Chromatin-Bound Protease: [^{3}H]Diisopropyl Fluorophosphate Labeling Patterns of Chromatin †

Donald B. Carter, Peggy H. Efird, and Chi-Bom Chae*

ABSTRACT: The nuclei and chromatin of rat liver contain three major proteins reacting with diisopropyl fluorophosphate (DFP). The molecular weights of the three proteins determined by gel filtration in the presence of sodium dodecyl sulfate and sodium dodecyl sulfate–polyacrylamide gel electrophoresis are 70 000, 60 000, and 25 000. The chromatin isolated from whole liver, instead of nuclei, contains an additional DFP-binding protein whose molecular weight is 100 000 in the presence of sodium dodecyl sulfate and β-mercaptoethanol. The small molecular weight DFP-binding protein can be fractionated from chromatin by 0.25 N HCl and was found to be a protease which is active in the most commonly used solution for chromatin dissociation, that is, 2–3 M NaCl–5 M urea. This enzyme appears to be the major DFP-binding chromatin-bound protease in the chromatin of most rat tissues. The acid-soluble protease is converted from a 25 000-dalton form to a 20 000-dalton form during 0.25 N HCl acid extraction from chromatin, which retains proteolytic activity.

Several investigators have reported the presence in chromatin of proteases which are inhibited by diisopropyl fluorophosphate (DFP), the active site serine phosphorylating reagent. Furlan and Jericijo (1967) isolated a protease from calf thymus chromatin having a molecular weight around 24 000 which is inhibited by DFP. Chong et al. (1974) have reported the isolation of a 200 000-dalton protease from rat liver chromatin prepared from whole tissue which is also inhibited by DFP. Carter and Chae (1976) have investigated the proteolytic degradation of chromatin in salt and urea by chromatin-bound protease and the proteolysis is inhibited by DFP. Also the proteolytic degradation of chromatin at an ionic strength close to that of the nucleus, that is 0.15–0.3 M (Siebert, 1966), is inhibited by DFP (Furlan et al., 1968).

These results suggest that the chromatin-bound protease possesses an active-site serine. The investigation of DFP inhibition of hydrolytic enzymes has shown that in every instance the exclusive phosphorylation of a single serine residue per catalytic site without reaction with other residues is concurrent with loss of enzyme activity (Cohen et al., 1967). Therefore, studies on the labeling patterns of chromatin with radioactive DFP will provide useful information on the possible multiplicity of chromatin-bound proteases.

In this report we examined the DFP-binding proteins of nuclei, chromatin prepared from nuclei and whole tissue, and chromatin isolated from various tissues. We demonstrated that one of the DFP-binding proteins, which is the major DFP-binding protein in the chromatin of most tissues, is a protease which is active in 2–3 M NaCl–5 M urea, a most commonly used solution for dissociation of chromatin.

Materials and Methods

Materials

Liver, thymus, kidney, testis, and lung were obtained from 2–3 month old Sprague-Dawley rats and Dr. H. P. Morris, Howard University, Washington, D.C., kindly supplied ACI rats bearing hepatoma 9121. All tissues were used fresh.

Bio-Gel A-5m (200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California; Sephadex G-75 was from Pharmacia Fine Chemicals, Piscataway, New Jersey; [^{3}H]-diisopropyl fluorophosphate (3.9 Ci/mmol) and NCS tissue solubilizer were obtained from Amersham/Searle Corporation, Arlington, Illinois; lipoxidase and bovine serum albumin were from Sigma Chemical Company, Saint Louis, Missouri, alcohol dehydrogenase and porcine RNase were from Worthington Biochemical Corporation, Freehold, New Jersey.

Methods

Isolation of Chromatin. Chromatin was prepared by four different methods. Three methods involve isolation of chromatin from purified nuclei.

Purification of Nuclei. Nuclei were prepared by centrifugation through a 2.3 M sucrose solution and subsequent washings in 1% Triton X-100 containing 0.25 M sucrose–3 mM $MgCl_2$–10 mM potassium phosphate, pH 6.5, as described previously (Smith and Chae, 1973).

Chromatin Prepared from Nuclei by the Method of Smith and Chae (1973). Nuclei were washed in 0.075 M NaCl–0.024 M EDTA, pH 7.0. The nuclear pellet was washed in 0.3 M NaCl, pH 6.0, and 5 mM potassium phosphate, pH 6.5. The final chromatin pellet was suspended in cold water, pH 8.0, and sheared at 50 V for 90 s in a Virtis homogenizer.

Chromatin Prepared from Nuclei by the Method of Huang and Huang. Nuclei were washed by homogenization in 0.075 M NaCl–0.024 M EDTA, pH 7.0. The nuclear pellet was washed in 50 mM Tris, pH 8.0, and 10 mM Tris, pH 8.0 according to Huang and Huang (1969). The final pellet was suspended in cold water and sheared in a Virtis homogenizer.

Chromatin Prepared by the Method of Stein et al. (1975). Briefly, liver was homogenized in 0.25 M sucrose containing 0.05 M KCl, 5 M $MgCl_2$, and 50 mM Tris, pH 7.4 and centrifuged at 2000*g*. After filtration through cheesecloth and

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[1] Abbreviations used are: DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

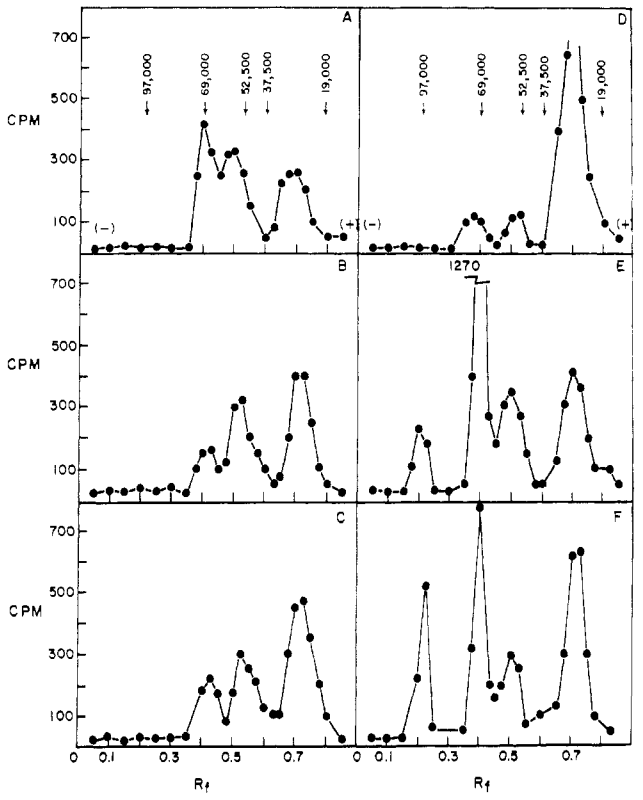

FIGURE 1: [^{3}H]DFP labeling of chromatin prepared by different methods. Proteins of rat liver nuclei and chromatin prepared and labeled with [^{3}H]DFP as described in Materials and Methods were electrophoresed on 7.5% polyacrylamide sodium dodecyl sulfate gels. The gels were sliced into 1.5-mm fractions and counted for radioactivity. The abscissa gives the ratio of the slice number counted to the slice number corresponding to the position of the tracking dye, bromphenol blue. Purified nuclei (A); chromatin prepared by the method of Smith and Chae (B), Huang and Huang (C), Stein (D), Elgin and Bonner, clear supernatant (E), and Elgin and Bonner, translucent supernatant (F). The ordinates are in counts per minute above background. The positions of molecular weight markers used in the same gel systems are indicated in frames A and D.

Miracloth, the crude nuclear pellet was washed with Earle's balanced salt solution (without phenol red) and 1% Triton X-100 containing 80 mM NaCl and 20 mM EDTA, pH 7.2. The nuclear pellet was then washed with 0.15 M NaCl–10 mM Tris, pH 8, suspended in cold water, and sheared.

Chromatin Prepared by the Method of Elgin and Bonner (1970). Chromatin was prepared from rat liver homogenized in 0.075 M NaCl–0.024 M EDTA, pH 7 by a Waring blender as described by Elgin and Bonner (1970).

Labeling with [^{3}H]DFP. Chromatin was adjusted to 0.75 mg of DNA/ml with 10 mM Tris, pH 8, and [^{3}H]DFP in propylene glycol was added. The ratio of [^{3}H]DFP added to DNA in chromatin was 67 μCi/mg DNA. The chromatin–[^{3}H]DFP mixture was incubated 15 h at 25 °C and excess label was removed by extensive dialysis against 1% sodium dodecyl sulfate–0.01 M sodium phosphate–0.1% β-mercaptoethanol, pH 7.

Disc Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out as described before on 7.5% polyacrylamide gels, pH 7.0 (Chae, 1975). Gel slices were incubated overnight at 37 °C in a scintillation cocktail consisting of 1 part NH_4OH, 5 parts NCS tissue solubilizer, and 50 parts toluene–1,4-bis[2-(5-phenyloxazolyl)]benzene–2,5-diphenyloxazole cocktail. The toluene–1,4-bis[2(5-phenyloxazolyl)]benzene–2,5-diphenyloxazole cocktail consists of 2.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, dissolved in 500 ml of toluene.

Extraction of Protease with 0.25 N HCl. Rat liver chromatin at a concentration of 0.75 mg of DNA/ml was adjusted to 0.25 N HCl with 1 N HCl at 4 °C. The acidified chromatin was stirred for 10 min in the cold and centrifuged for 5 min at 10 000 *g*. The supernatant was immediately brought to neutrality with 2 N NaOH and dialyzed against 0.1 M NaCl–0.01 M sodium acetate, pH 4.2 at 4 °C.

Chromatography on a Bio-Gel A-5m Column. Bio-Gel A-5m, 200–400 mesh, was equilibrated in 1% sodium dodecyl sulfate–10 mM Tris, pH 8.0, before packing into a 2 × 100 cm column. Chromatin and nuclei were labeled with [^{3}H]DFP and dialyzed exhaustively against 1% sodium dodecyl sulfate–1 mM β-mercaptoethanol–10 mM Tris, pH 8.0. The chromatin and nuclear solution were then centrifuged at 300 000 *g* for 5 h to remove DNA and layered on the column in 0.3 to 0.35 ml volumes. One-milliliter fractions were collected.

Assay for Proteolytic Activity. The assay for proteolytic activity was carried out as described before (Carter and Chae, 1976), with radioactive substrate, [^{3}H](F1 + F2B)histone, in the presence of 2 M NaCl–5 M urea. The acid-soluble radioactive peptides released were determined.

Results

Labeling of Rat Liver Nuclei and Chromatin Prepared by Different Methods. Rat liver nuclei and chromatin prepared by the procedures given in Materials and Methods were labeled by reaction with [^{3}H]DFP in order to compare the DFP-binding proteins of chromatin prepared by different methods. Rat liver nuclei labeled with [^{3}H]DFP as described in Materials and Methods show three peaks of radioactivity (A of Figure 1) corresponding to molecular weights of approximately 70 000, 60 000, and 25 000. Chromatin prepared from purified nuclei by the method of Smith and Chae (1973) and Huang and Huang (1969) (B and C of Figure 1) indicates qualitatively the same pattern of labeling that occurs with whole nuclei.

The relative amounts of radioactivity in the three proteins were somewhat variable between different preparations of nuclei and chromatin. The most typical labeling patterns of the several experiments are shown in Figure 1. In general the smallest molecular weight DFP-binding protein contained more radioactive DFP than two other proteins in the case of chromatin. Chromatin prepared by the method of Stein et al. (1975) shows a labeling pattern qualitatively similar to whole nuclei differing only in the relative quantity of high and low molecular weights proteins (D of Figure 1). Rat liver chromatin prepared from whole tissue by the method of Elgin and Bonner (1970), however, presents a different labeling pattern both qualitatively and quantitatively than chromatin prepared from purified nuclei (E and F of Figure 1). It was routinely observed during the preparation of chromatin according to Elgin and Bonner that the final step which consists of shearing and subsequent centrifugation at 12 000*g* results in a supernatant which is comprised of two layers. The top layer was clear and the bottom layer was translucent. Material from the top and bottom layers was labeled with [^{3}H]DFP and the labeling pattern of each is shown in Figure 1, frame E and F, respectively. One additional DFP-binding protein is present in Elgin and Bonner's chromatin as compared with purified rat liver nuclei, having a molecular weight of about 100 000. The molecular weight of this protein is identical with that of a subunit of the 200 000 protease isolated from Elgin and Bonner's rat liver chromatin by Chong et al. (1974). Additional evidence, supporting the observation that there are only three

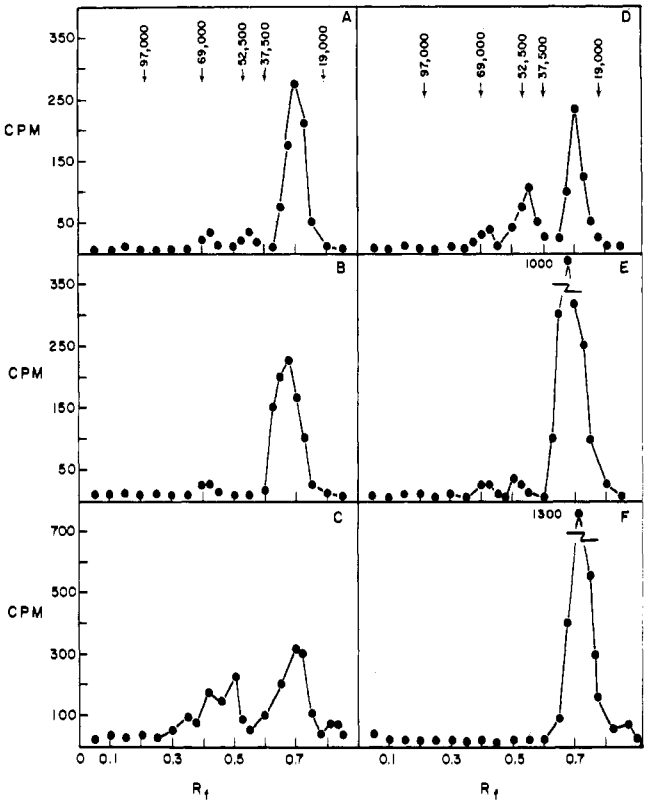

FIGURE 2: [^{3}H]DFP labeling of chromatin from different tissues. Chromatin prepared by the method of Huang and Huang (1969) from various tissues was labeled with [^{3}H]DFP as described in Materials and Methods and electrophoresed on 7.5% polyacrylamide sodium dodecyl sulfate gels. The gels were sliced into 1.5-mm fractions. The abscissa gives the ratio of the slice number counted to the slice number corresponding to the position of the tracking dye. Rat chromatins from the following tissues are displayed: spleen (A); kidney (B); Morris hepatoma 9121 (C); testis (D); lung (E); and thymus (F).

[^{3}H]DFP binding moieties present in purified nuclei and four in chromatin prepared according to Elgin and Bonner, was obtained by chromatography of samples of each labeled material on a Bio-Gel A-5m column. The elution profiles of labeled proteins from purified nuclei and Elgin and Bonner's chromatin are consistent with the spectra of labeled proteins found in the sodium dodecyl sulfate gels (not shown here).

Other investigators have shown that chromatin as prepared by Elgin and Bonner contains substantially more phospholipid fractions resembling those found in microsomal membranes than chromatin prepared from purified nuclei (Tata et al., 1972). Moreover, these investigators also show that Elgin and Bonner's chromatin possesses 26 times more cytochrome oxidase activity and 16 times more glucose-6-phosphatase activity than chromatin isolated from purified nuclei. The present data suggest that chromatin isolated from whole tissue may contain an extranuclear protein which binds DFP and possesses a molecular weight similar to the putative chromatin protease isolated by Chong et al. (1974).

The molecular weight of the low molecular weight DFP-binding protein by Bio-Gel A-5m chromatography is between 16 000 and 18 000, substantially smaller than the estimate of molecular weight from sodium dodecyl sulfate gels. It is known that highly charged proteins such as histones at pH 7.0 have anomalously slow electrophoretic mobilities on sodium dodecyl sulfate gels (Panyim and Chalkley, 1971). The data suggest that the low molecular weight DFP-binding protein may also possess a large number of positive charges at pH 7.0.

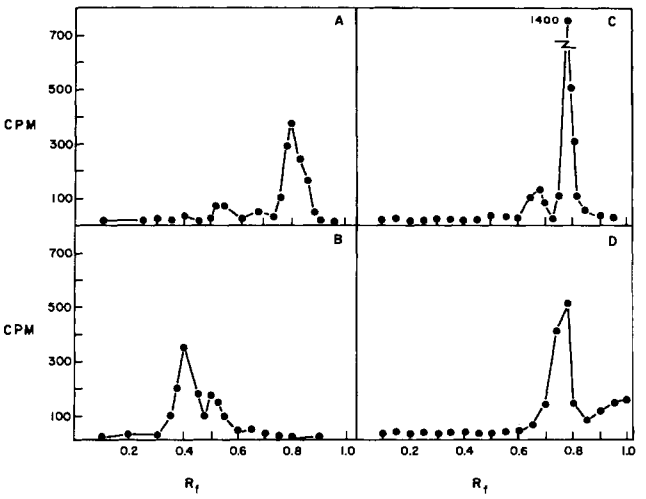

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of [^{3}H]DFP binding proteins in chromatin labeled prior to acid extraction, and labeled after acid extraction. Chromatin previously labeled with [^{3}H]DFP was extracted with 0.25 N HCl (A); the 0.25 N HCl insoluble proteins of chromatins labeled before acid extraction (B); the 0.25 N HCl soluble proteins of chromatin were labeled after acid extraction (C); the eluent containing proteolytic activity from the Sephadex G-75 column of Figure 4 was pooled and labeled with [^{3}H]DFP (D). The samples were prepared for electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gels as described in Materials and Methods.

[^{3}H]DFP Labeling of Chromatin from Different Tissues. Chromatin from various tissues was prepared by the method of Huang and Huang (1969) and labeled with [^{3}H]DFP (Figure 2). There are qualitative as well as quantitative differences in the DFP-binding proteins. However, all tissues examined thus far contain a DFP-binding protein of molecular weight 25 000 (by sodium dodecyl sulfate gel electrophoresis), which quantitatively is the predominant DFP-binding protein in the chromatins. Evidence will be presented which suggests that the low molecular weight DFP-binding protein is active proteolytically in 2 M NaCl–5 M urea, pH 8.0. The previous results indicate that the chromatin-bound protease active in NaCl and urea is a rather ubiquitous constituent of a variety of chromatins (Carter and Chae, 1976). However, there are interesting differences in the amounts of high molecular weight DFP-binding proteins present in the chromatin of various tissues. Although liver and testis appear very similar in the distribution of DFP-binding proteins, spleen, lung, thymus, and kidney chromatin appear to contain a relatively small quantity of the high molecular weight DFP-binding proteins. It is not certain at this time if these differences are due to the possible variations in the amount of the three DFP-binding proteins or due to the possible difference in the state of binding of the proteins in chromatins from different tissues.

Fractionation of Proteolytic Activity from Chromatin. Kurecki and Toczko (1972) reported that the calf thymus chromatin-bound protease could be efficiently removed from chromatin by 0.25 N HCl. Chromatin prepared by the method of Huang and Huang (1969) was labeled with [^{3}H]DFP as described before and then extracted with 0.25 N HCl as described in Materials and Methods. Sodium dodecyl sulfate gels were run for the acid extract, sliced, and counted for radioactivity (Figure 3A). The acid appears to extract little of the two high molecular weight DFP-binding proteins but extracts a large amount of a labeled material which migrates at an R_f of 0.78–0.82 (which corresponds to 20 000 dalton). This is in contrast to the molecular weight of the smallest DFP-binding protein in nonacid-treated chromatin (R_f 0.68–0.72, 25 000

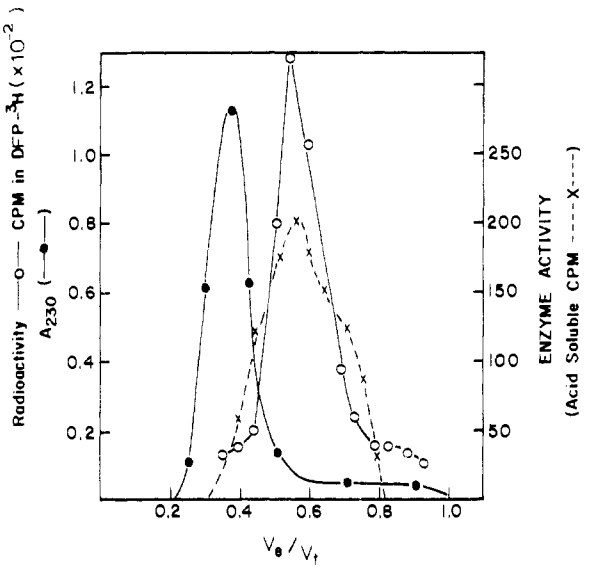

FIGURE 4: Elution diagram of protease extracted by 0.25 N HCl rat liver chromatin A on Sephadex G-75. Preparation of extract for chromatography is described in Materials and Methods. Elution was carried out with 0.1 M NaCl–0.01 M sodium acetate, pH 4.2. The acid extract containing 70 mg of protein was applied to a 45 × 3 cm column of Sephadex G-75. One milliliter fractions were collected under gravity flow at 4 °C. Proteolysis was determined by the capacity of aliquots from various fractions to hydrolyze the radioactive substrate ^{3}H-labeled (F1 + F2B) as described in Materials and Methods. The acid extract was also labeled with [^{3}H]DFP and applied to the same column. Absorbance at 230 nm (●), proteolytic activity (X), and radioactivity (O).

daltons). It appears that the 25 000-dalton DFP-binding protein is converted to a 20 000-dalton form during acid extraction. We rule out the possibility that the two high molecular weight DFP-binding proteins are also converted to a 20 000-dalton form since the acid-insoluble chromatin pellet contains the two high molecular weight DFP-binding proteins (Figure 3B).

The conversion of the 25 000-dalton DFP-binding protein to the 20 000-dalton form was further investigated by labeling the acid-extract of unlabeled chromatin and subsequent analysis by sodium dodecyl sulfate gel electrophoresis. Figure 3C shows that very little of the DFP-binding proteins in the extract migrate at an R_f of 0.7 and that most of the radioactivity migrates at an R_f of 0.78–0.82, confirming the results of Figure 3A. Barely detectable amounts of [^{3}H]DFP label are incorporated into the high molecular weight DFP binding proteins of chromatin, ostensibly due to their nonextractability by 0.25 N HCl. Since the neutralized acid extract of chromatin contains proteolytic activity (assayed as described in Materials and Methods), the possibility that the 20 000-dalton DFP-binding protein is a protease was studied.

Chromatography of the acid extract of chromatin was performed on a Sephadex G-75 column as described by Kurecki and Toczko (1972). The proteolytic activity eluting from the column was assayed by the ability of fractions to hydrolyze [^{3}H]-(F1 + F2B) histones in the presence of 2 M NaCl–5 M urea. As seen in Figure 4, the peak of proteolytic activity is separated from the main protein peak in complete agreement with Kurecki's results with calf thymus chromatin protease. The asymmetry of the peak of proteolytic activity eluting from the Sephadex G-75 column may imply the existence of multiple proteases bound to chromatin or may indicate the presence of autolyzed protease molecules which maintain activity after small peptides are removed from the protease molecule. It is also conceivable that there are proteases present in the fractions which are not labeled with DFP (e.g., particular sulfhydryl-dependent proteases), but which are active in 2 M NaCl–5 M urea. However, DFP completely inhibits the proteolysis of chromatin in 2 M NaCl–5 M urea and various sulfhydryl reagents have no effect on the proteolysis of chromatin in salt and urea (Carter and Chae, 1976). The specific activity of protease in the fractions from V_e/V_t = 0.55 to 0.8 was 30 times the specific activity in the crude acid extract. Fractions from V_e/V_t = 0.45 to 0.6 were pooled and incubated with [^{3}H]DFP in the presence of 2 M NaCl–5 M urea, pH 8.0. After removal of excess [^{3}H]DFP by extensive dialysis against 1% sodium dodecyl sulfate–10 mM $NaPO_4$–0.1% β-mercaptoethanol, pH 7.0, the labeled material was electrophoresed on sodium dodecyl sulfate gels and gel slices were counted as described (D of Figure 3). A peak of radioactivity was detected on the gels at an R_f of 0.77 (equivalent to 20 000 daltons) and a smaller portion (20%) of the recovered radioactivity was detected at the position of tracking dye. These results may indicate the presence of nondialyzable autolytic fragments of the protease containing [^{3}H]DFP label which may arise during the labeling period before complete inhibition is achieved.

In a separate experiment, the acid extract of unlabeled chromatin was labeled with [^{3}H]DFP and chromatographed as described above. The elution of radioactivity is plotted as a matter of convenience in Figure 4. The peaks of proteolytic activity and radioactivity coincide, but there is some apparent difference in the elution of proteolytic activity and radioactivity at the sides of the peak. These results may be explained by the arguments alluded to above, i.e., that there may be proteases present in fractions from the edge of the activity peak which are not labeled by [^{3}H]DFP, or that there may be autolyzed protease molecules present which retain activity.

Discussion

The labeling experiments with radioactive DFP show that there are three major DFP-binding proteins in liver nuclei and chromatin isolated from the same nuclei. The 25 000-dalton DFP-binding protein can be extracted from chromatin in a modified form by 0.25 N HCl and found to be a protease similar to the protease fractionated from calf thymus chromatin by Furlan and Jericijo (1967) and Kurecki and Toczko (1972). This enzyme is apparently responsible for the degradation of chromatin during dissociation and reconstitution of chromatin in the presence of salt and 5 M urea (Chae and Carter, 1974; Carter and Chae, 1976; Chae, 1975).

The small molecular weight protease appears to be the major DFP-binding chromatin-bound protease in most rat tissues. These experiments do not rule out the possibility that there are proteases present in rat chromatin which do not bind DFP. However, since proteolysis of chromosomal proteins is inhibited by DFP, non-DFP-binding proteases may not be principally responsible for the protein degradation seen in chromatin. Work is in progress to isolate the major DFP-binding protease in pure form. We have not yet been able to obtain the two other DFP-binding proteins in soluble form to determine whether or not these proteins are also proteases.

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Transfer Free Energies and Average Static Accessibilities for Ribonuclease A in Guanidinium Hydrochloride and Urea Solutions†

Martha Y. Schrier and Eugene E. Schrier*

ABSTRACT: Isopiestic vapor pressure measurements have been used to obtain free energies of transfer of ribonuclease A from dilute buffer to solutions of either urea or guanidine hydrochloride (GdnHCl) over a wide cosolute concentration range. The free energies of transfer vary monotonically from 0 to −8 kcal/mol in 8 M urea and to −18 kcal/mol in 6 M GdnHCl. These values are not large in relation to free energies of transfer of constituent groups of the protein from water to cosolute solutions of the same concentration. The assumption is made that the magnitude of the free energy of transfer of the protein is governed by the average static accessibility of its constituent groups to the solution. The free energies of transfer to different cosolute concentrations of a hypothetical 100% accessible ribonuclease A were determined using literature values of the free energies of transfer of constituent groups and the amino acid composition. The ratio of the experimentally determined free energy of transfer to the free energy of transfer of the 100% accessible protein gave 11% accessible surface area for the native protein in 1 M GdnHCl or 2 M urea. Additional considerations led to a value of 36% for the accessible surface area of the denatured protein in 6 M GdnHCl or 8 M urea.

Detailed consideration of solvation is of major importance in providing an accurate description of the conformation of a protein in solution. Only the preferential solvation, i.e., the preference of the protein for one or the other component of a mixed solvent, is completely defined by thermodynamic measurements. Numerical measures for it have been obtained in protein-cosolute-water systems (Noelken and Timasheff, 1967; Noelken, 1970; Span and Lapanje, 1973, Span et al., 1974) by a combination of equilibrium dialysis and differential refractometry and also (Hade and Tanford, 1967) by the use of isopiestic measurements. However, it has been difficult to utilize these values for further elaboration of protein conformation in solution (Tanford, 1970; Franks and Eagland, 1975).

The preferential solvation formalism conventionally adopted tends to obscure the fact that interactions between two solutes in aqueous solution can equally well be treated in terms of the free energies of transfer of the protein from dilute buffer to aqueous solutions of cosolutes of interest. Consideration of interactions in these terms has several advantages. For one thing, such free energy changes can readily be compared for different proteins and different cosolutes. In addition, comparison is also possible with the free energies of transfer of the constituent groups of the protein obtained in other experiments using small model compounds (Nozaki and Tanford, 1963, 1970).

In the work to be described, we have obtained free energies for the transfer of ribonuclease A from dilute buffer to solutions of urea and GdnHCl,[1] respectively, over wide cosolute concentration ranges. Combination of these results with previously obtained thermodynamic data (Nozaki and Tanford, 1963, 1970) allows an experimental estimate of the average static accessibility of the constitutent groups of both the native and unfolded protein to these solutions.

Experimental Section

Materials. Ribonuclease A was purchased from Sigma Chemical Co. While type IIA was used for most of the runs, the more extensively chromatographed type XIIA was also employed in a few runs to test the effect of ribonuclease A homogeneity on the results. Within experimental error, no difference was found between runs with the two types of protein. The protein was dialyzed at 2 °C against three changes of 0.1 M KCl and, subsequently, against three changes of doubly distilled water. The dialyzed solutions were passed through a mixed-bed ion-exchange column (Rohm and Haas Amberlite MB-1). The pH of the protein solution as it came off the column was found to be 9.6–9.7, in agreement with the value of 9.6 reported previously (Tanford and Hauenstein, 1956). The protein solutions were lyophilized at concentrations

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[1] Abbreviation used: GdnHCl, guanidine hydrochloride.