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Ribosomal proteins S2, S6, S10, S14, S15 and S25 are localized on the surface of mammalian 40 S subunits and stabilize their conformation

A study with immobilized trypsin

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Trypsin immobilized on collagen membranes has been used to digest accessible ribosomal proteins of rat liver 40 S subunits. Six proteins (S2, S6, S10, S14, S15 and S25) have been found to be highly exposed on the surface of 40 S particles. They appear to be in close physical contact and localized in the same region of the subunit, most likely protruding at its surface. Electric birefringence reveals that digestion of these proteins results in unfolding of subunits: the birefringence of 40 S particles becomes negative, like that of RNA, the relaxation time undergoes a 15-fold decrease and the mechanism of orientation is drastically modified.

Ribosomal protein; Ribosomal 40 S subunit; Trypsin; Immobilized enzyme; Electric birefringence; (Rat liver)

1. INTRODUCTION

To understand clearly the role of ribosomal proteins in the structure and function of ribosomes, their spatial arrangement within the subunits must be determined as a priority. In comparison with bacterial ribosomes, information on the protein topography of mammalian ribosomes is scarce, essentially because their reconstitution intact from proteins and RNA has been unsuccessful until now. Most available data derive from immune electron microscopy observations and from cross-linking experiments, with their inherent drawbacks. An experimental approach which has proved valuable in ascertaining the accessible histone surfaces of chromatin fibers is the use of immobilized proteases [1–5]. In our laboratory, ribosomal proteins were recently localized on the surface of 60 S mammalian subunits by trypsin covalently bound on collagen membranes and a model of protein topography was proposed [6]. Here, we investigate using this method the accessibility of proteins in 40 S rat liver ribosomal subunits, and show that six proteins are highly exposed at the surface of the particles. In addition, we have used the sensitive electric birefringence technique to demonstrate that drastic structural changes occur in the ribosomes upon digestion of these proteins.

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2. EXPERIMENTAL

Ribosomal subunits from Sprague-Dawley rat livers were prepared according to Blobel and Sabatini [7] as described in [8]. Before the experiments, solutions of 40 S ribosomal subunits were dialyzed at 4°C for 18 h vs PKM buffer (1 mM potassium buffer, 30 mM KCl, 2 mM $MgCl_2$, pH 7.4) and centrifuged at 5000 × *g* for 5 min to remove aggregates.

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Trypsin (EC 3.4.21.4, Sigma) was covalently bound to the surface of water-insoluble collagen films (Centre Technique du Cuir, Lyon) as in [9]. The activity of the immobilized trypsin, determined by the rate of hydrolysis of TAME in PKM buffer, varied from 180 to 750 nmol/min.

Tryptic digestion was initiated by dipping the enzymatic membranes into 10 ml of the 40 S subunit solution, adjusted to a concentration of $5A_{260}$ units/ml, and stopped by removing the membrane. Digestions were performed at 4°C, the reaction medium being maintained under gentle agitation. Aliquots were removed at increasing incubation times for protein analysis. $5A_{260}$ units of 40 S subunits were precipitated by treatment with 10% trichloroacetic acid for 20 min at 4°C. Proteins were extracted via the acetic acid procedure at high magnesium concentration [10] and analyzed by two-dimensional electrophoresis, at pH 8.6 in the first dimension and pH 4.5 in the second [11]. Proteins were stained with Coomassie brilliant blue R250 in 50% methanol and 10% acetic acid.

The apparatus and procedures for transient electric birefringence experiments have been described [12,13].

3. RESULTS AND DISCUSSION

Tryptic digestion of proteins within the 40 S ribosomal subunits could be perfectly modulated by using trypsin-bound membranes of different specific activities. In all cases, the order of degradation of the proteins was the same. Fig.1 shows typical protein electrophoretic patterns for incubation of the subunits with membranes of specific activity equal to 180 nmol/min.

From the initial stages of incubation, proteins S10 and S6 were attacked by the immobilized trypsin: the S10 spot disappeared progressively and S6 was transformed into S6*, S10 probably being degraded more rapidly. Between 20 and 40 min, a spot derived from S2 appeared below S2 while a new spot arising from S15 was observed below S14 and S15. After 70 min, S6 was semi-transformed into S6*, both spots being of nearly equal intensity; a spot, S14*, appeared behind S14, most likely being derived from S14. An electropherogram of the 40 S subunit proteins at this stage of digestion is shown in fig.1b. After 90 min digestion, S10 was no longer visible on the gel and S6 was virtually completely transformed into S6*; the S2*, S14* and S15* spots were as intense as the corresponding original spots; S25 began to grow fainter (fig.1c). After 150 min, the S25 spot was very faint, while S2*, S14* and S15* were still as intense as S2, S14 and S15; thus, S25 was attacked after these proteins, but was degraded more rapidly. Finally, when digestion was prolonged for 300 min, the spots corresponding to S2, S6, S10, S14, S15 and S25 were scarcely detectable if at all while several other proteins were in turn digested, such as S3a, S3b, S7, S8, S17 and S20 (fig.1d).

These results are in relative agreement with previous data obtained with soluble trypsin. According to Arpin et al. [15], the most sensitive proteins are S6, S10, S14-S15, S25, S26 and S28, and proteins S2, S3-S3a-S3b, S5, S6, S7, S10 and S14 in the work of Henkel et al. [16], who observed that S2, S4, S6, S7 and S9 are digested into large resistant peptides. However, under our conditions, S26 and S28 do not undergo degradation and S3a, S3b and S7 are clearly degraded after S2, S6, S10, S14, S15 and S25. Moreover, S25, classified as a 'less sensitive' protein by Henkel et al. [16], is readily accessible to the insoluble enzyme. This discrepancy undoubtedly arises from the different experimental approaches used with either soluble or immobilized trypsin.

Moreover, antigenic determinants of S2 and S6 have been localized on the 'head' and in the groove between the head and the 'body', one epitope of S6 being found in the body of the 40 S ribosomal subunits [17,18]. In contrast, S3-S3a, S5, S7, S9, S17 and S21, localized on the surface of ribosomes by immune electron microscopy, are not found to be easily accessible to immobilized enzymes, suggesting that their proteolytic sites are not exposed at the surface.

Our results fit perfectly with cross-linking data [19,20]; thus, it appears that S2, S6, S10, S14, S15 and S25, the most trypsin-exposed proteins, are all closely adjacent proteins; both sets of data suggesting the presence of a large region protruding from the surface of the 40 S subunits, and composed of at least these six proteins. On the other hand, S2, S6, S14 and S25 have been found to be cross-linked in the ribosomes, to the 28 S RNA [21], or to proteins of the large subunit [22]. Therefore, the bulk formed by the most exposed proteins is located partly on the side of the 40 S subunit involved in the reassociation of both subunits. Moreover, S2, S6, S15 and S25 are cross-linked to 18 S RNA [21,23,24]; hence, the RNA and the proteins appear to be closely intermingled in the protruding region of the 40 S subunits. It is also striking that most of the proteins which are degraded later, such as S3a, S7, S8, S17 and S20, are cross-linked directly to the exposed proteins

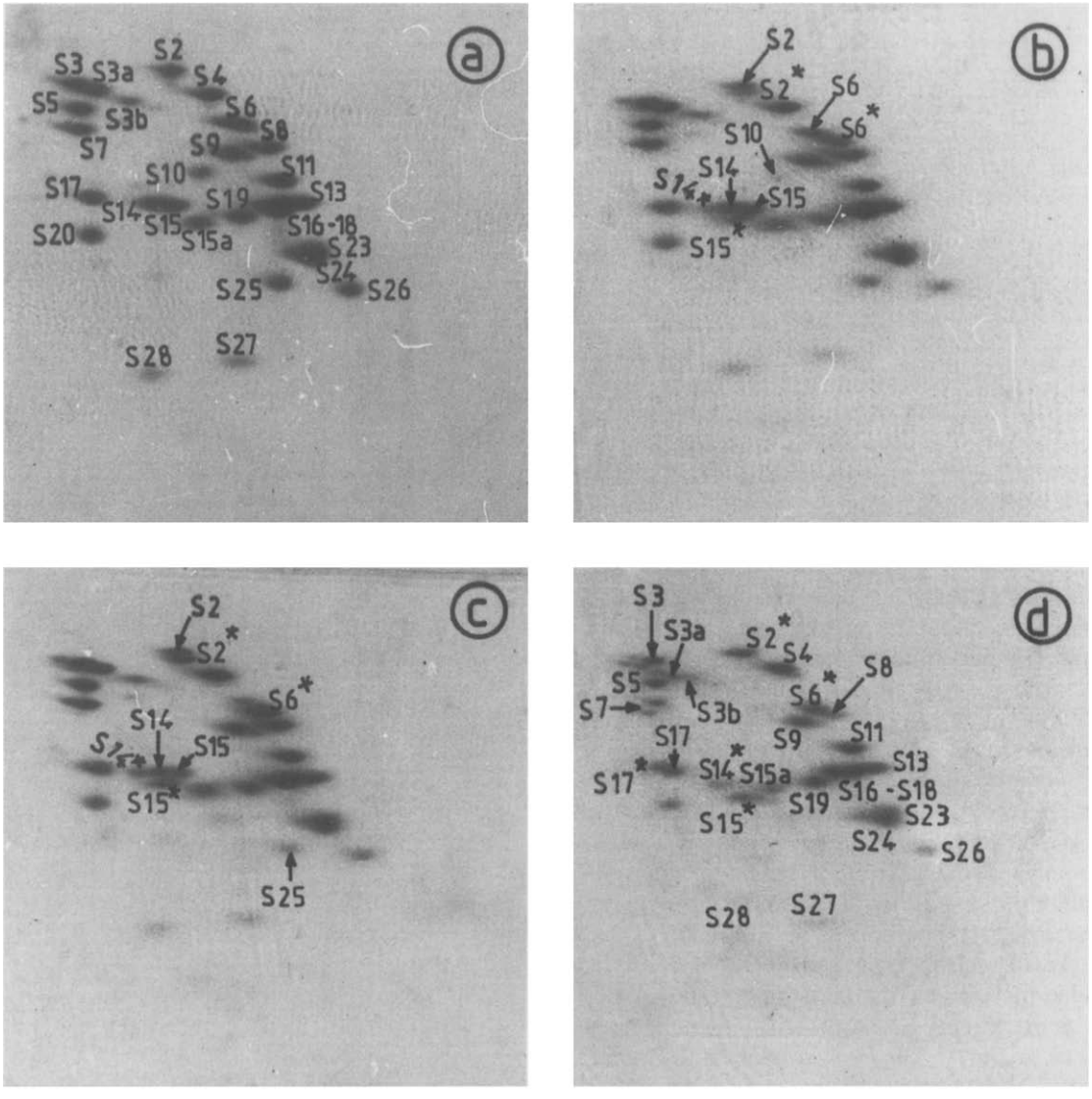

Fig.1. Two-dimensional electrophoresis of proteins extracted from 40 S ribosomal subunits treated with immobilized trypsin, for 0 (a), 70 (b), 90 (c) and 300 min (d). In (a), all 40 S ribosomal proteins are numbered according to the standard nomenclature in [14]. Arrows and stars respectively indicate proteins attacked by the enzyme and the polypeptides which presumably derive therefrom, both being designated by the same number.

[19,20]. Although a cross-link between two proteins does not necessarily mean that they are in close physical contact [19], it does suggest that the most exposed proteins form a protective shield and must therefore be degraded before the second protein layer can be attacked. Our results are thus consistent with the model proposed by Gross et al. [20].

Given the importance of this finding, it was of particular importance to determine whether the removal of these proteins may affect the conformation of subunits. The failure to detect small morphological differences between subunits by physical methods currently used to analyze ribosome structure prompted us to initiate conformation studies via electric birefringence [25]. In contrast to numerous studies on DNA and chromatin, electro-optical techniques have had lit-

tle use in RNA and ribosome research thus far [12,25–31] and to our knowledge data on 40 S subunits have not been reported. As shown for 60 S subunits [10,32], the anisotropy of 40 S particles is clearly positive (fig.2c). It is likely that such an orientation arises from a preferential orientation of the rRNA double-helical segments within the particles [25,27,30]. The Kerr law ($\Delta n_{eq.}$ vs E^2) is obeyed throughout the entire range of applied fields, the specific Kerr constant B having a value of 0.81×10^{-2} esu. The relaxation time is found to be about 31.5 μs (fig.3), reflecting the overall particle orientation, and the curve for the rise kinetics is quite symmetrical with respect to its relaxation counterpart (fig.2c), indicating an orientation due to a pure induced dipole moment. Three criteria characterizing a change in ribosome conformation are revealed upon digestion (figs 2d,3): (i) the birefringence becomes negative ($B = -1.26 \times 10^{-2}$), like that observed for 18 S rRNA but with a much higher amplitude (about 5-fold); (ii) the relaxation time decreases to 2.5 μs; (iii) the mechanism of orientation is due to a nearly pure permanent dipole moment, as shown by a comparison of the rise and decay curves. Degradation of ribosomal proteins S2, S6, S10, S14, S15 and S25 then results in a segmentary orientation of 40 S subunits. This unfolding suggests that accessible proteins play a key role in the organization of 40 S subunits. One can then suppose that any factor that modifies the conformation of these proteins and/or alters protein-RNA interactions should contribute to the tendency to unfold. This includes protein modifications such as phosphorylation of S6 and binding to proteins of tRNA and initiation or elongation factors. This finding is particularly interesting, since there is evidence that the translating ribosome is a dynamic structure and that changes mainly affect the structure of the small subunit, at least for *E. coli* ribosome [33,34].

The goal of future studies is to correlate the surface topology of proteins with their structural role and functional participation in various activities. Digestion under various conditions (activity and specificity) as well as a more functional approach are now required. At this stage we can only state that the 40 S subunits retained about 30% of the initial value of the peptide chain elongation activity after the longest incubation time with immobilized trypsin.

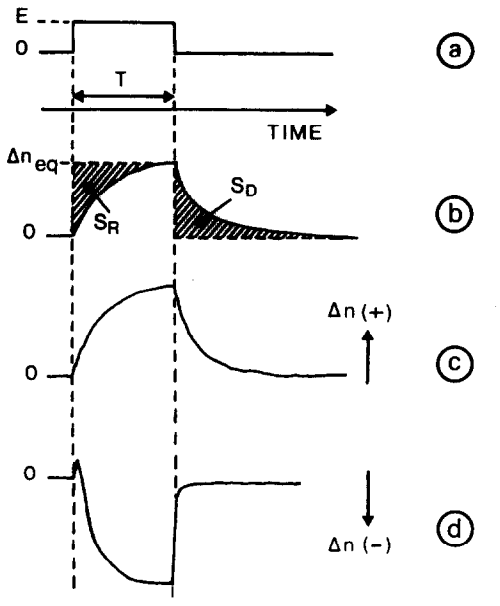

Fig.2. Electric birefringence of 40 S rat liver ribosomal subunits. (a) Electrical rectangular pulse with transition times of 50 ns. (b) Schematic illustration of a birefringence signal; the ratio of the areas under the rise (S_R) and decay (S_D) curves can be related to the mechanism of polarization. S_R/S_D reaches a maximum value of 4 for a pure permanent moment, and is equal to unity for pure induced polarization. (c) Typical birefringence signal observed for 40 S subunits in PKM buffer. (d) Birefringence signal observed for subunits digested for 300 min with immobilized trypsin. Field applied was 3 kV/cm. Pulse duration, T, was equal to 60 (c) or 20 μs (d).

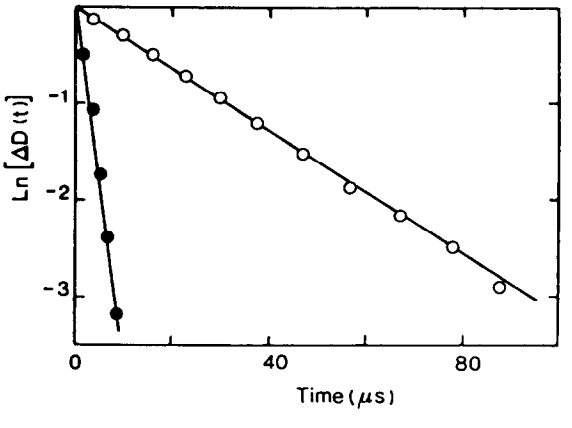

Fig.3. Analysis of the decay of birefringence signals and determination of orientational relaxation times for 40 S ribosomal subunits treated for 0 (○) and 300 min (●) with immobilized trypsin.

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REFERENCES

[1] Marion, C., Roux, B., Pallotta, L. and Coulet, P.R. (1983) Biochem. Biophys. Res. Commun. 114, 1169–1175.
[2] Saccone, G.T.P., Skinner, J.D. and Burgoyne, L.A. (1983) FEBS Lett. 157, 111–114.
[3] Dimitrov, S.I., Apostolova, T.M., Makarov, V.L. and Pashev, I.G. (1986) FEBS Lett. 200, 322–326.
[4] Couturier, R., Hacques, M.F., Ville, A. and Marion, C. (1988) Life Sci. Adv., in press.
[5] Hacques, M.F., Muller, S., De Murcia, G., Van Regenmortel, M.H.V. and Marion, C. (1988) submitted.
[6] Marion, M.J. and Marion, C. (1987) Biochem. Biophys. Res. Commun. 149, 1077–1083.
[7] Blobel, G. and Sabatini, D.D. (1971) Proc. Natl. Acad. Sci. USA 68, 390–394.
[8] Marion, M.J. and Reboud, J.P. (1981) Nucleic Acids Res. 9, 4325–4338.
[9] Coulet, P.R., Julliard, J. and Gautheron, D.C. (1974) Biotechnol. Bioeng. 16, 1055–1068.
[10] Sherton, C.G. and Wool, I.G. (1974) Mol. Gen. Genet. 135, 97–112.
[11] Madjar, J.J., Arpin, M., Buisson, M. and Reboud, J.P. (1979) Mol. Gen. Genet. 171, 121–134.
[12] Marion, M.J. and Marion, C. (1984) Biochimie 66, 111–114.
[13] Marion, C., Roux, B. and Bernengo, J.C. (1984) Makromol. Chem. 185, 1649–1664.
[14] MacConkey, E.H., Bielka, H., Gordon, J., Lastick, S.M., Lin, A., Ogata, K., Reboud, J.P., Traugh, J.A., Traut, R.R., Warner, J.R., Welfle, H. and Wool, I.G. (1979) Mol. Gen. Genet. 169, 1–6.
[15] Arpin, M., Reboud, J.P. and Reboud, A.M. (1975) Biochimie 57, 1177–1184.
[16] Henkel, B., Welfle, H. and Bielka, H. (1974) Acta Biol. Med. Germ. 33, 691–698.
[17] Lutsch, G., Noll, F., Theise, H., Enzmann, G. and Bielka, H. (1979) Mol. Gen. Genet. 176, 281–291.
[18] Bommer, U.A., Noll, F., Lutsch, G. and Bielka, H. (1980) FEBS Lett. 111, 171–174.
[19] Tolan, D.R. and Traut, R.R. (1981) J. Biol. Chem. 256, 10129–10136.
[20] Gross, B., Westermann, P. and Bielka, H. (1983) EMBO J. 2, 255–260.
[21] Nygard, O. and Nika, H. (1982) EMBO J. 1, 357–362.
[22] Uchiumi, T., Kikuchi, M. and Ogata, K. (1986) J. Biol. Chem. 261, 9663–9667.
[23] Uchiumi, T., Terao, K. and Ogata, K. (1983) Eur. J. Biochem. 132, 495–499.
[24] Westermann, P., Nygard, O. and Bielka, H. (1981) Nucleic Acids Res. 9, 2387–2396.
[25] Fredericq, E. and Houssier, C. (1973) Electric Dichroism and Electric Birefringence, Clarendon, Oxford.
[26] Spirin, A.S. (1963) Prog. Nucleic Acid Res. Mol. Biol. 1, 301–345.
[27] Morgan, R.S. (1963) Biophys. J. 3, 253–259.
[28] Golub, E.I., Gauze, C.G., Dvorkin, G.A. and Spirin, A.S. (1963) Dokl. Akad. Nauk. SSSR 149, 446–456.
[29] Golub, E.I. and Nazarenko, V.G. (1967) Biophys. J. 7, 13–24.
[30] Schoentjes, M. and Fredericq, E. (1972) Biopolymers 11, 361–374.
[31] Revzin, A. and Neumann, E. (1974) Biophys. Chem. 2, 144–150.
[32] Marion, M.J. and Marion, C. (1988) submitted.
[33] Spirin, A. (1987) Biochimie 69, 949–956.
[34] Guérin, M.F. and Hayes, D.H. (1987) Biochimie 69, 965–974.