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A ^{1}H NMR Probe for Mobility in the Reactive Center Loops of Serpins: Spin-Echo Studies of Native and Modified Forms of Ovalbumin and α_1-Proteinase Inhibitor†

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Received April 18, 1991; Revised Manuscript Received June 28, 1991

ABSTRACT: It has recently been proposed that the expression of inhibitory activity in serine protease inhibitors (serpins) is a function of the mobility of the extended α-helical reactive center loop [Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990) *Nature 347*, 99-102]. We have employed solution ^{1}H NMR methods, including the Carr-Purcell-Meiboom-Gill (CPMG) and Hahn spin-echo pulse sequences, to try to identify such regions by virtue of their anticipated longer T_2 relaxation times in two of the best characterized members of the serpin superfamily, ovalbumin and α_1-proteinase inhibitor. The CPMG spectra of native ovalbumin reveal the presence of long-lived resonances from the methyl protons of alanine residues and the CH_3 protons of leucine or valine residues as well as the acetyl and ring methine protons of the carbohydrate moieties. Following reaction of ovalbumin with subtilisin Carlsberg to generate plakalbumin [where excision from within the reactive center loop homologue of a hexa- or heptapeptide, with sequence (E)-A-G-V-D-A-A, occurs], its CPMG spectrum retained almost all of the originally present long-lived resonances. Concurrent with the retention of these mobile resonances in plakalbumin is the appearance of two additional resonances consistent with the formation of new C and N termini. On the basis of the proposed mobility of the reactive center loop, it had been expected that removal of the alanine-rich hexapeptide would result in loss of some or all of the long-lived alanine methyl resonances. Similar CPMG experiments were conducted with native, proteolytically (papain) modified, and N-chlorosuccinimide-oxidized α_1-proteinase inhibitor, where a very similar set of long-lived (mobile) resonances was identified, which persisted within each form of the inhibitor. The results lead us to conclude that the extended α-helical loop of serpins is not unusually mobile. These findings and their significance are discussed in terms of the instances of segmental internal motion (i.e., mobility and flexibility) within polypeptide domains demonstrated by NMR spectroscopy, and also with regard to the recently proposed ovalbumin-based model for the mobile reactive center loops of serpins.

The elucidation of the X-ray crystallographic structure of cleaved (Wright et al., 1990) and native ovalbumin (Stein et al., 1990) has further stimulated an already active interest (Huber & Carrell, 1989) in serpin structure and function. This pair of native and proteolytically modified structures serves as a critical point of reference in our attempts to understand structure-function relationships within the serpin superfamily.

In the structure of native ovalbumin, the reactive center loop was found to exist as an extended α-helix in which the Debye-Waller B factors for residues in the helix relative to residues in the protein core show some degree of elevation. This was interpreted as an indication of a flexible, mobile structure for the α-helix (Stein et al., 1990). Since a computer-simulated reconstruction of the cleaved α_1-proteinase inhibitor (α_1-PI)[1] reactive center loop also indicates the presence of an α-helix, it has been proposed that such a flexible structural element may play a critical role in the general process of recognition of the reactive site of serpins by target proteases and their successful inhibition.

One method that has proved to be extremely useful and sensitive for probing discrete domains of proteins that possess

† This work was supported by National Institutes of Health Grants RO1 HL32595 and T32 ES 07028. D.B.H. is supported by NSF Postdoctoral Fellowship DIR-9102057. The NMR facility is supported in part by the Center in Molecular Toxicology through Grant ES 00267 from the National Institutes of Health.

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[1] Abbreviations: CPMG, Carr-Purcell-Meiboom-Gill; α_1-PI, α_1-proteinase inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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different mobilities is solution ^{1}H NMR spectroscopy. A good example of the segmental, independent mobility of domains within a protein, deduced by ^{1}H NMR spectroscopy, was seen with the multidomain fibrinolytic protein urokinase (Oswald et al., 1989). The M_r 54000 protein consists of two functional domains, an epidermal growth factor like (EGF) kringle domain and a serine protease domain. Each domain was found to contain distinct resonances with broad and narrow line widths for the EGF–kringle domain and the serine protease domain, respectively. This effect is due to substantial independent motion within and between the individual domains of the protein. In the case of protease inhibitors, Gettins and Cunningham (1986), using ^{1}H NMR CPMG spin-echo pulse trains, demonstrated that the narrow, sharp resonances in the aromatic region of the spectrum of human α_2-macroglobulin originate from the proteolytically sensitive bait region, in which there is a high degree of independent mobility. More recently, Sommerville et al. (1990) showed that the narrow resonances in the ^{1}H NMR spectrum of gizzard myosin originate predominantly from the mobile myosin heads. Using spin-echo ^{1}H NMR, they were able to show that localized changes in the myosin heads are detectable as part of the 6S (extended) to 10S (folded) conformational transition of gizzard myosin.

In the present study, we have used ^{1}H NMR spectroscopic methods, particularly the CPMG experiment, to obtain mobility information on the reactive center loops of ovalbumin and the archaetype of the serpin superfamily, α_1-PI, to test the hypothesis of Stein et al. (1990), since, if their hypothesis is correct, it would afford a convenient means of focusing on the NMR resonances from the reactive center loop regions of serpins in general. A series of CPMG spectra of native ovalbumin obtained with increasing delay times between the initial 90° excitation pulse and data collection revealed the presence of a number of highly mobile aliphatic side chains, including those from alanine and leucine or valine residues. However, after removal of a large portion of the reactive center loop, containing three alanine residues and one valine, by limited proteolysis with subtilisin Carlsberg, almost no long-lived resonance intensity was lost. With α_1-PI, the CPMG spectra of the oxidized protein show the presence of two highly mobile methyl groups from the two reactive center methionine sulfoxide residues that are formed by treatment of the protein with *N*-chlorosuccinimide. In the native protein, the unoxidized methionine precursor residues do not possess the same high degree of mobility. Furthermore, protease treatment, which results in integration of the exposed loop region into the protein core, does not result in the loss of resonance intensity from slowly relaxing nuclei. It is concluded that for neither ovalbumin nor α_1-PI is there evidence in solution for unusually high mobility of the reactive center loops. These findings are discussed in terms of the magnitude of the differences in *B* structure factors reported by Stein et al. (1990).

Materials and Methods

Plakalbumin was prepared from hen ovalbumin (Sigma, grade VI) by reaction with subtilisin Carlsberg at pH 8.5 in 50 mM Tris-HCl for 2 h using a protease:protein ratio of 1:8000 (45 pmol:360 nmol ratio). The protease was removed from the reaction mixture by repeated cycles of dilution and reconcentration in an Amicon ultrafiltration cell using a PM-30 membrane. The initial dilution–reconcentration step was sufficient to remove most of the subtilisin, and after repeated cycles of dilution, the amount of protease was less than 0.1% of that initially used. This ensured that subsequent nonspecific cleavage of plakalbumin by trace amounts of protease would be insignificant.

α_1-PI was purified by the method of Travis and Johnson (1981). The purity of the final preparation was checked by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% slab gels according to the procedure of Laemmli (1970). The heterogeneous preparation migrated characteristically as two bands (M_r 52000) due to the microheterogeneity of the oligosaccharide moieties (Carrell et al., 1982). The inhibitory activity of this pool was assayed against porcine pancreatic elastase (Sigma) by using the substrate Succ-$(Ala)_3$-pNA (Sigma) (Bieth et al., 1974) and was found to be 100% active, using an extinction coefficient of $E^{1\%}_{280nm}$ = 5.3 (Travis & Johnson, 1981). The α_1-PI pool was dialyzed versus 0.1 M Tris-HCl, pH 8.0, and concentrated to 9.43 mg/mL; 1-mL aliquots were stored at −70 °C until needed.

Proteolytically modified α_1-PI was prepared according to the method of Johnson and Travis (1977). Briefly, to 178 nmol of α_1-PI was added 890 pmol of papain, previously activated in 20 mM sodium phosphate, 5 mM cysteine, and 10 mM EDTA, pH 7.0, giving a total volume of 3 mL. The mixture was allowed to incubate for 4 h at 25 °C in 10 mM NH_4HCO_3 buffer, pH 6.5, containing 1 mM EDTA and 2 mM β-mercaptoethanol. Following incubation, the reaction mixture was immediately freeze-dried. The modified inhibitor was recovered from the reaction mixture by chromatography on Sephadex G-50 (Pharmacia) in 0.1 M NH_4HCO_3, pH 8.0. An SDS–PAGE examination of native inhibitor versus an aliquot of the void volume fraction revealed a shift in the molecular weight of the void volume material from 53000 to 48000, indicative of P_1–$P_{1'}$ reactive center loop cleavage (Johnson & Travis, 1979).

N-Chlorosuccinimide-oxidized α_1-PI was prepared according to the method of Johnson and Travis (1979). At a 20:1 molar ratio of *N*-chlorosuccinimide to α_1-PI, two methionine residues per mole of α_1-PI are oxidized to the sulfoxide species, those residues being the P_8 and P_1 methionines of the extended α-helical reactive center loop. Briefly, to 178 nmol of α_1-PI was added 3560 nmol of *N*-chlorosuccinimide (from a 50 mM stock solution in 50 mM Tris-HCl, pH 8.0), and the oxidation was continued for 20 min at 25 °C. The reaction mixture was immediately chromatographed on Sephadex G-25 (Pharmacia) in 0.1 M NH_4HCO_3, pH 8.0 (Shechter et al., 1975). The oxidized inhibitor eluted in the void volume of the column. SDS–PAGE of incubations of native and oxidized inhibitor with porcine pancreatic elastase (1:2 inhibitor:protease molar ratio) revealed that native inhibitor formed SDS-stable complexes with porcine pancreatic elastase whereas the oxidized inhibitor formed no complex at all and was converted to a lower molecular weight (48000) modified form.

The methionine sulfoxide content of the *N*-chlorosuccinimide-oxidized preparation was determined by amino acid analysis under alkaline conditions in 1 M KOH for 24 h at 110 °C according to the Pico-Tag method for methionine sulfoxide determination (Cohen et al., 1989). The oxidized preparation was analyzed for the PTH derivative of methionine sulfoxide referenced against an external standard of the PTH derivative of authentic methionine sulfoxide (Sigma) and the PTH derivatives of the standard amino acid H preparation (Pharmacia). In this procedure, PTH methionine sulfoxide is resolved as an earlier separate component immediately prior to arginine. Quantitation demonstrated the presence of 2 mol of the methionine sulfoxide PTH derivative per mole of oxidized α_1-PI, with a 94% recovery of the PTH sulfoxide derivative.

Protease concentrations were determined spectrophotometrically by using extinction coefficients for subtilisin

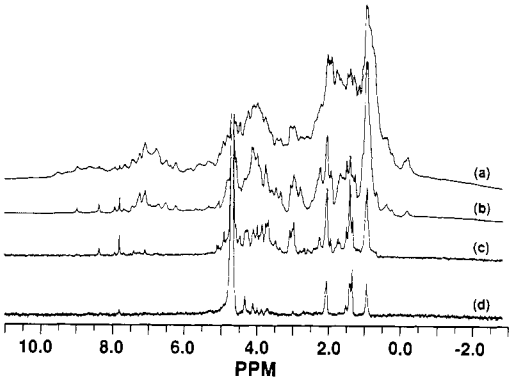

FIGURE 1: 400-MHz ^{1}H NMR spectra of 0.36 mM ovalbumin. (a) Normal spectrum; (b–d) Carr–Purcell–Meiboom–Gill spectra of the same sample with total delays between the 90° excitation pulse and data collection of (b) 16, (c) 64, and (d) 240 ms. Spectra were recorded at 303 K and represent the average of 2000 scans. The vertical scale of (d) is twice that of (c), which is twice that of (b).

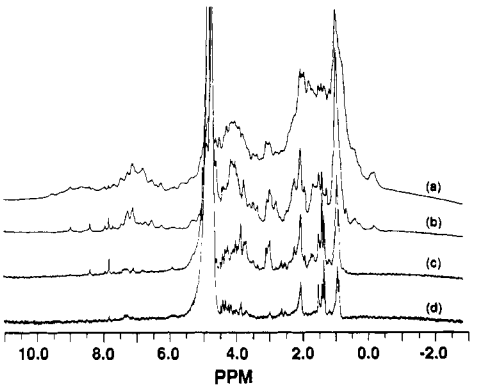

FIGURE 2: 400-MHz ^{1}H NMR spectra of 0.36 mM plakalbumin. (a) Normal spectrum; (b–d) Carr–Purcell–Meiboom–Gill spectra of the same sample with total delays of (b) 16, (c) 64, and (d) 240 ms. Spectra were recorded at 303 K and represent the average of 2000 scans. The vertical scale of (d) is twice that of (c), which is twice that of (b).

Carlsberg of $E^{1\%}_{280nm}$ = 9.6 (Ottensen & Svendsen, 1970), papain $E^{1\%}_{280nm}$ = 25.0 (Glazer & Smith, 1961), and porcine pancreatic elastase $E^{1\%}_{280nm}$ = 22.0 (Pannell et al., 1974).

Ovalbumin, plakalbumin, native α_1-PI, proteolytically modified α_1-PI, and *N*-chlorosuccinimide-oxidized α_1-PI were prepared for NMR analysis by transferring from H_2O buffer to D_2O (99.9 atom %, Sigma) buffer by several cycles of dilution with D_2O in a 3-mL Amicon ultrafiltration cell fitted with a PM-30 membrane. The D_2O buffer contained 0.1 M NaCl and 20 mM sodium phosphate, pH 7.25.

^{1}H NMR spectra were recorded at 400 MHz on a narrow-bore Bruker AM 400 spectrometer equipped with a 5-mm ^{1}H probe. Sample size was 400 μL in all experiments. The probe temperature was maintained at 303 K. Acquisition of normal spectra used at 70° pulse (5.8 μs), a sweep width of 6024 Hz, and a data block size of 8K points. In the case of native α_1-PI experiments, it was necessary to reduce the residual HOD resonance by a low-power presaturation pulse prior to the observation pulse. Hahn spin-echo *J*-modulated spectra were recorded with a 90°–τ–180°–τ pulse sequence and a τ value of 60 ms which results in complete inversion of doublets for which J = 8 Hz. Carr–Purcell–Meiboom–Gill spectra were recorded with the pulse sequence 90°-(τ–180°–τ)$_n$ (Campbell & Dobson, 1979) using a value for τ of 1 ms. Chemical shifts are referenced to an external standard of 2,2-dimethyl-2-silapentane-5-sulfonate (Sigma) at 0 ppm.

RESULTS

T_2 Relaxation Studies on Ovalbumin and Plakalbumin. Subtilisin Carlsberg excises the P_6–P_1 hexapeptide,[2] A-G-V-D-A-A, in converting ovalbumin to its proteolytically modified form, plakalbumin (Ottesen & Wollenberger, 1956). If the reactive center loop region of native ovalbumin possesses much greater mobility than the rest of the molecule, the result of the proteolytic conversion into plakalbumin should be manifested by a loss of three mobile alanine resonances in the ^{1}H NMR spectrum, as well as of resonances from one each of glycine, valine, and aspartate.

The 400-MHz ^{1}H NMR spectrum of native ovalbumin at a concentration of 0.36 mM is shown in Figure 1a. Also shown are CPMG spectra of the same sample obtained with total delays between the 90° excitation pulse and observation of 16, 64, and 240 ms. Examination of these spectra shows a dramatic loss of resonance intensity after only 16 ms (Figure 1b), corresponding to relaxation of resonances from the majority of residues, which have little or no motion independent of the overall rotation of the protein. After 64 ms (Figure 1c), only a few narrow resonances remain in the aromatic region, while in the aliphatic region there are resonances at the positions expected for the ring CH and acetyl CH_3 protons of the carbohydrate (3.5–4.5 and 2.1 ppm, respectively), as well as for the ϵ-CH_2 groups of lysine (3.0 ppm) and the methyls of alanine (1.3 ppm) and valine, or isoleucine (0.9 ppm). By 240 ms, only the most mobile, and therefore the most slowly relaxing, nuclei give significant resonance intensity in the CPMG spectrum (Figure 1d). A discrete set of resonances remains at 0.95, 1.3–1.39, and 2.0 ppm, together with a few resonances from the carbohydrate CH protons between 3.6 and 4.35 ppm. To confirm that the resonances between 1.30 and 1.39 ppm were the doublets expected for alanine methyl resonances, Hahn spin-echo spectra, which exhibit phase modulation dependent on the coupling constant J, were recorded, with τ values from 30 to 120 ms. These gave the expected inversion for these resonances for a total delay, 2τ, of 120 ms (data not shown). The T_2 values of these alanine resonances, calculated from the full set of CPMG spectra obtained with total delays of 4, 16, 64, 160, and 240 ms, were found to be on the order of 35 ms, representing slower relaxation and therefore higher mobility than expected for a protein of the size of ovalbumin.

Figure 2a shows the 400-MHz ^{1}H NMR spectrum of plakalbumin obtained under the same conditions as for the sample of ovalbumin shown in Figure 1a. The corresponding CPMG spectra with total delays of 16, 64, and 240 ms are shown in Figure 2b–d, respectively. These spectra show great overall similarity to the spectra of ovalbumin in Figure 1. Closer comparison of the spectra in Figures 1d and 2d shows, however, that there is only a slight reduction in the intensity of the alanine resonances, rather than the major change expected if the long-lived resonances originated from alanines in the reactive center loop. This can be more clearly visualized in a difference spectrum between the 240-ms CPMG spectra of plakalbumin (Figure 2d) and ovalbumin (Figure 1d), which is shown in Figure 3. This difference spectrum clearly shows not only that the mobile alanine methyl resonances are retained in plakalbumin but also that new long-lived resonances appearing at 0.89 and 0.94 ppm are in the expected chemical shift regions for the methyl protons of isoleucine or of leucine or valine residues. The resonance at 1.48 ppm appears to be

[2] Depending on conditions, treatment of ovalbumin has been reported to result in excision of either a hexapeptide or a heptapeptide, with the glutamate at position P7 being the extra residue.

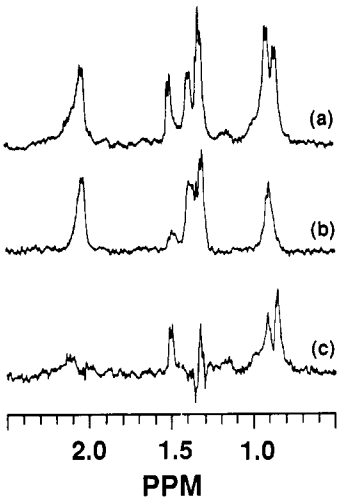

FIGURE 3: Effect of excision of the hexapeptide (heptapeptide) from the reactive center loop of ovalbumin on the aliphatic region of the CPMG ^{1}H NMR spectrum. (a) Plakalbumin; (b) ovalbumin; (c) difference spectrum of (a) − (b). The spectra in (a) and (b) are from Figures 2d and 1d, respectively.

a doublet and could arise from alanine or possibly threonine (Bundi & Wüthrich, 1979). These new resonances arise as a result of proteolytic cleavage and may therefore originate from residues at, or adjacent to, the newly formed N- and C-termini located within the reactive center region. The small negative peak in the difference spectrum at 1.39 ppm represents some loss of intensity from alanine methyl groups, presumably resulting from loss of the three reactive center alanine residues. However, the magnitude of this negative peak is much less than expected if the reactive center alanines were mobile, as can be judged by comparison with the intensity of the new resonance at 1.48 ppm, which most probably represents a single methyl group. Thus, the discrete set of long-lived, mobile resonances in the CPMG spectra of ovalbumin and plakalbumin cannot arise from that region of the protein that has been proposed to exhibit a high degree of mobility based on its X-ray crystal structure.

α_1-PI ^{1}H NMR Relaxation Studies. Ovalbumin is one of the few nonhibitory members of the serpin superfamily. As such, it may possess structural features, absent or different in inhibitory serpins, that preclude the positive stabilization factors necessary for expression of inhibitory activity (Wright et al., 1990). Additionally, it has been suggested that the extended α-helical reactive center loop in the inhibitory members of the serpin superfamily is most likely more mobile than that of the noninhibitory members (Stein et al., 1990). For these reasons, similar experiments to those described for ovalbumin were performed on three different forms of α_1-PI to try to detect resonances from mobile residues in the reactive center loop. Since α_1-PI undergoes a major conformational change upon proteolysis, thereby making the assignment strategy for reactive center loop resonances adopted for ovalbumin invalid, an *N*-chlorosuccinimide-oxidized form of α_1-PI was examined in addition to the native and proteolyzed forms.

Figure 4 shows the 400-MHz ^{1}H NMR spectra of native α_1-PI at a concentration of 0.18 mM (Figure 4a), together with CPMG spectra obtained with total delays of 16 (Figure 4b), 128 (Figure 4c), and 240 ms (Figure 4d). As was seen with ovalbumin, the great majority of resonance intensity has decayed within 16 ms, as evidenced by the CPMG spectrum in Figure 4b. After a total delay of 240 ms, only a discrete set of long-lived resonances from mobile residues remains (Figure

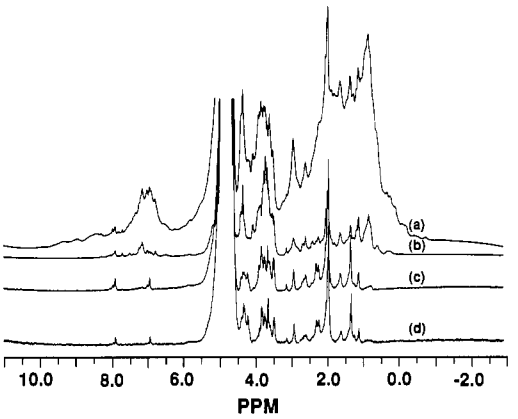

FIGURE 4: 400-MHz ^{1}H NMR spectra of 0.18 mM native α_1-PI. (a) Normal spectrum; (b–d) CPMG spectra with total delays of (b) 16, (c) 64, and (d) 240 ms. Spectra were recorded at 303 K and represent the average of 2100 scans each. The vertical scale of (d) is twice that of (c), which is twice that of (b).

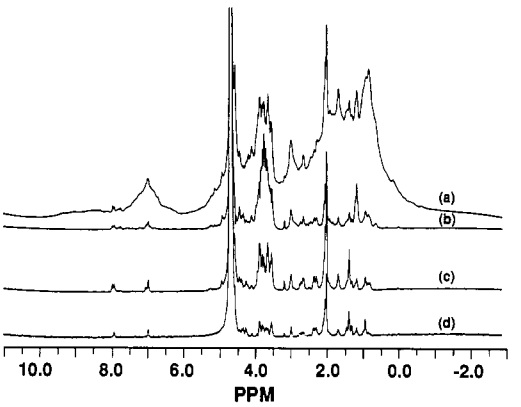

FIGURE 5: Effect of P_1–$P_{1'}$ reactive center loop cleavage of α_1-PI by papain on the 400-MHz ^{1}H NMR spectra. The protein concentration was 0.18 mM. Spectra were recorded at 303 K and represent the average of 3000 scans each. (a) Normal spectrum; (b–d) CPMG spectra with total delays of (b) 16, (c) 64, and (d) 240 ms. The vertical scale of (d), is twice that of (c), which is twice that of (b).

4d). This includes resonances from alanine CH_3 protons at 1.39 ppm, acetyl CH_3 protons at 2.0 ppm, lysine ϵ-CH_2 protons at 3.0 ppm, carbohydrate ring methine protons between 3.5 and 3.9 ppm, and the C(4)H and C(2)H protons from a histidine residue at 7.0 and 8.0 ppm, respectively. A prominent difference from the CPMG spectra of ovalbumin is the appearance of a pair of resonances at 2.2–2.3 ppm, which might arise from methionine CH_3 protons.

Although the effects of proteolysis are more complicated than with ovalbumin, because of the conformational change in α_1-PI, it might still be expected that, if any of the resonances present in the spectrum shown in Figure 4d are from amino acids in or around the reactive site loop, proteolysis within the loop would, at the very least, cause significant perturbations of these resonances.

The 400-MHz ^{1}H NMR spectrum of α_1-PI, proteolytically modified by reaction with papain, and at a concentration of 0.18 mM, is shown in Figure 5a. Papain catalytically cleaves α_1-PI at its Met P_1–Ser $P_{1'}$ reactive center peptide bond, and in doing so inactivates the inhibitor (Johnson & Travis, 1977). This proteolytically modified form is accepted to be representative (Smith et al., 1990) of the form which was crystallized subsequent to release from complex with chymotrypsin (Löberman et al., 1984). CPMG spectra with total delays of 24, 64, and 240 ms are shown in Figure 5b–d, respectively. A comparison of Figure 4d with Figure 5d shows that the set

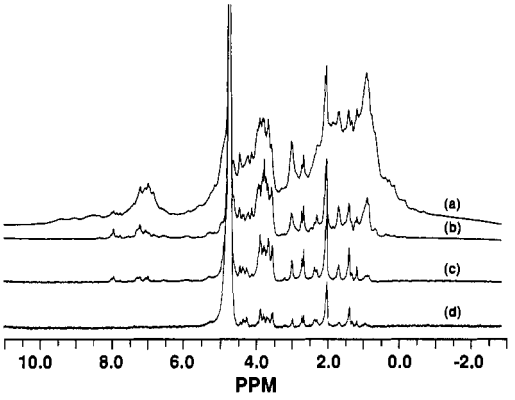

FIGURE 6: Effect of *N*-chlorosuccinimide oxidation of the P_1 and P_8 methionine residues of α_1-PI on the ^1H NMR spectrum. The protein concentration was 0.18 mM. Spectra were recorded at 303 K and represent the average of 2500 scans each. (a) Normal spectrum; (b–d) CPMG spectra with total delay times of (b) 16, (c) 64, and (d) 240 ms. The vertical scale of (d) is twice that of (c), which is twice that of (b).

of long-lived resonances are virtually identical in native and proteolytically modified α_1-PI. There is, however, one additional resonance in the proteolytically modified form at 0.89 ppm, which is at the expected chemical shift for the γ-CH_3 protons of Ile residues. This resonance may arise from Ile-360, which, following cleavage in the reactive center loop by papain, is the residue penultimate to the newly generated N-terminus and, as such, might be expected to exhibit increased mobility. Nonetheless, the appearance of this resonance in the modified form of the inhibitor with no perturbations or losses of any of the former resonances indicates that the long-lived resonances arise from residues elsewhere within the molecule and not from within or around the extended α-helical reactive center loop.

An alternative to proteolysis in attempting to identify reactive site resonances is chemical modification. A very well characterized modified species is the product of oxidation by *N*-chlorosuccinimide. This reaction has been shown to oxidize the P_8 and P_1 methionine residues of the reactive center loop, converting them to the sulfoxide species (Johnson & Travis, 1979). In doing so, the oxidized inhibitor is rendered inactive and subsequently fails to form complexes with porcine pancreatic elastase, which instead converts it to a lower molecular weight form (see Materials and Methods). Since it was expected that the chemical shifts of the methionine methyl resonances would be sensitive to the conversion to sulfoxide and thus provide a means of resonance assignment, this chemically modified species was chosen for examination.

Figure 6 shows the 400-MHz ^1H NMR spectra of oxidized α_1-PI together with CPMG spectra obtained with total delays of 16, 64, and 240 ms. Although these spectra are very similar to those of native α_1-PI (Figure 4), there is a striking difference of two additional resonances at 2.65 and 2.70 ppm. From model compound studies in which methionine was oxidized with 3% hydrogen peroxide, these resonances have been assigned to the ϵ-CH_3 protons of methionine sulfoxide. This assignment was supported by the results obtained from a Hahn spin-echo spectrum, which is shown in Figure 7. This spectrum was obtained under conditions for which doublet resonances, such as those of alanine, would be inverted, while singlet resonances, such as those of methionine or methionine sulfoxide CH_3 groups, would be positive. The expected inversion of the alanine methyl doublet at 1.39 ppm is clearly seen, while the two resonances at 2.65 and 2.70 ppm have normal phase. That the resonances at 2.65 and 2.70 ppm are singlets was confirmed by the absence of phase modulation in spin-echo spectra collected for delay times other than 120 ms (data not shown).

From the prominence of these resonances in the CPMG spectrum after a delay of 240 ms, it seems that the methyl groups in the P_8 and P_1 methionine sulfoxides have high mobility. Importantly, there does not appear to be a major loss of resonance intensity from the chemical shift position of the methyl resonances of unoxidized methionine (2.1 ppm), so that one effect of the oxidation appears to be an increase in mobility of the side chains of these residues.

Discussion

This NMR study of two of the best characterized members of the serpin superfamily, ovalbumin and α_1-PI, was undertaken to obtain evidence in support of the proposal of Stein et al. (1990) that segmental motion is present in the α-helical reactive center loop region of serpins in solution. These authors had further proposed that such flexibility is necessary for the interaction of these serpins with their target proteases and their successful inhibition. Such obligate flexibility not only would be of great mechanistic importance but also would result in a convenient way of detecting NMR signals selectively from the reactive center regions in these otherwise dauntingly large proteins, by using pulse sequences such as the CPMG experiment, which emphasizes resonances from mobile residues, which have long-lived excited states.

CPMG spectra of native ovalbumin (Figure 1) succeed in distinguishing slowly relaxing mobile nuclei from more rigidly held and therefore more rapidly relaxing nuclei. Not surprisingly, the carbohydrate located on the protein surface is found to contribute significantly to the CPMG spectrum obtained after the longest delay times between excitation and data collection (Figure 1d). Relatively few amino acid side chains possess comparably slow relaxing nuclei, though alanine residues are among the most prominent. However, it is clear from the CPMG spectra of subtilisin Carlsberg treated ovalbumin (Figure 2) that removal of a large portion of the reactive center loop, including three alanine side chains, does not lead to a major loss of resonance intensity from the CPMG spectra. This is true not only for the longest delay time of 240 ms but also for delay times as short as 16 ms. It must be concluded, therefore, that the three alanine residues in the reactive center loop of native ovalbumin do not possess motional flexibility significantly greater than the bulk of the protein and that the alanine residues that possess slowly relaxing side chain nuclei are located elsewhere, at unidentified positions. This conclusion is reinforced by the appearance of *additional* slowly relaxing resonances in the spectrum of cleaved ovalbumin (Figure 3) that are most probably at or very close to the newly created N- and C-termini within the reactive center loop, which were immobile prior to excision of the hexapeptide but gained mobility by removal of the intervening stretch of polypeptide.

Although the proposal of reactive center loop flexibility was based on the X-ray crystal structures of native and cleaved ovalbumin, we were concerned that ovalbumin, being one of the few noninhibitory serpins, might be atypical in the properties of its reactive center loop, so that the conclusion of no additional flexibility found here in our NMR studies might not have general applicability to inhibitory serpins. For this reason, α_1-PI was also examined by similar NMR methods.

As was found with ovalbumin, the carbohydrate moieties as well as a comparatively small number of amino acid side chains give rise to slowly relaxing resonances in α_1-PI (Figure 4). Because of the potential complication in interpretation of

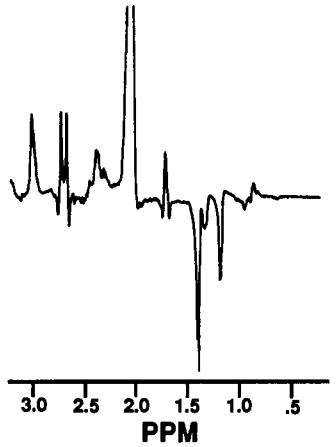

FIGURE 7: 400-MHz ^{1}H NMR Hahn spin-echo spectrum of the aliphatic protons of *N*-chlorosuccinimide-oxidized α_1-PI. The sample is the same as the one shown in Figure 4. The spectrum was recorded with a 90°–τ–180°–τ pulse sequence and a value for τ of 60 ms. The spectrum was recorded at 303 K and represents the average of 6000 scans.

the NMR spectra arising from the proteolytically induced conformational rearrangement, it was fortunate that an oxidized species, involving transformation of two reactive center loop methionines into methionine sulfoxides, has been previously well characterized (Johnson & Travis, 1977), since this afforded a conservatively modified form of the native protein for NMR spectral comparison. Two new long-lived resonances at 2.65 and 2.70 ppm in the CPMG spectra of the oxidized form (Figure 6) were assigned on the basis of chemical shift and spin multiplicity to the methionine sulfoxide methyl groups of the P_1 and P_8 residues from the reactive center loop. The slow relaxation of these resonances indicates high mobility of the methyl groups in the oxidized form of the protein. However, their appearance as long-lived resonances in the spectra of the oxidized form of α_1-PI is not parallelled by a corresponding loss of two long-lived resonances at the chemical shift expected for the methyl groups of normal methionine residues (2.1 ppm). Such a loss would be expected if the mobility of the unoxidized methionine residues were as high as that of the sulfoxides. The conclusion from the present studies about the mobility of the reactive center loop in α_1-PI is thus the same as for ovalbumin, viz, that there is no significantly higher flexibility for this region in the native protein. This is further supported by the data on the cleaved form of α_1-PI, since the concern about the effects of the conformational change per se turned out not to be important. Thus, the CPMG spectra of cleaved α_1-PI (Figure 5) showed no loss of slowly relaxing nuclei when compared with those of native α_1-PI (Figure 4).

It has already been pointed out that NMR spectroscopy, particularly through the exploitation of CPMG experiments (Gettins & Cunningham, 1986; Sommerville et al., 1990) or spin diffusion effects (Arata et al., 1980; Arakawa et al., 1986), is a successful means of identifying regions of proteins that have differential mobility. The failure to find evidence in the present study for high mobility for the reactive center loop regions of either ovalbumin or α_1-PI is therefore unlikely to be due to use of an inappropriate method.

The mean square displacements, $\langle x^2 \rangle$, calculated from the *B* factors of native ovalbumin reported by Stein et al. (1990) for the extended α-helical reactive center loop relative to the protein core are 0.35 and 0.28 Å^2, respectively. In the context of studies on other proteins, this variation does not represent a major difference. Thus, in hen egg white lysozyme, it was found that the regions bounding the sugar binding site had considerably greater mobility than the remainder of the protein. This was evidenced by $\langle x^2 \rangle$ values for the former as high as 0.7 Å^2, while the rest of the protein had $\langle x^2 \rangle$ values close to 0.23 Å^2 (Artymuik et al., 1979). The differences in $\langle x^2 \rangle$ observed for lysozyme are thus very much greater than the difference of 0.07 Å^2 seen for ovalbumin.

SUMMARY

Using CPMG NMR measurements, which have been shown in other proteins to be sensitive to differential mobility, we found no evidence for unusually high flexibility or segmental motion associated with the reactive center loop regions of either the noninhibitory serpin ovalbumin or the inhibitory serpin α_1-PI. It is concluded that high mobility for this region cannot be a necessary prerequisite for successful inhibition of target proteases.

ACKNOWLEDGMENTS

We thank Dr. Leon Cunningham for helpful discussions and Mr. Thomas Porter for carrying out the amino acid composition analysis of the oxidized α_1-PI species.

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Thermodynamics of Condensation of Nuclear Chromatin. A Differential Scanning Calorimetry Study of the Salt-Dependent Structural Transitions†

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Received May 17, 1991

ABSTRACT: We present a detailed thermodynamic investigation of the conformational transitions of chromatin in calf thymus nuclei. Differential scanning calorimetry was used as the leading method, in combination with infrared spectroscopy, electron microscopy, and techniques for the molecular characterization of chromatin components. The conformational transitions were induced by changes in the counterion concentration. In this way, it was possible to discriminate between the interactions responsible for the folding of the higher order structure and for the coiling of nucleosomal DNA. Our experiments confirm that the denaturation of nuclear chromatin at physiological ionic strength occurs at the level of discrete structural domains, the linker and the core particle, and we were able to rule out that the actual denaturation pattern might be determined by dissociation of the nucleohistone complex and successive migration of free histones toward native regions, as recently suggested. The sequence of the denaturation events is (1) the conformational change of the histone complement at 66 °C, (2) the unstacking of the linker DNA at 74 °C, and (3) the unstacking of the core particle DNA, that can be observed either at 90 or at 107 °C, depending on the degree of condensation of chromatin. Nuclear chromatin unfolds in low-salt buffers, and can be refolded by increasing the ionic strength, in accordance with the well-known behavior of short fragments. The process is athermal, therefore showing that the stability of the higher order structure depends on electrostatic interactions. The transition between the folded conformation and the unfolded one proceeds through an intermediate condensation state, revealed by an endotherm at 101 °C. The analysis of the thermodynamic parameters of denaturation of the polynucleosomal chain demonstrates that the wrapping of the DNA around the histone octamer involves a large energy change. The most striking observation concerns the linker segment, which melts a few degrees below the peak temperature of naked DNA. This finding is in line with previous thermal denaturation investigations on isolated chromatin at low ionic strength, and suggests that a progressive destabilization of the linker occurs in the course of the salt-induced coiling of DNA in the nucleosome.

In a recent paper (Balbi et al., 1989), we reported on a differential scanning calorimetry (DSC)[1] study of chromatin within rat liver nuclei. Between 70 and 110 °C, the thermal profile of this material showed three main, well-separated endotherms (Nicolini et al., 1983; Balbi et al., 1989), and we sought their relationship to the melting of the structural domains of the polynucleosomal chain. The thermal peaks observed at 68 and 85 °C for micrococcal nuclease digested nuclei were attributed to the linker and core particle DNA; more importantly, for native material, the major heat investments occurred at 107 and 90 °C. Since the equilibrium unfolding of the higher order structure resulted in the transfer of denaturation heat from the former endotherm to the latter (Balbi et al., 1988, 1989), we concluded that DSC can be regarded as a leading method for investigating the conformational changes underlying transcription and replication. Besides this perspective, the calorimetric approach can be extended in order to assess the role of condensation phenomena in gene regulation. Indeed, a DSC characterization of the organization of chromatin in transformed rat hepatocytes (Balbi et al., 1990) revealed the occurrence of an extensive decondensation process, and we were in consequence struck by the identification of a protein responsible for the packaging of the chromatin fiber into heterochromatin in position-effect variegation (Reuter et al., 1990).

† This work was supported by the Italian National Research Council, Special Project "Oncology" (Grant 104348.44.8808213), and the EEC [Grant EV4V-0036-I(A)]. M.P. thanks the Italian Association for Cancer Research for a fellowship.
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[1] Abbreviations: DSC, differential scanning calorimetry; IR, infrared; mol bp, moles of base pairs; DM, dissociation medium; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); kb, kilobase(s); DNase, deoxyribonuclease.

0006-2960/91/0430-9060$02.50/0