CHEMICAL EVIDENCE SHOWING INVOLVEMENT OF SULFHYDRYL GROUPS IN PROTEIN SYNTHESIS WITH E. coli SYSTEM

Koichi Kimura, Akira Watanabe*, Minoru Machida and Yuichi Kanaoka

Faculty of Pharmaceutical Sciences, Hokkaido University

Sapporo, Japan

Received April 8, 1971

Summary The activity of E. coli ribosomes to synthesize polyphenylalanine under the direction of polyuridylic acid is inhibited by certain maleimide derivatives. Using a fluorescence-labeled sulfhydryl reagent, N-(p-(2-benzimidazolyl)phenyl)maleimide, it is confirmed that the inhibition takes place through covalent bond formation of this reagent with cysteine-sulfhydryl groups of the ribosomal proteins.

Since Schweet et al. (1,2) have reported that the poly-U directed binding of Phe-tRNA to ribosome was inhibited by various sulfhydryl reagents including N-ethylmaleimide (NEM) with a reticulocyte system, results have been accumulated to indicate that the ribosomal sulfhydryl groups, as well as those in E. coli systems, are important in the protein synthesis (3,4,5). However, it has been difficult to directly prove the participation of the cysteine-sulfhydryl groups in this process. In the present paper, we describe new chemical evidence that certain maleimide derivative inhibits cell-free synthesis of polyphenylalanine in a E. coli system under the conditions in which the reagent combines with cysteine-sulfhydryl group of the ribosomal proteins by covalent bond formation.

Materials and Methods

Exponential phase cells of E. coli strain Q13 were used. The extrac-

---

* On leave from Department of Botany, Faculty of Science, The University of Tokyo; Present address : Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya, Japan.

tion was made with 60 mM KCl-10 mM $MgCl_2$-10 mM Tris/HCl buffer, pH 7.6, and ribosomes were isolated by repeated differential centrifugations (6). Transfer RNA and soluble enzyme fractions were prepared from the post ribosomal supernatant through chromatography on a DEAE-cellulose column. In this case, 12 mM β-mercaptoethanol was added to the medium throughout the preparation procedure. N-(p-(2-benzimidazolyl)phenyl)maleimide (BIPM) and N-(p-(2-benzimidazolyl)phenyl)succinimide (BIPS) were synthesized as described previously (7).

Treatment of ribosomes with NEM or BIPM was carried out in a reaction mixture containing 200 OD_{260} units/ml ribosomes, 10 mM $MgCl_2$ and 10 mM Tris /HCl buffer, pH 7.0, with certain amount of monoglyme solution of the maleimides, at 37°C in the dark for 30 minutes. After stopping the reaction by chilling, the mixture was dialyzed at 2°C against 10 mM $MgCl_2$-10 mM Tris/HCl buffer, pH 7.6, to remove excess of the unreacted reagent. Ultraviolet absorption and fluorescence of the product were measured with a Hitachi EPS-3T spectrophotometer and MPF-2A spectrofluorophotometer, respectively.

The activities of the maleimide-treated ribosomes were examined by a cell-free system for poly-U directed polyphenylalanine synthesis. Intact preparations of tRNA and enzymes were used in this system. After incubation, four volumes of cold 0.5 N perchloric acid was added to each reaction mixture. The precipitates were collected, washed, and hydrolyzed in the same acid at 70°C for 15 minutes. The insoluble residue was collected quantitatively on a filter paper disc and counted for the radioactivity of incorporated phenylalanine. A windowless type, low background gas-flow counter, Aloka LBC-22B was used for the radioassay.

Results and Discussion

Addition of BIPM to a solution of *E. coli* ribosomes produced a strong fluorescence. Fig.1 presents the fluorescence spectrum of the reaction product of the ribosomes with BIPM as well as the absorption and fluores-

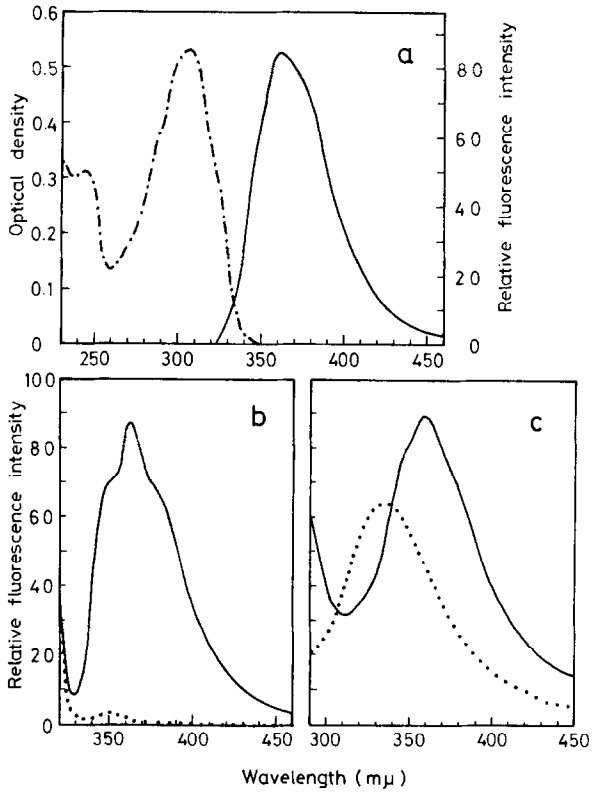

Fig.1. Ultraviolet absorption and fluorescence spectra. (a) Ultraviolet absorption spectrum of BIPS (•—•—•) : Concentration, 2×10^{-5} M in 0.1 M phosphate buffer, pH 7.0. Fluorescence spectrum of BIPS (———) in 0.1 M phosphate buffer, pH 7.0. Excitation wave length, 310 mμ; OD_{310}, less than 0.2 unit/ml. (b) Fluorescence spectra of BIPS-ribosomes (———) and intact ribosomes (•••••). Excitation wave length, 310 mμ; OD_{260}, 0.8 unit/ml. (c) Fluorescence spectra of BIPS-ribosomes (———) and intact ribosomes (•••••). Excitation wave length, 260 mμ.

cence spectra of the related systems. Comparison of the fluorescence spectrum (b) with that of BIPS (a), a reference compound of the adduct of BIPM with a sulfhydryl substrate (7), indicates that the dramatic increase in the fluorescent intensity of the ribosomes is apparently due to incorporation of BIPM molecule into the ribosomes. The inference that the reagent has been introduced into the ribosomes was further confirmed by the fact that the spectrum (b) is essentially the same on variation of excitation wave-lengths along with the wave region of the absorption of the chromophore of the reagent (a). Fig.1 shows also the fluorescence spectra of ribosomes

excited at 260 mμ before and after the treatment with BIPM (c). The former had the maximum at 337 mμ, whereas the latter had the maximum at the same wave length (362 mμ) as excited at 310 mμ. It has been amply shown that BIPM, being non-fluorescent, reacts specifically and quantitatively with sulfhydryl groups to form characteristic fluorescent adducts (7,8). The above results thus well demonstrate that this reagent has now been introduced at sulfhydryl groups of ribosomes with covalent bond formation.

Fig.2 presents the stoichiometry of the reaction of BIPM with the ribosomes. The cysteine content of the ribosome sample employed was calculated to be approximately 0.18 mM based on reported values (9). The content of sulfhydryl groups in the ribosome proteins obtained by the fluorometrical titration with BIPM as above was close to this expected value. These results may give an additional support of the stoichiometric reaction of BIPM with cysteine residues in the ribosomal proteins.

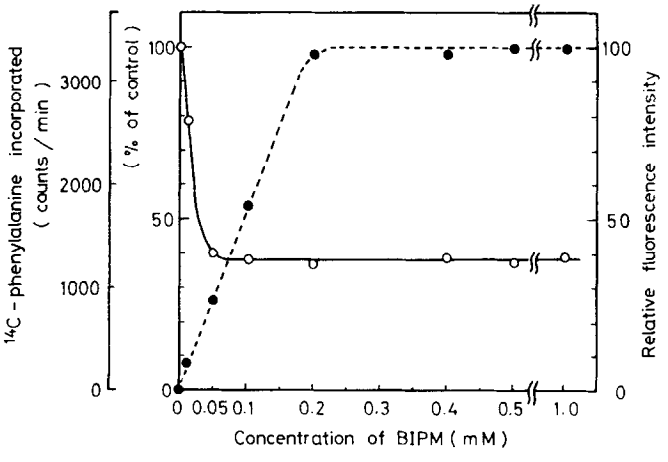

Fig.2. Incorporation of BIPM into the ribosomes and the effect on polyphenylalanine synthesis. Fluorescence intensity (-•---•-) was determined with the diluted BIPM-ribosome solution of OD_{260} 1.0 unit/ml. Excitation wave length, 310 mμ. Assay for the activity of ribosomes in polyphenylalanine synthesis (-o—o-) was made as mentioned in Table I.

Poly-U dependent synthesis of polyphenylalanine by cell-free system of E. coli was also remarkably inhibited by preincubation of the ribosomes with BIPM. The time required to produce a 50% reduction in synthetic activ-

ity was 7-10 minutes, and the enhancement of this inhibition ratio as well as that of fluorescent intensity of ribosomes levelled off entirely after 30 minutes of preincubation. The dotted line in Fig.2 shows the inhibition levels as a function of the amount of BIPM added. When approximately 0.25 molar equivalent of BIPM to the cysteine content was incorporated, the initial rapid loss in the activity reached a limiting value of approximately 40% of the intact level and stayed unchanged on further addition of the reagent. This direct correlation between the inhibition of polyphenylalanine synthesis and the specific modification provides chemical evidence for involvement of cysteine-sulfhydryl groups in cell-free protein synthesis in E. coli system.

It was reported that NEM causes the dissociation of ribosomes into their subunits at millimolar concentrations of the reagent (10). Table I shows the effect of pre-treatment of ribosomes with the concentrations

Table I. Effects of pre-treatment of ribosomes with BIPM or NEM upon the activity in polyphenylalanine synthesis.

Condition of preincubation	^{14}C-phenylalanine incorporated	
	counts/min/mg ribosome	% of control
Ribosomes only	6,735	100
Ribosomes in 10 % monoglyme	6,876	102
Ribosomes plus 1 mM BIPM	2,670	39.6
Ribosomes plus 2.5 mM BIPM	2,709	40.2
Ribosomes plus 5 mM BIPM	1,457	21.6
Ribosomes plus 1 mM NEM	4,093	60.7
Ribosomes plus 2.5 mM NEM	3,935	58.4
Ribosomes plus 5 mM NEM	3,538	52.5
Ribosomes plus 10 mM NEM	2,250	33.4

Ribosomes were pre-treated with or without maleimides under the conditions described in the text, and then analyzed for the ability to synthesize polyphenylalanine. The reaction mixtures of 0.5 ml contained 60 mM Tris-HCl, pH 7.6, 50 mM NH_4Cl, 12 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 1 mM ATP, 0.03 mM each of GTP and CTP, 5 mM phosphoenolpyruvate, 20 µg/ml pyruvate kinase, 40 µg/ml poly-U, 0.05 mM ^{14}C-phenylalanine (10 mC/mmole, The Radiochemical Centre, Amersham, England), 0.05 mM each of other 19 non-radioactive amino acids, OD_{280} 2 units/ml supernatant enzymes, OD_{260} 30 units/ml tRNA's and OD_{260} 20 units/ml control or maleimide-treated ribosomes. Incubation was done at 37 °C for 30 minutes.

higher than 1 mM of BIPM together with the data obtained using NEM. The inhibitory effect by the maleimides, once being saturated, began to increase progressively at concentrations higher than 2.5 mM for BIPM, and 5 mM for NEM. This increase in inhibition may be ascribed to the dissociation of ribosome monomers. Preliminary sedimentation analyses of these ribosome samples showed that this is indeed the case. However, the dissociation has never been observed at the concentrations of NEM and BIPM below 1 mM. These results confirmed that the inhibition of polyphenylalanine synthesis with BIPM at such low concentration as shown in Fig.2 is not due to the dissociation of the ribosomes. Further experiments to elucidate the nature of the modification of ribosomal proteins with the reagent are under way in our laboratory.

Acknowledgement ——— We are grateful to Prof. T. Sekine for his advice, and to Miss C. Obara for her help in some experiments. This work was supported in part by grants from the Takeda Science Foundation and the Commemorative Foundation of Kaiun Mishima.

REFERENCES

1. Heintz,R.L., McAllister,H.C., Arlinghaus,R. and Schweet,R.S., Cold Spr.Harb.Symp.Quant.Biol., 31, 633 (1966).
2. McAllister,H.C. and Schweet,R.S., J.Mol.Biol., 34, 519 (1968).
3. Trout,R.R. and Haenni,A.L., European J. Biochem., 2, 64 (1967).
4. Retsema,J.A. and Conway,R.W., Biochim.Biophys.Acta, 179, 369 (1969).
5. Dunn,A.J., Arch.Biochem.Biophys., 136, 203 (1970).
6. Tissieres,A., Watson,J.D., Schlessinger,D. and Hollingworth,B.R., J. Mol.Biol., 1, 221 (1959).
7. Kanaoka,Y., Machida,M., Ando,K. and Sekine,T., Biochim.Biophys.Acta, 207, 269 (1970).
8. Kanaoka,Y., Machida,M., Kokubun,H. and Sekine,T., Chem.Pharm.Bull. (Tokyo), 16, 1747 (1968).
9. Spahr,P.F., J.Mol.Biol., 4, 395 (1962).
10. Tamaoki,T. and Miyazawa,F., J.Mol.Biol., 23, 35 (1967).