RAT LIVER DNA BINDING PROTEINS: PHYSIOLOGICAL VARIATIONS

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Received 12 July 1979
Revised version received 20 August 1979

1. Introduction

Purification, physical and functional properties of regenerating rat liver helix destabilizing (HD) protein (also called unwinding protein) have been described [1–3]. In these studies, the protein (25 000 dalton subunit) was found to destabilize the double helix of poly[d(A–T) · d(T–A)] and of some other synthetic nucleic acids, and to stimulate homologous DNA polymerases α and β [2]. These properties allowed us to compare the protein to other known HD proteins from eukaryotes and from prokaryotes (reviewed [3–11]).

The in vitro stimulation of DNA polymerases argued in favor of the intervention of the protein in DNA replication processes. If so, since regenerating liver cells are in an active phase of DNA synthesis, it could be first predicted that the biosynthesis of the protein should be stimulated in these rapidly dividing cells. The question arose whether or not the protein was synthetized in stationary phase cells of rat liver.

A single strand specific DNA binding protein with the same subunit molecular weight was actually found (in similar amounts) in normal rat liver.

Here, we describe preliminary results about the structure and the properties of this protein, called S25, as compared to the protein isolated from regenerating rat liver (HD25).

2. Materials and methods

2.1. *DNA binding protein, enzymes, nucleic acids and nucleotides*

Rat liver HD protein and DNA polymerases α and β were purified as in [1,3,13]. HD protein was >97% pure as judged by SDS–polyacrylamide gel electrophoresis. DNA polymerases α and β were obtained at spec. act. 30 000 units/mg and 20 000 units/mg, respectively. One unit is defined as the amount of DNA polymerase able to convert one nanomole of total nucleotide per hour at 37°C into insoluble material with poly(dC) · oligo(dG) as a template.

Poly[d(A–T) · d(T–A)] was purchased from Boehringer (Mannheim). Poly- and oligo-deoxyribonucleotides were from P.L. Biochemicals (USA). Poly(dC) · oligo(dG) and poly(dA) · oligo(dT) were prepared by hybridization. ^{3}H-Labelled deoxyribonucleoside triphosphates were obtained from New England Nuclear (USA).

2.2. *Sucrose velocity sedimentation*

Protein samples (100 μl) equilibrated by dialysis against buffer A (50 mM Tris–HCl (pH 7.6), 2 mM 2-mercaptoethanol) containing 0 or 200 mM KCl, were layered onto 5 ml of 5–20% sucrose gradients prepared in the same buffers and sedimented at 45 000 rev./min for 16 h at 4°C in a SW50.1 rotor; 180 μl fractions were collected, dialyzed against buffer A, and analyzed for their capacity to bind the DNA to nitrocellulose filters. Alcohol dehydrogenase (7.4 S), detected by enzymatic activity, bovine serum albumin (4.7 S) and chymotrypsinogen (2.5 S), detected by their A_{280} were used as markers.

2.3. *Acid–urea gel electrophoresis*

The technique used was a modification of Panyim and Chalkley's method for high resolution gel electrophoresis of histones [12]. The solutions used gave final conc. 15% acrylamide and 2.5 M urea. Electro-

 Elsevier/North-Holland Biomedical Press

phoresis was performed at room temperature with 0.9 N acetic acid as a running buffer in a standard slab gel apparatus. After a 6 h prerun at 100 V, gels were run at 150 V for 7 h and stained with Coomassie Blue R250.

2.4. *Assays*

2.4.1. Poly[d(A–T) · d(T–A)] melting assay

This was performed essentially as in [1] in 250 μl total vol. containing 5 mM KH_2PO_4/NaOH (pH 7.9), 10% glycerol, 1 μg poly[d(A–T) · d(T–A)] in 20 μl 5 mM Tris/HCl (pH 8.8), 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 50 or 500 μM $MgCl_2$, and 0–15 μg HD protein. Absorbances in microcuvettes were monitored at 260 nm in a Gilford 240 Spectrophotometer; temperature within the cuvettes was recorded on the same chart.

2.4.2. DNA polymerase assay

This was performed as in [2].

2.5. *Proteolytic analysis of purified proteins*

Cleavage of protein samples was carried out at 37°C for 30 min by addition of increasing amounts of *Staphylococcus aureus* protease [14] in 10 mM phosphate buffer (pH 7.9). The reaction was stopped by addition of 2-mercaptoethanol and SDS to final conc. 10% and 2%, respectively, and the samples boiled for 2 min. Of each sample 4–5 μg were loaded onto a 4–20% acrylamide gradient gel containing SDS. Electrophoresis was performed as in [15].

3. Results

3.1. *Purification and native state of the protein from normal rat liver (S25)*

Using the purification procedure described for regenerating rat liver [1] (i.e., elution through double-stranded then single-stranded DNA cellulose columns) we selected from normal rat liver a protein for its specific affinity for single-stranded DNA. The subunit molecular weight of this protein was 25 000 precisely, as was that of the protein from regenerating liver. Moreover, the elution patterns from phosphocellulose columns were also identical. Finally, both proteins seemed to be present in similar amounts in

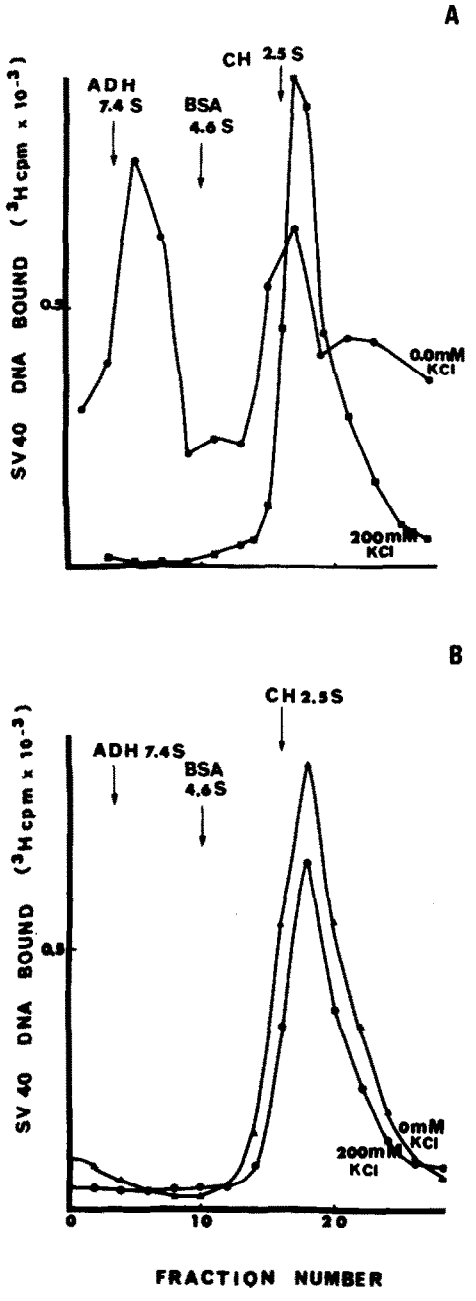

Fig.1. Sucrose velocity sedimentation of HD25 and S25 at various ionic strengths. Protein samples, 5 μg HD25 or S25, 200 μg alcohol dehydrogenase (ADH), 500 μg bovine serum albumin (BSA) and 500 μg chymotrypsinogen (CH) were sedimented for 16 h at 45 000 rev./min in a SW50.1 rotor (section 2). Fractions were collected from the bottom to the top. (A) HD25: (●——●) 0 mM KCl; (■——■) 200 mM KCl. (B) S25: (▲——▲) 0 mM KCL; (●——●) 200 mM KCl.

normal and regenerating liver cells (10^6 copies/cell). It had been shown that the protein from regenerating liver (HD25) exists, at low ionic strength, as a tetramer in equilibrium with a monomer [1]. In order to look at the native state of S25, the molecule was sedimented in parallel with HD25 for 16 h at 45 000 rev./min in a neutral sucrose gradient (5–20%). As shown in fig.1, no tetrameric form was observed for S25: even at low ionic strength, the protein, in contrast with HD25, remained in a monomeric state.

3.2. *Thermal melting of poly[d(A–T) · d(T–A)]*

Double helix destabilization in the presence of regenerating rat liver HD protein has been described [1]. Strand separation was monitored by the increase in A_{260}, as temperature increased. In salt and magnesium concentrations used here (section 2), the melting of poly[d(A–T) · d(T–A)] normally occurred at 58°C (fig.2). In the presence of HD25, denaturation occurred at 45°C, and was reversible when temperature decreased. In contrast (fig.2), in the presence of S25, destabilization was not observed. Moreover, melting took place at 61°C, providing that

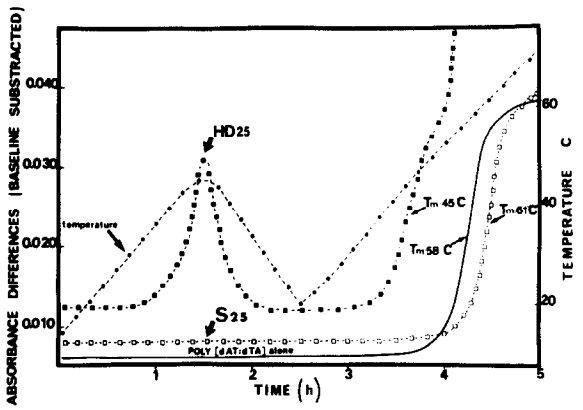

Fig.2. Thermal denaturation of poly[d(A–T) · d(T–A)] in the presence of normal or regenerating rat-liver DNA-binding proteins. Final conc. $MgCl_2$ 0.5 mM. Cuvette 1 contained in a 250 μl total vol. all components (section 2) except nucleic acid and protein. Cuvettes 2, 3 and 4 contained 1 μg poly[d(A–T) · d(T–A)] and, respectively, 0, 10 μg S25 and 10 μg HD25. The absorbance of cuvette 1 (260 nm) was subtracted and that of other cuvettes was arbitrarily shifted to clarify the diagram. (•——•) Temperature program; (——) hyperchromicity of poly[d(A–T) · d(T–A)] alone; (□——□) hyperchromicity of poly[d(A–T) · d(T–A)] in the presence of S25; (■——■) hyperchromicity in the presence of HD25.

poly[d(A–T) · d(T–A)] is slightly stabilized in these conditions.

In other experiments (not shown) where poly[d(A–T) · d(T–A)] was melted at 43°C (50 μM Mg^{2+}), denaturation occurred at only 59°C in the presence of S25.

3.3. *In vitro stimulation of DNA polymerases*

The in vitro stimulation of DNA synthesis observed in the presence of an homologous helix destabilizing protein HD25 was reported [2,3]. Both α- and β-polymerases were stimulated on the synthetic templates used, but the extent of stimulation observed was critically dependent on the HD protein/DNA ratio and the nature of the template-initiator. In the same conditions, S25 was found to inhibit DNA polymerase-α on poly(dA) · oligo(dT) and poly(dC) · oligo(dG) templates. The inhibition by S25 was a non-linear function of binding protein/DNA ratio. DNA polymerase-α activity decreased rapidly at low protein/DNA ratios (0–0.5, w/w) and more slowly at higher ratios (>0.5, w/w). A slight stimulation of DNA polymerase-β was still detected in the presence of poly[d(A–T) · d(T–A)] but the β-enzyme was

Table 1
Effect of rat liver DNA-binding proteins on DNA synthesis catalyzed by DNA polymerase-α and DNA polymerase-β

	DNA polymerase activities (%)		
Template	α	α + HD25	α + S25
Poly(dA) · oligo(dT)	100	–	10
Poly(dC) · oligo(dG)	100	140	35
	β	β + HD25	β + S25
Poly[d(A–T) · d(T–A)]	100	280	124
Poly(dC) · oligo(dG)	100	142	40

Reaction mixture contained, in 30 μl total vol.: 50 mM Tris–HCl (pH 8.6) (or pH 7.0 for poly(dA) · oligo(dT)); 1 mM $MgCl_2$; 1 mM KCl; 50 μg/ml bovine serum albumin; 200 μM d[^{3}H]TTP or d[^{3}H]GTP (1 μCi/assay); 50 μm poly(dA) · oligo(dT), 50 μM poly[d(A–T) · d(T–A)] or 100 μM poly(dC) · oligo(dG). After addition of 1 μg HD25 or S25 protein, the mixture was incubated for 10 min at 4°C. DNA polymerases α or β (0.1–0.25 units) was added and the mixture further incubated for 30 min at 37°C. 10 μl mixture was collected on glass fiber filters and the acid-insoluble material was counted

inhibited by S25 on a poly(dC) · oligo(dG) template as was the α-enzyme (table 1). Thus, the difference between HD25 and S25 properties was also revealed by these experiments.

3.4. *Subspecies in HD25 and S25*

Keeping in mind the large discrepancy in some of the functional properties of HD25 and S25, we have investigated the electric charge of both proteins in their native state. On a non-denaturing 10% polyacrylamide gel, run at pH 8.0, HD25 and S25 migrated as a single-band, nearly at the same position. At pH 2.7 in 2.5 M urea (fig.3), we found two main subspecies in HD25, an acidic and a basic one, and a main basic species in S25 that comigrated with the basic subspecies of HD25. The acidic species present in HD25 accounted for 10–30% of the total protein, depending on the preparation.

Protein bands were cut out of the gel and submitted separately or as a mixture to SDS–polyacrylamide gel electrophoresis. In all cases a single protein band at 25 000 daltons was revealed.

In order to determine if HD25 and S25 were related, both proteins were submitted to limited proteolysis (section 2) and the products analyzed in SDS–polyacrylamide gel electrophoresis. As shown in fig.4, the pattern of proteolytic fragments was the same for HD25 and S25, but, in the latter, the acidic band was minor, so that the experiment only revealed the analogy between the basic species of HD25 and S25.

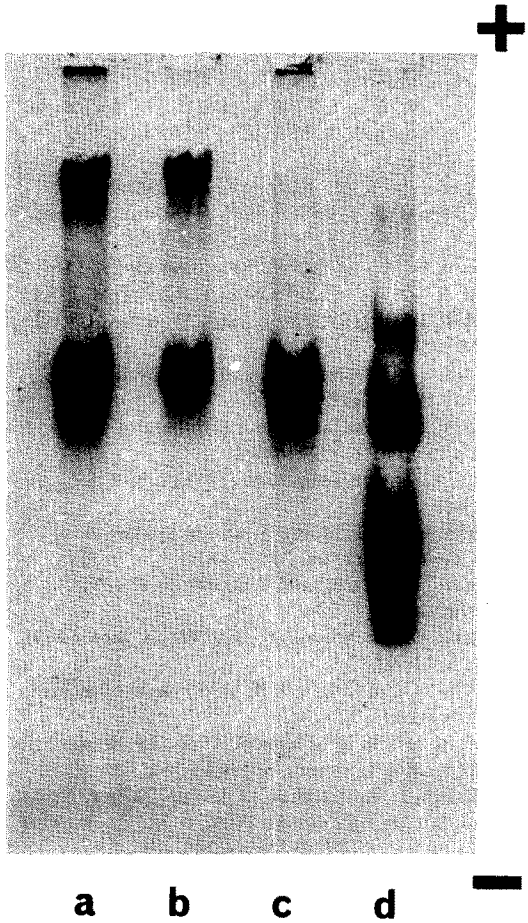

Fig.3. Acetic acid–urea polyacrylamide slab-gel electrophoresis. HD25 (7–10 μg) and S25 (7 μg) were submitted simultaneously to electrophoresis under the conditions in section 2. (a,b) HD25; (c) S25; (d) rat liver histones.

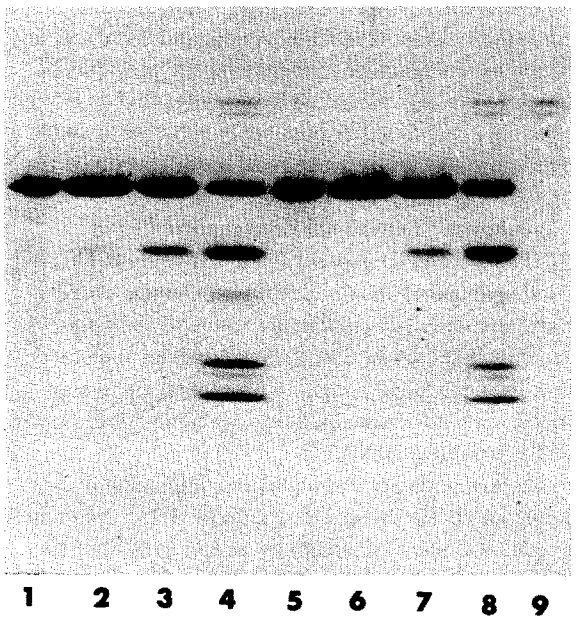

Fig.4. Digestion patterns of HD25 and S25. HD25 and S25, at 0.1 mg/ml were incubated at 37°C for 30 min with increasing concentrations of *Staphylococcus aureus* protease as indicated. Of each sample 40 μl were loaded onto an SDS gel (5–15% acrylamide). (1) S25 without treatment; (2–4) digestions of S25 with final conc. 0.1 μg/ml, 1 μg/ml, 10 μg/ml of protease; (5) HD25 without treatment; (6–8) digestions of HD25 with final conc. 0.1 μg/ml, 1 μg/ml, 10 μg/ml of protease; (9) *Staphylococcus aureus* protease at 10 μg/ml (0.5 μg).

4. Discussion

The experiments described here revealed the existence of a single-strand-specific DNA binding protein in normal rat liver cells (S25). In contrast with the HD protein isolated from regenerating liver by the same purification procedure [1], S25 does not exhibit the usual main properties of a helix destabilizing protein (i.e., lowering the T_m of DNA and stimulating homologous DNA polymerases). Moreover, it is able, in some experimental conditions, to stabilize the double helix and to inhibit the action of DNA polymerases. These effects may reflect an in vivo function of protein S25 or be only due to the binding of the protein to DNA. In the latter case, the protein from normal rat liver may be involved in another function than DNA replication, for instance (since it is rather abundant in the cell and found in nuclear extracts) it can play a structural role within chromatin.

Our results indicate that HD25 and S25 share a common basic polypeptide. Acidic and basic species found in regenerating liver may be the products of unrelated genes, the acidic being synthetized only in dividing cells. Another possibility is the occurrence of the acidic species by a chemical modification of the preexisting basic polypeptide.

A similar situation has been reported for a 24 000 dalton HD protein from calf thymus [11,16] where subspecies bearing different electric charges were revealed. The similarities in tryptic peptide maps of these acidic and basic subspecies indicated that they are closely related. Moreover, these subspecies in calf thymus had not the same effect on the melting point of DNA.

Experiments are in progress to determine if the acidic and basic subspecies found in HD25 are related or not, and the mechanism by which an active HD protein occurs when rat liver cells enter a phase of DNA synthesis.

Acknowledgements

This work was supported by grants from the Délégation à la Recherche Scientifique et Technique (A 650 1524), from the Centre National à la Recherche Scientifique (ATP A 655 3724) and from the Institut National de la Santé et de la Recherche Médicale (ASR no.-1-016). We thank Dr Bruce Alberts, University of California, San Francisco, for his helpful suggestions. DNA polymerase-α and DNA polymerase-β were generous gifts from Dr M. Méchali and Dr J.-M. Rossignol, respectively.

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