A Study of the Non-histone Proteins of the Nucleo-Chromatin Complex

Progress Report

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This request for continuation of my research contract to study eukaryotic DNA-unwinding proteins (DUP) describes the work to be done during the coming year. I propose to: (a) continue the physico-chemical characterization of the 29000 dalton DUP purified from rat liver during the previous year, (b) identify and purify other species of DUPs, and examine their structural relationships by an immunological approach using the antiserum already prepared against the 29K-DUP, and (c) continue characterization of the DNA binding and unwinding reaction with specific emphasis on natural DNAs.

The above abstract is reproduced from the request for continuation of this contract submitted in May 1978. During the past 18 months we have accomplished the proposed objectives in greater detail than anticipated, and have generated additional data that will enable us to take multiple approaches to elucidate the function and regulation of the DNA unwinding protein. Recently, the investigators in this field have felt that the term Helix Destabilizing Protein (HDP) reflects the activity of this protein more accurately. Therefore, the term HDP will be employed henceforth instead of DUP. Our work on HDP, described in the previous progress report (SRO-644-18) and accomplished to date, has resulted in two presentations at national meetings (see SRO-644-19 submitted last year and DOE/EV/00644-21 accompanying this report) and four manuscripts to be submitted together for publication. These manuscripts concern: (1) purification and characterization of HDP from soluble and particulate phases of normal and regenerating rat liver; (2) parameters and specificities of the nucleic acid helix destabilizing activity of this protein; (3) interrelationships between amino acid side-chain modifications and subunit dissociations with the activity of the protein; and (4) development and applications of a radioimmunoassay (RIA) and other immunological techniques for quantitation, cellular localization, and determination of tissue and species specificity of HDP. These data are summarized below.

Purification and properties of HDP

We have purified HDP to molecular homogeneity from soluble and particulate (presumably bound to chromatin) phases of both normal and regenerating adult rat liver. The procedure employed is simple and rapid, and yields large amounts of pure protein. The various HDP preparations appear to be similar with respect to the following properties.

(a) Molecular weight and other physical parameters - The native HDP has a molecular weight of 110,000 as determined by sedimentation equilibrium analysis (Model E Spinco Ultracentrifuge) and 117,000 as determined by gel filtration on Sephadex G150. Following denaturation in sodium dodecylsulfate and reduction with mercaptoethanol the protein behaves as a homogeneous polypeptide of 30,000 daltons. Thus, the native HDP is a tetramer of about 120,000 daltons with identical subunits. The subunits exhibit tight interaction since even in 2M NaCl the HDP has a molecular weight of the tetramer. From Sephadex G150 chromatography we calculate its Stokes radius to be 42.5 Å and diffusion coefficient to be 1.33.

In equilibrium isoelectric focusing the HDP bands in the basic region. However, we have been unable to determine its exact pI since the linearity of the pH gradient breaks down in the band area. In non-equilibrium pH gradient gels also it bands as a single component. Based on these data and on its insolubility at pH 8.6 we estimated its pI to be ~8.6. Basic nature of HDP is also corroborated by its mobility in cationic nondenaturing gels; these data have also shown that it is not any of the histones.

(b) Amino acid composition - HDP contains a preponderance of acid over basic residues; but since in native form it is slightly basic, some of its acidic residues must exist in their amidated forms. Other composition features of interest are, (i) presence of four tryptophans, which

enable exploitation of fluorescence quenching in DNA interaction analysis (currently in progress in collaboration with Dr. Richard Karpel); (ii) presence of five methionines, which enable generation of limited peptide fragments by cyanogen bromide; and (iii) other amino acids with side chains that allow in vitro chemical modifications (see below). Also, 40% of residues are hydrophobic, which may be responsible for the stability of the tetrameric form and play a role in nonelectrostatic interactions with DNA.

(c) Other properties - (i) The HDP preparations do not exhibit any measurable nuclease activity. This was analyzed by monitoring release of radioactivity by their action on ^{32}P -DNA labelled to very high specific activity. This was an important control to rule out DNA degradation as explanation for the observed hyperchromicity in their activity assay (see below). (ii) We have also ascertained that the HDP is not phosphorylated in vitro by cyclic AMP and GMP dependent kinases. (iii) It is water soluble and fully active after lyophilization.

Helix destabilizing activity

Preferential binding to single stranded DNA is a property common to all HDP. Presumably this affinity shifts the helix $\leftrightarrow$ coil equilibrium of duplex in favor of the denatured conformation. The helix destabilizing activity is most commonly monitored by following protein induced depression of thermal denaturation temperature (T_m) of DNA. We have reported previously (SRO-644-18) that our HDP depresses the T_m of synthetic poly d(AT) far below room temperature. This allows us to monitor the protein activity by following hyperchromic shifts ($\Delta A_{260\text{nm}}$) upon its mixing with poly d(AT). We have measured the HDP activity on various synthetic and natural DNA and

report the following observations:

(a) It binds poly d(AT) cooperatively, and melts the duplex. Under similar conditions it does not bind poly d(G-C) at all. HDP binds and melts poly d(AT), poly d(A)·d(T), and poly r(A-U) equally.

(b) Although it does not melt natural DNAs at room temperature, it interacts loosely and lowers their T_m by about 20°C; the exact value being a function of the G+C content of the DNA. Six different DNAs with G+C content in the range of 25-72% were tested. It appears that the protein binds preferentially to A·T rich sequences in DNA, and facilitates initiation of duplex melting.

(c) The depression of T_m is dependent upon mono and divalent cations. This is most dramatic with melted d(AT)-HDP complex, which upon addition of salt exhibit rapid hypochromic shift. This was previously interpreted to result from dissociation of HDP and renaturation of poly d(AT). However, more recent data suggest that the cations may bring about steric changes in the complex such that the bases are free to pair without dissociation of the HDP from poly d(AT).

Structure-function relationships

The question of structure-function relationships is universal in molecular biology. We have recently begun to examine the effects of side-chain modification of HDP on its activity, and to relate this information to the alterations in its subunit structure. Preliminary data obtained so far showed the following.

(a) Acylation of ϵ -NH₂ groups (acetylation and succinylation) lowers the activity of the protein; acetylation being more effective than succinylation. Even with maximal modification, however, the protein retains

tetrameric conformation. Thus changes in activity are probably due to minor local modifications in change and conformation rather than due to subunit dissociation.

(b) Reductive methylation has no effect on structure and activity.

(c) Selective iodination of tyrosine residues also inactivate the protein; the degree of inactivation depending upon the fraction of tyrosines affected. Contrary to acylation, gross structural changes (aggregation as well as subunit dissociation) are apparent in iodinated protein.

(d) In native protein only three of a total of four tryptophans are accessible to oxidation by N-bromosuccinimide. This modification also results in drastic inactivation.

(e) In contrast to the above, the protein is refractive to treatment with sulfhydryl reagents. Thus the six cysteins are either "buried" or in disulfide links.

(f) Inactivation of the protein with urea and guanidine hydrochloride causes dissociation of subunits only at high concentrations of the reagent. For example, 1M guanidine-HCl inactivates the protein but retains it in tetrameric state. Therefore, as with acylation (see above), local and minor conformational alterations suffice to inactivate it.

These initial studies suggest that the activity of HDP depends on a two step process: (i) binding via electrostatic interaction between cationic residues of protein and anionic phosphates of DNA, and (ii) helix destabilization due to intercalation of aromatic amino acids at specific base pairs. Clearly these are only preliminary indications that will require detailed study in the future.

Immunological studies

We have succeeded in immunizing two rabbits with HDP. The two anti-HDP sera cross react with normal and regenerating HDP and show them to be immunologically identical (no spurs in Ouchterlony immunodiffusion precipitin lines). The anti-sera also react with mouse liver single-stranded DNA binding proteins (DBP), but the data indicate the mouse and rat HDP to be not identical (spurs). It appears that the two antisera are directed against two different antigenic determinants since, while both cross react with rat and mouse DBP, only one of them cross reacts with drosophila embryo and human (HeLa) cell DBP. These differences will aid in our future studies on species specificity of HDP.

We have also employed these sera to develop a highly sensitive radio-immunoassay. The two sera differ in their titer, which are 1:250,000 and 1:80,000 (dilutions that give 50% binding to ^{125}I -HDP). With the high titer anti-serum we can detect as little as 1 mg/ml of HDP in total extracts. Application of this RIA to quantitate HDP in various rat tissues showed dramatic variability among muscle, liver, spleen, brain, kidney and testis. Functional significance of this is not clear at present. Quantitation of HDP in liver regenerating over a 48 hr period showed no significant variability in total tissue. Experiments in progress will tell us whether there are changes in subcellular compartmental distribution; for example, cyto $\rightarrow$ nuclear $\rightarrow$ chromatin translocations.

The immunological methods we have adapted will enable us to explore quantitative alterations, tissue and species specificities, intracellular localization, and turn-over rates of HDP in hepatocytes under varying metabolic conditions.

Other studies

We have supplied our HDP to: Dr. S.H. Kim (U. Calif., Berkeley), who is attempting to crystallize it; Dr. R. Karpel (U. MD), who is comparing it to bacteriophage T4-HDP by fluorescence quenching and circular dichroism; Dr. R. Wartell (Ga. Tech.), who is using it in Laser-Raman spectroscopy; and Dr. D. Vizard (Univ. Texas), who has computer technology for fine structure resolution of HDP induced changes in thermal denaturation profiles of DNA.

In summary, I feel this has been a very productive contract period during which some segments of our project were completed, and new information was obtained to provide basis for future evolution of this problem. Specific work to be undertaken is described in the accompanying proposal for continuation of this contract.