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Formation and properties of S-protein complex with S-peptide-containing fusion protein

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Abstract

A fusion protein (FP) comprised of the RNase A S-peptide and human epidermal growth factor was shown to form a stable noncovalent catalytically active complex with the RNase A S-protein at a stoichiometric ratio 1:1 with $K_{diss} = 5.0 \times 10^{-7}$ M. The S-protein complex with FP exhibits the pyrimidine specificity toward substrates in both reactions catalyzed by RNase S, transesterification and hydrolysis. The fusion protein can be determined specifically and quantitatively in the presence of S-protein by RNase activity assays. The possibility of effective purification of S-peptide-containing proteins by affinity chromatography on an S-protein-Sepharose column has been demonstrated.

Key words: S-peptide and S-protein of RNase A; Human epidermal growth factor; Fusion protein

1. Introduction

The problem of 'modular activity' of fused proteins has both a theoretical and an obviously practical significance. The present work deals with the study of the S-peptide recognition by S-protein when the former is inserted into a recombinant protein, containing the human epidermal growth factor sequence (hEGF). Limited proteolysis of pancreatic ribonuclease (RNase A) by subtilisin results in cleavage of a peptide bond (20–21), and fragments (1–20)-S-peptide and (21–124)-S-protein are held together by noncovalent interactions [1]. The modified protein called RNase S, retains the catalytic properties of RNase A. Acid treatment of RNase S results in formation of catalytically inactive components. At neutral pH restoration of the catalytically active complex, RNase S′, takes place. The RNase S crystal structure study shows that the C-terminus of S-peptide and N-terminus of S-protein of RNase S are at a distance of 27 Å from each other (Fig. 1) [2]. Consequently, one may assume that the attachment of a protein to the C-terminus of the S-peptide should not interfere with its specific interaction with the S-protein. However, the catalytic properties of a conjugate (if any) might be affected. The fusion protein hEGF (53 amino acids) with one molecule of S-peptide-(FP) was obtained using conventional DNA manipulations techniques [3].

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Abbreviations: RNase A, pancreatic ribonuclease (EC 3.1.27.5); hEGF, human epidermal growth factor; FP-fusion protein made up of one molecule of hEGF with one molecule of S-peptide.

Footnote: The main results of this work were reported at the International Conference 'Modern Enzymology: Problems and Trends', (1992), St-Peterburg; at the 3rd International Meeting 'Ribonucleases: Chemistry, Biology, Biotechnology' (1993), Capri, Italy.

2. Materials and methods

2.1. Materials

The earlier described technique [3] was used to prepare a crude extract (periplasmic fraction) of *E. coli* cells transformed by a plasmid, carrying the FP encoding gene. S-protein was obtained by RNase A (Sigma, USA) selective hydrolysis with subtilisin according to [4]. Homopolymers of ribonucleotides and 2′,3′-nucleoside cyclophosphates were from Sigma; yeast RNA and cyanogen bromide were from Serva; Sepharose CL-4B, and the LMW Calibration Kit were from Pharmacia LKB Biotechnology Inc. The electrophoretically homogeneous FP and polyclonal antibodies against hEGF were kindly supplied by A.V. Azhayev and N.V. Batchicova. The conjugate S-protein–Sepharose 4B was obtained as described [5].

2.2. Purification of fusion protein (FP)

30 ml of periplasmic fraction preliminarily diluted by half with 0.1 M ammonium-acetate buffer, pH 6.0, were applied to a column (0.5 × 4 cm), filled with S-protein–Sepharose CL-4B (total S-protein content 7 mg) and equilibrated with the same buffer. The column was washed with the equilibration buffer containing 2.0 M NaCl. Elution of FP was performed with 1% acetic acid at the flow rate of 16 ml/h, $\lambda = 280$ nm.

2.3. Determination of fusion protein concentration

The concentration of electrophoretically homogeneous FP was determined by Bradford's method [6]. Quantitative determination of FP concentration in the crude extract was carried out using the RNA or 2′,3′-cCMP hydrolysis initial rate dependence upon the purified FP concentration.

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2.4. SDS-gel electrophoresis

Electrophoresis was performed in a 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate using the buffer system of Laemmli [7]. Band positions were determined by using a Molecular Dynamics Computing Densitometer.

2.5. Enzyme activity assays of S-protein–FP complex

The RNase activity was determined by a modification of the method of Kunitz (yeast RNA hydrolysis) [8] or according to Crook et al. (2′,3′-cCMP as substrate) [9], using a constant concentration of S-protein and various concentrations of S-peptide or FP. The evaluation of the S-protein–FP complex dissociation constant K_{diss} is based on the application of the mass action law [8].

2.6. Determination of the complex stoichiometry by HPLC

An excess of S-protein was added to FP and the mixture was kept overnight at pH 7.0, 4°C. Aliquots (20 μl), containing about 40 μg of protein were applied to a Diasorb 400 DIOL (0.8 × 25 cm) column (Elsika, Russia). Elution was performed with 25 ml of HEPES (Na) buffer, pH 7.3, at a flow rate of 1 ml/min, 20°C, λ = 280 nm. A mixture of standard proteins was separated under the same conditions as a reference.

2.7. Immunoassay

Determination of hEGF antigens in periplasmic fractions and FP samples was carried out using the classic indirect ELISA.

3. Results and discussion

S-peptide in a neutral aqueous solution is known to form a specific noncovalent complex with S-protein (K_{diss} = 10^{-5}–10^{-9} M). The catalytic properties this complex are practically the same as those of a native enzyme [1,8,10,11]. One may expect that the S-protein interaction with the fusion protein (FP) would result in formation of a catalytically competent complex, resembling by its properties those of RNase S′. The FP addition to S-protein gives rise to restoration of the specific enzyme activity of RNase S′. It was demonstrated that for S-protein or FP alone no detectable enzymatic activity was found under the same conditions. Consequently, one may conclude that the S-peptide attached to hEGF, interacts with S-protein forming a functionally productive noncovalent complex.

3.1. Stoichiometry of S-protein–FP complex

The FP mixture with an excess of S-protein has been separated by HPLC into two main components. The first component corresponds to a polypeptide with the molecular mass of about 21–22 kDa (Fig. 2, peak 1), and the second one to a polypeptide of 13–15 kDa (Fig. 2, peak 2). The components were collected separately, concentrated and tested for ribonuclease activity. Component 1 was found to be active, whereas component 2 was devoid of it, but the activity appeared after addition of S-peptide to the solution. These results allow us to identify the first component as an FP complex with S-protein (1:1) and the second one as S-protein. Besides these, a small amount of FP (< 5%) was detected.

3.2. Properties of the complex formed by FP with S-protein

The FP complex with S-protein is as effective in 2′,3′-cCMP hydrolysis as RNase S′, whereas 2′,3′-cAMP is resistant to both RNase S′ and the complex (Table 1). Although catalytic properties of the complex are rather close to those of RNase S′ there are several distinctions between them. For example, practically complete hydrolysis of poly(C) by RNase S′ requires two hours, whereas a significant amount of 2′,3′-cCMP has been detected in the reaction mixture even after two days of incubation

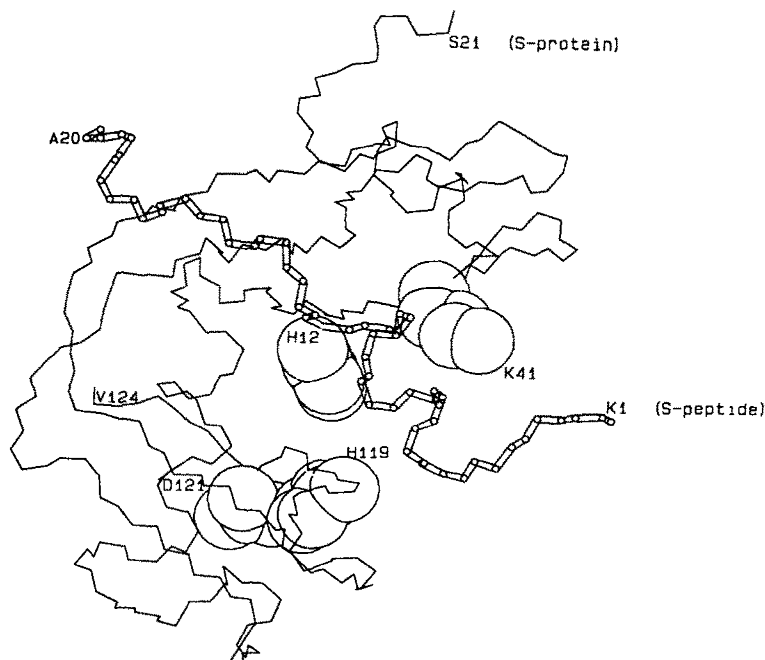

Fig. 1. Schematic representation of a molecular three-dimensional structure of RNase S′.

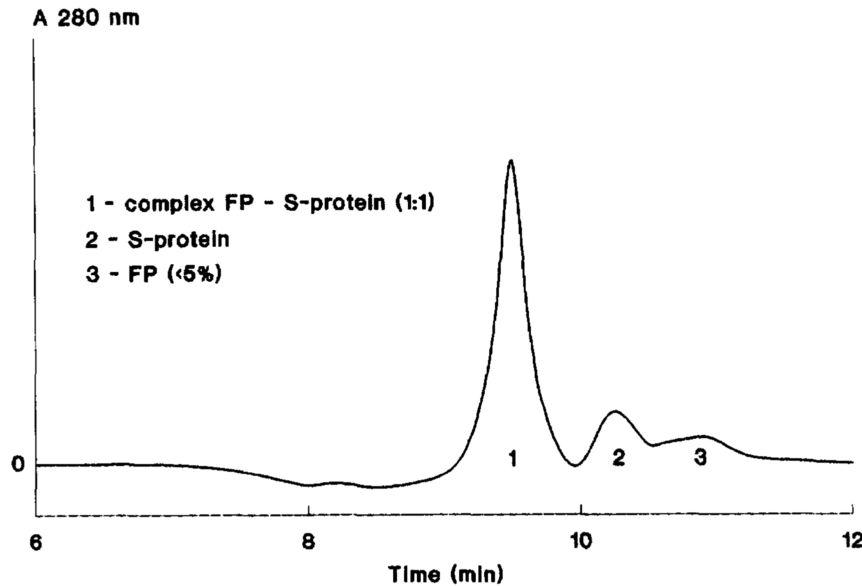

Fig. 2. Determination of stoichiometry between S-protein and FP by the size exclusion HPLC on the Diasorb 400 DIOL column. Separation of the FP–S-protein complex (1) and S-protein (2).

in the presence of the complex. When poly(A) was incubated in the presence of RNase S′ the hydrolysis products (olygonucleotides, 2′,3′-cAMP, 3′-AMP) appeared already the next day. Poly(A) was found to be stable for two days in the presence of the complex and only then traces of oligonucleotides and 2′,3′-cAMP appeared. One may assume that such differences are due to inactivation of the complex. In spite of those differences, the results indicate that pyrimidine specificity of RNase A was still retained by the complex.

Dissociation (binding) constants, free binding energies ($\Delta G°$) and relative catalytic activities of the complex and RNase S′ were determined (Fig. 3, Table 2). It is of interest that the maximum rate value is the same provided that S-protein is saturated with S-peptide. The results were shown to be reproducible with various polypeptide preparations.

3.3. *The perspectives of application*

The results obtained suggest certain prospects for their practical applications. The first self-evident consequence would be to use affinity chromatography with a matrix, containing immobilized S-protein for purification of FP with S-peptide attached to its N-terminal part. The validity of such an approach was demonstrated by FP isolation from the crude extract using affinity chromatography on the S-protein–Sepharose column. Fig. 4 shows SDS-PAGE separation of hEGF and FP from periplasmic fractions. The estimated FP molecular mass value 9.1 kDa is close to that calculated from the amino acid composition (8.8 kDa). Eluted from the column, FP gave a single band with the same molecular mass value on SDS-PAGE (data not shown). Recently the FP construction of β-galactosidase with the truncated S-peptide (1–15 amino acid residues) has been reported [13]. This

Table 1
TLC analysis of a mixture resulted from substrates treatment with RNase S′ and FP–S-protein complex

	RNase S′		Complex FP–S-protein	
Substrate	Time of reaction	Products identified	Time of reaction	Products identified
2′,3′-cCMP	20 min	3′-CMP (50%)	20 min	3′-CMP (~ 50%)
	24 h	3′-CMP (100%)	24 h	3′-CMP (100%)
2′,3′-cAMP	3 h	none	20 min	none
	24 h	none	24 h	none
	48 h	traces of 3′-AMP	48 h	traces of 3′-AMP
Poly(C)	30 min	2′,3′-cCMP (50%) and 3′-CMP (50%)	60 min	2′,3′-cCMP (~ 50%) and 3′-CMP (~ 50%)
	2 h	3′-CMP (100%)		
Poly(A)	30 min	none	60 min	none
	24 h	traces of oligonucleotides and 2′,3′-cAMP	48 h	traces of oligonucleotides and 2′,3′-cAMP

Conditions: 0.1 M Tris-HCl, pH 7.0, 0.1 M NaCl.

Table 2
Binding properties and activities of S-protein–S-peptide system at pH 5.0, 20°C, (substrate–RNA)

Peptide	K_{diss} (M)	K_b (M^{-1})	$-\Delta G°$ (kcal/mol)	A_p^{max} / A_s^{max}
S-peptide	5.7×10^{-8}	1.8×10^{7}	10.2	1.0
FP	5.0×10^{-7}	2.0×10^{6}	8.8	1.0

A_s^{max}, maximum activity of RNase S′ at the S-protein saturation with S-peptide. A_p^{max}, maximum activity of FP–S-protein complex.

study has shown that the interaction between the S-peptide portion of FP and immobilized S-protein allowed for affinity purification of FP and quantitative determination of FP concentration by the restoration of RNase activity after activation with S-protein.

Thus, S-protein recognize S-peptide even when the latter is incorporated into the fusion protein. It should be noted that, according to the immunoassay, hEGF in the FP retains properties of native hEGF. This means that the S-peptide fragment of the hybrid protein is still available for interaction with S-protein even when the polypeptide chain acquires the conformational characteristic of native hEGF.

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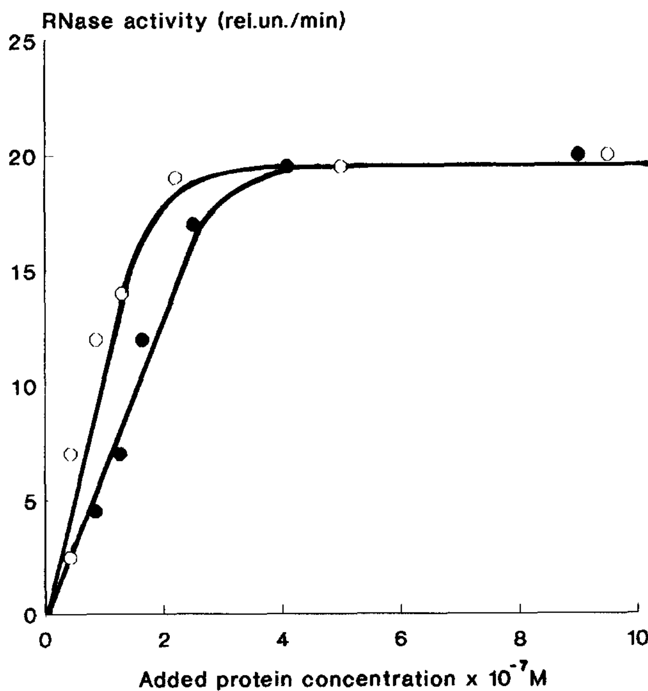

Fig. 3. The enzymatic activity development resulted from addition of either S-peptide (○) or the fusion protein (●) to S-protein. Conditions: 0.1% solution of RNA in 0.1 M NaAc, pH 5.0, S-protein concentration: 3.0×10^{-7} M, 20°C.

Fig. 4. SDS-acrylamide gel electrophoresis of crude extract containing hEGF (lane 2) and fusion protein (lane 3). Lanes 1 and 4, LMW markers (kDa): 17.2; 14.6; 8.2; 6.38; 2.56

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