for each peptide. This amino acid sequence corresponds unambiguously to residues 25–27 of

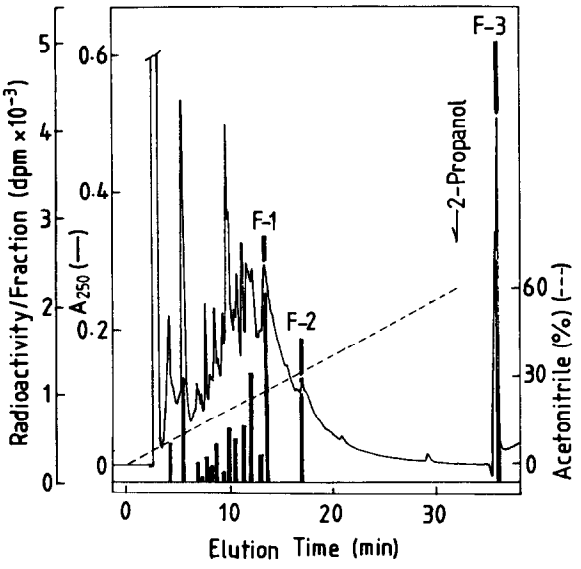

Fig.2. Separation of peptic peptides of [^{14}C]PGO-modified A-chain by reverse-phase HPLC. A peptic digest of [^{14}C]PGO-modified A-chain was chromatographed on a Jasco Fine SIL C_8 column (4.6 × 250 mm) using a linear gradient of acetonitrile in 5 mM potassium phosphate buffer, pH 6.0. The histogram shows the radioactivity determined for each fraction.

the A-chain. Amino acid analysis revealed that all of these peptides contained six arginine residues, apparently corresponding to positions 26, 29, 31, 39, 48 and 56 in the N-terminal region of the A-chain. Fractions F-1 and F-2 were eluted as broadened peaks and were not well separated from each other. They were, therefore, mixed and subjected to rechromatography with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. This HPLC run provided one major radioactive fraction. N-terminal sequencing of this fraction gave a main sequence of Ile-Phe-Pro-, which corresponds to the N-terminal sequence of intact A-chain. These results of peptide analysis demonstrate that some of the six arginine residues in the N-terminal region of A-chain react with PGO faster than other arginine residues.

The A-chain has long been known to inactivate catalytically the eucaryotic 60 S ribosomal subunit with no added cofactors [3]. However, the precise substrate and molecular mechanism for the inactivation of the 60 S subunit have not yet been elucidated in spite of several efforts. The present

Table 1

Effect of modifications of arginine residues on the inhibition of protein synthesis by A-chain

Reagent	No. of modified residues	Activity (%)
Phenylglyoxal	2.9	6.0
1,2-Cyclohexanedione	7.6	0.9
1,2-Cyclohexanedione + hydroxylamine	1.9	100

results strongly suggest that definite arginine residue(s) in the N-terminal region of the A-chain are located at the active site and are involved in the inhibition of cell-free protein synthesis. Further confirmation of the position(s) of functional arginine residue(s) is now in progress.

Arginine residues appear to play an important role in the active site of many enzymes, acting as positively charged recognition groups for negatively charged substrates or anionic cofactors [17,18]. Although the role of arginine residues in the action of the A-chain has not been elucidated from the present data, involvement of functional arginine residue(s) in the recognition of negatively charged substrate on the 60 S subunit seems to be one possibility.

REFERENCES

[1] Funatsu, G. and Funatsu, M. (1977) Agric. Biol. Chem. 41, 1211–1215.
[2] Olsnes, S., Refsnes, K. and Pihl, A. (1974) Nature 249, 627–631.
[3] Sperti, S., Montanaro, L., Mattioli, A. and Stirpe, F. (1973) Biochem. J. 136, 813–815.
[4] Sperti, S., Montanaro, L., Mattioli, A. and Testoni, G. (1975) Biochem. J. 148, 447–451.
[5] Carrasco, L., Fernandez-Puentes, C. and Vazquez, D. (1975) Eur. J. Biochem. 54, 499–503.
[6] Funatsu, G., Yoshitake, S. and Funatsu, M. (1978) Agric. Biol. Chem. 42, 501–503.
[7] Taira, E., Yoshizuka, N., Funatsu, G. and Funatsu, M. (1978) Agric. Biol. Chem. 42, 1927–1932.
[8] Sandvig, K., Olsnes, S. and Pihl, A. (1978) Eur. J. Biochem. 84, 323–331.
[9] Watanabe, K. and Funatsu, G. (1984) J. Fac. Agr. Kyushu Univ. 28, 201–211.
[10] Hara, K., Ishiguro, M., Funatsu, G. and Funatsu, M. (1974) Agric. Biol. Chem. 38, 65–70.
[11] Ono, M., Kuwano, M., Watanabe, K. and Funatsu, G. (1982) Mol. Cell. Biol. 2, 599–606.
[12] Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179.
[13] Noller, C.R. and Adams, R. (1924) J. Am. Chem. Soc. 46, 1889–1896.
[14] Patthy, L. and Smith, E.L. (1975) J. Biol. Chem. 250, 557–564.
[15] Takahashi, K. (1977) J. Biochem. 81, 403–414.
[16] Chang, J.Y., Brauer, D. and Wittmann-Liebold, B. (1978) FEBS Lett. 93, 205–214.
[17] Riordan, J.F., McElvany, K.D. and Borders, C.L. jr (1977) Science 195, 884–885.
[18] Riordan, J.F. (1979) Mol. Cell. Biochem. 26, 71–92.