Articles

Self-Association of the Globular Domain of Histone H5†

Joseph D. Maman,‡ Thomas D. Yager,§ and James Allan*

Department of Biochemistry, Edinburgh University, Hugh Robson Building, George Square, Edinburgh EH8 9XD, United Kingdom

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ABSTRACT: The capacity of the globular domain of the chicken erythrocyte linker histone H5 (GH5) to self-associate in solution has been demonstrated by chemical cross-linking with dimethyl 3,3′-dithiobis-(propionimidate) (DTBP), dithiobis(succinimidyl propionate) (DSP), and 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP). Several observations suggest that the GH5–GH5 interactions that mediate self-association are specific: (a) Incubation with each of the above reagents produces a discrete and characteristic pattern of cross-linked products; (b) GH1, the related peptide from chicken erythrocyte H1, is not cross-linked under the same conditions; (c) GH5 is not cross-linked with disuccinimidyl tartarate (DST), which has a shorter cross-linking span (6.4 Å) than the other reagents (12 Å); and (d) analysis of cross-linking as a function of peptide concentration provides an equilibrium constant for GH5 self-association of (4.8 ± 1.3) × 10^3 M^{-1}. The ability of GH5 to specifically self-associate is compatible with the proposal [Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell Biol. 83,* 403–427] that linker histone globular domains occupy an axial position within the higher order chromatin fiber; the spatial juxtaposition of the GH5 domains at this location would be expected to promote their association and exert a stabilizing effect upon higher order chromatin structure.

The DNA in most eukaryotic cells is packaged, by association with histone proteins, into the form of a higher order chromatin fiber (van Holde, 1988). Recent evidence suggests that even transcriptionally active regions of the genome are only transiently unfolded from this state (Nacheva et al., 1989; Ericsson et al., 1990). Although the structure of the basic unit of the chromatin fiber, the core particle, is understood at relatively high resolution (Richmond et al., 1984), the nature of the higher order chromatin fiber remains poorly understood and controversial (Felsenfeld & McGhee, 1986; van Holde, 1988; Williams & Langmore, 1991): in structural terms we can be relatively confident only about its average diameter (30 nm), its helical structure, and that the nucleosomes in the fiber are radially arranged with their flat faces approximately parallel to the axis (McGhee et al., 1980). The absence of a precise model for the higher order chromatin fiber arises because we have been unable to identify (i) the path of the linker DNA as it passes from one nucleosome to its neighbors and (ii) the location of the linker histone, H1, within the fiber and the manner in which this protein determines and maintains the folded state.

The canonical linker histone comprises three domains: a globular domain, which is highly conserved, is flanked at its NH-terminus by a short, unstructured tail, which varies extensively in length between H1 subtypes, and at its COOH-terminus by a long tail, which contains a very high proportion of basic residues (Hartman et al., 1977). The structure of the globular domain of the erythroid-specific linker histone H5 was deduced from two-dimensional nuclear magnetic resonance studies (Zarbock et al., 1986; Clore et al., 1987) and, more recently, solved to 2.5-Å resolution by crystal diffraction (Ramakrishnan et al., 1993).

We have previously shown that the globular domains of H1 and H5 bind at the dyad axis of the nucleosome where the DNA enters and exits from the restraint of the core histone octamer, thus sealing two complete turns of DNA within this structure (Allan et al., 1980). The location of the COOH-terminal tail of H1, which is instrumental in folding chromatin into higher order structure, is determined by the binding of the globular domain (Allan et al., 1986). Within the higher order chromatin fiber, the location of the globular domain, and therefore the COOH-terminal tail, remains unknown.

Attempts to locate linker histones within the higher order chromatin fiber have employed probes directed toward the globular domains of H1 and H5 (GH1 and GH5, respectively); in contrast to the long and unstructured COOH-terminal tails, these might be expected to occupy a discrete location within the folded fiber. The use of antibodies to GH1 and GH5 has provided conflicting data, suggesting on the one hand that the globular domain is sequestered at an inaccessible location within the higher order chromatin fiber (Dimitrov et al., 1987; Cattini & Allan, 1988), while other studies have suggested that higher order folding makes little difference to accessibility (Russanova et al., 1987). Studies in which chymotrypsin was used as a probe suggested that the globular domain of H1 was relatively inaccessible, and therefore centrally located, within the rat liver higher order chromatin fiber (Losa et al., 1984).

An axial location for the globular domain of H1 is an integral feature of the solenoid model for the higher order chromatin fiber proposed by Thoma et al. (1979). They argued that polymerization of globular domains at the axis of the fiber could play an important part in determining and stabilizing

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* Author to whom correspondence should be addressed.

‡ Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

§ Present address: Research Institute and Division of Nephrology, Hospital for Sick Children, Toronto, Ontario, Canada MSG 1X8.

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higher order structure. Cross-linking studies of linker histones in free solution (Thomas & Khabaza, 1980; Russo et al., 1983) and within the chromatin fiber (Lennard & Thomas, 1985) have, however, failed to demonstrate any significant tendency for the linker histones to self-associate via their globular domains.

In this study, we have investigated the self-association properties of the isolated globular domains of linker histones H1 and H5. Using cross-linking in free solution, we demonstrate that GH5 does self-associate to form polymers, while GH1 remains largely monomeric within the accessible concentration range. These observations are discussed in terms of the structure, stability, and functional properties of the higher order chromatin fiber.

MATERIALS AND METHODS

(a) Sample Preparation. Chicken erythrocyte H5 and H1 were prepared as described previously (Harborne & Allan, 1986). The globular domains (GH5 and GH1) were prepared by limited trypsin digestion and purified according to the method of Aviles et al. (1978).

(b) Chemical Cross-Linking. Chemical cross-linking of GH5 or GH1 was performed with dimethyl 3,3′-dithiobis-(propionimidate) (DTBP), dithiobis(succinimidyl propionate) (DSP), 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), and disuccinimidyl tartarate (DST) obtained from Pierce Ltd. Immediately before use, DSP and DST were dissolved in *N*,*N*-dimethylformamide to 5 mg/mL; DTBP was dissolved in 50 mM triethanolamine (TEA) (pH 8.5), 1 mM EDTA, and 0.25 mM phenylmethanesulfonyl fluoride (PMSF) to 10 mg/mL; and DTSSP was dissolved in 20 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, and 0.25 mM PMSF to 4.5 mg/mL. GH5 or GH1, at 0.2 mg/mL (0.1 mg/mL for DTBP cross-linking), was cross-linked with DSP or DST at 0.25 mM in 1 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, and 0.25 mM PMSF (Clark & Thomas, 1988) or with DTBP at 2 mg/mL in 50 mM TEA (pH 8.5), 1 mM EDTA, and 0.25 mM PMSF. GH5 was cross-linked as a function of concentration (0.1–3.0 mg/mL, 0.012–0.36 mM) with equimolar DTSSP in 20 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, and 0.25 mM PMSF; the sodium phosphate concentration was raised to 20 mM in these reactions to maintain the pH at 7.0–7.5. Cross-linking reactions were supplemented with NaCl as appropriate. Reactions were conducted at room temperature and were terminated by the addition of glycine to 50 mM. Reaction products were either loaded directly onto gels after the addition of concentrated gel-loading buffer (minus β-mercaptoethanol) or, after adjustment to equal volume and 250 mM NaCl, precipitated with 6 vol of acetone and collected by centrifugation. Pellets were washed with acetone, dried in vacuo, redissolved in gel-loading buffer (minus β-mercaptoethanol), and examined on 15% SDS–polyacrylamide gels (Laemmli, 1970). Proteins were detected by silver staining according to Wray et al. (1981) and analyzed by densitometry (Molecular Dynamics digital scanner, Imagequant software).

(c) Circular Dichroism. Circular dichroism (CD) spectra between 250 and 200 nm wavelengths were measured using a Cary 61 spectropolarimeter. GH5 and GH1 concentrations, in 6 M guanidine hydrochloride, were determined by UV spectroscopy assuming molar absorptions of 4500 M cm^{-1} for GH5 and 1500 M cm^{-1} for GH1: these values are based on the molar extinction coefficient of an *N*-acetyl-L-tyrosyl ethyl ester moiety at 275.5 nm in 6 M guanidine hydrochloride (pH 6.5) (Edelhoch, 1967), and they reflect the fact that GH5 contains three tyrosines (Briand et al., 1982) and GH1 one tyrosine (Sugarman et al., 1983). The molecular weights of GH5 and GH1 were calculated from their amino acid compositions (Briand et al., 1982; Sugarman et al., 1983). CD spectra were measured, at 0.5 mg/mL peptide, in 1 mM sodium phosphate (pH 7.4) and at various NaCl concentrations, at room temperature in 2-mm path length cells. The mean residue ellipticity ($[\theta]$) is expressed in deg cm^2 $dmol^{-1}$; the error for $[\theta]$ was ±5% at 222 nm.

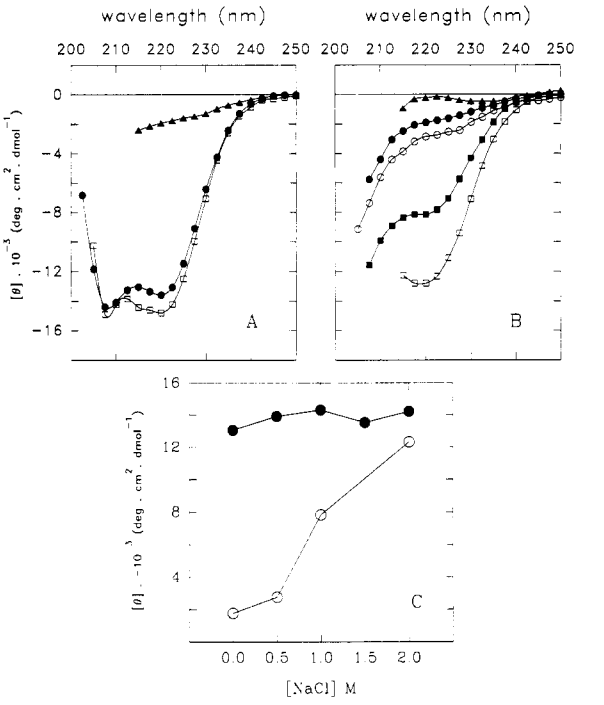

FIGURE 1: CD spectra of GH5 (A) and GH1 (B) in 6.0 M guanidine chloride (▲) and in 1 mM sodium phosphate (pH 7.4) containing 0.0 (●), 0.5 (○), 1 (■), or 2 M (□) NaCl. In C are shown the mean residue ellipticities at 222 nm for GH5 (●) and GH1 (○) in 1 mM sodium phosphate (pH 7.4) as a function of NaCl concentration.

(d) Trypsin Digestion. H5, GH5, and GH1, at 0.2 mg/mL, were digested with trypsin at 37 °C at an enzyme:substrate ratio of 1:250 (w:w) in 1 mM sodium phosphate (pH 7.4) and 0.2 mM EDTA. Reactions were supplemented with NaCl at various concentrations as appropriate. Digestion was stopped by the addition of PMSF to 1 mM. Samples were either loaded directly onto 18% SDS–polyacrylamide gels or first precipitated with 6 vol of acetone, collected by centrifugation, and the dried pellets resuspended in loading buffer containing 0.5% β-mercaptoethanol. Proteins were detected by silver staining.

RESULTS

(a) Folding of the Linker Histone Globular Domain. Prior to studying the self-association of linker histone globular domains in free solution, we examined their folding in 1 mM sodium phosphate (pH 7.4) as a function of NaCl concentration, in order to ensure that cross-linking, and other studies, would be carried out on the folded peptide. Both circular dichroism (CD) and resistance to trypsin were used to assess folding.

The CD spectra obtained from analyses of the globular domains of chicken erythrocyte H5 (GH5) and H1 (GH1) are shown in Figure 1. The data obtained for GH5 (Figure 1A) indicated that the peptide was almost fully folded in 1 mM sodium phosphate and that the addition of NaCl to 2 M made little further difference to the spectra. The capacity of

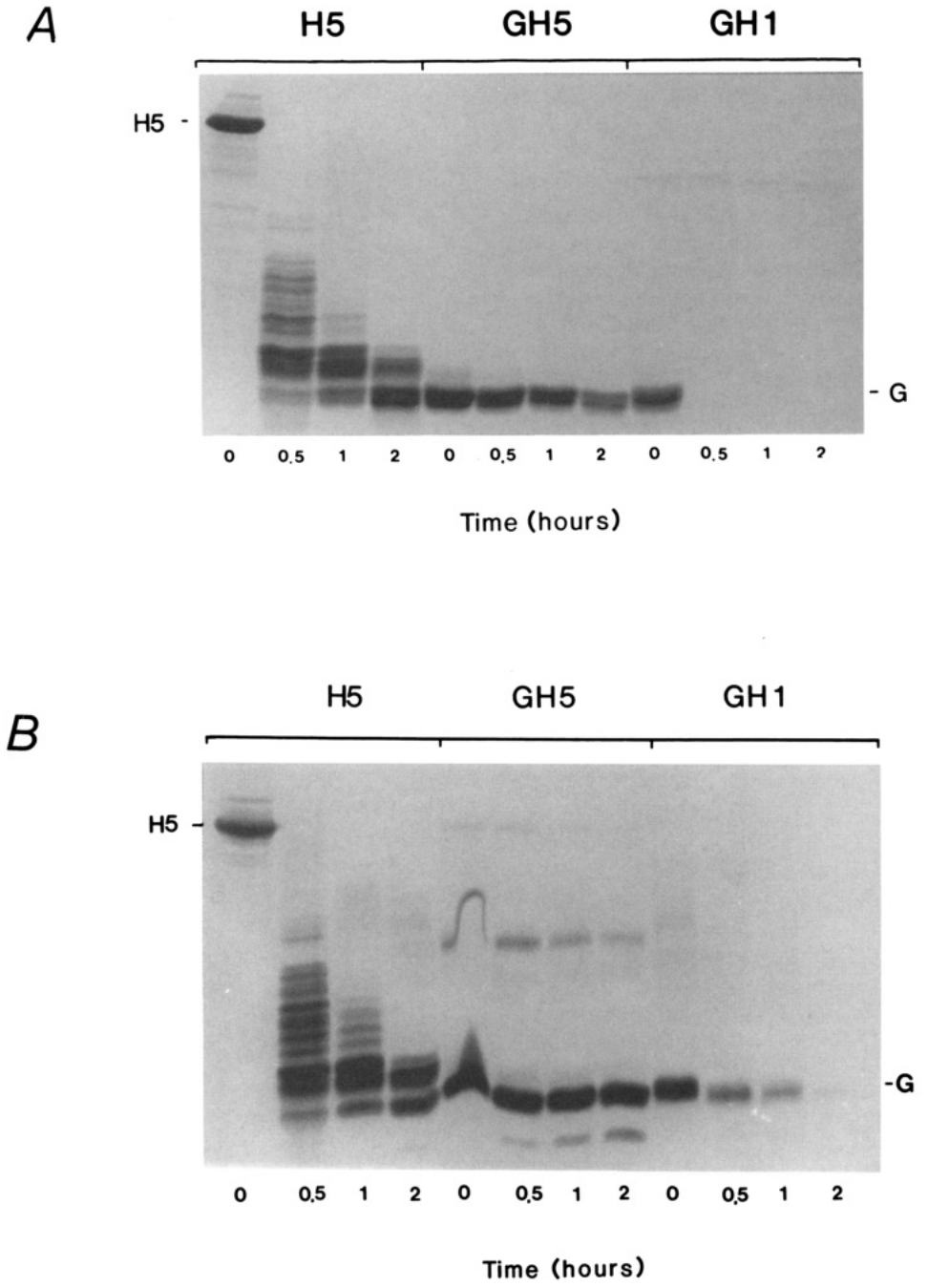

FIGURE 2: Trypsin digestion time courses for H5, GH5, and GH1 in 1 mM sodium phosphate (pH 7.4) containing 250 mM (A) or 2.0 M (B) NaCl (18% SDS–polyacrylamide gels). G indicates the position of the intact globular domain.

tetrahedral polyanions, particularly phosphates, to induce H5 and GH5 folding has been noted previously (De Petrocellis et al., 1986; Clark et al., 1988). From the mean residue ellipticity at 222 nm, an estimate of the number of globular domain amino acid residues in the α-helical state can be made (Clark et al., 1988): our data (Figure 1) gave a value of 33 residues, which compares favorably with the 32 residues estimated by Clore et al. (1987), but is lower than the 39 residues determined by Ramakrishnan et al. (1993).

In contrast to GH5, the CD spectra obtained for GH1 (Figure 1B) were strongly NaCl-concentration-dependent: in the absence of NaCl, GH1 was essentially devoid of tertiary structure, and complete folding was achieved only at 2 M NaCl. Clearly, phosphate ions are selective for GH5 with respect to their capacity to induce the folding of these peptides. When fully folded in 2 M NaCl, we calculate that 27 GH1 residues are in α-helical conformation, a value lower than the 34 residues reported for intact H1 by Clark et al. (1988). The profile of ellipticity as a function of NaCl concentration reveals that helicity is attained with GH1 only above 0.5 M NaCl (Figure 1C).

As an additional criterion of folding, the susceptibility of GH5 and GH1 (with chicken erythrocyte H5 as a control substrate) to trypsin degradation in the presence of 1 mM sodium phosphate was studied, again as a function of NaCl concentration. The results of representative experiments carried out in the presence of 250 mM and 2 M NaCl are shown in Figure 2. GH5 was essentially resistant to trypsin attack at both ionic strengths; GH1, however, was completely degraded by trypsin at the earliest time point (0.5 h) in 250 mM NaCl, and although the presence of 2 M NaCl substantially increased resistance, GH1 was completely digested after 2 h. These observations support those obtained by CD analysis, but they further indicate that the maximally folded form of GH1 is substantially less stable than that of GH5.

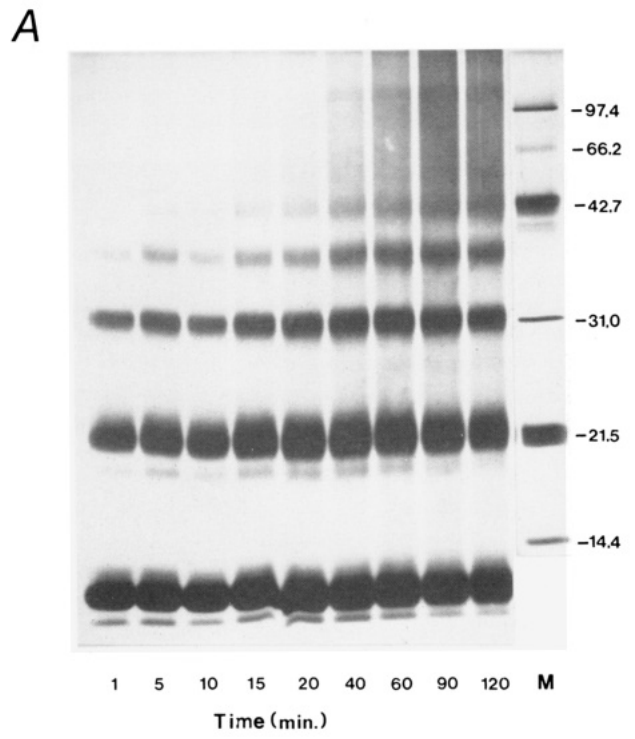

FIGURE 3: Cross-linking of GH5 with DTBP as a function of time (A) or NaCl concentration (B) (15% SDS–polyacrylamide gels). M is a low molecular weight protein marker; lane a in B is un-cross-linked GH5. The product X is referred to in the text.

(b) GH5 Cross-Linking in Solution. Dimethyl 3,3′-dithiobis(propionimidate) (DTBP), a homobifunctional imido ester (Wang & Richards, 1974) that reacts with ϵ-amino groups in proteins (Davis & Stark, 1970), has been used extensively in the study of histone–histone interactions in chromatin (Ring & Cole, 1979; Thomas & Khabaza, 1980). We have used DTBP to study the cross-linking of GH5 in free solution. Reactions were carried out in 50 mM TEA (pH 8.5), 1 mM EDTA, 0.25 mM PMSF, and 2 M NaCl with DTBP and GH5 at 6.5 mM (2 mg/mL) and 0.012 mM (0.1 mg/mL), respectively. Aliquots were quenched with glycine

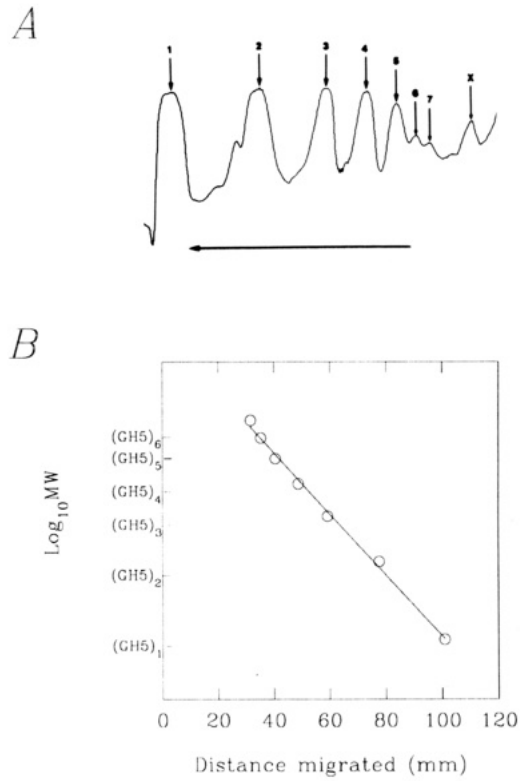

FIGURE 4: (A) Densitometry scan of cross-linked GH5 (0.5 M NaCl lane from Figure 3B). The horizontal arrow indicates the direction of electrophoresis. The numbered vertical arrows indicate the positions of GH5 oligomers. (B) A semilog plot of the predicted molecular weights of GH5 oligomers versus the relative mobility of the corresponding peaks from A.

at various times, acetone-precipitated, and analyzed on SDS–polyacrylamide gels.

DTBP was found to progressively cross-link GH5 into a set of discrete oligomers (Figure 3A). By the end of the reaction, dimers to pentamers constituted the most prominent of these species; relatively weaker bands corresponding to a hexamer and a heptamer were also discernible, together with a relatively strong band of slightly lower mobility than the 97.4-kDa marker. This basic pattern of cross-linking was not affected by NaCl (Figure 3B; here the high molecular weight product is labeled X). Strong staining at the top of these gels was indicative of material that could not enter the gel. When precipitated from 2 M NaCl, un-cross-linked GH5 was consistently observed to generate a small proportion of dimer and sometimes trimer (Figures 2B and 3B).

Densitometry of cross-linked GH5 (0.5 M NaCl lane in Figure 3B) indicated that most of the peptide was in the form of oligomers (Figure 4A). When the log of the molecular weights of the GH5 oligomers (monomer to heptamer) is plotted against the electrophoretic mobilities of the corresponding cross-linked products, a linear relationship is displayed (Figure 4B); by extrapolation, the high molecular weight band (X, Figure 3B) contained 12–14 cross-linked GH5 monomers.

The depletion of active DTBP, which has a half-life of about 5 min at pH 8.5 (Brown & Kent, 1975), is probably partly responsible for the cross-linking reaching an end point after about 90 min. In contrast to imido esters, NHS esters have a long half-life (4–5 h) at near-physiological pH (7.4) (Lomant & Fairbanks, 1976). Dithiobis(succinimidyl propionate) (DSP) is a bifunctional NHS ester with a maximum cross-linking distance of 12 Å (DTBP, 11.9 Å). DSP has previously been used to investigate the head-to-tail arrangement of H5 polymers in chromatin (Lennard & Thomas, 1985) and the

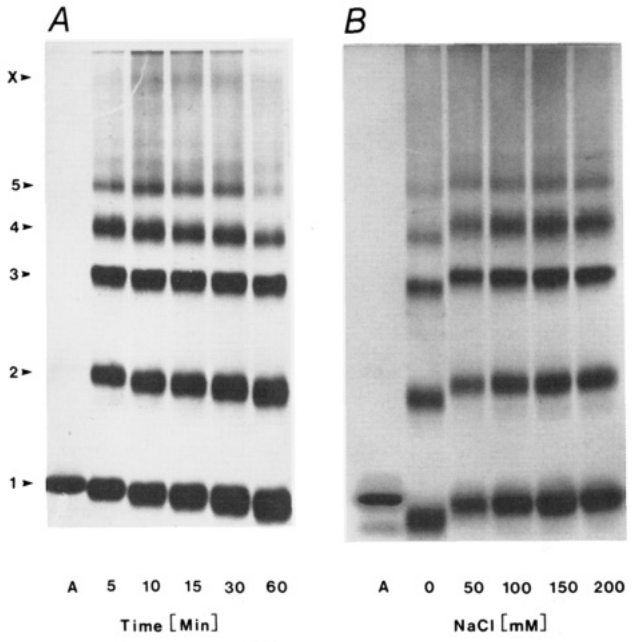

FIGURE 5: Cross-linking of GH5 with DSP as a function of time (A) or NaCl concentration (B) (15% SDS–polyacrylamide gels). Lane A contains un-cross-linked GH5. The product X is referred to in the text.

interaction of H1, GH1, and GH5 bound to DNA (Clark & Thomas, 1986, 1988; Thomas et al., 1992).

GH5, at 0.2 mg/mL (0.024 mM), was cross-linked with DSP at 0.1 mg/mL (0.25 mM) in 1 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, 0.25 M NaCl, and 0.25 mM PMSF; this equates to about one DSP molecule per available ϵ-amino group. Aliquots, withdrawn and quenched at timed intervals, were applied directly to SDS–polyacrylamide gels after the addition of concentrated loading buffer. The extent and pattern of GH5 cross-linking obtained with DSP after 10 min (Figure 5A) were equivalent to those with DTBP after 90 min (Figure 3A): strong bands up to a pentamer, weak hexamer and heptamer bands, and a discrete high molecular weight product (X) were again observed. Reaction times in excess of 10 min did not alter the distribution, although incubation for more than 30 min resulted in the gradual disappearance of higher oligomers, while lower oligomers displayed both band-broadening and an increased electrophoretic mobility. These effects probably reflect extensive intramolecular modification of GH5 by DSP, changing both the charge and conformation of the cross-linking products to affect both their solubility and electrophoretic mobility.

In the absence of NaCl, GH5 was less effectively cross-linked by DSP, and the products displayed an increased electrophoretic mobility (Figure 5B); this could again reflect extensive intramolecular modification and suggests that the self-association of GH5 is relatively weak under these conditions.

(c) GH5 Cross-Linking as a Function of Peptide Concentration. The results described above indicate that, in solution, GH5 oligomers are cross-linked with high efficiency, with the particular pattern of the cross-linked products suggesting, furthermore, that GH5 monomers self-associate in a specific manner. The reaction should therefore be characterized by one or more self-association equilibrium constants.

DSP was inappropriate for quantitative studies because its low solubility precluded the maintenance of a constant cross-linker to GH5 ratio at high GH5 concentrations. 3,3′-Dithiobis(sulfosuccinimidyl propionate) (DTSSP) is a highly water-soluble cross-linking reagent, analogous to DSP (Staros, 1982): it has the same maximum cross-linking distance (12 Å) and contains, at both ends of the molecule, a DSP-reactive group (*N*-hydroxysuccinimide) modified by a hydrophilic sulfonate group (Staros, 1982). DTSSP was therefore chosen for studying the concentration dependency of GH5 cross-linking.

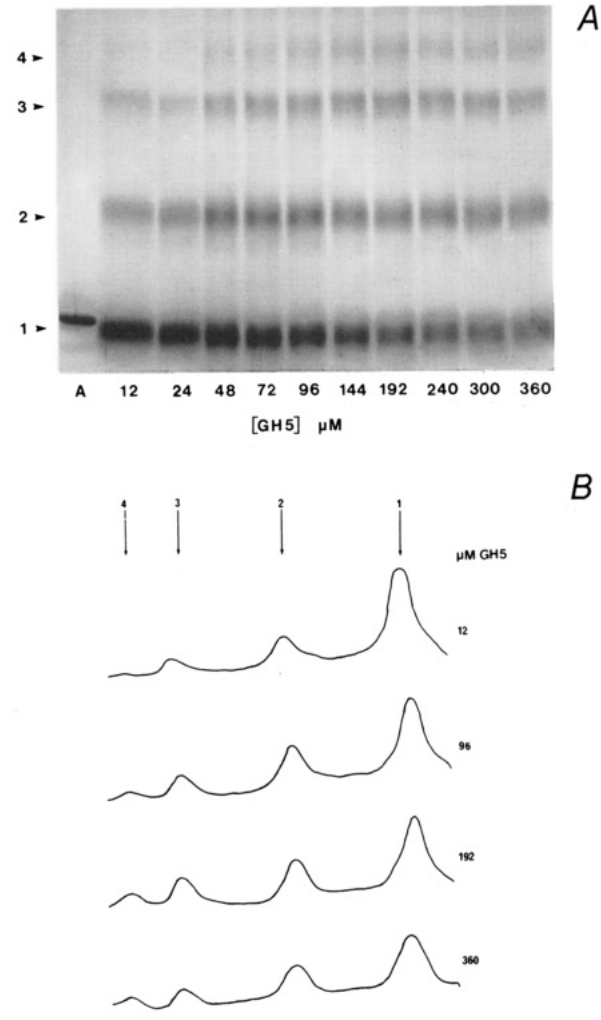

FIGURE 6: (A) Cross-linking of GH5 with DTSSP as a function of peptide concentration (15% SDS–polyacrylamide gel). Lane A is un-cross-linked GH5. The sizes of the GH5 oligomers are indicated. (B) Densitometry of selected lanes from A. Electrophoresis was from left to right. The numbered vertical arrows indicate the positions of the GH5 oligomers.

GH5, at 0.1–3.0 mg/mL (0.012–0.36 mM), was cross-linked with DTSSP at a ratio of one cross-linker molecule per available ϵ-amino group in 20 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, 250 mM NaCl, and 0.25 mM PMSF. After 10 min, the reactions were quenched, concentrated loading buffer was added, and 3-μg aliquots from each reaction were run on SDS–polyacrylamide gels. As illustrated in Figure 6A, as the concentration of GH5 was increased, a gradual decrease in the concentration of monomeric GH5, concomitant with an increase in the higher oligomers, was observed; the dimer peaked at around 72 μM and the trimer and tetramer at proportionately higher GH5 concentrations. These data have been evaluated quantitatively by densitometry (Figure 6B).

The data presented in Figure 6 were employed to estimate the self-association equilibrium constant of GH5 in solution. If we assume the reaction to be isodesmic (Adams et al., 1978), i.e., that it involves a linear stepwise association in which the equilibrium constant for adding a single monomer to the end of a growing chain is independent of its length, then the

molar concentration of the ith GH5 oligomer, C_i, can be expressed in terms of the molar concentration of free monomer (C_1):

$$C_2 = K_a C_1^2$$

$$C_3 = K_a^2 C_1^3$$

$$C_i = K_a^{i-1} C_1^i \quad (1)$$

where K_a is the association constant for the addition of a GH5 monomer to the end of any oligomer. The molar concentration of an oligomer (C_i) in monomer units is then

$$C_i = iK_a^{i-1} C_1^i \quad (2)$$

Summation over all oligomers (i = 0 to ∞) gives the total protein concentration (C_0) in monomer units:

$$C_0 = \frac{C_1}{(1 - K_a C_1)^2} \quad (3)$$

Thus, the total concentration being known, the determination of C_1 allows the computation of K_a.

Integration of the areas under the densitometric peaks corresponding to each oligomer zone (Figure 6B) gave the concentrations of all of the resolved components. A quantitative relationship between the peak area and GH5 concentration was established by scanning a gel containing a concentration gradient of un-cross-linked GH5: the stain response was found to be linear across the range of gel loadings used for cross-linking.

In Figure 7A, the fractional weight concentration of remaining monomer is plotted against the total concentration. As the GH5 concentration increases, the proportion of the monomer decreases, tending to a plateau value of about 0.35. A nonlinear best fit to the raw data was obtained, using a computer-based algorithm, by fitting the monomer concentration to the raw data of monomer and total concentration, calculating the ratios, and fitting them to the raw data plot:

$$\sqrt{C_1/C_0} = (1 - K_a C_1) \quad (4)$$

The best-fit data points fall on a line calculated for an isodemic model self-association constant of 4×10^3 M^{-1}; the calculated value for the constant is 3900 ± 460 (standard deviation). Thus, the data are satisfactorily accounted for by an isodesmic model for the self-association of GH5.

In Figure 7B, the ratio of the concentration of dimers, trimers, and tetramers (expressed in monomer units, eq 2) to the total GH5 concentration is expressed relative to the concentration of free monomer. The relative abundance of dimers and trimers shows the same trend of an initial rise to a maximum and then a fall, with the trimer peak lagging behind that of the dimer, in quantitative accordance with the characteristics of an isodesmic system. The tetramer profile shows a slow increase throughout the concentration range, probably because of base-line uncertainty due to the low intensitiy of the tetramer bands and to increased background noise of the scans.

Theoretical curves (Figure 7B) were obtained from a modified form of eq 2 (eq 5) in which arbitrary values for C_1 and different values for K_a were employed:

$$\frac{C_i}{C_0} = \frac{iK_a^{i-1} C_1^i}{C_0} = iK_a^{i-1} C_1^{i-1} (1 - K_a C_1)^2 \quad (5)$$

A nonlinear best-fit analysis of the raw data for each species (Figure 7B) was also obtained using eq 5. There is generally good agreement between the best-fit and theoretical curves for each species, although for the tetramer a best-fit constraint of $K_a > 2000$ had to be applied. The values for the binding constants obtained for each species are shown in Table 1.

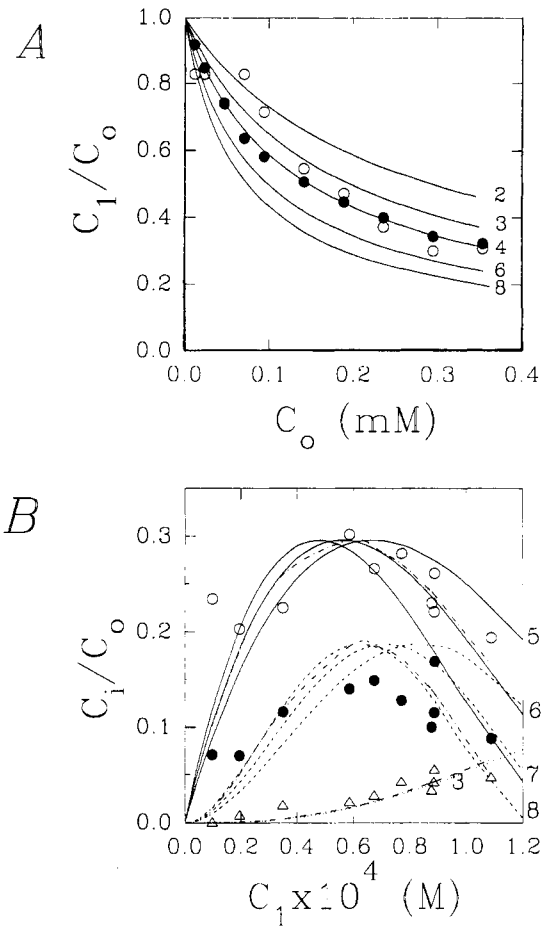

FIGURE 7: Quantitative analysis of the GH5 self-association data presented in Figure 6. The ratio of the free monomer concentration (C_1) to the total GH5 concentration (C_0) as a function of C_0 is shown (A). Raw data (○) and a nonlinear best-fit analysis (●) are presented together with a set of theoretical curves (—) calculated for reactions with self-association constants (in units of 10^3 M^{-1}) indicated at the ends of the lines. In B the ratio of the concentration (in monomeric units) of dimers (○), trimers (●), and tetramers (△) to the total GH5 concentration is plotted as a function of the free monomer concentration. Here, the theoretical curves for dimers (—), trimers (- - -), and tetramers (···), together with the nonlinear best-fit analyses (- · -),are shown.

Rather than assuming an isodesmic model, we may instead calculate successive association constants individually:

$$C_n + C_1 \Leftrightarrow C_{n+1} \quad [C_{n+1}] = K_n[C_n][C_1] \quad (6)$$

Equilibrium constants can be determined from plots of $[C_{n+1}]$ against $[C_n][C_1]$, which should give a linear slope of K_n passing through the origin. Equilibrium constant values determined in this manner (data not shown) are presented in Table 1. These do not differ significantly from one another, again showing that the isodesmic model applies.

(d) A Comparison between GH5 and GH1 Cross-Linking. GH5 and GH1, at 0.2 mg/mL, were cross-linked with 0.25 mM DSP in 1 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, and 0.25 mM PMSF containing either 0.25 or 2.0 M NaCl. Aliquots were removed at timed intervals, quenched, precipitated, and run on SDS–polyacrylamide gels (Figure 8). In the presence of 0.25 M NaCl, GH5 showed high oligomer concentrations up to a pentamer, with weaker hexamer and heptamer and strong 12–14-mers. In contrast,

Table 1: GH5 Self-Association Constants (in Units of 10^3 M^{-1})

method of analysis	K_a monomer	dimer	trimer	tetramer
[monomer]/[total] vs [total]	3.9 ± 0.5[a]			
[*i*-mer]/[total] vs [monomer]		5.9 ± 0.4[a]	7.8 ± 0.4[a]	3.1 ± 0.1[a]
[dimer] vs [monomer][monomer]		5.6 ± 0.5		
[trimer] vs [monomer][dimer]			5.5 ± 0.2	
[4-mer] vs [monomer][trimer]				3.5 ± 0.5
[4-mer] vs [dimer][dimer]				3.3 ± 0.7
mean	3.9 ± 0.5	5.8 ± 0.4	6.6 ± 0.3	3.3 ± 0.3

[a] Analysis assumes an isodesmic model.

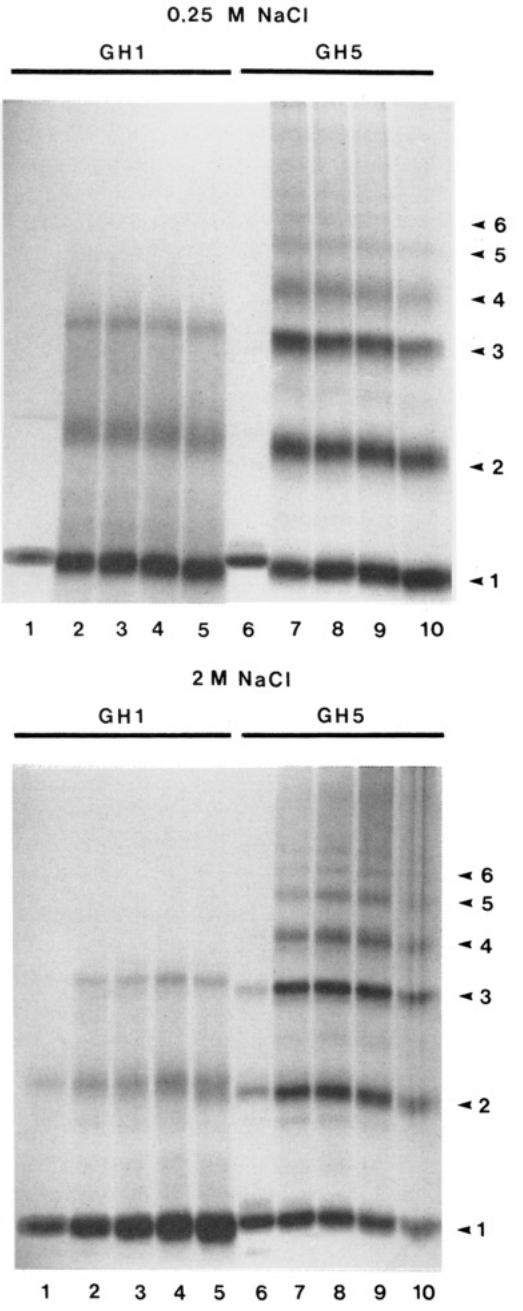

FIGURE 8: Cross-linking of GH1 and GH5 with DSP in the presence of 0.25 M or 2.0 M NaCl as a function of time (15% SDS–polyacrylamide gels). Lanes 1 and 6 contain un-cross-linked GH1 and GH5, respectively. Cross-linking was for 1 (lanes 2 and 7), 2.5 (lanes 3 and 8), 5 (lanes 4 and 9), and 10 (lanes 5 and 10) min.

the products obtained with GH1 extended only up to a trimer. This pattern did not change significantly even in the presence of 2 M NaCl.

On the basis of the lysine residue content, one would expect GH1 (12 lysines) to be cross-linked more readily than GH5 (9 lysines), if the reaction were nonspecific in nature. Evidence to the contrary thus supports our contention that GH5–GH5 self-association is specific. As GH1 is essentially "unfolded" in the presence of 1 mM sodium phosphate and 250 mM NaCl and "fully folded" only when the salt concentration is raised to 2.0 M (Figure 1B,C), the absence of any significant difference between the cross-linking pattern of GH1 when it is in these substantially different conformations suggests that the formation of GH1 dimers and trimers may well arise through nonspecific associations.

An interaction between two biological macromolecules may be defined as specific when the interacting species show a significantly greater preference to bind to each other in a unique orientation. When examined by chemical cross-linking, only a unique set of lysine residues would thus be in the correct orientation to participate in such cross-links. For DTBP, DSP, and DTSSP, the maximum cross-linking distance between the participating ϵ-amino groups is 12 Å. If the cross-linking pattern for GH5 is specific, then it could be altered if a reagent with a different cross-linking span were employed (Chalkley, 1975). We used disuccinimidyl tartarate (DST) to investigate this possibility; this reagent has the same reactive group as DSP, but a maximum cross-linking distance of only 6.4 Å (Smith et al., 1978).

GH5 and GH1, at 0.2 mg/mL, were cross-linked with 0.25 mM DST in 1 mM sodium phosphate (pH 7.4), 0.2 mM EDTA, and 0.25 mM PMSF in the presence of 2.0 M NaCl, and the products were analyzed in SDS–polyacrylamide gels (Figure 9). With DST, GH5 is no longer cross-linked more extensively than GH1: in neither case were cross-linked products larger than a trimer observed, and only a small proportion of GH5 formed oligomers. The similarity between the cross-linking patterns of GH1 obtained with DSP (Figure 8) and DST (Figure 9) supports the inference that the oligomers of GH1 result from nonspecific interactions. The failure of GH5 oligomers to be effectively cross-linked by DST suggests that a unique set of ϵ-amino groups is involved in their cross-linking by DTBP, DSP, and DTSSP and that these residues are separated by a distance greater than 6.4 Å but no more than 12 Å.

DISCUSSION

(a) Self-Association of GH5. Four features of its cross-linking suggest that GH5 self-associates in a specific manner: (1) Three different cross-linking reagents (DTBP, DSP, and DTSSP) each generate from GH5, under all conditions tested, a characteristic set of oligomers consisting of strong bands up to a pentamer, underrepresented hexamers and heptamers, and a discrete product estimated as a 12–14-mer. The invariance of this distinctive pattern suggests that these reaction products are structurally unique and do not arise from nonspecific interactions. (2) The extent of GH5 cross-linking is dependent upon the concentration of the peptide, and its self-association is governed by an equilibrium constant,

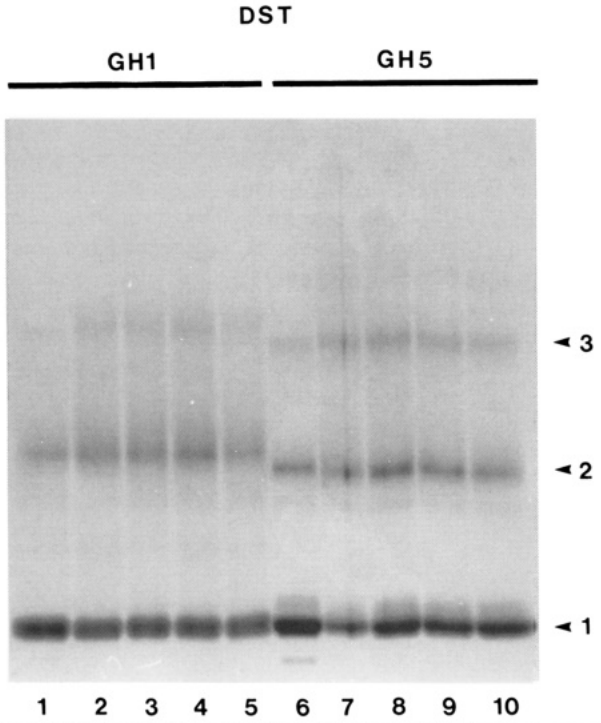

FIGURE 9: Cross-linking of GH1 and GH5 with DST in the presence of 2.0 M NaCl as a function of time (15% SDS–polyacrylamide gels). Lanes 1 and 6 contain un-cross-linked GH1 and GH5, respectively. Cross-linking was for 1 (lanes 2 and 7), 2.5 (lanes 3 and 8), 5 (lanes 4 and 9), and 10 (lanes 5 and 10) min.

the value of which falls within the range of 3×10^3 to 7×10^3 M^{-1}. (3) The products obtained by cross-linking GH5 are very distinct from those of GH1; GH5 is also more readily cross-linked despite having a lower content of ϵ-amino groups, a feature incompatible with cross-linking through nonspecific interactions. (4) GH5 gives no covalent products when cross-linked with the NHS ester DST, which has a maximum cross-linking distance (6.4 Å) about one-half that of DSP and DTBP (12 Å). A unique set of ϵ-amino groups thus appears to be involved in the cross-linking reaction, presumably at or near the interface between subunits in the quaternary structure.

Numerous studies have shown that cross-linking can be used to study the association state of proteins and that collisional encounters in solution do not lead to cross-linking (Davis & Stark, 1970; Carpenter & Harrington, 1972; Sutoh & Harrington, 1977; Herrmann et al., 1981). This is confirmed in our system by the notable reduction in cross-linking when the GH5 monomers are largely dissociated in low salt (Figure 5B). Furthermore, where studied, quantitative data on the association provided by cross-linking have been in agreement with those derived from hydrodynamic methods (Calvert et al., 1980; Herrmann et al., 1981). Although, in principle, cross-linking might be expected to disturb the equilibrium of an associating protein, driving it toward a higher state of association, other factors are likely to compensate for or inhibit this tendency. For example, unreacted protein and reagent will be greatly diluted with time, and the rate of reaction will become very slow after the initial phase. In addition, the blocking of ϵ-amino groups by intramolecular cross-linking and monofunctional modification events (Davis & Stark, 1970) will reduce and ultimately stop the reaction. In general, moreover, the association of proteins is not rapid by reason of orientational requirements and can be slower than the cross-linking reaction (Northrup & Erickson, 1992). Our results thus indicate that GH5 associates in solution in a sterically specific manner and according to an isodesmic scheme. If cross-linking does displace the equilibrium during the period of the reaction, our association constant would reflect an upper limit.

The self-association in solution of histones H1, H5, and their globular domains has been studied previously by Russo et al. (1983), using formaldehyde as a cross-linker. Extensive cross-linking was observed only with intact H1 and H5. GH1, but not GH5, did however give a pattern [Figure 3 in Russo et al. (1983)] with some features similar to that which we obtained with GH5. However, the extensive conditions used to cross-link GH1 (1% formaldehyde for 18 h) complicate a direct comparison with our own data. The authors correlated the extent of cross-linking to the number of lysine residues in each peptide, concluding that self-association under these conditions was nonspecific. However, as the cross-linking distance of formaldehyde is only about 2 Å (Jackson, 1978), while our results suggest that a cross-linker span of greater than 6.4 Å is required to detect specific self-association, the results of Russo et al. (1983) are not necessarily contrary to our own.

Thomas et al. (1992), in examining the cooperative nature of the binding of GH5 and GH1 to DNA, cross-linked GH5 and GH1 in solution with DSP and observed no cross-linking to oligomers for GH1 and only about 5% for GH5. This qualitative distinction between GH1 and GH5 agrees with our results, and the quantitative difference in the extent of GH5 polymerization may be explained through differences in the peptide concentrations used. Employing the upper limit of the self-association constant derived for GH5 in our study (7×10^3 M^{-1}) in eq 3, we calculate the amount of multimeric material expected to exist at the GH5 concentration of 12 μg/mL used by Thomas et al. (1992) to be about 2%. Thus, our results are not inconsistent with their observations.

In the structure of GH5, recently solved to 2.5-Å resolution (Ramakrishnan et al., 1993), the asymmetric crystal unit consists of a dimer of GH5 molecules. The area of the interface between the two monomers in the crystal is relatively small, suggesting that self-association through interaction of these surfaces should be weak. Ramakrishnan et al. (1993) also pointed out that, of three residues (Tyr 53, Tyr 58, and His 57) involved in solvent-mediated interactions at the interface of the GH5 molecules in the crystal, only one (Tyr 58) is conserved in the globular domain of chicken H1. These observations are not inconsistent with the suggestion that the surfaces of the molecules which interact in the crystal may also be involved in the self-association of GH5 in solution.

Given that GH5 is such a small molecule and has a large charge, and therefore a large second viral coefficient, the difficulties of obtaining sufficiently precise hydrodynamic data to define the equilibrium are considerable. Aviles et al. (1978) reported that the sedimentation coefficient of GH5 in 1 M NaCl at pH 3.0 is independent of the peptide concentration, concluding that GH5 is essentially monomeric under these conditions. However, at pH 3.0 the majority of basic residues in GH5 would be fully charged and self-association is thus disfavored through charge-repulsion effects. Furthermore, the scatter in the sedimentation data presented by Aviles et al. (1978) does not in fact preclude self-association at a level similar to that which we determined. Draves et al. (1992) have also studied the self-association of GH5 in solution by measuring its translational diffusion coefficient as a function of protein and salt concentrations. Their estimate for the association constant of GH5 is approximately an order of magnitude lower than ours although, again, the data used to derive this value show substantial scatter.

(b) GH5 Self-Association and Higher Order Chromatin Structure. The packing of chromatin into a 30-nm higher

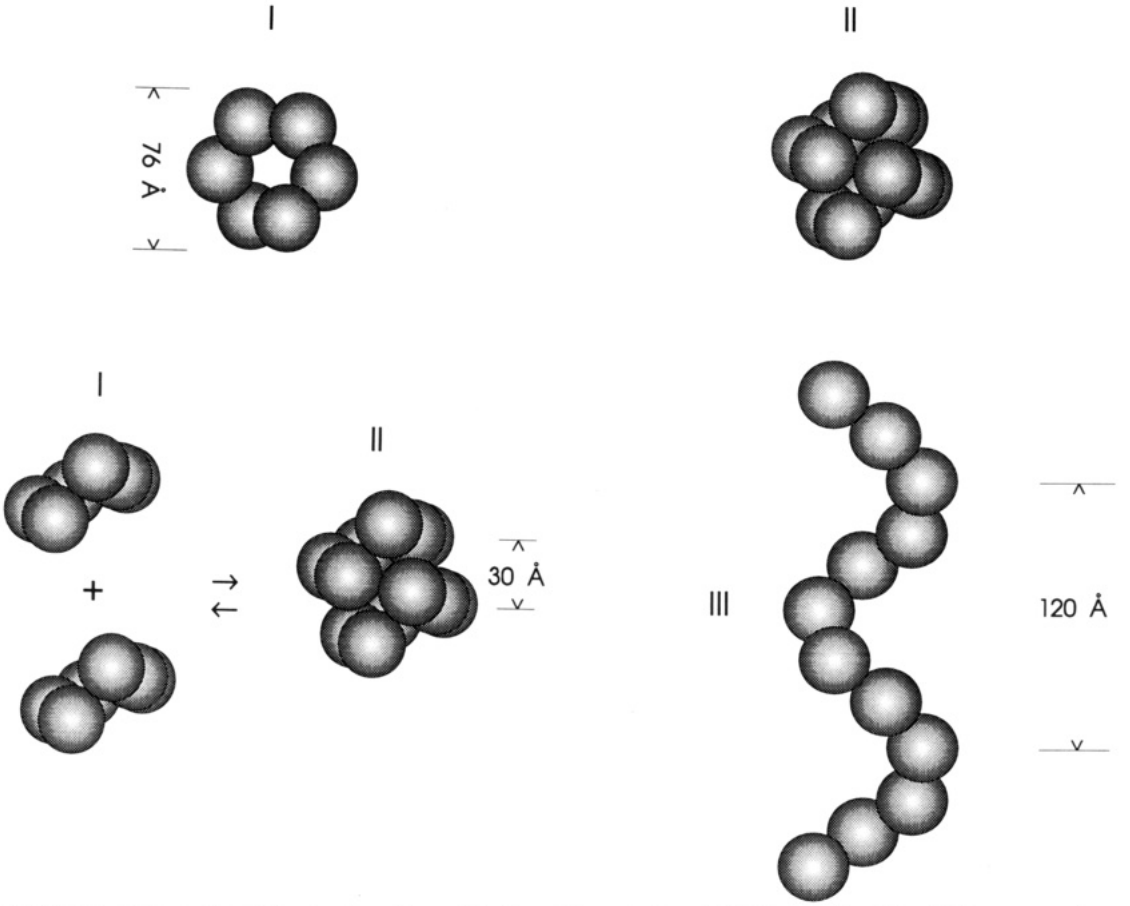

FIGURE 10: Hypothetical models of the structures formed by the self-association of GH5 in solution. The GH5 monomer is represented by a 30-Å diameter sphere. The helical 6-mer (I) is shown from two perspectives, and the assembly of a 12-mer (II) from two 6-mers is illustrated (A, left). The manner in which the 12-mer (or component 6-mers) might be related to the structure envisaged to be formed in the axis of the higher order chromatin fiber (III) is shown (B, right).

order fiber must involve factors that contribute both to the thermodynamic driving force and to the specificity of correct folding. In vitro, this process is critically dependent upon ionic conditions and the presence of linker histone (H1/H5) (Thoma et al., 1979; Allan et al., 1981). Clark & Kimura (1990) have, through the application of polyelectrolyte theory, proposed that monovalent cations act in conjunction with the highly basic, polycationic COOH-terminal tail domain of histone H1 to reduce the electrostatic free energy of the linker DNA, enabling it to become closely packed in the higher order fiber. These activities could clearly contribute to the thermodynamic driving force for chromatin folding. However, those factors that affect the specificity of folding have been less easily identified. Condensation and the packing of the linker DNA bring neighboring nucleosomes close together, and their capacity to interact with each other could contribute toward their adopting a particular spatial relationship in the folded fiber (Worcel & Benyajati, 1977; Dubochet & Noll, 1978). Chromatin condensation may also bring the globular domains of linker histones into close proximity. In unfolded chromatin, the linker histone globular domain is located on the dyad axis of the nucleosome where the DNA enters and exits from the core particle (Allan et al., 1980); thus, if the radially arranged nucleosomes (Thoma et al., 1979; McGhee et al., 1980) in the higher order chromatin fiber are consistently oriented with the DNA entry/exit point facing toward the axis of the fiber (Thoma et al., 1979), the linker histone globular domains will be brought close together. Under these circumstances, the domains could polymerize to form an axial core, and the resulting structure might serve to stabilize the associated nucleosomes, as originally suggested (Thoma et al., 1979).

The cross-linking pattern of GH5 is distinctive in that, although multimers up to a pentamer are strongly displayed, the amount of hexamer (and heptamer) is relatively underrepresented. This phenomenon is concomitant with the formation of a high molecular weight product estimated to contain 12–14 monomeric units. These features can be interpreted by a model in which the formation of a hexamer is followed by the association of two hexamers to form a dodecamer, in preference to the continued addition of monomers to the hexamer. Figure 10A illustrates one possibility as to the nature of the products formed during cross-linking. Here, the globular domain of GH5 is modeled as a sphere of 30-Å diameter (Aviles et al., 1978; Clore et al., 1987; Ramakrishnan et al., 1993). The sequential addition of GH5 monomers to a growing chain results in a closed ring, which may be helical, with about six monomeric units per turn (form I). The dimerization of two rings results in a structure (form II) consisting of two turns of a helical array with about 5.7 GH5 monomers per turn, a pitch of 30 Å, and a cross-sectional diameter of 76 Å. Additional helical rings could be stacked onto this structure, which might account for the high molecular weight material that does not enter the cross-linking gels (see Figures 3 and 5, for example).

The formation in solution of a helical ring containing six polymerized GH5 monomers is equivalent to the number of nucleosomes per turn in the higher order chromatin fiber (Thoma et al., 1979; McGhee et al., 1980). However, if the linker histone globular domain is located near the nucleosomal dyad axis in this fiber, it follows that the pitch of the hypothetical globular domain polymer should follow the pitch of the nucleosomes; the anticipated structure, shown in Figure 10B (form III), constitutes a helix with a pitch of 120 Å.

Conversion of the form II helix to the form III helix is analogous to stretching a spring and need only slightly reduce the cross-sectional diameter. A globular domain helix with a cross-sectional diameter of 76 Å would, of course, fit neatly within the central axial hole of the solenoid.

The above model for a globular domain polymer (form III) is compatible with the single-start solenoid model of Thoma et al. (1979), with some variations on this model (Butler, 1984), and also with the two-start helix cross-linker model of Williams et al. (1986). The model is difficult to reconcile with the solenoid model suggested by McGhee et al. (1980) or the twisted zig-zag ribbon models of Worcel et al. (1981) & Woodcock et al. (1984), because in these latter structures the linker histone is not located in the axial cavity of the fiber.

In order to substantiate the above proposal, it will be necessary to demonstrate that the globular domain of H5 interacts within the chromatin fiber in vivo. This is a problem that we are now attempting to investigate, but there are difficulties inherent in employing a cross-linking approach for such experiments. Although intact linker histones have been shown to interact extensively within chromatin in vivo, the interactions are predominantly between the tails of the molecules (Thomas & Khabaza, 1980; Lennard & Thomas, 1985). However, the relatively mild cross-linking conditions that were required to demonstrate these strong, tail–tail interactions may not have favored the detection of weak globular domain contacts. For this purpose, extensive cross-linking conditions may be required, but these would promote substantial linker histone–core histone cross-linking. Under these circumstances, the detection globular domain interactions will be difficult but not insurmountable.

(c) Biological Significance of the GH5 Self-Association Constant. The mid-range self-association constant (K_a) determined for GH5 is 5000 M^{-1}, corresponding to a standard free energy change ($\Delta G°$) for GH5 polymerization of about −5 kcal/mol. The magnitude of ΔG is low, but well within the range associated with important biological processes such as protein folding (Creighton, 1984) and, perhaps more saliently in terms of its significance for DNA structure and stability, with base-stacking.

If linker histone globular domains do polymerize to form an axial core in the chromatin fiber, one would expect the self-association constant for the polymerization to be necessarily low. This is particularly pertinent when one considers that the effective protein concentration generated on placing the linker histone globular domains at this restricted location could be as high as 16.5 mM (140 mg/mL). Under these circumstances, even a very small self-association constant would dictate extensive polymerization; at this concentration and with a K_a of 5000 M^{-1}, the extent of GH5 polymerization calculated from eq 3 is 99%. It should be stressed, however that this interpretation would apply only in solution, for in the context of the chromatin fiber the linker histone globular domains would be restricted in terms of their locality and relative orientation by virtue of their association with the nucleosome. Under these circumstances, the weak interactions seen in free solution could become very strong, and the combined effect of a high local concentration of reactant, a structure in which interaction sites are in close proximity, and a great reduction of the unfavorable entropic contribution of immobilization and orientation could substantially increase the effective K_a. In another system it has been estimated that such factors can increase the likelihood of dimer formation between proteins localized in membranes by 10^6-fold (Grasberger et al., 1986); such a prospect provides at least a qualitative understanding of how GH5 self-association could contribute to the stability of the chromatin fiber.

Our failure to detect specific GH1 cross-linking in solution suggests that self-association between GH1 domains, if it occurs, is much weaker than that exhibited by GH5. Histone H1 is known to be associated with active, as well as inactive, regions of the chromosome (Nacheva et al., 1989; Ericsson et al., 1990), and the necessity to transiently disrupt such chromatin may demand a relatively low degree of self-association. In contrast, the association of H5 with the chromatin fiber in terminally differentiated red blood cells is correlated to, and indeed may be responsible for, the cessation of both transcription and replication in this cell type. Such effects may be due to an H5-dependent increase in higher order chromatin fiber stability (Bates et al., 1981), which our results suggest may be brought about by a weak, but significant, ability of the globular domain of H5 to self-associate.

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