Denaturation Behavior of Antithrombin in Guanidinium Chloride. Irreversibility of Unfolding Caused by Aggregation†

Wayne W. Fish,*,‡ Åke Danielsson,§ Kerstin Nordling,§ Scott H. Miller,‡ Chan F. Lam,∥ and Ingemar Björk§

Departments of Biochemistry and Biometry, Medical University of South Carolina, Charleston, South Carolina 29425, and Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, S-75123 Uppsala, Sweden

Received July 13, 1984

ABSTRACT: The structural stability of the protease inhibitor antithrombin from bovine plasma was examined as a function of the concentration of guanidinium chloride (GdmCl). A biphasic unfolding curve at pH 7.4, with midpoints for the two phases at 0.8 and 2.8 M GdmCl, was measured by far-ultraviolet circular dichroism. Spectroscopic and hydrodynamic analyses suggest that the intermediate state which exists at 1.5 M GdmCl involves a partial unfolding of the antithrombin molecule that exposes regions of the polypeptide chain through which slow, intermolecular association subsequently takes place. The partially unfolded molecule can be reversed to its fully functional state only before the aggregation occurs. Upon return of the aggregated state to dilute buffer, the partially unfolded antithrombin remains aggregated and does not regain the spectroscopic properties, thrombin-inhibitory activity, or heparin affinity of the native inhibitor. This behavior indicates that the loss of the functional properties of the proteins is caused by the macromolecular association. Comparative experiments gave similar results for the human inhibitor. Analyses of bovine antithrombin in 6 M GdmCl indicated that the second transition reflects the total unfolding of the protein to a disulfide-cross-linked random coil. This transition is spectroscopically reversible; however, on further reversal to dilute buffer, the molecules apparently are trapped in the partially unfolded, aggregated, intermediate state. The results are consistent with the existence of two separate domains in antithrombin which unfold at different concentrations of GdmCl but do not support the contention that the thrombin-binding and heparin-binding regions of the protein are located in different domains [Villanueva, G. B., & Allen, N. (1983) *J. Biol. Chem. 258*, 14048–14053].

Several noteworthy features of one of the plasma protein inhibitors of serine proteases, antithrombin, have emerged from recent extensive investigations of the functions of this inhibitor. First, the antithrombin–protease complexes are only kinetically stable and dissociate slowly, i.e., with a half-life of several days, to free enzyme and a modified inhibitor that is cleaved at a specific, reactive-site bond (Jesty, 1979a,b; Fish & Björk, 1979; Danielsson & Björk, 1980, 1982; Longas & Finlay, 1980; Björk et al., 1982). The inhibition is thus akin to a normal proteolytic reaction, one of the intermediate steps of which is very slow. Furthermore, these complexes exhibit a strong tendency to aggregate (Pepper et al., 1977; Danielsson & Björk, 1983), and the mechanism for this aggregation is presently not understood. Second, the heparin-binding site on antithrombin appears to be intimately linked to the protease-reactive site of the inhibitor (Nordenman & Björk, 1978b; Jordan et al., 1979; Björk & Fish, 1982). Third, as first pointed out by Hunt & Dayhoff (1980), antithrombin belongs to a family of proteins which exhibit pronounced amino acid sequence homology (Peterson et al., 1979) to ovalbumin (McReynolds et al., 1978). Other members of this family include α_1-proteinase inhibitor (Carrell et al., 1980; Kurachi et al., 1981), another plasma protein that binds heparin tightly, namely, histidine-rich glycoprotein (Koide et al., 1982; Lijen et al., 1983), α_1-antichymotrypsin (Chandra et al., 1983), and the hormone precursor angiotensinogen (Doolittle, 1983). Finally, a recent report from Villanueva's laboratory describes a biphasic denaturation behavior for human antithrombin (HAT)[1] upon its exposure to the denaturant GdmCl (Villanueva & Allen, 1983a). The investigators further reported that after HAT had undergone the first of the two conformational transitions and was returned to dilute buffer, only the integrity of its thrombin-inhibitory site was preserved; the heparin-binding site was reported to be irreversibly lost with the unfolding of the first domain (Villanueva & Allen, 1983b).

We too have examined the stability of antithrombin [in this case bovine antithrombin (BAT)] to various concentrations of GdmCl. Although our results agree with those of Villanueva and Allen regarding the biphasic nature of the denaturation process (Villanueva & Allen, 1983a), there is marked disagreement between our results and theirs with respect to two very critical points. First, we observe a simultaneous loss of heparin-binding activity and thrombin-inhibitory activity rather than a differential loss as reported by Villanueva & Allen (1983b). Second, our data indicate that the irreversible loss of the two antithrombin functions occurs as a result of aggregation of the partially unfolded protein, rather than from the unfolding itself. In contrast, aggregation was reported not to occur in the earlier study (Villanueva & Allen, 1983b). Our results thus provide no evidence for the proposal that the thrombin-binding and heparin-binding sites of antithrombin

† This investigation was supported by grants from the National Institutes of Health (HL 26445), the Swedish Medical Research Council (4212), and the Swedish Council for Forestry and Agricultural Research (A5861).

‡ Department of Biochemistry, Medical University of South Carolina.
§ Swedish University of Agricultural Sciences.
∥ Department of Biometry, Medical University of South Carolina.

[1] Abbreviations: GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; BAT, bovine antithrombin; HAT, human antithrombin; HPLC, high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; B.P., British Pharmacopoeia.

0006-2960/85/0424-1510$01.50/0

are located on separate domains (Villanueva & Allen, 1983b).

Experimental Procedures

GdmCl was obtained from Heico Inc., Delaware Water Gap, PA. Concentrations of GdmCl stock solutions were determined by densitometry (Kawahara & Tanford, 1966) and/or by refractive index measurements (Nozaki, 1972).

Bovine antithrombin was isolated by affinity chromatography on heparin–agarose (Miller-Andersson et al., 1974). The concentration of the protein was determined from absorption measurements at 280 nm with the use of an absorption coefficient of 0.67 Lg^{-1} cm^{-1} (Nordenman et al., 1977). A molecular weight of 56 000 was used in calculations of molar concentrations (Nordenman et al., 1977). Human antithrombin (lot MDPH) was a generous gift from Dr. Milan Wickerhauser of the American Red Cross. The sample was ≃95% of one electrophoretic component. The thrombin used was a preparation of bovine α-thrombin which was a gift from Dr. Craig Jackson, Washington University, St. Louis, MO; its properties have been described previously (Carlström et al., 1977).

Commercial heparin, from pig intestinal mucosa (stage 14; Inolex Pharmaceutical Division, Park Forest South, IL), was purified and coupled to cyanogen bromide activated agarose by procedures described earlier (Nordenman & Björk, 1978a,b). The purified material was also fractionated by gel chromatography and separated into fractions with high and low affinity for antithrombin (Höök et al., 1976; Nordenman & Björk, 1978b; Danielsson & Björk, 1981). The high-affinity fraction used has an average molecular weight of 13 200, as determined by sedimentation equilibrium, and an anticoagulant activity of 207 B.P. units/mg (Danielsson & Björk, 1981).

Circular dichroic spectra were measured at room temperature (22 ± 2 °C) with a Jasco J41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) or a Cary 60 spectropolarimeter with a CD attachment. Measurement in the far-UV (200–250 nm) wavelength region employed cells of 0.05–0.1-cm path length and protein concentrations of about 0.2 g/L, while cells of 1-cm path length and protein concentrations of about 1.5 g/L were used in the near-UV (250–310 nm) regions. The results were expressed as mean residue ellipticities in the far-UV region and as molar ellipticities in the near-UV region. A mean residue weight of 112 was used for the peptide moiety of antithrombin (Nordenman et al., 1977).

UV difference absorption and fluorescence were measured as described earlier (Nordenman & Björk, 1978a; Nordenman et al., 1978; Björk & Fish, 1982).

Sedimentation velocity measurements were made by standard procedures on a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with a photoelectric scanner as well as schlieren optics. Sedimentation equilibrium measurements were made by the long-column meniscus-depletion method of Chervenka (1970). A partial specific volume of 0.72 mL/g was used for antithrombin both in buffer and in GdmCl (Nordenman et al., 1977). Densities and relative viscosities of GdmCl solutions were taken from Kawahara & Tanford (1966).

Low-pressure gel chromatography in 6 M GdmCl was performed on a Sephacryl S-300 column (1.5 cm × 100 cm; Pharmacia, Uppsala, Sweden). Elution positions from the column were calibrated with respect to equivalent hydrodynamic radii in the usual fashion (Fish, 1975). Size exclusion by HPLC was performed on an Altex TSK 3000 SW column (30 cm × 7.5 mm with no precolumn) at room temperature (ca. 22 °C). The remainder of the isocratic system consisted of a Beckman A-110 pump, a single-wavelength detector (254 nm), and a strip-chart recorder. The pump rate was calibrated and maintained at 1 mL/min during sample runs; a complete run required about 15 min. Elution times for the emergence of peak solute concentrations were utilized in the calculation of K_d values for column calibration. From the data for a number of commonly employed protein standards, the Stokes radius could be estimated for each chromatographic species. Changes in the relative proportions of the various chromatographic species were quantitated by deconvolution of the peaks. The continuous concentration data recordings were first digitized by means of a bit pad interfaced to an Apple II computer. The resolution of the bit pad is 0.9 mm which resulted in an average of 175 digitized data points per curve. The digitized data were then fitted to the following Gaussian curve:

$$C_j = \sum_{i=1}^{N} A \exp\{-[(x_i - \mu_i)^2/2\sigma_i^2]\}$$

by means of a least-squares fit or by minimizing the residue

$$\min R = \sum_{j=1}^{M} (O_j - C_j)^2$$

where C_j = the calculated concentrations at point j, O_j = the observed concentration at point j, A_i = the peak amplitude of the ith Gaussian peak, x_i = the location (i.e., elution position) of the observed concentration, μ_i = the location (i.e., elution position) of the ith Gaussian peak, σ_i = the standard deviation (width) of the ith Gaussian peak, N = the number of overlapped Gaussian peaks, and M = the number of data points. The residue of the sum of squares was minimized by means of a simplex procedure (Lam et al., 1979; Nelder & Mead, 1965) which has been used successfully to deconvolute overlapped pulse height spectra from analytical scanning electron microscopy (Lam et al., 1979). The minimization procedure does not require derivatives of the residue function with respect to the parameters to be estimated.

Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate followed the method described by Weber & Osborn (1969) or Laemmli (1970).

Spectrophotometric assays of antithrombin activity against thrombin in the absence or presence of heparin routinely utilized a chromogenic tripeptide substrate, S2238, and followed procedures which have been detailed previously (Björk & Nordenman, 1976; Björk & Nordling, 1979). In several instances, thrombin and antithrombin activities were measured by using the clotting assay described by Fenton & Fasco (1974).

Results

Unfolding to and Attempted Refolding from 6 M GdmCl. The unfolding of BAT at increasing concentrations of GdmCl was monitored by far-UV CD (Figure 1). Two well-resolved stages of the unfolding process were evident. The first stage exhibited a transition midpoint at 0.8 M GdmCl and was complete at 1.2–1.5 M, while the second stage exhibited a midpoint at a denaturant concentration of 2.8 M and was complete at 4.0 M. Only the transition occurring at the higher GdmCl concentrations appeared reversible when the concentration of the denaturant was lowered from 6.0 M.

Spectroscopic and hydrodynamic analyses indicated that BAT was completely unfolded to a disulfide-cross-linked random coil in 6 M GdmCl. The far-UV and near-UV CD spectra thus were those of a typical randomly coiled polypeptide (Greenfield & Fasman, 1969; Cortijo et al., 1973). Moreover, analyses of the denatured, unreduced protein by gel chromatography in 6 M GdmCl (Fish et al., 1969) gave

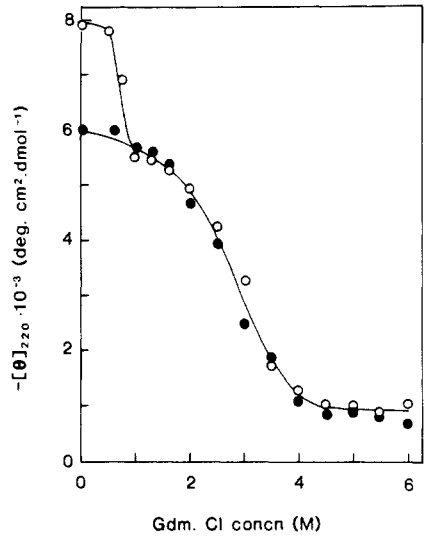

FIGURE 1: Unfolding of antithrombin at different concentrations of GdmCl and refolding of the denatured protein from 6 M GdmCl, as monitored by CD. (○) Unfolding: Antithrombin was mixed with buffer and 7.8–8.2 M GdmCl to the appropriate concentration of denaturant and to a final protein concentration of 0.22–0.25 g/L. Measurements were made after 24 h. (●) Refolding: Antithrombin, at a concentration of 2.2 g/L, was kept in 6.0 M GdmCl for 4 h and was then diluted with buffer and 6.0 M GdmCl to the appropriate concentration of denaturant. The reversal to 0 M GdmCl, however, was done by gel chromatography on Sephadex G-25. In both cases, the final protein concentration was 0.22 g/L. The samples were measured 24 h after their transfer to the lower GdmCl concentration. All solutions in both the unfolding and refolding experiments contained 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.4, as buffer. All points are averages of two separate analyses. The unit on the ordinate is the mean residue ellipticity.

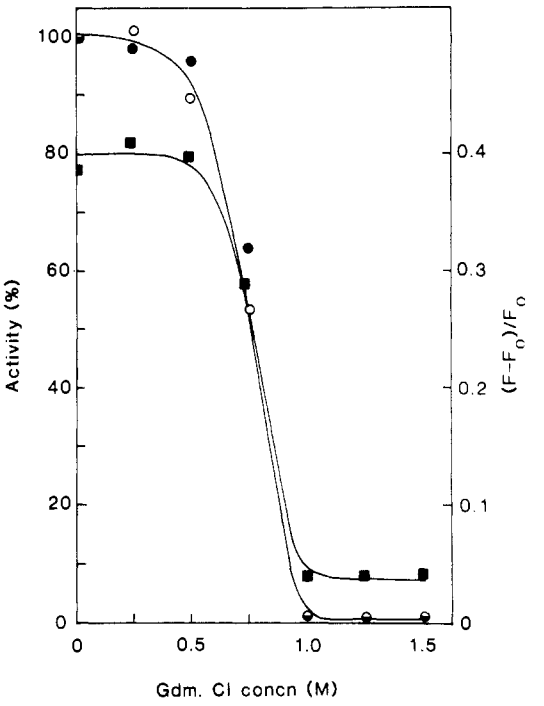

FIGURE 2: Thrombin-inhibiting activity and heparin-induced fluorescence increase of antithrombin refolded from different concentrations of GdmCl. Antithrombin (2.0 g/L) was kept for 24 h in 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.4, containing GdmCl at the different concentrations. The denaturant was then removed by gel chromatography on Sephadex G-25, and the analyses were made after an additional 24 h. (○) Activity in the absence of heparin; (●) activity in the presence of an excess of unfractionated heparin. Activities were measured by a spectrophotometric assay (Björk & Nordenman, 1976; Björk & Nordling, 1979) and were expressed as the percent of the activity of native antithrombin under identical conditions. (■) Fluorescence increase caused by high-affinity heparin in a molar ratio to the protein of 1.5. The analyses were done as in Nordenman et al. (1978) at a protein concentration of 0.11 g/L. F, fluorescence of the protein in the presence of high-affinity heparin; F_0, fluorescence of the protein at the same concentration without heparin.

a Stokes radius of 6.4 ± 0.1 nm (this corresponds to an $[\eta]$ of ~29.5 mL/g). This value for the Stokes radius is in good agreement with the value of 6.2 nm that can be estimated for a randomly coiled polypeptide having the chain length and disulfide bond pattern of antithrombin (Tanford, 1968; Leach & Fish, 1977; Peterson et al., 1979). In keeping with the latter result, sedimentation equilibrium measurements of disulfide-cross-linked BAT in 6 M GdmCl yielded a molecular weight of 53 500 which indicates that the denatured protein is monomeric under these conditions.

Both the far-UV and near-UV CD spectra of BAT which had been returned to buffer from 6 M GdmCl showed that the conformation of this form of the protein is distinctly different from that of native BAT, in agreement with the refolding curve of Figure 1. Moreover, sedimentation velocity analyses showed the refolded BAT to be aggregated with a weight-average sedimentation coefficient of about 17 S. Upon reexposure to 6 M GdmCl, the refolded protein eluted at the same position during gel chromatography in this solvent as BAT which was exposed to the denaturant for the first time. This indicates that the aggregation was not caused by disulfide interchange during the unfolding–refolding process and that the aggregate is held together through noncovalent interactions.

Further studies showed that the form of BAT that was refolded from 6 M GdmCl had no activity against thrombin in a spectroscopic assay with a chromogenic tripeptide substrate, in either the absence or the presence of heparin. Moreover, it eluted from matrix-linked heparin at ≤0.3 M NaCl, in contrast to the native inhibitor which elutes at ~0.8 M NaCl (Miller-Andersson et al., 1974; Nordenman & Björk, 1978b). As judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, reaction with thrombin resulted in no detectable formation of complex between the two proteins but resulted instead in extensive degradation of the refolded antithrombin. Together, all these experiments lead to the conclusion that the native, monomeric, functional conformation of antithrombin is not regained after exposure of the protein to 6 M GdmCl and subsequent removal of the denaturant.

Characterization of the Initial Unfolding Step. The susceptibility of BAT to partial unfolding at low GdmCl concentrations and the existence of an apparent intermediate state of unfolding at 1 M GdmCl prompted a more detailed analysis of this initial denaturation step.

Equilibrium Measurements. Analyses of the recovery of the activity and of the heparin-binding ability of BAT (as reflected by the fluorescence increase of the protein in the presence of high-affinity heparin; Einarsson & Andersson, 1977; Nordenman et al., 1978) after reversal from 24 h in increasing GdmCl concentrations showed that both functional properties of the inhibitor were irreversibly lost in a narrow concentration interval between 0.5 and 1.0 M GdmCl (Figure 2). The loss of the heparin-binding capacity was also shown by affinity chromatography on heparin–agarose, in which the protein that was refolded from 1.5 M GdmCl eluted at 0.35 M NaCl. In addition, the absorbance difference spectrum between the refolded protein in the presence of high-affinity heparin and the refolded protein alone had only ~15% of the magnitude of the corresponding spectrum for native antithrombin (Nordenman & Björk, 1978a). Thus, it appeared that the apparent irreversibility of BAT refolding was asso-

ciated with the unfolding transition centered at ~0.8 M GdmCl. On the basis of the equilibrium results from the spectral and activity measurements, further characterization of the intermediate state was made at 1.5 M GdmCl, i.e., at about the concentration where the initial transition is complete and the putative intermediate probably is in highest concentration.

Circular dichroism analyses showed pronounced differences between BAT after 24 h in 1.5 M GdmCl and the native protein, in both the far-UV and near-UV wavelength regions. UV difference spectra between the partially unfolded and the native inhibitor showed a red shift accompanied by an increase in absorption with spectral characteristics typical of transfer of tryptophanyl residues to a more hydrophobic environment. Moreover, BAT in 1.5 M GdmCl had ~12% lower fluorescence than the native protein. Sedimentation velocity analysis of disulfide-cross-linked BAT in 1.5 M GdmCl demonstrated that over 90% of the protein was aggregated after 24 h in this concentration of denaturant. A weight-average sedimentation coefficient of 14 S was estimated for this aggregated state. The presence of 0.25% Nonidet P-40 in 1.5 M GdmCl had little or no effect on the state of aggregation.

Circular dichroism spectra also showed minimal regain of the spectral characteristics of native BAT upon its return to buffer after exposure to 1.5 M GdmCl for 24 h. Moreover, sedimentation velocity analysis indicated that antithrombin remained aggregated after reversal from 1.5 M GdmCl; a weight-average sedimentation coefficient of 14.8 S was measured.

Kinetic Measurements. To better understand the events which led to loss of thrombin-inhibitory activity and to aggregation of BAT on exposure to 1.5 M GdmCl, the changes in a number of properties were followed as a function of time. These properties included the circular dichroic properties, the molecular size, and the thrombin-inhibitory activity of the protein after its reversal into dilute buffer.

Upon subjection of BAT to 1.5 M GdmCl, pH 7.4 at 25 °C, the amplitude of the mean residue ellipticity at 220 nm decreased to a relatively stable value of about −5500 deg cm^2 dmol^{-1} in less than 1 min. This value is about the same as that measured after a 24-h exposure of the protein to the denaturant (Figure 1). Analysis of the CD data according to the method of Siegel et al. (1980) suggests that this change might be produced by a loss in the periodic elements of secondary structure such as the proportion of α-helix going from 26% to 15%. Such a change in ordered structure might also be reflected by an increase in the effective hydrodynamic size of the BAT molecule. As shown in Figure 3A for the first time point, this is indeed the case. A chromatographic species of BAT of R_e = 43 Å (compared to an R_e = 34 Å for the native state) was the only form of the molecule existing after a few minutes in the 1.5 M GdmCl. This form remained the predominant chromatographic species throughout the first hour in the denaturing solvent (Figure 3A). When BAT which had been exposed to 1.5 M GdmCl for less than 45 min was subjected to sedimentation velocity analysis while still in the denaturant, it sedimented as a single boundary with an $s^0_{20,w}$ of 2.9 ± 0.3 S. These sedimentation velocity results support the notion that the 43-Å chromatographic species is partially unfolded monomer since a dimeric form of BAT which exhibited a Stokes radius of 43 Å would be expected to exhibit a sedimentation coefficient of ca. 6.3 S.

At the time this initial unfolding transition in 1.5 M GdmCl was complete (i.e., <1 min), ≥90% of the heparin-dependent and heparin-independent thrombin-inhibitory activities of BAT

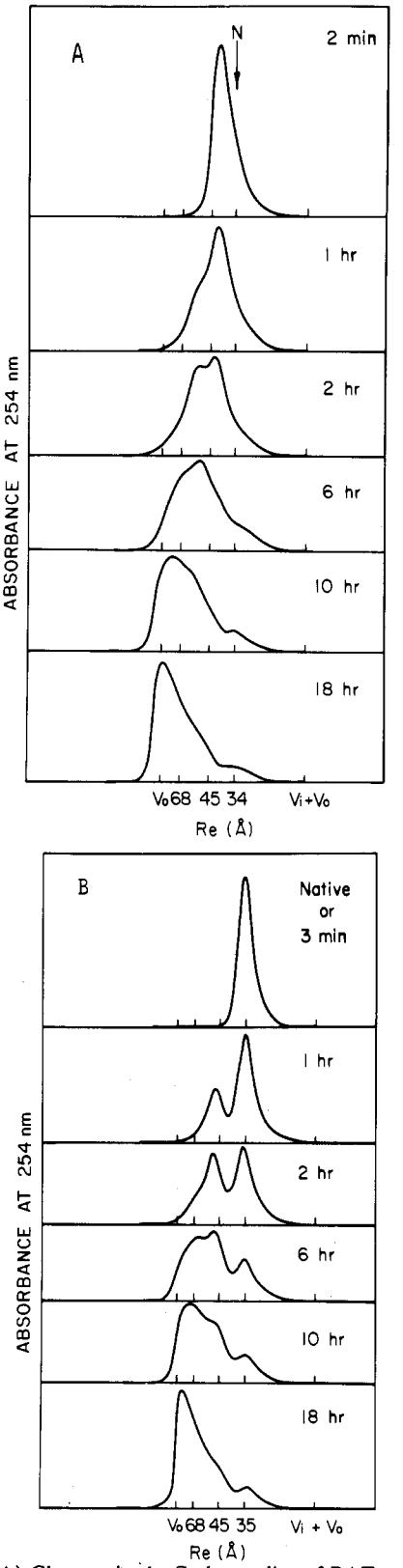

FIGURE 3: (A) Changes in the Stokes radius of BAT as a function of time of incubation in 1.5 M GdmCl, pH 7.4 at 25 °C. Size-exclusion chromatography in the presence of 1.5 M GdmCl was performed as described under Experimental Procedures. The times indicated in the figure refer to the times BAT was exposed to 1.5 M GdmCl before injection onto the column. Depending on its state, the protein spent an additional 6–9 min in the presence of 1.5 M GdmCl while traveling through the column. (B) Changes in the Stokes radius of BAT upon its return to dilute buffer after various times of incubation in 1.5 M GdmCl, pH 7.4 at 25 °C. Size-exclusion chromatography was performed in the presence of 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4. The times indicated in the figure refer to the times BAT was exposed to 1.5 M GdmCl before injection onto the column.

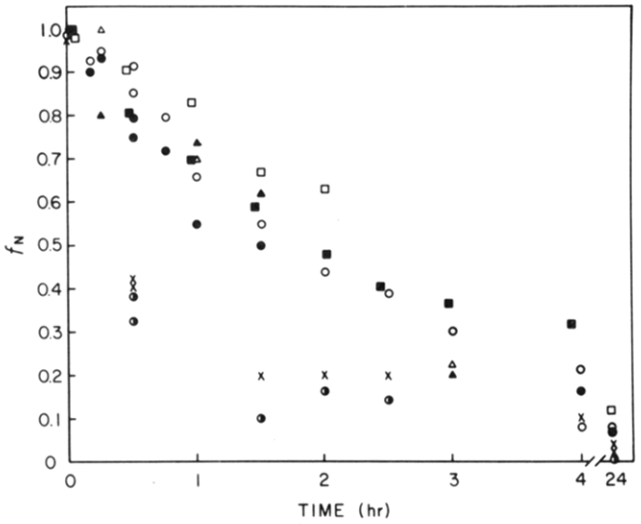

FIGURE 4: Loss of antithrombin's native properties upon its exposure to 1.5 M GdmCl, pH 7.4 at 25 °C, for various periods of time. (○) Heparin-dependent antithrombin activity of BAT as measured with a chromogenic peptide substrate after return of the protein to buffer. (●) Heparin-independent (i.e., progressive) antithrombin activity of BAT as measured with a chromogenic peptide substrate after return of the protein to buffer. (△) Heparin-dependent antithrombin activity of BAT as measured by a clotting assay after return of the protein to buffer. (▲) Heparin-independent (i.e., progressive) antithrombin activity of BAT as measured by a clotting assay after return of the protein to buffer. (□) BAT of Stokes radius = 43 Å (i.e., monomer) as estimated by size-exclusion HPLC in 1.5 M GdmCl. (■) BAT of Stokes radius = 34 Å (i.e., same as that of native BAT) as estimated by size-exclusion HPLC in dilute buffer. (×) Heparin-dependent antithrombin activity of HAT after return of the protein to buffer. (◑) Heparin-independent (i.e., progressive) antithrombin activity of HAT after return of the protein to buffer. Most of the assays for the thrombin-inhibitory activity of HAT employed a chromogenic peptide substrate, but those samples which were also checked with the clotting assay yielded identical results with both assay procedures. The symbol f_N on the ordinate represents the response on a fractional basis expressed by the treated sample when compared to the same response expressed by native antithrombin.

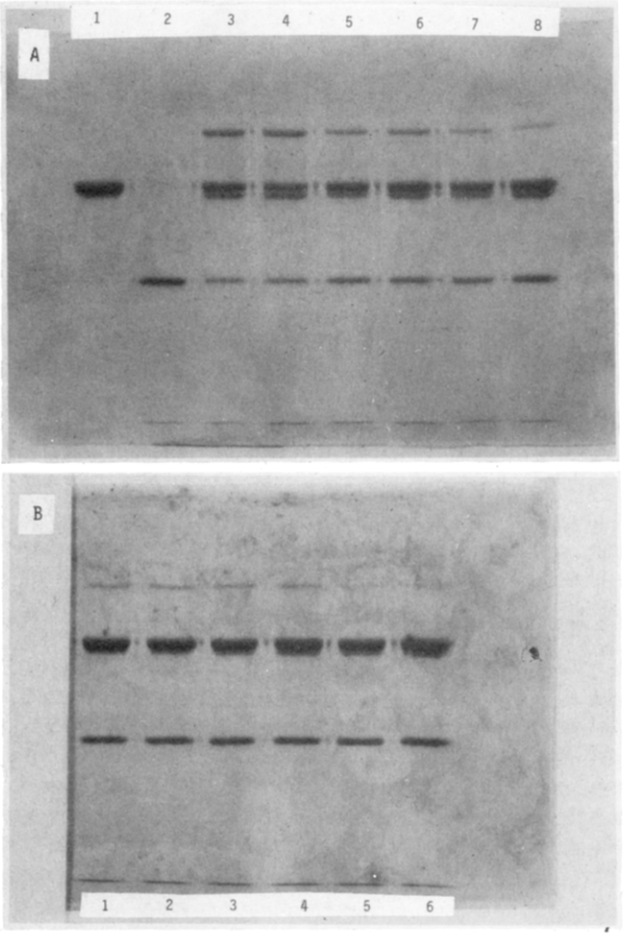

FIGURE 5: SDS–PAGE of products of the reaction of bovine thrombin with BAT upon reversal of BAT into buffer after various lengths of time in 1.5 M GdmCl, pH 7.4 at 25 °C. (A) Reactions in the presence of heparin for 30 s. Lane 1, native BAT only; lane 2, native bovine α-thrombin only. Molar ratios of the components in the reaction mixtures applied to lanes 3–8 were ~3 mol of heparin, 1.6 mol of BAT, and 1 mol of bovine thrombin. Lane 3, native BAT; lane 4, BAT in 1.5 M GdmCl, 30 s; lane 5, BAT in 1.5 M GdmCl, 1 h; lane 6, BAT in 1.5 M GdmCl, 2 h; lane 7, BAT in 1.5 M GdmCl, 4 h; lane 8, BAT in 1.5 M GdmCl, 8 h. (B) Reactions in the absence of heparin for 30 s. Molar ratios of the reactants were the same as in (A). Lane 1, native BAT; lane 2, BAT in 1.5 M GdmCl, 30 s; lane 3, BAT in 1.5 M GdmCl, 1 h; lane 4, BAT in 1.5 M GdmCl, 2 h; lane 5, BAT in 1.5 M GdmCl, 4 h; lane 6, BAT in 1.5 M GdmCl, 8 h.

could be recovered upon dilution of the protein from the denaturant into buffer. These same results were obtained regardless of whether a chromogenic peptide or fibrinogen was used as the substrate for the assay (Figure 4). Furthermore, as illustrated in Figure 3B, immediate return to buffer of the chromatographic species of BAT of R_e = 43 Å not only permitted its regain of activity but also resulted in the protein behaving hydrodynamically like its native state (R_e = 34 Å), and its far-UV CD spectrum again was identical with that of native BAT. These results suggest that the partially unfolded, monomeric protein returns to its native conformation upon removal of the denaturant.

Over the succeeding 24 h of exposure of BAT to 1.5 M GdmCl, pH 7.4 at 25 °C, the recovery of both the heparin-dependent and heparin-independent thrombin inhibitory activities of BAT progressively decreased in a parallel fashion (Figure 4). The results were the same whether BAT was reversed by a 40-fold dilution into buffer or by desalting on a PD-10 column. This loss of activity was also verified by SDS–PAGE analysis of the products of the interaction of bovine thrombin with BAT which had been exposed to 1.5 M GdmCl for various lengths of time before its return to buffer. As illustrated by Figure 5, three observations were made. First, the amount of complex formed diminished with the length of time BAT was exposed to 1.5 M GdmCl. Second, the rate of complex formation for each BAT treatment was faster in the presence of heparin than in its absence. This observation suggests that the heparin-binding site was functional in those BAT molecules in which the thrombin-inhibitory site was functional. Third, as suggested by the appearance of an electrophoretic component smaller than BAT, limited proteolysis of the BAT polypeptide chain without complex formation occurred in those samples which were exposed to 1.5 M GdmCl for longer periods of time. Concomitant with the decrease in the recovery of thrombin-inhibitory activity, a further increase in the molecular size of BAT was observed by HPLC size-exclusion chromatography (Figure 3A,B). After about an hour in 1.5 M GdmCl, a chromatographic species of 53 ± 3 Å Stokes radius appeared and became the predominant species during the next 2–6 h (Figure 3A). Upon its return to dilute buffer, this species appeared to maintain its integrity (cf. 3-h patterns of Figure 3A,B). Sedimentation velocity of BAT after it had been in 1.5 M GdmCl for 3 h yielded an $s^0_{20,w}$ of 4.8 S in 1.5 M GdmCl for the faster, major species. With the assumption that this particular ultracentrifuge species was also the major chromatographic species of 53 Å, a combination of the two physical parameters (Fish, 1975) suggests that the predominant molecular state of BAT

at this time is that of a dimer. After 5–6 h, larger aggregates became more prominent (Figure 3A,B). Digitization of the HPLC size-exclusion data and subsequent deconvolution of the peaks made it possible to quantitatively examine the rate of disappearance of the monomeric form of BAT, both while in 1.5 M GdmCl and after reversal into buffer. As shown in Figure 4, these data appeared to parallel the rate of loss of reversible thrombin-inhibitory activity, suggesting that this loss is due to the intermolecular association. It is probably these various aggregated states in which the BAT molecule is trapped upon its return to buffer from 6 M GdmCl.

Comparative Experiments with HAT. Obviously, the behavior we observed for BAT in the presence of low concentrations of GdmCl was markedly different from that reported for HAT under similar conditions (Villanueva & Allen, 1983b). In an attempt to test the possibility that there is a species difference in the way the products of the first unfolding transition of antithrombin behave, we made an examination of the activity loss and the state of aggregation of HAT upon its exposure to 1.5 M GdmCl. As illustrated in Figure 4, we observed a simultaneous loss of heparin-dependent and heparin-independent thrombin-inhibitory activities regardless of whether a chromogenic peptide or fibrinogen served as the thrombin substrate. In fact, data with HAT suggested that it irreversibly lost its activities faster than did BAT. Furthermore, size-exclusion HPLC of the HAT samples demonstrated that formation of chromatographic species of larger Stokes radii occurred concomitantly with the loss of thrombin-inhibitory and heparin-binding activities (data not shown). Finally, the presence of aggregates was also verified by electrophoresis in dilute buffer of samples of BAT or HAT which were returned to buffer after 2 or 20 h of exposure to 1.0 or 1.5 M GdmCl. Up to eight or nine discrete polymer bands were observed for the treated samples (Figure 6). Thus, this polymerization was observed to occur under conditions that were reported to give monomeric antithrombin which no longer possessed a functional heparin-binding domain but which still inhibited thrombin (Villanueva & Allen, 1983b). Thus, in our hands, HAT and BAT behaved similarly upon exposure to GdmCl, and we were unable to observe a monomeric form of antithrombin which had irreversibly lost only its heparin-binding domain (Villanueva & Allen, 1983b).

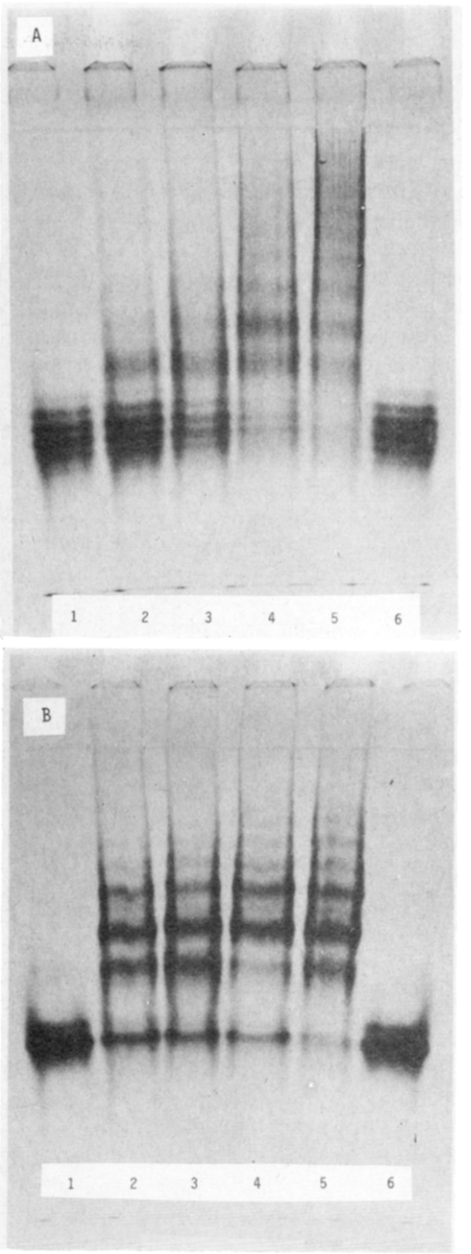

FIGURE 6: Polyacrylamide gel electrophoresis of BAT and HAT upon reversal of each into buffer after its exposure to low concentrations of GdmCl, pH 7.4 at 25 °C. The electrophoresis buffer system was that of Laemmeli (1970) without SDS. Gels were 5% acrylamide. (A) Bovine antithrombin: lane 1, native BAT; lane 2, BAT in 1.0 M GdmCl, 2 h; lane 3, BAT in 1.5 M GdmCl, 2 h; lane 4, BAT in 1.0 M GdmCl, 20 h; lane 5, BAT in 1.5 M GdmCl, 20 h; lane 6, native BAT. (B) Human antithrombin: lane 1, native HAT; lane 2, HAT in 1.0 M GdmCl, 2 h; lane 3, HAT in 1.5 M GdmCl, 2 h; lane 4, HAT in 1.0 M GdmCl, 20 h; lane 5, HAT in 1.5 M GdmCl, 20 h; lane 6, native HAT.

Discussion

The results of this investigation show that BAT undergoes a biphasic unfolding transition when exposed to increasing concentrations of the denaturant GdmCl. The first phase of denaturation occurs with a midpoint at ~0.8 M GdmCl, i.e., at a concentration at which most proteins are unaffected by the denaturant (Tanford, 1968). This initial transition apparently involves a partial unfolding of the antithrombin molecule which leads to changes in both its tertiary and its secondary structure. A result of these conformation changes is most likely exposure of hydrophobic regions on the molecule through which subsequent, slow, intermolecular association takes place. The aggregated state which results is refractory to refolding to the native, active conformation. The irreversibility of BAT unfolding is apparently a consequence of aggregation since it is possible to regain fully active protein if reversal is conducted while the partially unfolded protein is in the monomeric state. The second unfolding transition of BAT, which has a midpoint at ~2.8 M GdmCl and is completed at ~4 M, primarily reflects the total unfolding of the remainder of the protein's structure to the final state which is a disulfide-cross-linked random coil. Analyses suggest that this second transition is reversible; however, after reversal of this transition, the molecules apparently are trapped in an aggregated state than cannot refold to the functional conformation. A similar situation has been documented for the behavior of phosphorylase *b* in urea (Chignell et al., 1972). It should be emphasized that the inability of antithrombin to refold to its native, active state from that of a disulfide-cross-linked random coil is not merely a consequence of the size of the polypeptide chain. Certainly, equally large and complicated proteins can be induced to refold to their active conformations (Johanson et al., 1981; Teipel & Koshland, 1971a,b; Zettlmeissl et al., 1981).

Biphasic equilibrium denaturation curves, though not commonly observed for small globular proteins (Tanford, 1968),

are not without precedence [see reviews by Wetlaufer (1981) and Kim & Baldwin (1982)]. Several of the proteins showing such behavior, e.g., penicillinase (Carrey & Pain, 1978), the α-subunit of *Escherichia coli* tryptophan synthetase (Yutani et al., 1979, 1980), bovine serum albumin (Teale & Benjamin, 1976; Johanson et al., 1981), and ovomucoid (Masroor et al., 1978; Kato et al., 1978), have been shown to possess domains which are folded independently of one another. Hence, a multiphasic equilibrium unfolding transition is generally considered to be indicative of a modular assembly of the polypeptide chain, i.e., folding by parts (Kim & Baldwin, 1982). The unfolding of antithrombin in GdmCl suggests that separate domains also exist in this protein. The two phases of the observed denaturation curve probably reflect the independent unfolding of such domains at different concentrations of denaturant. An alternative mechanism which involves a two-step unfolding of the entire molecule with the first step affecting primarily the tertiary structure of the protein is unlikely since marked changes of both the secondary and the tertiary structure are observed to occur during the initial phase. Unfortunately, however, the aggregation of antithrombin on reversal from the fully unfolded state in 6 M GdmCl precludes a rigorous characterization of the mechanism of refolding of the protein.

The susceptibility of antithrombin to partial unfolding at unusually low concentrations of GdmCl is paralleled by the instability of the inhibitor under acid conditions. Bovine antithrombin thus irreversibly loses both its thrombin-inhibitory ability and its high affinity for heparin after exposure to pH values below 5.5 (Nordenman & Björk, 1981). Moreover, both activities also are lost after thermal denaturation of HAT at only moderate temperatures (Busby et al., 1981; Mitra et al., 1982). As first illustrated by Busby et al. (1981) for the thermally denatured protein, a common feature of all these reactions is that the inhibitor is aggregated after reversal from the respective denatured states. The native structure of antithrombin is thus easily perturbed by environmental changes, and the resulting conformational changes apparently lead to exposure of the same or similar sites on the protein through which intermolecular association occurs. Interestingly, the complex between antithrombin and thrombin has a strong tendency to aggregate (Pepper et al., 1977; Fish & Björk, 1979; Danielsson & Björk, 1983). Possibly, similar sites also are exposed on the inhibitor following formation of the thrombin–antithrombin complex.

Finally, our data do not support the claim by Villanueva and Allen that the domain of antithrombin which unfolds at the lower GdmCl concentrations is the domain which is essential only for heparin binding (Villanueva & Allen, 1983b; Villanueva, 1984). Quite the contrary, in all instances with both BAT and HAT, we observe the irreversible losses of heparin binding and thrombin inhibition to occur simultaneously. Furthermore, the simultaneous loss of these two functions coincides with an irreversible aggregation of the partially unfolded antithrombin.

Registry No. GdmCl, 50-01-1; antithrombin, 9000-94-6.

REFERENCES

Baig, M. A., & Salahuddin, A. (1978) *Biochem. J. 171*, 89–97.

Björk, I., & Nordenman, B. (1976) *Eur. J. Biochem. 68*, 507–511.

Björk, I., & Nordling, K. (1979) *Eur. J. Biochem. 102*, 497–502.

Björk, I., & Fish, W. W. (1982) *J. Biol. Chem. 257*, 9487–9493.

Björk, I., Jackson, C. M., Jörnvall, H., Lavine, K. K., Nordling, K., & Salsgiver, W. J. (1982) *J. Biol. Chem. 257*, 2406–2411.

Busby, T. F., Atha, D. H., & Ingham, K. C. (1981) *J. Biol. Chem. 256*, 12140–12147.

Carlström, A.-S., Liedén, K., & Björk, I. (1977) *Thromb. Res. 11*, 785–797.

Carrell, R. W., Boswell, D. R., Brennan, S. O., & Owen, M. C. (1980) *Biochem. Biophys. Res. Commun. 93*, 399–402.

Carrey, E. A., & Pain, R. H. (1978) *Biochim. Biophys. Acta 533*, 12–22.

Chandra, T., Stackhouse, R., Kidd, V. J., Robson, K. J. H., & Woo, S. L. C. (1983) *Biochemistry 22*, 5055–5061.

Chervenka, C. H. (1970) *Anal. Biochem. 34*, 24–29.

Chignell, D. A., Azhir, A., & Gratzer, W. B. (1972) *Eur. J. Biochem. 26*, 37–42.

Cortijo, M., Panijpan, B., & Gratzer, W. B. (1973) *Int. J. Pept. Protein Res. 5*, 179–186.

Danielsson, Å., & Björk, I. (1980) *FEBS Lett. 119*, 241–244.

Danielsson, Å., & Björk, I. (1981) *Biochem. J. 193*, 427–433.

Danielsson, Å., & Björk, I. (1982) *Biochem. J. 207*, 21–28.

Danielsson, Å., & Björk, I. (1983) *Biochem. J. 213*, 345–353.

Doolittle, R. F. (1983) *Science (Washington, D.C.) 222*, 417–419.

Einarsson, R., & Anderson, L.-O. (1977) *Biochim. Biophys. Acta 490*, 104–111.

Fenton, J. W., II, & Fasco, M. J. (1974) *Thromb. Res. 4*, 809–817.

Fish, W. W. (1975) *Methods Membr. Biol. 4*, 198–276.

Fish, W. W., & Björk, I. (1979) *Eur. J. Biochem. 101*, 31–38.

Fish, W. W., Mann, K. G., & Tanford, C. (1969) *J. Biol. Chem. 244*, 4989–4994.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Höök, M., Björk, I., Hopwood, J., & Lindahl, U. (1976) *FEBS Lett. 66*, 90–93.

Hunt, L. T., & Dayhoff, M. O. (1980) *Biochem. Biophys. Res. Commun. 95*, 864–871.

Jesty, J. (1979a) *J. Biol. Chem. 254*, 1044–1049.

Jesty, J. (1979b) *J. Biol. Chem. 254*, 10044–10050.

Johanson, K. O., Wetlaufer, D. B., Reed, R. G., & Peters, T., Jr. (1981) *J. Biol. Chem. 256*, 445–450.

Jordan, R., Beeler, D., & Rosenberg, R. D. (1979) *J. Biol. Chem. 254*, 2902–2913.

Kato, I., Kohr, W. J., & Laskowski, M. (1978) *FEBS-Symp. 47*, 197–206.

Kawahara, K., & Tanford, C. (1966) *J. Biol. Chem. 241*, 3228–3232.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Koide, T., Odani, S., & Ono, T. (1982) *FEBS Lett. 142*, 222–224.

Kurachi, K., Chandra, T., Degen, S. J. F., White, T. T., Marchioro, T. L., Woo, S. L. C., & Davie, E. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6826–6830.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lam, C. F., Forst, A., & Bank, H. (1979) *Appl. Spectrosc. 33*, 273–278.

Leach, B. S., & Fish, W. W. (1977) *J. Biol. Chem. 252*, 5239–5243.

Lijen, H. R., Hoylaerts, M., & Collen, D. (1983) *Thromb. Res. 29*, 443–446.

Longas, M. O., & Finley, T. H. (1980) *Biochem. J. 189*, 481–489.

McReynolds, L., O'Malley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Field, S., Robertson, M., & Brownlee, G. G. (1978) *Nature (London) 273*, 723–728.
Miller-Andersson, M., Borg, H., & Andersson, L.-O. (1974) *Thromb. Res. 5*, 439–452.
Mitra, G., Schneider, P. M., & Lundblad, J. L. (1982) *Biotechnol. Bioeng. 24*, 97–107.
Nelder, J. A., & Mead, R. (1965) *Comput. J. 8*, 308–313.
Nordenman, B., & Björk, I. (1978a) *Biochemistry 17*, 3339–3344.
Nordenman, B., & Björk, I. (1978b) *Thromb. Res. 12*, 755–765.
Nordenman, B., & Björk, I. (1980) *Thromb. Res. 19*, 711–718.
Nordenman, B., & Björk, I. (1981) *Biochim. Biophys. Acta 672*, 227–238.
Nordenman, B., Nystrom, C., & Björk, I. (1977) *Eur. J. Biochem. 78*, 195–203.
Nordenman, B., Danielsson, Å., & Björk, I. (1978) *Eur. J. Biochem. 90*, 1–6.
Nozaki, Y. (1972) *Methods Enzymol. 26*, 43–50.
Pepper, D. S., Banhegyi, D., & Cash, J. D. (1977) *Thromb. Haemostasis 38*, 494–503.
Peterson, T. E., Dudek-Wojeciechowska, G., Sottrup-Jensen, L., & Magnusson, S. (1979) in *The Physiological Inhibitors of Blood Coagulation and Fibrinolysis* (Collen, D., Wiman, B., & Verstraete, M., Eds.) pp 43–54, Elsevier/North-Holland, Amsterdam.
Siegel, J. B., Steinmetz, W. E., & Long, G. L. (1980) *Anal. Biochem. 104*, 160–167.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122–275.
Teale, J. M., & Benjamin, D. C. (1976) *J. Biol. Chem. 251*, 4609–4615.
Teipel, J. W., & Koshland, D. E., Jr. (1971a) *Biochemistry 10*, 792–798.
Teipel, J. W., & Koshland, D. E., Jr. (1971b) *Biochemistry 10*, 798–805.
Villanueva, G. B. (1984) *J. Biol. Chem. 259*, 2531–2536.
Villanueva, G. B., & Allen, N. (1983a) *J. Biol. Chem. 258*, 11010–11013.
Villanueva, G. B., & Allen, N. (1983b) *J. Biol. Chem. 258*, 14048–14053.
Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61–92.
Yutani, K., Ogasahara, K., Suzuki, M., & Sugino, Y. (1979) *J. Biochem. (Tokyo) 85*, 915–921.
Yutani, K., Ogasahara, K., & Sugino, Y. (1980) *J. Mol. Biol. 144*, 455–465.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1981) *Eur. J. Biochem. 121*, 169–175.

New Heterobifunctional Protein Cross-Linking Reagent That Forms an Acid-Labile Link†

Walter A. Blättler,* Bernard S. Kuenzi, John M. Lambert, and Peter D. Senter
Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115
Received September 17, 1984

ABSTRACT: A new heterobifunctional protein cross-linking reagent has been synthesized. The reagent is based on 2-methylmaleic anhydride (citraconic anhydride) which forms an acid-labile link upon reaction with amino groups. The second reactive group of the heterobifunctional reagent is a maleimido group. The novel reagent has been used to form a cross-link between two different proteins, a murine monoclonal antibody, J5, and the ribosome-inactivating protein gelonin. Gelonin was first modified by reaction with the anhydride, and the maleimido groups so introduced were allowed to react with antibody that had been modified with 2-iminothiolane to introduce sulfhydryl groups. The conjugate was stable above neutral pH. Incubation of the purified conjugate at mildly acidic pH (pH 4–5) resulted in the release of fully functional native gelonin. The amide bond between the reagent and gelonin was cleaved with about the same sensitivity to acid as that described for 2-methylmaleic anhydride.

Bifunctional cross-linking reagents are extremely useful tools for investigation of the number and arrangement of subunits in complex biological structures (Davies & Stark, 1970; Peters & Richards, 1977; Ji, 1983). The development of heterobifunctional reagents has increased the versatility of protein cross-linking techniques. These reagents are widely used to introduce into proteins various reactive functional groups such as maleimido groups (Yoshitake et al., 1979; Liu et al., 1979), sulfhydryl groups (Traut et al., 1973; Perham & Thomas, 1971), 2-pyridyldithio groups (Carlsson et al., 1978), and azido groups (Ji, 1977). Such materials allow two different proteins to be modified with complementary reactive groups, for example, maleimido groups and sulfhydryl groups, and then subsequently to be cross-linked in a separate reaction that yields only the heterodimer in high yield. Reagents of this type have found widespread use in linking enzymes or toxins to immunoglobulins (Yoshitake et al., 1979; Youle & Neville, 1980; Vitetta et al., 1983) and in the study of biological structures (Rinke et al., 1980; Ji et al., 1980).

Complex structures containing many different polypeptide chains are difficult to characterize when simple bifunctional cross-linking reagents are used since it becomes difficult to identify the components of a cross-linked species on the basis of molecular weight alone. Analysis was made possible by the development of reagents with an easily cleavable bond, and valuable information about the arrangement of proteins in

† This work was supported by a grant from ImmunoGen Inc.

0006-2960/85/0424-1517$01.50/0