Structural Studies on Histones H1. Circular Dichroism and Difference Spectroscopy of the Histones H1 and Their Trypsin-Resistant Cores from Calf Thymus and from the Fruit Fly *Ceratitis capitata*†

José L. Barbero, Luis Franco,* Francisco Montero, and Federico Morán

ABSTRACT: A peptide containing the globular region of the histone H1 from the fruit fly *Ceratitis capitata* has been isolated after limited tryptic digestion of insect H1. The composition of this trypsin-resistant core resembles that of the homologous peptide from calf thymus H1, although the insect H1 core possesses one cysteine, two tyrosines, one histidine, and more isoleucine and less glycine and leucine than the calf thymus H1 core. Circular dichroism measurements indicate that all the fragments that possess an ordered secondary structure (~11% in both calf thymus H1 and *Ceratitis* H1) are present in the trypsin-resistant cores. Both calf thymus and *Ceratitis* H1 and their trypsin-resistant cores fold cooperatively on titration with NaOH, though the folding of the cores is less cooperative than that for the parent molecules. On the other hand, salt-induced folding of both cores and intact molecules is noncooperative. The environment of the tyrosyl residues in both calf thymus and *Ceratitis* H1 has been studied by circular dichroism in the region 250–300 nm and by difference spectroscopy; their pK_a' values have also been determined. The results suggest that one of the tyrosyl residues of *Ceratitis* H1 is buried in the hydrophobic core, in an environment similar to that of calf thymus tyrosine-72, while the second tyrosyl residue of the insect H1 molecule, which titrates with a lower pK_a' value (~9.30 in the absence of salt and ~9.80 in the presence of 0.3 M KF), is on the surface of the trypsin-resistant core. Due to the limited number of aromatic residues in the histone molecules, the above-mentioned techniques proved to be useful tools to study conformational transitions.

The structural roles of histones H2A, H2B, H3, and H4, or "inner histones" (Isenberg, 1976), are now fairly well understood [for a review, see Felsenfeld (1978)]. On the other hand, the precise role of the very lysine-rich histone H1 is not known. Histone H1 is not required to form the nucleosome core, and it has been reported to be associated in some way with linker DNA, i.e., DNA between two adjacent nucleosome cores (Noll & Kornberg, 1977). It is also widely accepted that histone H1 contributes to the higher order compaction of nucleosomes to form the chromatin fiber (Littau et al., 1965; Bradbury et al., 1973; Finch & Klug, 1976; Renz et al., 1977) but, in spite of the efforts of several authors to describe the mode of binding of H1 to DNA and to the chromatin fiber (Vogel & Singer, 1976; Christiansen & Griffith, 1977; Gaubatz et al., 1977; Singer & Singer, 1978), its precise mode of action remains unclear.

Taking into account structure–function correlations, it is clear that a better knowledge of H1 structure may lead to a better understanding of its function. Histones H1 consist of three structural domains. The N-terminal region, of ~40 residues, is highly variable in sequence (Rall & Cole, 1971). The next region, of ~80 residues in H1 from mammals, is more conservative and contains most of the hydrophobic residues of the histone molecule (Jones et al., 1974; Isenberg, 1979). Finally, the C-terminal region contains some 90 residues, which are nearly 90% lysine, alanine, and proline (Cole, 1977), and it has been shown to be the most important DNA-binding domain in the H1 molecule (Bradbury et al., 1975a).

Structural studies on histones have to be carried out in solution. This kind of approach is not, of course, aimed at an exact definition of the tertiary structure, but it can lead to a better understanding of some aspects of secondary and tertiary structure of histones. Bradbury and his colleagues first demonstrated that part of the H1 molecule can adopt a globular structure under physiological conditions (Bradbury et al., 1975b; Chapman et al., 1976), and they were able to show that the globular region extends from residues 39 ± 4 to 116 ± 4 in the calf thymus histone, whereas the N-terminal and C-terminal regions are unstructured in solution (Hartman et al., 1977). They also succeeded in isolating, by limited trypsin digestion, a peptide containing the globular region (Hartman et al., 1977), whose structure in solution has been studied by ^{1}H NMR[1] (Chapman et al., 1978a). This technique has proved to be a useful tool to examine some structural details of histone H1 (Bradbury et al., 1975a,b) and of other lysine-rich histones (Puigdomênech et al., 1975; Chapman et al., 1978b; Avilés et al., 1978, 1979). Circular dichroism has also been used to study structural transitions in histone H1 (Bradbury et al., 1975a,b; Smerdon & Isenberg, 1976), in histone H5 (Avilés et al., 1978), and in the marine invertebrate sperm histone ϕ1 (Puigdomênech et al., 1975). These circular dichroism studies were carried out in the far-UV region, so that only structural changes which affect the conformation of peptide bonds have been examined.

All the above-mentioned studies on H1 have been carried out with the calf thymus histone. Based on sequence homologies, it has been proposed that the globular region of histone H1 remains largely conserved (Isenberg, 1979), but it is of interest to study the structure of histones H1 from organisms other than mammals. We have previously reported the isolation (Franco et al., 1974) and purification (Franco et al., 1977) of the histone H1 from the fruit fly *Ceratitis capitata*. This histone exhibits several differences from the H1 of higher organisms. The most striking one is the presence of a single residue of cysteine in the histone molecule, which can form intermolecular disulfide bridges, leading to histone aggregation

† From the Department of Biochemistry, Faculty of Sciences, Universidad Complutense, Madrid-3, Spain. *Received December 18, 1979.* This work has been supported in part by a grant from the Comisión Asesora de Investigación Científica y Técnica (Spain).

[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/80/0419-4080$01.00/0

under oxidizing conditions. Moreover, *Ceratitis* H1 contains more than one tyrosyl residue, a single methionyl residue, and less alanine and more serine than mammalian H1 histones (Franco et al., 1977).

The aim of the present work is the comparative study of some structural features of calf thymus and *Ceratitis* H1 histones and their trypsin-resistant cores. To this end, we have mainly used difference spectroscopy and circular dichroism in the near-UV region. These techniques proved to be suitable for the study of histones H1 because of the limited number of aromatic residues in their molecules. Both calf thymus and *Ceratitis* H1 lack tryptophan and they possess respectively one and two tyrosines, so that the contribution of the ^1L_a and ^1L_b transitions of the tyrosine chromophores to the circular dichroism spectrum could be readily assigned.

Materials and Methods

Isolation and Purification of Histones H1. Calf thymus histone H1 was isolated by the method of Johns & Butler (1962). *C. capitata* H1 was isolated as described elsewhere (Franco et al., 1974). Both calf thymus and *Ceratitis* histones were purified by carboxylmethylcellulose chromatography (Johns, 1964), as previously described (Franco et al., 1977).

Preparation of the Trypsin-Resistant Cores of Histones H1. The trypsin-resistant cores of calf thymus and *Ceratitis* H1, which contain the globular region of the molecules, were prepared by a modification of the procedure of Hartman et al. (1977). Histones were dissolved in 1.0 M NaCl and 0.3 M phosphate buffer, pH 8.0, to a concentration of 10 mg/mL. TPCK-treated trypsin (Worthington) was then added to obtain an enzyme/histone ratio of 1:1000 (w/w), and the solution was incubated at 22 °C. Under these conditions the optimum digestion time was 90 min (see Results). Reaction was stopped by adding soybean trypsin inhibitor to give an inhibitor/trypsin ratio of 2:1 (w/w). Cold trichloroacetic acid (100% w/v) was then added to a final concentration of 18%, and the precipitate was spun down and washed once with 18% trichloroacetic acid, once with acetone–1 N HCl, and 3 times with acetone. The final precipitate, which mainly consisted of the trypsin-resistant core, was dried under vacuum and purified by gel filtration on Sephadex G-75, according to Hartman et al. (1977).

Absorption Spectra. Absorption spectra were recorded in a Cary 118 spectrophotometer. Both absorbance and the first derivative were routinely recorded from 350 nm downwards. Most of the samples exhibited no turbidity, as shown by their negligible absorbance between 350 and 310 nm. In some instances, however, there was an apparent increase in absorbance between 350 and 310 nm, due to sample turbidity, which was corrected according to Donovan (1969).

Salt-induced difference spectra of histones and their trypsin-resistant cores were recorded within a concentration range of (3.5–9.0) $\times 10^{-5}$ M protein, depending on the molecular weight of the peptide or histone. The samples were dissolved in water at pH 6.0; two accurately measured aliquots of 3 mL were placed in two matched quartz cells of 1-cm optical path. Solvent–solvent and protein–protein base lines were identical, showing that the sample absorption was still low enough to enable the photomultiplier to give an adequate response. Aliquots of a 3.0 M solution of KF at pH 6.0 were added to the first cell with a syringe microburet. Identical aliquots of water were added to the second cell, which was used as the reference cell. Spectra were recorded at 22 °C after the samples were thoroughly mixed, at a sensitivity of 0.05–0.1 absorbance unit full-scale between 320 and 260 nm in the autoslit mode. Gain was adjusted so that the slit varied between 0.03 and 0.2 mm. Samples were dissolved in water at pH 2.8 within a concentration range as above to obtain pH-difference spectra. The pH of the samples was raised by adding ≤3-μL aliquots of 2, 0.5, or 0.1 N NaOH, depending on the pH range. Identical aliquots of water at pH 2.8 were added to the reference cell, and the spectra were recorded as described above. The sensitivity was in the range 0.2–0.5 absorbance unit full-scale between 350 and 260 nm and 1–2 absorbance units between 260 and 230 nm.

Circular Dichroism Spectra. Circular dichroism spectra were recorded in a Mark III dicrograph (Jobin-Yvon). For spectra in the near-UV region (320–245 nm) cells of 1-cm optical path were used and the instrument was operated at a sensitivity of 1×10^{-6} ΔA mm^{-1}. Cells of 0.05 cm optical path were used and the sensitivity was set at 5×10^{-6} ΔA mm^{-1} to obtain spectra in the far-UV region (250–190 nm). Ionic strength and pH were adjusted as described above. For measurements in the near-UV, proteins were dissolved to a concentration as above, whereas for measurements in the far-UV region, solutions were diluted 10-fold. Results are expressed in molar ellipticities with the dimensions of deg × cm^2 × (dmol of residue)$^{-1}$ in the far-UV region or deg × cm^2 × (dmol of protein)$^{-1}$ in the near-UV region. For an estimation of the contributions of α helix, β structure, and random coil to the secondary structure, the method of Smerdon & Isenberg (1976) was used.

Analytical Techniques. Electrophoresis was carried out in 15% polyacrylamide gels in the presence of 2.5 M urea (Panyim & Chalkley, 1969). Gels were stained with Coomasie Blue as described by Avilês et al. (1978). Molecular weights were estimated by the electrophoretic method of Panyim & Chalkley (1971). Hydrolysis of protein and peptides was carried out at 105 °C in constant-boiling HCl, containing 0.1% (w/v) phenol, in evacuated sealed tubes during 24 h. Amino acid analyses were performed with a Durrum Model D-500 amino acid analyzer. Cysteine was determined as (carboxymethyl)cysteine (Craven et al., 1965). Protein concentration was determined spectrophotometrically in 0.01 N HCl at two wavelengths, 205 and 210 nm ($E_{205}^{1\%} = 310$; $E_{210}^{1\%} = 205$).

Results

Isolation and Purification of Trypsin-Resistant Cores from Histones H1. Figure 1 shows the time course of the tryptic digestion of histones H1 from calf thymus and *C. capitata*. From a kinetic point of view, the tryptic digestion of *Ceratitis* H1 was similar to that of calf thymus H1; i.e., large histone fragments appeared early during the digestion and they were hydrolyzed to a resistant peptide more slowly. This behavior had already been described by Hartman et al. (1977) in calf H1. Under the conditions used here, the trypsin-resistant core of insect H1 appeared as a minor band very early during the course of the digestion (Figure 1) and it constituted the major band in the 60-min digest, but we found that continuing the digestion to 90 min, when the trypsin-resistant core was almost the only band visible in the gels (Figure 1), was advantageous in order to obtain purer preparations.

Some small peptides were also present in the 90-min digest, but the precipitation step and the chromatography in Sephadex G-75 provided a final purification of the trypsin-resistant core, which electrophoresed as a single band (Figure 1, k).

It is also evident from Figure 1 that the electrophoretic mobility of fly H1 trypsin-resistant core is smaller than that of the homologous fragment from calf H1. This may result from differences in molecular weight, because the electrophoretic determination gave an estimate of 10 100 for the *Ceratitis* trypsin-resistant core and of 9800 for the corresponding calf H1 core.

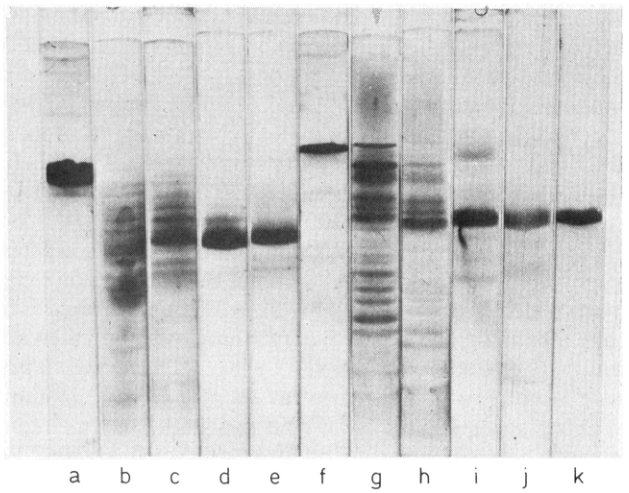

FIGURE 1: Electrophoretic separation of the fragments obtained by limited tryptic digestion of histones H1 from calf thymus (b–e) and *C. capitata* (g–k). Aliquots were taken from the reaction mixture at (b and g) 6, (c and h) 35, (d and i) 60, and (e and j) 90 min. Gels a and f show the electrophoretic image of intact H1 from calf thymus and *Ceratitis*, respectively. The electrophoretic analysis of the trypsin-resistant core from *C. capitata* H1 recovered from gel filtration is shown in lane k. Migration is from top to bottom in 15% polyacrylamide gels in the presence of 2.5 M urea.

Table I: Amino Acid Composition of the Trypsin-Resistant Cores from *C. capitata* and Calf Thymus Histones H1[a]

amino acid	*Ceratitis*	calf thymus
aspartic acid	5.9	6.1
threonine	6.8	5.5
serine	10.6	10.6
glutamic acid	6.8	7.6
proline	5.9	4.7
glycine	6.2	11.7
alanine	16.5	16.2
cysteine[b]	0.7	0.0
valine	6.7	6.7
methionine	0.7	0.0
isoleucine	5.6	2.3
leucine	6.6	9.5
tyrosine	2.0	1.0
phenylalanine	2.0	1.4
lysine	13.2	14.4
histidine	1.1	0.0
arginine	2.5	2.4

[a] Data are given as mole percent of total amino acid content. [b] Determined as (carboxymethyl)cysteine.

The amino acid composition of the trypsin-resistant core from *Ceratitis* H1 is shown in Table I together with the composition of the homologous peptide isolated from calf thymus H1. The composition of the latter is very similar to that reported for the fragment 35–120 of calf thymus H1 by Hartman et al. (1977). We have every reason to believe that in spite of the differences between our method of isolation and that of Hartman et al. (1977), both our "trypsin-resistant core" and their "Tr Tr fragment" actually refer to the same region in calf thymus H1.

It has been previously reported that *C. capitata* H1 possesses cysteine, methionine, and histidine (Franco et al., 1977). All of these amino acids are present in the trypsin-resistant core, as Table I shows. There also are some other differences between calf and insect histone cores: the *Ceratitis* H1 core has less glycine and leucine and more isoleucine and tyrosine (two residues). In spite of these differences, it is clear that the compositions of calf thymus and insect H1 cores are very similar and that the larger differences found between the intact parent molecules (Franco et al., 1977) must, therefore, occur in the N-terminal and/or C-terminal regions.

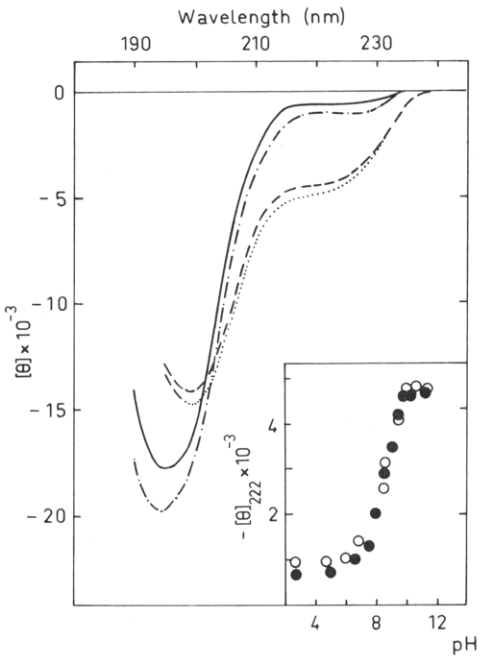

FIGURE 2: Circular dichroism spectra (far-UV) of 0.5×10^{-5} M solutions of histones H1 in water at several pH values. (—) Calf thymus H1 at pH 2.8; (–·–) *C. capitata* H1 at pH 2.8; (- - -) calf thymus H1 at pH 10.6; (···) *Ceratitis* H1 at pH 10.6. The spectra were recorded in a 0.05-cm path length cell as described in the text, and the results are expressed as molar ellipticity, with the dimensions of deg × cm^2 × (dmol of residue)$^{-1}$. Inset: variation of $[\Theta]_{222}$ with pH for (●) calf thymus H1 and (○) *Ceratitis* H1.

Table II: Secondary Structure of Folded H1 Histones and Their Trypsin-Resistant Cores

histone	conditions of folding: pH	conditions of folding: KF (M)	% of: α helix[b]	% of: β structure[b]	% of: random coil[b]
calf thymus H1	10.2	0.0	8	3	89
	6.0	0.3	8	3	89
Ceratitis H1	10.2	0.0	8	1	91
	6.0	0.3	9	4	87
calf thymus TRC[a]	10.2	0.0	22	0	78
Ceratitis TRC[a]	10.2	0.0	22	0	78

[a] TRC = trypsin-resistant core from H1. [b] These percentages were obtained as the coefficients in the theoretical equations which better fitted the experimental curves. The errors of the figures range from about 10% for α helix and random coil to almost 50% for β sheet. Probably no significance can be attached to the presence of β structure.

Circular Dichroism in the Far-UV Region. Calf thymus and *C. capitata* histones H1 show no noticeable variations in their secondary structure between pH 2.8 and 6.5 (Figure 2). Their circular dichroism spectra are very similar, and they are typical of random-coiled polypeptides. However, a sharp conformational transition takes place in both histones between pH 7.0 and 10.0; the midpoint of this transition may be estimated at pH 8.3 from the data shown in Figure 2. Obviously, the titration of lysyl residues may account for this structural transition. Our results are in good agreement with those of Smerdon & Isenberg (1976).

The conformational transitions in both calf thymus and *Ceratitis* H1 were complete by pH 10.0 (Figure 2). The contributions of α helix, β structure, and random coil to the secondary structure are given in Table II. On the other hand,

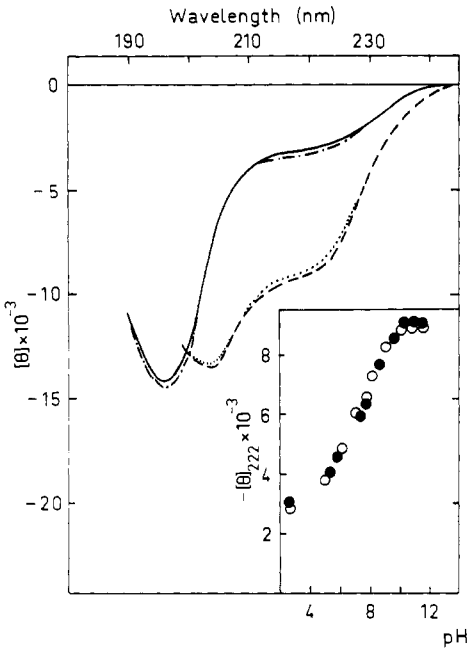

FIGURE 3: Circular dichroism spectra (far-UV) of 0.8 × 10^{-5} M solutions of trypsin-resistant cores from histones H1 in water at several pH values. (—) Calf thymus H1 core at pH 2.8; (-·-) *Ceratitis* H1 core at pH 2.8; (---) calf thymus H1 core at pH 10.6; (···) *Ceratitis* H1 core at pH 10.6. Spectra were recorded and expressed as described in Figure 2. The inset shows the variation of $[\theta]_{222}$ with pH; (●) calf thymus H1 core; (○) fly H1 core.

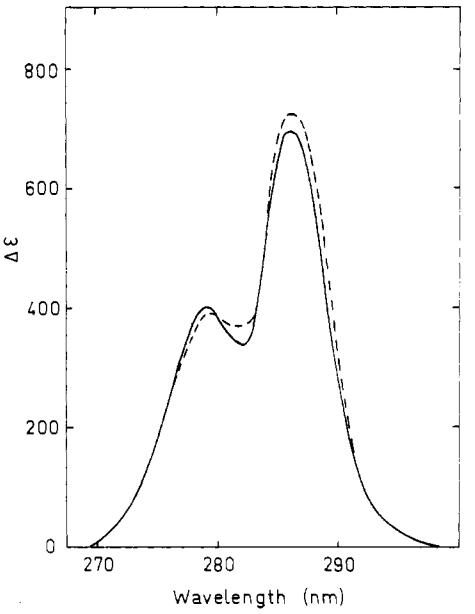

FIGURE 4: Difference absorption spectra between folded (0.3 M KF, pH 6.0) and unfolded (water, pH 2.8) trypsin-resistant cores from calf thymus (—) and *Ceratitis* (---) H1. Spectra were recorded at a concentration of 8 × 10^{-5} M as described in the text.

the ionic strength induced conformational transitions were noncooperative; plots of $[\theta]_{222}$ against KF concentration gave hyperbolic curves. These salt-induced transitions in both calf thymus and insect histones at pH 6.0 were complete by 0.3 M, and the estimated secondary structure of these salt-folded histones is also given in Table II. Differences between both histones are not significant irrespective of the method of folding.

The structural transitions in the trypsin-resistant cores in going from pH 2.8 to 12.0 are less cooperative than those in the intact molecules, as evidenced by the relative smoothness of the $[\theta]_{222}$ vs. pH plots given in Figure 3. On the other hand, the folded trypsin-resistant cores exhibited a large α-helix contribution to their secondary structure, as expected in peptides containing the globular region of the molecule.

The structural transitions induced in H1 cores by increasing ionic strength at pH 6.0 were noncooperative, and they were also complete when the KF concentration was 0.3 M, as found in the intact molecules.

Ultraviolet Absorption Studies. The experimental extinction coefficients at 275 nm of calf thymus H1 and its trypsin-resistant core in water were 1360 and 1340 cm^{-1} M^{-1}, respectively. The difference between these two values is not significant, and they are consistent with the theoretical value of 1345 cm^{-1} M^{-1}, calculated from the amino acid composition (Smerdon & Isenberg, 1976). The corresponding experimental value for both *Ceratitis* H1 and its trypsin-resistant core was 2720 cm^{-1} M^{-1}. These results are in accordance with the presence of two tyrosyl residues in the insect H1 molecule, both of them being present in the core. The presence of tryptophan in fly H1 could be ruled out by examining the first derivative of the spectrum, which showed no peak nor shoulder at 290 nm. Moreover, the curve obtained by subtracting the absorption spectrum of calf thymus H1 from that of fly H1 closely fitted the spectrum of one tyrosine. This was also found when the spectra were recorded in 0.3 M KF, pH 6.0.

As pointed out before, both H1 histones are folded in 0.3 M KF, pH 6.0. Bradbury and his colleagues (Chapman et al., 1978a) have demonstrated that the tyrosyl residue of calf thymus H1 is buried in the globular region, and Smerdon & Isenberg (1976) showed that both fluorescence anisotropy and intensity of the tyrosyl residue change during salt-induced folding of H1. It would be reasonable that salt-induced folding also had an influence on the absorption spectrum. Difference spectra between salt-folded and unfolded calf and insect H1 histones and their cores were recorded as described under Materials and Methods to check this possibility. Figure 4 shows the difference spectra of the trypsin-resistant cores; the curves obtained with the intact molecules virtually coincided with those given in Figure 4. As expected, the difference spectrum of the trypsin-resistant core from calf thymus H1 corresponds with the transition of a tyrosyl residue from a polar environment to a nonpolar one. The magnitude of the difference spectrum ($\Delta\epsilon_{278}$ = 700 cm^{-1} M^{-1}) agrees with the estimated change in molar absorbance produced by transfer of a phenol chromophore from water to the interior of a protein (Donovan, 1969). Although no difference spectra were described, a red shift in the absorption spectra of the four major calf thymus H1 subfractions upon addition of salt had been previously reported by Smerdon & Isenberg (1976).

However, the most interesting feature of Figure 4 is that the difference spectrum of the trypsin-resistant core from insect histone is very similar, in both shape and magnitude, to that of the calf H1 core. Taking into account that *Ceratitis* H1 contains two tyrosyl residues, located in the trypsin-resistant core, we can explain the similarity between the difference spectra depicted in Figure 4 in two ways: (a) one of the tyrosyl residues undergoes a change of environment in going from water to 0.3 M KF similar to that of the tyrosine of calf thymus H1, while the environment of the second tyrosyl residue of the fly histone does not appreciably change during salt-induced folding; (b) the two tyrosyl residues of *Ceratitis* H1 move to somewhat nonpolar environments upon protein folding, and the resulting red shift of both residues equals that of the single tyrosine of calf H1. While the first possibility seems to be more probable, the second one cannot be ruled

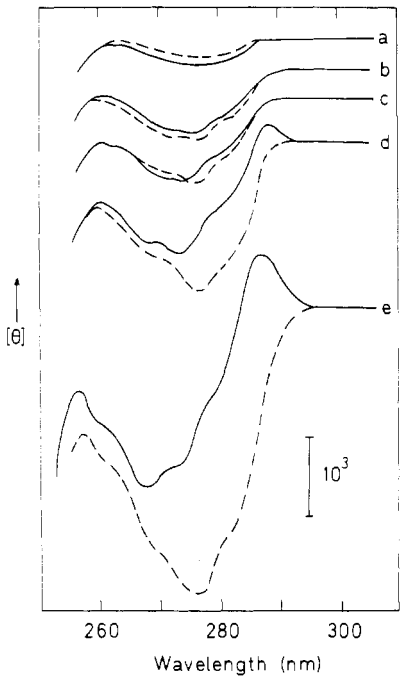

FIGURE 5: Influence of ionic strength on the circular dichroism of calf thymus H1 (—) and *Ceratitis* H1 (- - -) in the near-UV region. Solutions were 5×10^{-5} M protein in the following: (a) water, pH 2.8; (b) 0.04 M KF, pH 6.0; (c) 0.08 M KF, pH 6.0; (d) 0.15 M KF, pH 6.0; (e) 0.30 M KF, pH 6.0. Spectra were recorded in a 1-cm path length cell as described under Materials and Methods, and the results are expressed as molar ellipticities with the dimensions of deg × cm² × (dmol of protein)$^{-1}$.

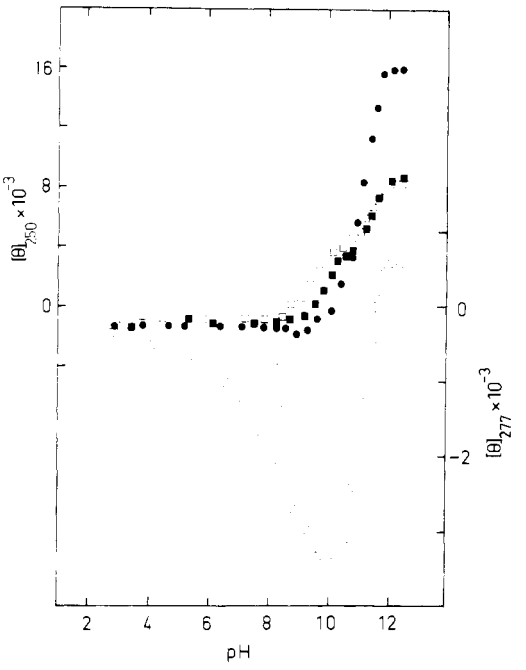

FIGURE 6: Molar ellipticities [deg × cm² × (dmol of protein)$^{-1}$] of 5×10^{-5} M solutions of histones H1 and their trypsin-resistant cores at two wavelengths (250 and 277 nm) as a function of pH. Filled circles: $[\theta]_{250}$ of the trypsin-resistant core from calf thymus H1 in water. Open squares: $[\theta]_{250}$ of the trypsin-resistant core from *Ceratitis* H1 in water. Filled squares: $[\theta]_{250}$ of the trypsin-resistant core from *Ceratitis* H1 in the presence of 0.3 M KF. Open circles: $[\theta]_{277}$ of calf thymus H1 in water. Open triangles: $[\theta]_{277}$ of the trypsin-resistant core from calf thymus H1 in water. Solutions were titrated with NaOH as described under Materials and Methods. Note that the scales of $[\theta]_{250}$ (left-side ordinate) and of $[\theta]_{277}$ (right-side ordinate) are different.

out without further experimental evidence. This will be provided below.

Apart from the results given in Figure 4, difference spectra were also recorded at several values of KF concentration between 0 and 0.5 M. The magnitude of $\Delta\epsilon_{278}$ showed a hyperbolic increase with ionic strength and reached its maximum value in 0.3 M KF. This behavior may be clearly correlated with the structural transitions observed in the circular dichroism experiments.

Circular Dichroism in the Near-UV Region. Figure 5 shows the circular dichroism spectra in the near-UV region of histones H1 from calf thymus and *C. capitata* at several ionic strength values. Although not depicted in Figure 5, the spectra of the trypsin-resistant cores were very similar to those of their parent molecules. The rotational strength of tyrosyl residues is very small in the unfolded molecules (Figure 5, a), as expected of randomly orientated isolated chromophores without intrinsic optical activity. The folding of the proteins in 0.3 M KF, pH 6.0, induced the appearance of rotational strength (Figure 5, e), but the spectra of calf thymus H1 and *Ceratitis* H1 differ quantitatively and qualitatively. The single tyrosyl residue of calf thymus H1 gives rise to a negative dichroic band, but the circular dichroism spectrum of folded *Ceratitis* H1 exhibits a small maximum at 287 nm and a minimum at 266 nm. This fact will be discussed below. It is also evident from Figure 5 that the circular dichroism spectra of both calf thymus and *Ceratitis* histone solutions are virtually identical up to 0.08 M KF. The influence of the second tyrosine of the insect histone on the circular dichroism spectrum became apparent when salt concentration was raised to 0.15 M KF (Figure 5, d).

A plot of $[\theta]_{277}$ against KF concentration (not shown) gave a hyperbolic curve in the case of calf thymus H1. This result provides additional evidence of noncooperativity in the salt-induced folding of H1. The presence of two tyrosines in fly H1 causes difficulties in the analysis of circular dichroism data, except at low KF concentrations (≤0.08 M), owing to the influence of the second tyrosyl residue on the spectrum.

Titration of the Tyrosyl Residues. The above results on the pH-induced folding of histones H1 and their trypsin-resistant cores, detected in our far-UV circular dichroism measurements, suggest that a similar effect might be observed when the pH dependence of the tyrosine ^1L_b transition is studied. Figure 6 shows that this possibility actually occurs. The plot of calf thymus H1 $[\theta]_{277}$ vs. pH shows a sharp change between pH 8 and 10, which agrees with the results of Smerdon & Isenberg (1976), and it may be due to a progressive decrease of tyrosine mobility as the protein is folded. A similar plot for the trypsin-resistant core of calf H1, also depicted in Figure 6, shows that the transition is less cooperative than that in the intact molecule. This fact is in accordance with the results shown in Figure 3, and it may be explained in a similar way (see Discussion).

The circular dichroism signals at 277 nm began to diminish at pH 10; this is obviously due to the ionization of the tyrosine phenolic group, because histones H1 and their trypsin-resistant cores are still folded at pH 12 (Figures 2 and 3). Taking advantage of this fact, we have determined the pK_a' values of the tyrosyl residues, as shown in Table III. These values have also been determined from the pH dependence of the ellipticity at 250 nm (Figure 6), a wavelength region where the ^1L_a band of tyrosine is shifted upon ionization of the phenolic group (Pflumm & Beychok, 1969). The results of this determination are also shown in Table III. Additional checking of these calculations was provided by pH-difference absorption measurements at 243 and 293 nm. Table III shows the close correspondence of the pK_a' values determined by these four

Table III: pK_a' Values of the Phenolic Groups of Calf Thymus and *C. capitata* Tyrosyl Residues

		pK_a'			
		first tyrosine		second tyrosine	
histone	method	no salt	0.3 M KF	no salt	0.3 M KF
calf thymus H1	$[\Theta]_{250}$	11.20	11.25		
	$[\Theta]_{277}$	11.15	11.15		
	ΔA_{243}	11.15	11.30		
	ΔA_{293}	11.30	11.30		
calf thymus TRC[a]	$[\Theta]_{250}$	11.20	11.30		
	$[\Theta]_{277}$	11.15	11.15		
	ΔA_{243}	11.30	11.30		
	ΔA_{293}	11.25	11.35		
Ceratitis H1	$[\Theta]_{250}$	11.40	11.30	9.40	nd[b]
	$[\Theta]_{277}$	11.20	11.20	nd[b]	nd[b]
	ΔA_{243}	11.30	11.20	9.20	9.70
	ΔA_{293}	11.30	11.20	9.30	9.80
Ceratitis TRC[a]	$[\Theta]_{250}$	11.25	11.40	9.20	9.90
	$[\Theta]_{277}$	11.20	11.25	nd[b]	nd[b]
	ΔA_{243}	11.30	11.40	9.20	9.80
	ΔA_{293}	11.20	11.30	9.30	9.80

[a] TRC = trypsin-resistant core. [b] Not determined.

separate measurements both in the presence and absence of 0.3 M KF. It is evident from the data shown in Table III that the pK_a' of the phenolic group of the single tyrosyl residue of calf thymus H1 is abnormally high.

The titration of the tyrosyl residues of *Ceratitis* H1 by circular dichroism at 250 nm (Figure 6) gave a two-step curve, showing that each tyrosine titrates independently. The ellipticity at 250 nm at the end point of the titration curve (pH 12.5) is smaller than that in the calf H1 trypsin-resistant core curve. This is not surprising, because of the relatively large contributions of the tails of the peptide chromophore to the ellipticity at 250 nm. The possibility that the sign of rotational strength is opposite for the two tyrosinates can be ruled out by two facts: the two clearly visible steps in the curves and the continuous increase in ellipticity in going to higher pH values.

One of the tyrosyl residues of *Ceratitis* H1, referred to as "first tyrosine" in Table III, behaves in every way like the tyrosyl residue of calf thymus H1, and this fact may be taken as indirect proof of hypothesis a (see above). The behavior of the extra tyrosyl residue of *Ceratitis* H1, further referred to as "second tyrosine", is, however, markedly different. It titrates with a low pK_a', which largely depends on ionic strength. These facts may indicate that the second tyrosine is on the surface of the trypsin-resistant core of the insect H1.

Were the above assumptions correct, the *Ceratitis* H1 tyrosyl residue whose environment is similar to that of tyrosine-72 in calf thymus histone would titrate with the same pK_a', whereas the second tyrosyl residue would be the one which titrates with $pK_a' = 9.2$. The experiment described in Figure 7 proves that this assumption is right. In the calf thymus H1 there is a small change in $[\Theta]_{277}$ in going from pH 6.0 to 10.2 in the presence of 0.3 M KF, which is due to the partial ionization of the phenolic group. It is important to note that in the present experiment the saline concentration was maintained at 0.3 M KF in order to keep the protein folded while the pH is raised; otherwise the structural transitions that follow the increase of pH (see above) would conceal the changes due to the ionization of tyrosine phenolic groups. As far as the calf thymus H1 is concerned, the ionization of the tyrosyl residue at pH 10.2 would only have proceeded to 10%, as calculated by using the Henderson–Hasselbach equation

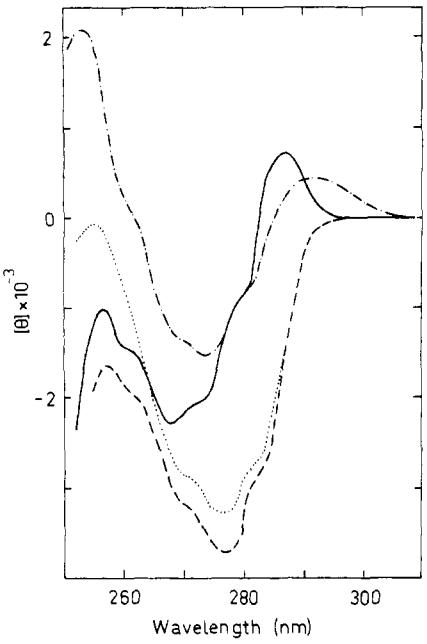

FIGURE 7: Circular dichroism spectra (near-UV) of 5×10^{-5} M solutions of histones H1 in the presence of 0.3 M KF. (- - -) Calf thymus H1 at pH 6.0; (···) calf thymus H1 at pH 10.2; (—) *Ceratitis* H1 at pH 6.0; (-·-) *Ceratitis* H1 at pH 10.2. Spectra were recorded and expressed as in Figure 5.

with $pK_a' = 11.25$ (Table III). The ^1L_b transition of the tyrosinate chromophore does not possess optical activity, as revealed by the absence of bands at ~290 nm. On the contrary, the ^1L_a transition actually is active, as denoted by the increase in the ellipticity at 250 nm. This increase was, in fact, used to obtain titration curves, as depicted in Figure 6.

Figure 7 shows some interesting features in the behavior of insect H1. It should be pointed out that at pH 10.2 the second tyrosine is almost completely ionized; assuming $pK_a' = 9.90$ (Table III), its ionization can be estimated at 85%. On the other hand, the first tyrosine, whose pK_a' is identical with that of calf thymus H1 tyrosine, is ionized to a much lesser extent (10%). It is then evident from Figure 7 that upon ionization of the second tyrosyl residue, the small maximum at 287 nm shifts to 293 nm, indicating that its tyrosinate form does possess optical activity. The shape of the fly H1 spectrum at pH 10.2 around 275 nm closely resembles that of the calf thymus H1 curve. There is, of course, a discrepancy in magnitude that may surely result from the partial overlapping of both the ^1L_a and ^1L_b transitions of the second tyrosyl residue in its tyrosinate form, but these results seem to indicate that the environment of the first tyrosyl residue is largely similar to that of the calf thymus H1 tyrosine, in accordance with hypothesis a (see above).

Discussion

The behavior of the trypsin-resistant cores from histones H1 clearly supports the assumption that they contain the globular region of the parent molecules. Apart from the reasons given above, further evidence comes from the method of isolation itself, because tryptic digestions were carried out in the presence of 1.0 M NaCl and we have shown that the maximum degree of folding in both calf thymus and *Ceratitis* H1 is reached in 0.3 M KF. A more direct proof, however, is given by the fact that all the α-helix segments of the intact molecules are found in the trypsin-resistant cores. This statement comes from a simple calculation from the sizes of the intact molecules and the trypsin-resistant cores and their respective content of α-helical structure.

Results given in Figures 2 and 3 show that pH-induced folding is more cooperative in the intact molecules than in the trypsin-resistant cores. The curve of $[\Theta]_{222}$ vs. pH for the intact molecules (Figure 2) is sharper than that of the cores (Figure 3), and the midpoint occurs at a higher pH value (8.3 in the intact molecules and 7.3 in the cores). Obviously, the presence of the N-terminal and C-terminal regions in the intact molecule causes it to fold at higher pH values and be more cooperative. This may be explained by the fact that the region 35–120 of the molecule contains 17 basic amino acids and 6 acidic residues, whereas the content of basic amino acids in the whole molecule amounts to 66 and that of acidic residues to only 9 (these figures have been taken from the data on CTL1 subfraction; Cole, 1977). This means that at neutral pH values the trypsin-resistant cores would have a net positive charge of ~11, while the charge of the intact molecules would be as high as +57. Bearing this in mind, it is easy to understand that the cores can start folding at lower pH values, but the folding of intact molecules would require higher pH values in order to minimize the electrostatic repulsions. However, once the folding had started, it would proceed with higher cooperativity.

The coarse aspects of secondary and tertiary structure of the calf thymus and insect histones seem to be very similar and so do those of the trypsin-resistant cores. There are some differences in composition between both H1 cores (Table I) which do not affect that level of structural organization, but they do have an influence on the fine details of structure, as discussed below. In any case, the resemblance between the H1 cores themselves is higher than that between the intact molecules [see Franco et al. (1977) for the composition of *Ceratitis* H1] and this fact may be in accordance with the assumed conservativity of the globular region of H1 histones. However, the conservativity may not be too strict, because preliminary evidence shows that the fingerprints and initial sequence of the trypsin-resistant core of *Ceratitis* H1 differ to some extent from those of the calf H1 core (J. L. Barbero and R. Rodríguez, unpublished results).

The results reported in this paper support the idea that the environment of the first tyrosyl residue of *Ceratitis* H1 is similar to that of the calf thymus H1 tyrosine-72, which is buried in the hydrophobic core (Chapman et al., 1978a). The similarity of the difference spectra (Figure 4) and the close correspondence of their pK_a' values (Table III) are the more evident facts to support the hypothesis. On the other hand, results shown in Figure 5 seem to indicate that the initial folding of *Ceratitis* H1 (up to 0.08 M KF) results in the moving of just the first tyrosyl residue to an anisotropic environment, this being an indirect proof of its inner location.

The second tyrosyl residue of the *Ceratitis* histone is on the surface of the trypsin-resistant core, as shown by its pK_a' value and its dependence on ionic strength (Table III and Figure 6). This residue moves to an anisotropic environment in a late stage of *Ceratitis* H1 folding (Figure 5) as expected of a residue located on the surface of the molecule. The optical activity of the second tyrosine chromophore does affect at this stage the activity of the first one (Figures 5 and 7). The possibility that the Cotton effects of both tyrosines in fly H1 are not independent because of their relative proximity should be kept in mind. Coupling of transitions between aromatic residues might result in exciton splitting, leading to a combination of positive and negative dichroic bands (Sears & Beychok, 1973). Similar interactions between chromophores, involving tyrosine transitions, have been suggested to explain the shape of the circular dichroism spectrum of human lysozyme (Halper et al., 1971). Whatever the nature of the reciprocal influences between tyrosyl chromophores might be, their differences of environment seem to be evident. Very recently, Padrós and Palau (J. Palau, personal communication) have shown that the histone ϕ1 from the sperm of the sea urchin *Arbacia lixula* also contains two tyrosines; these authors have been able to demonstrate by thermal denaturation studies that one of these residues is included in a nonpolar environment, while the second one seem to be exposed to the solvent. This structural feature might then be characteristic of some very lysine-rich histones from invertebrates.

Acknowledgments

We are very grateful to Professor A. M. Municio for his invaluable suggestions and helpful advice during the course of this research. We are also very indebted to Dr. J. G. Gavilanes for helpful discussions and to G. González de Buitrago for excellent technical assistance. We also thank Dr. J. Palau for communicating unpublished data.

References

Avilés, F. J., Chapman, G. E., Kneale, G. G., Crane-Robinson, C., & Bradbury, E. M. (1978) *Eur. J. Biochem. 88*, 363–371.

Avilés, F. J., Danby, S. E., Chapman, G. E., Crane-Robinson, C., & Bradbury, E. M. (1979) *Biochim. Biophys. Acta 578*, 290–296.

Bradbury, E. M., Carpenter, B. G., & Rattle, H. W. E. (1973) *Nature (London) 241*, 123–126.

Bradbury, E. M., Chapman, G. E., Danby, S. E., Hartman, P. G., & Riches, P. L. (1975a) *Eur. J. Biochem. 57*, 521–528.

Bradbury, E. M., Cary, P. D., Chapman, G. E., Crane-Robinson, C., Danby, S. E., Rattle, H. W. E., Boublik, M., Palau, J., & Avilés, F. J. (1975b) *Eur. J. Biochem. 52*, 605–613.

Chapman, G. E., Hartman, P. G., & Bradbury, E. M. (1976) *Eur. J. Biochem. 61*, 69–75.

Chapman, G. E., Hartman, P. G., Cary, P. D., Bradbury, E. M., & Lee, D. R. (1978a) *Eur. J. Biochem. 86*, 35–44.

Chapman, G. E., Avilés, F. J., Crane-Robinson, C., & Bradbury, E. M. (1978b) *Eur. J. Biochem. 90*, 287–296.

Christiansen, G., & Griffith, J. (1977) *Nucleic Acids Res. 4*, 1837–1851.

Cole, R. D. (1977) in *The Molecular Biology of the Mammalian Genetic Apparatus* (Ts'o, P. O. P., Ed.) Vol. I, pp 93–104, North-Holland Publishing Co., Amsterdam.

Craven, G. R., Steers, E., & Anfinsen, C. B. (1965) *J. Biol. Chem. 240*, 2468–2477.

Donovan, J. W. (1969) *J. Biol. Chem. 244*, 1961–1967.

Felsenfeld, G. (1978) *Nature (London) 271*, 115–122.

Finch, J. T., & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1897–1901.

Franco, L., Montero, F., Navlet, J. M., Perera, J., & Rojo, M. C. (1974) *Eur. J. Biochem. 48*, 53–61.

Franco, L., Montero, F., & Rodríguez-Molina, J. J. (1977) *FEBS Lett. 78*, 317–320.

Gaubatz, J., Hardison, R., Murphy, J., Eichner, M. E., & Chalkley, R. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 265–272.

Halper, J. P., Latovitzki, N., Bernstein, H., & Beychok, S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 517–521.

Hartman, P. G., Chapman, G. E., Moss, T., & Bradbury, E. M. (1977) *Eur. J. Biochem. 77*, 45–51.

Isenberg, I. (1976) in *Search and Discovery*, a volume dedicated to Albert Szent-Gyorgy (Kaminer, B., Ed.) Academic Press, New York.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159–191.
Johns, E. W. (1964) *Biochem. J. 92*, 55–59.
Johns, E. W., & Butler, J. A. V. (1962) *Biochem. J. 82*, 15–18.
Jones, G. M. T., Rall, S. C., & Cole, R. D. (1974) *J. Biol. Chem. 249*, 2548–2553.
Littau, V. C., Burdick, C. J., Allfrey, V. G., & Mirsky, A. E. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1204–1212.
Noll, M., & Kornberg, R. D. (1977) *J. Mol. Biol. 109*, 393–404.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.
Panyim, S., & Chalkley, R. (1971) *J. Biol. Chem. 246*, 7557–7560.
Pflumm, M. N., & Beychok, S. (1969) *J. Biol. Chem. 244*, 3982–3989.
Puigdomênech, P., Cabrê, O., Palau, J., Bradbury, E. M., & Crane-Robinson, C. (1975) *Eur. J. Biochem. 59*, 237–243.
Rall, S. C., & Cole, R. D. (1971) *J. Biol. Chem. 246*, 7175–7190.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879–1883.
Sears, D. W., & Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry, Part C* (Leach, S. J., Ed.) pp 445–593, Academic Press, London.
Singer, D. S., & Singer, M. F. (1978) *Biochemistry 17*, 2086–2095.
Smerdon, M. J., & Isenberg, I. (1976) *Biochemistry 15*, 4233–4242.
Vogel, T., & Singer, M. F. (1976) *J. Biol. Chem. 251*, 2334–2338.

Small-Angle X-ray Studies of the Quaternary Structure of the *lac* Repressor from *Escherichia coli*†

Ingrid Pilz,* Karin Goral, Otto Kratky, R. P. Bray, N. G. Wade-Jardetzky, and Oleg Jardetzky

ABSTRACT: The quaternary structures of the *lac* repressor molecule from *Escherichia coli* and its tetrametic core, which can be derived from it by proteolytic cleavage, were studied in dilute solutions by small-angle X-ray scattering. The dimensions and general shape of the *lac* repressor and of the tetrameric core are reported. The core itself appears to be an elongated structure, and in the intact repressor the headpieces are located at its ends. The results are derived from model calculations and from the following molecular parameters determined from the scattering curve and the pair distance distribution function: for lac repressor, radius of gyration 5.30 ± 0.02 nm, radius of gyration of the cross section 2.20 ± 0.03 nm, maximum diameter 18.0 ± 0.5 nm, hydrated volume 329 ± 20 nm^3, relative molecular mass 149 000 ± 15 000; for tetrameric core, radius of gyration 4.92 ± 0.02 nm, radius of gyration of the cross section 2.24 ± 0.03 nm, maximum diameter 16.0 ± 0.5 nm, hydrated volume 278 ± 15 nm^3, relative molecular mass 120 000 ± 10 000.

The *lac* repressor is a regulatory protein of *Escherichia coli* originally isolated and characterized by Gilbert & Muller-Hill (1966). It has a molecular weight of 154 500 and is a tetramer of four identical subunits, each with a known sequence of 360 amino acids (Beyreuther, 1978; Farabaugh, 1978). Its function is to control the expression of the *lac* operon by forming a complex with the operator region of its DNA. Some of the physical properties of the repressor have been studied (Barkley et al., 1975), but little is known about its tertiary and quaternary structures. In the present paper we report the dimensions and the general shape of the repressor molecule and of the tetrameric core that can be derived from it by proteolytic cleavage, as determined by small-angle X-ray scattering.

Materials and Methods

The *lac* repressor (LR)[1] was prepared from cultures of the SQ mutant of *E. coli*, as previously described (Matthews et al., 1977). The tryptic core (T-core) and headpiece (HP) fragments of LR have been prepared according to the procedure of Geisler & Weber (1977). LR, at a protein concentration of 15 mg/mL in 1 M Tris-HCl buffer (pH 7.6), 30% glycerol, and 3×10^4 M dithiothreitol, was digested with 1.5% of its weight of *N,N*-dicyclohexylcarbodiimide-treated trypsin (Sigma Chemical Company) for 2 h at 20 °C. The trypsin was then inactivated by a threefold excess of soybean trypsin inhibitor (Sigma Chemical Company). Under these conditions, peptide bond hydrolysis at lysyl residue 59 goes to completion, while a partial cleavage occurs at arginyl residue 51. The monomeric HP (residues 1–51 and 1–59) and the tetrameric T-core (residues 60–347) have been purified at 5 °C by gel filtration on a column of Sephadex G-150 eluted with 0.2 M ammonium bicarbonate with 3×10^{-4} M dithiothreitol.

For diffraction studies LR, T-core, and HP were extensively dialyzed against a 0.3 M potassium phosphate buffer containing 0.2 M KCl, 1×10^{-4} M dithiothreitol, and 1×10^{-4} M ethylenedinitrilotetraacetic acid, pH 7.7. The homogeneity of the LR and T-core preparations was checked by NaDodSO$_4$–gel electrophoresis, as shown in Figure 1.

A highly stabilized X-ray generator (Philips PW 1140) with a copper target tube (50 kV, 30 mA) and a camera with slit

† From the Institut für Physikalische Chemie der Universität Graz, A-8010 Graz, Austria (I.P. and K.G.), the Institut für Röntgenfeinstrukturforschung der Österreichischen Akademie der Wissenschaften und des Forschungszentrums Graz, A-8010 Graz, Austria (O.K.), and the Stanford Magnetic Resonance Laboratory, Stanford University, Stanford, California 94305 (R.P.B., N.G.W.-J., and O.J.). *Received November 19, 1979; revised manuscript received May 5, 1980.* We thank the Österreichischen Fonds zur Förderung der wissenshaftlichen Forschung and the National Institutes of Health (GM18098) for generous support.

[1] Abbreviations used: LR, *lac* repressor; T-core, tryptic core; HP, headpiece; NaDodSO$_4$, sodium dodecyl sulfate.

0006-2960/80/0419-4087$01.00/0