THE INTERACTION OF AURINTRICARBOXYLIC ACID WITH RIBOSOMAL PROTEINS AND ITS APPLICATION TO GEL ELECTROPHORESIS

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1. Introduction

In recent years the classical Al^{3+} reagent, aurintricarboxylic acid (ATA), has found a new application as an inhibitor of chain initiation in protein and RNA synthesis [1,2]. As might be anticipated from the chemical properties of this reagent, its selectivity as an initiation inhibitor depends on the concentration [1,2], and the effect is also influenced by the composition of the system [3,4]. It is usually held that in mammalian systems protein initiation is selectivily inhibited by 10^{-5}–10^{-4} M ATA. At 10^{-3} M, chain elongation is already strongly affected, and the sedimentation properties of the ribosomes are modified [2].

The present experiments indicate that conformational alterations of mammalian ribosomes are induced by ATA at remarkably low concentrations (well below 10^{-4} M), apparently due to the affinity of the reagent to basic ribosomal proteins. These alterations are not necessarily deleterious to individual ribosomal functions. The strong interaction of ATA with basic proteins in solution can be utilized in gel electrophoresis, allowing even minor bands to be detected without conventional staining, and excised with great accuracy for further processing.

2. Materials and methods

2.1. *ATA and ribosomal conformation*

Rat liver ribosomes [5] were suspended in a medium of 75 mM KCl, 5mM $MgCl_2$, 20 mM Tris–HCl buffer (pH 7.7) and 1 mM mercaptoethanol, centrifuged for 7 min at 15 000 *g* and adjusted to 5.5 A_{260} units/ml. After incubation for 10–15 min (35°C) with ATA at different concentrations, the suspensions were diluted with 1.5 volumes of plain medium and centrifuged for 60 min (4°C) at 50 000 rev/min (Spinco rotor 50 Ti). The pellets were suspended in the same medium at 40–50 A_{260} units/ml, and tested for the unmasking of ribosomal proteins by treatment with chymotrypsin (5 mg/ml, 20 min 0°C) as described previously [6]. Proteins were extracted with 0.2 M HCl and analyzed by electrophoresis in 12.5% polyacrylamide gel, pH 4.3 [6–9].

2.2. *ATA as an internal stain in cathodic gel electrophoresis*

Polyacrylamide gel electrophoresis of ribosomal proteins was carried out in 5 × 150 mm columns of 7.5% or 12.5% polyacrylamide gel pH 4.3 [6–9], or (for preparative purposes) in 4 × 100 × 200 mm 7.5% gel slabs. All gels contained 7 M urea. ATA ($2.5–25 \times 10^{-5}$ M, depending on the amounts of protein added) was included from the beginning in the cathode buffer (70 mM β-alanine in 27.5 mM acetic acid). Electrophoresis was for 16 hr at 6–8 V/cm and about 4°C. For preparative purposes the sharp protein bands were excised, homogenized with equal volumes of 3 M HAc and left overnight at 0–5°C. The proteins were recovered electrophoretically [7,10].

Two-dimensional electrophoresis of 7.5% gel columns (or gel sections) containing ATA-protein bands was carried out as described previously [6], using 10% polyacrylamide gel (pH 7.2) with 0.1% sodium dodecyl sulfate in the second (anodic) direction.

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2.3. *Peptidyl transferase assay*

The synthesis of puromycin peptides was analyzed in a system which, in final volumes of 120 μl, contained 4.0 A_{260} units/ml of rat liver ribosomes, 0.1–1.75 M KCl [11,12], 8.5 mM $MgCl_2$, 16.5 mM Tris–HCl buffer (pH 7.7 at 20°C), 1 mM mercaptoethanol and 10^{-5}–10^{-3} M ATA. After pre-incubation for 10–15 min at 35°C, 0.6 μM [^{3}H]puromycin (Amersham, 3.7 Ci/mol) was added, and the complete system was incubated for 4 min at 20°C. Polypeptide-bound puromycin was determined as described by Pestka [13]. Puromycin-induced release of nascent peptides from prelabelled rat liver ribosomes was determined as described previously [11,12] after pre-incubation with ATA as specified above.

3. Results and discussion

When exposed to ATA at increasing concentrations, rat liver ribosomes showed progressive structural disarrangement, as indicated by the unmasking of normally shielded proteins (fig.1). At low ATA concentrations the unmasking primarily involved a protein

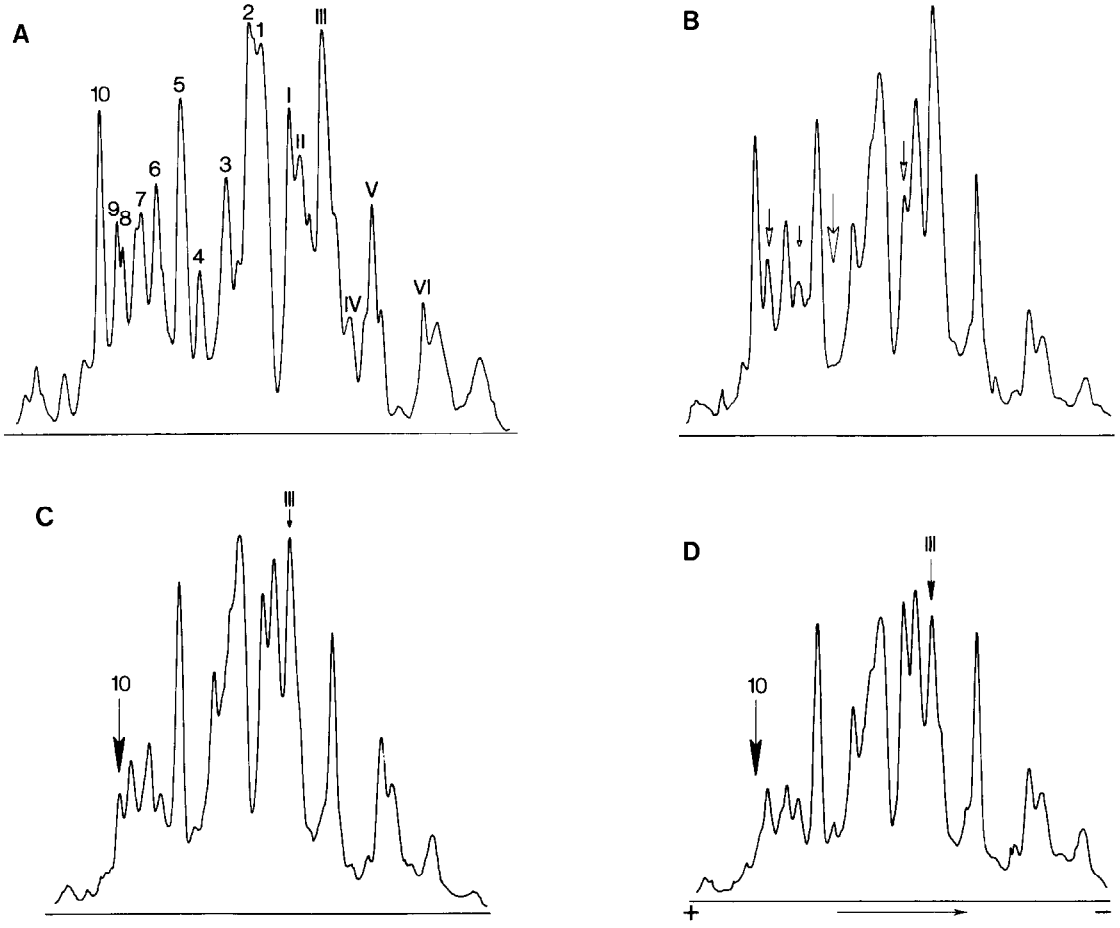

Fig.1. Unmasking of ribosomal proteins by ATA. Rat liver ribosomes were incubated for 15 min. (35°C) with ATA at the concentrations indicated. After repelleting, the ribosomes were tested for protein unmasking by treatment with chymotrypsin (5 μg/ml, 20 min at 0°C). The proteins were extracted and analyzed by electrophoresis in 12.5% polyacrylamide gel (80 μg protein per column). A, B, Control ribosomes before and after chymotrypsin treatment; C, ribosomes incubated with 10^{-4} M ATA; D, ribosomes incubated with 3×10^{-4} M ATA. Black arrows indicate unmasking.

('protein 10') in the large subunit, characterized by particularly high conformational flexibility [6,7]. Under the conditions described, the unmasking of this protein was already noticeable at 10^{-5} M ATA. Although the rate of unmasking increased with increasing temperature, the reaction was not strictly temperature-dependent (cf. [6,7]), and was only moderately influenced by alterations in the K^+ and Mg^{2+} concentrations.

At limiting ATA concentrations the extent of unmasking was inversely correlated with the ribosome concentration. The reaction was also counteracted by added soluble proteins, suggesting that the concentration of free reagent was effectively reduced by interaction with positively charged polypeptide sequences.

This was confirmed by preliminary double-diffusion experiments in agarose gel, which indicated that ATA formed very sharp precipitation lines with basic proteins, e.g. ribosomal protein extracts. A pattern of similar precipitates was obtained in cathodic gel electrophoresis of ribosomal proteins , when ATA was included in the cathode buffer (fig.2). The pattern was very similar to that observed after conventional staining and destaining, except for a general increase in staining intensity towards the cathodic end of the pattern (figs.1A, 2A,C). The sensitivity of detection was comparable to that of conventional methods (figs.1A, 2B,C). From 7.5% gel slabs the coloured bands could be excised with great accuracy, and the proteins recovered on a preparative scale (fig.2A,B). The ATA–protein complex was dissociated by SDS, and after equilibration with SDS-buffer 7.5% gels could be used directly for two-dimensional electrophoresis [6].

The difference between free and structure-associated ribosomal proteins in their susceptibility to ATA may reflect the extensive shielding of positively charged groups in the intact ribosomes [9]. The restricted availability of internal protein sites is further illustrated by the fact that neither puromycin binding, nor puromycin-induced release of nascent peptides was impaired after incubation of rat liver ribosomes with ATA under the conditions described in fig.1. On the contrary, there was a noticeable stimulation (data not shown). The preferential inhibition of initiation by ATA at low concentrations may be related to the basic character of at least one of the initiation factors [14] in combination with their less effective structural shielding.

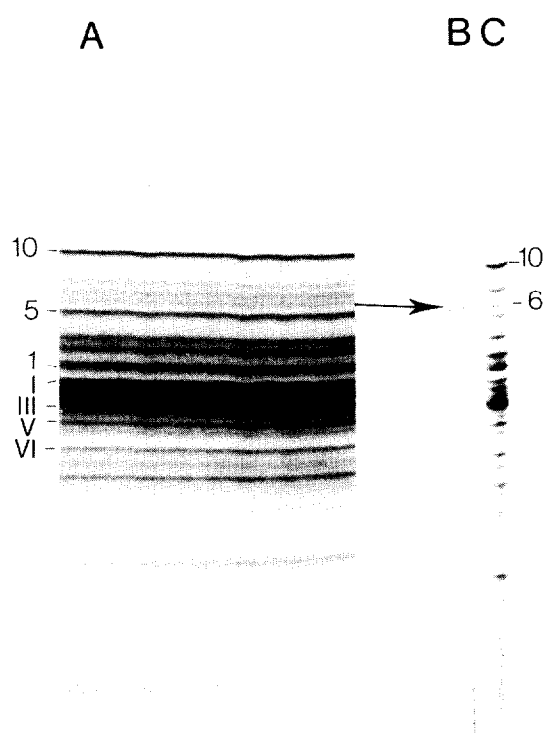

Fig.2. The use of ATA as an internal stain in cathodic gel electrophoresis. A, Section of 7.5% gel slab used for the preparation of rat liver ribosomal proteins; B, sample (1 μg) of protein recovered from the excised, minor band '6', and analyzed by electrophoresis in 12.5% gel; C, control gel (12.5%) with 50 μg ribosomal protein. The concentration of ATA in the cathod buffers was 25×10^{-5} M (A) and 2.5×10^{-5} M (B,C).

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References

[1] Mathews, M. B. (1971) FEBS Lett. 15, 201.
[2] Blumenthal, T. and Landers, T. A. (1973) Biochim. Biophys. Res. Commun. 55, 680.
[3] Huang, M. T. and Grollman, A. P. (1972) Mol. Pharmacol. 8, 111.
[4] Leader, D. P. (1972) FEBS Lett. 22, 245.
[5] Rendi, R. and Hultin, T. (1960) Exp. Cell Res. 19, 253.
[6] Hultin, T. and Sjöqvist, A. (1971) Comp. Biochem. Physiol. 40B, 1011.
[7] Hultin, T. (1972) Biochim. Biophys. Acta 269, 118.
[8] Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) Nature 195, 281.
[9] Östner, U. and Hultin, T. (1968) Biochim. Biophys. Acta 154, 376.
[10] Kaltschmidt, E. and Wittman, H. G. (1969) Anal. Biochem. 30, 132.
[11] Hultin, T. (1962) Biochim. Biophys. Acta 61, 916.
[12] Hultin, T. (1966) Biochim. Biophys. Acta 123, 561.
[13] Pestka, S. (1972) Proc. Natl. Acad. Sci. U.S. 69, 624.
[14] Staehelin, T. (in press) Acta Biol. Med. Germ.