Krystal, G., Rattner, J. B., & Hamkalo, B. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4977-4981.
Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass.) 13*, 1-8.
Margolis, R. L., & Rauch, C. T. (1981) *Biochemistry 20*, 4451-4458.
Margolis, R. L., Job, D., Pabion, M., & Rauch, C. T. (1986a) *Ann. N.Y. Acad. Sci. 466*, 306-321.
Margolis, R. L., Rauch, C. T., & Job, D. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 639-643.
Pabion, M., Job, D., & Margolis, R. L. (1984) *Biochemistry 23*, 6642-6648.
Schulze, E., & Kirschner, M. W. (1986) *J. Cell Biol. 102*, 1020-1031.
Sheir-Neiss, G., Lai, M. H., & Morris, N. R. (1978) *Cell (Cambridge, Mass.) 15*, 639-647.
Spurck, T. P., & Pickett-Heaps, J. D. (1987) *J. Cell Biol. 105*, 1691-1705.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Webb, B. C., & Wilson, L. (1980) *Biochemistry 19*, 1993-2001.
Wehland, J., & Weber, K. (1987) *J. Cell Sci. 88*, 185-203.

Reversible Unfolding of the Gelatin-Binding Domain of Fibronectin: Structural Stability in Relation to Function†

Benjamin S. Isaacs,‡ Shelesa A. Brew, and Kenneth C. Ingham*
Biochemistry Laboratory, American Red Cross Biomedical Research & Development, 15601 Crabbs Branch Way, Rockville, Maryland 20855

Received June 16, 1988; Revised Manuscript Received August 17, 1988

ABSTRACT: Fibronectin, a large multidomain glycoprotein, binds denatured collagen (gelatin) and mediates cell attachment and spreading on collagen-coated surfaces. Despite the high affinity, binding to gelatin is disrupted by relatively mild conditions. We have examined the effects of denaturants on the structure and function of a 42-kDa gelatin-binding fragment (GBF) isolated from chymotryptic and thermolytic digests of the parent protein. Application of linear gradients to GBF-loaded gelatin–agarose columns resulted in peak elution of the fragment at pH 5.2 or 10.2, at 0.4 M dimethylformamide, 0.9 M GdmCl, or 2.0 M urea, conditions far short of those required to induce structural changes detectable by fluorescence or circular dichroism. Solvent perturbation, fluorescence quenching, and chemical modification experiments indicate that about half of the 8 tryptophans, one-third of the 21 tyrosines, and all of the 9 lysine residues are solvent-exposed in the native protein and that 1 or more of the latter are directly involved in binding to gelatin, most likely through a hydrogen-bonding mechanism. Titration with GdmCl produced a single unfolding transition centered near 2.5 M GdmCl as monitored by changes in fluorescence and circular dichroism. This transition was fully reversible with complete recovery of structural parameters and gelatin binding. Treatment with disulfide reducing agents caused rapid irreversible changes in structure similar to those produced by GdmCl with concomitant loss of gelatin binding. Thus, tertiary and secondary structures are important for binding, but binding can be disrupted without perturbing those structures.

Fibronectin (Fn),[1] a large multidomain glycoprotein, is found in plasma and other body fluids, in the extracellular matrix, and on the surfaces of numerous types of cells (Furcht, 1983; Hynes, 1985; Yamada, 1983; Akiyama & Yamada, 1987). It functions primarily as a cell adhesion protein, mediating the attachment and spreading of cells on various surfaces. Fn binds to several types of denatured collagen (gelatin) and mediates cell attachment to and spreading on collagen-coated surfaces (Kleinman et al., 1976, 1978). The binding site for gelatin has been localized to an ~42-kDa fragment near the N-terminus of each of the two similar but nonidentical polypeptide chains (Hahn & Yamada, 1979). This fragment contains six disulfide-bonded homologous repeat structures, each encoded by a single exon (Patel et al., 1987). Four of these are of the "type I" variety also found in other parts of the molecule, and two are of the "type II" variety unique to the gelatin-binding domain (Skorstengaard et al., 1986). The importance of both types of units for gelatin binding was recently demonstrated by means of fusion proteins expressed in *Escherichia coli* (Owens & Baralle, 1986). Structures having varying degress of homology with the type II units have been identified in bovine seminal fluid protein PDC-109 (Esch et al., 1983), blood coagulation factor XII (Cool et al., 1985), the kringle domain found in several proteins of the blood coagulation/fibrinolytic system (Patthy et al., 1984), the insulin-like growth factor/mannose-6-PO_4 receptor (Lobel et al., 1987), and type IV collagenase (Collier et al., 1988). A three-dimensional structure for the Fn type II unit was recently proposed on the basis of modeling after the X-ray crystal structure of the kringle I domain of bovine prothrombin (Holland et al., 1987).

† Supported in part by Grant HL21791 from the National Institutes of Health.
* To whom correspondence should be addressed.
‡ Present address: Genetics Institute, 87 Cambridge Dr., Cambridge, MA 02140.

[1] Abbreviations: DMF, dimethylformamide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; Fn, fibronectin; GBF, 42-kDa gelatin-binding fragment of Fn; GdmCl, guanidinium chloride; TBS, Tris-buffered saline; TNBS, trinitrobenzenesulfonate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/89/0428-0842$01.50/0

An intriguing aspect of the interaction of Fn with collagen was the early observation that denatured collagen appeared to have a much stronger affinity for Fn than did native collagen (Kleinman et al., 1976; Engvall & Ruoslahti, 1977; Jilek & Hormann, 1979). This suggested that the binding determinants are at least partially masked in native collagen, becoming accessible only after unfolding of the triple-helical structure (Engvall et al., 1981; Ingham et al., 1985). Indeed, purified α chains, as well as certain fragments thereof, have been shown to be potent inhibitors of Fn-mediated attachment of cells to collagen-coated surfaces (Kleinman et al., 1976, 1978) and to bind Fn with high affinity, i.e., $K_d \sim 10$–30 nM (Dessau et al., 1978; Mosher, 1980; Ingham et al., 1983, 1988). Since these isolated chains and fragments of collagen are presumably devoid of secondary and tertiary structure, the results suggest that each collagen chain contains one or more specific sequences of amino acids that are recognized by a structural determinant within the gelatin-binding domain of Fn.

The aim of the present study was to learn more about the structure and stability of the gelatin-binding domain of Fn and to examine the relation between stability and function. For this purpose, we have chosen the above-mentioned 42-kDa fragment (GBF) isolated from thermolytic and/or chymotryptic digests of the parent protein. This is the smallest gelatin-binding fragment so far characterized. Despite the high affinity of the Fn/gelatin interaction, the binding can be disrupted by relatively mild conditions including low concentrations of chaotropic agents, polyamines, and dilute acid (Klebe et al., 1980; Vuento & Vaheri, 1978; Miekka et al., 1982). This is in contrast to antigen–antibody interactions of comparable affinity where it is often difficult to determine conditions for elution of the antigen from immobilized antibody without denaturation of one or the other (Goding, 1986). In the case of Fn/gelatin, these agents presumably act directly by disrupting noncovalent bonds between the interacting molecules as opposed to indirectly by destroying secondary and/or tertiary structures that are important for binding. Previous studies suggested that the gelatin-binding domain is remarkably stable toward heat, withstanding exposure to 70 °C without effect on the dissociation constant for gelatin (Ingham et al., 1984). However, the stability toward denaturants has not been examined. We therefore attempted to ascertain whether changes in conformation of GBF could be detected under denaturant conditions sufficient to disrupt noncovalent bonds between the interacting molecules. At the same time, we sought to determine if more than one unfolding transition could be detected in response to higher concentrations of denaturants, a result which might suggest differential stability of the type I and type II homologous repeat units within GBF. The spectroscopic properties of the intrinsic tyrosine and tryptophan residues were used to assess their exposure to solvent in the native protein and to detect possible conformational changes. In addition, we present the results of some chemical modification experiments designed to elucidate the role of specific residues of GBF in the binding to gelatin.

Materials and Methods

Chemicals. *N*-Acetyltryptophanamide, TNBS (2,4,6-trinitrobenzensulfonic acid), tetranitromethane, citraconic anhydride, CNBr, NaI, and EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride) were obtained from Sigma. CNBr-activated Sepharose was from Pharmacia. *N*-Acetylimidazole was purchased from Pierce Biochemicals. High-purity urea was from Schwarz/Mann, and GdmCl was from Heico. All other reagents were reagent grade or better. Thioredoxin was purchased from Calbiochem.

Proteins. Human plasma fibronectin was isolated as described by method B of Miekka et al. (1982). The 42-kDa gelatin-binding fragment was obtained either by a limited chymotryptic digestion of human fibronectin immobilized on gelatin–Sepharose (Forastieri & Ingham, 1985) or by a thermolysin digest of whole fibronectin (Zardi et al., 1985). The gelatin-binding fragments were isolated and purified from the proteolytic digests by affinity chromatography on gelatin–Sepharose. The bound fragment was eluted with 6 M urea or by lowering the pH of the buffer to pH 5.0. Some preparations contained small amounts of a slightly larger ($\sim$56 kDa) gelatin-binding fragment that was easily removed by dialyzing the material against low ionic strength 0.02 M Tris buffer and passing it through a small column of heparin–Sepharose. The unadsorbed 42-kDa fragment (GBF) was dialyzed into TBS (0.02 M, Tris, 0.15 M NaCl, and 0.02% NaN_3, pH 7.3) and stored at −70 °C. It was homogeneous by exclusion chromatography on Superose-12 (Pharmacia) and by SDS–PAGE in the Pharmacia PhastGel system using 8–25% gradient gels with Coomassie and/or silver stain. Most of the data presented here were obtained with the thermolytic fragment whose N-terminal sequence was heterogeneous, about two-thirds of the molecules beginning at Ala^{261} and one-third at Val^{262}. Concentrations of GBF are based on an extinction coefficient of 7.3×10^4 L mol^{-1} cm^{-1}, calculated by the method of Edelhoch (1976) assuming 8 Trp, 21 Tyr, and 12 disulfides (Skorstengaard et al., 1986; Kornblihtt et al., 1985).

Affinity Chromatography. Swine skin gelatin (Sigma type I) was coupled to CNBr-activated Sepharose as described earlier (Miekka et al., 1982). In a typical experiment, 50 μL of GBF (1 mg/mL) was applied to a 1.5-mL column of gelatin–Sepharose equilibrated in TBS at room temperature at a constant flow rate of 1.0 mL/min using the Pharmacia FPLC system. The elution profile was monitored by the absorbance at 280 nm and by the intrinsic fluorescence (280/350 nm) on a Shimazdu variable-wavelength fluorescence monitor. The bound protein was routinely eluted from the column by application of a linear gradient of GdmCl or urea, and the fraction bound was determined from the integrated area under the eluted peak relative to the total area. In some experiments (Figure 1), the protein was eluted with a linear gradient of DMF or by raising or lowering the pH, whose value was monitored by direct measurement of collected fractions.

Fluorescence measurements were made with an SLM-8000C spectrofluorometer controlled with an IBM personal computer and thermostated at 25 °C. Anisotropy measurements were made in the T-format utilizing two emission monochromators to isolate the vertical and horizontal emission components. All intensity values were corrected when necessary for a protein-free blank. To monitor the spectral shift in response to changing conditions, the instrument was programmed to automatically switch back and forth between emission wavelengths of 320 and 350 nm, calculating the ratio of the intensity at 350 nm to that at 320 nm, and averaging the resulting values until no further change occurred. Plotted values of anisotropy and intensity ratios are the average of at least 15 measurements under each set of conditions. The excitation wavelength for these experiments was 295 nm. Titrations were performed by sequential addition of small volumes of concentrated stock solutions, correcting for dilution where appropriate. In the case of the alkaline titrations, the entire spectrum was recorded at each pH, exciting at 270 nm in order to observe both Trp and Tyr fluorescence. Quantum

yields were determined by the comparison method using the relationship $Q_1/Q_2 = (F_1/F_2)(A_2/A_1)$, where Q_1 is the quantum yield of the Trp residues in GBF (excited at 295 nm), Q_2 is the quantum yield of the reference compound, *N*-acetyltryptophanamide in TBS, F_1 and F_2 are the integrated areas of the uncorrected emission spectra of the GBF and reference compound, respectively, and A_1 and A_2 are the corresponding absorbances at the excitation wavelength. A value of 0.13 was assumed for Q_2 (Chen et al., 1969). Iodide quenching measurements were made and analyzed according to the method of Lehrer (1971) as previously described (Ingham et al., 1976).

Differential absorption measurements were made as described by Herskovits (1967) and Donovan (1969) using an AVIV 118DS spectrophotometer. The solvent perturbation experiment utilized matched tandem 0.5-cm path-length Telfon-stoppered cuvettes containing equal volumes of GBF (7.6 μM) in one chamber and 40% glycerol in the other, both in TBS. After a base line was obtained, the cell in the sample compartment was inverted and the contents were thoroughly mixed before the difference spectrum was recorded several times and averaged. The changes in the extinction coefficient at 286 and 292 nm were used to estimate the extent of exposure of Tyr and Trp as described by Herskovits (1967). For the alkaline titrations, two identical 1 cm^2 cuvettes containing GBF (7.6 μM) in 0.005 M TBS were used to obtain a base line. The sample cuvette was then removed, and a small volume of 1 M KOH added to increase the pH by 0.3–0.5 unit. An equal (small) volume of buffer was added to the reference cuvette to compensate for slight dilution, and the difference spectrum was recorded. The number of Tyr ionized was calculated from the magnitude of the difference peak near 293 nm assuming a value of 2500 L mol^{-1} cm^{-1} for the difference in extinction of Tyr vs Tyr^- (Edelhoch, 1967) and the above-calculated extinction coefficient for GBF. The agreement between the maximum number of titratable tyrosines and the known number of tyrosines in the amino acid sequence provides a measure of the internal consistency of the optical measurements and the extinction coefficients.

Circular dichroism was measured at room temperature on a JASCO 500C spectropolarimeter in 1.0-mm path-length cells. The protein concentration was 0.2 mg/mL in TBS. Each sample was scanned eight times at 20 nm/min with 0.2-nm resolution, and the resulting spectra were averaged and smoothed. The blank substracted value of the ellipticity at 225 nm was used in monitoring the effect of the various denaturants (GdmCl, acid and alkaline pH) on the secondary and tertiary structure.

Lysine Modification. GBF in 0.15 M NaCl and 0.2 M $NaHCO_3$, pH 8.5, was exposed to a 100-fold excess of citraconic anhydride (reagent to Lys residues) for 2 h at room temperature (Habeeb & Atassi, 1970). Lys residues were quantified by using TNBS (trinitrobenzenesulfonic acid) as described by Habeeb (1967). The modification was reversed by dialyzing for 10 h at room temperature against a buffer containing 0.15 M NaCl and 0.1 M sodium acetate, pH 5.0.

Tyrosine Modification. Tyrosines were modified with *N*-acetylimidazole as described by Riordan and Vallee (1972) and Myers and Glazer (1971). The decrease in absorbance at 278 nm was monitored continuously with time after addition of the reagent, blanking against reagent alone, to provide an estimate of the number of tyrosines acetylated at the end of the 2-h reaction period. Reversal was accomplished by addition of NH_2OH (1 M) and was monitored by the increase in absorbance at 278 nm. Control experiments with ribonuclease gave results in excellent agreement with Simpson and Vallee (1966). Tyrosines were also modified with tetranitromethane according to Sokolovsky et al. (1966), monitoring the extent of the reaction by the absorbance at 428 nm. After 90 min, the reacted material was passed over Sephadex G-25 to remove excess reagent. The extent of aggregation was determined by exclusion chromatography with the FPLC system using a Superose-12 column.

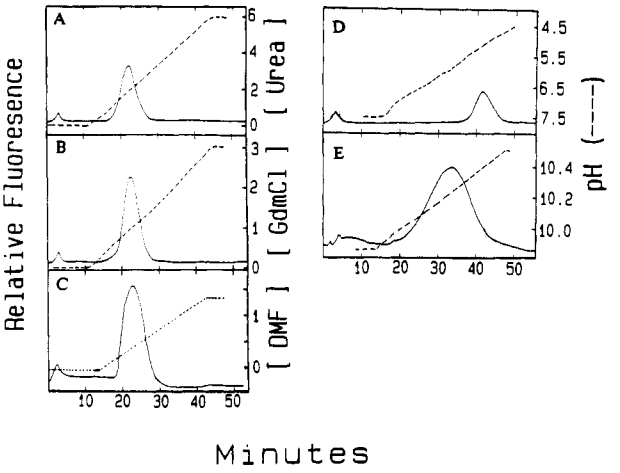

FIGURE 1: Affinity chromatography of the 42-kDa gelatin-binding fragment (GBF) of fibronectin on gelatin–Sepharose. GBF (50–150 μg in TBS) was applied to a 1.5-mL column of gelatin–Sepharose equilibrated in TBS at room temperature and a flow rate of 1.0 mL/min. The bound protein was eluted with a linear gradient (---) of (A) urea, (B) GdmCl, (C) DMF, (D) acid pH, or (E) alkaline pH. Elution was monitored by fluorescence. Although the amount of protein applied and the scale settings varied between experiments, full recovery was obtained, in each case, as determined by subsequent application of 6 M GdmCl.

Carboxyl group modification was carried out according to Yamada et al. (1981). An aqueous solution of GBF (8.2 μM) containing 0.3 M methylamine, 0.1 M NaCl, and 0.02% NaN_3 was adjusted to pH 4 prior to adding EDC to a final concentration of 0.1 M. After 3 h at 37 °C, the solution was neutralized and tested for binding to gelatin–Sepharose.

Results

Affinity Chromatography. The 42-kDa gelatin-binding fragments (GBF) derived from chymotryptic and thermolytic digests of human plasma fibronectin were indistinguishable in terms of the conditions required to elute them from gelatin–Sepharose. Typical results are shown in Figure 1. Application of a linear gradient caused the fragment to elute in a sharp peak centered at 0.9 M GdmCl (panel A), 2.0 M urea (panel B), or 0.4 M dimethylformamide (DMF, panel C). Similarly, when the pH was changed, the fragment eluted at pH 5.2 (panel D) or pH 10.2 (panel E). Note that in the latter case, the sample was applied at pH 10.0 and was completely eluted by pH 10.4, indicating a sharp dependence on hydroxide ion concentration. Because of an earlier report (Agin & Gartner, 1982) that Fn could be eluted from immobilized gelatin by glucose, we tested this compound also. No elution of GBF could be detected at concentrations of glucose up to 2 M. Galactose, the other sugar found in collagen, was also ineffective at this concentration.

Exposure of Tryptophans and Tyrosines. The wavelength of maximum Trp fluorescence in proteins ranges from greater than 350 nm for a fully exposed residue to as low as 315 nm for a residue which is completely buried in the nonpolar interior of a protein (Teale, 1960; Finazzi-Agro' et al., 1970; Eftink & Ghiron, 1976). Figure 2A shows the fluorescence spectrum of GBF when excited at 295 nm. The quantum yield is rather

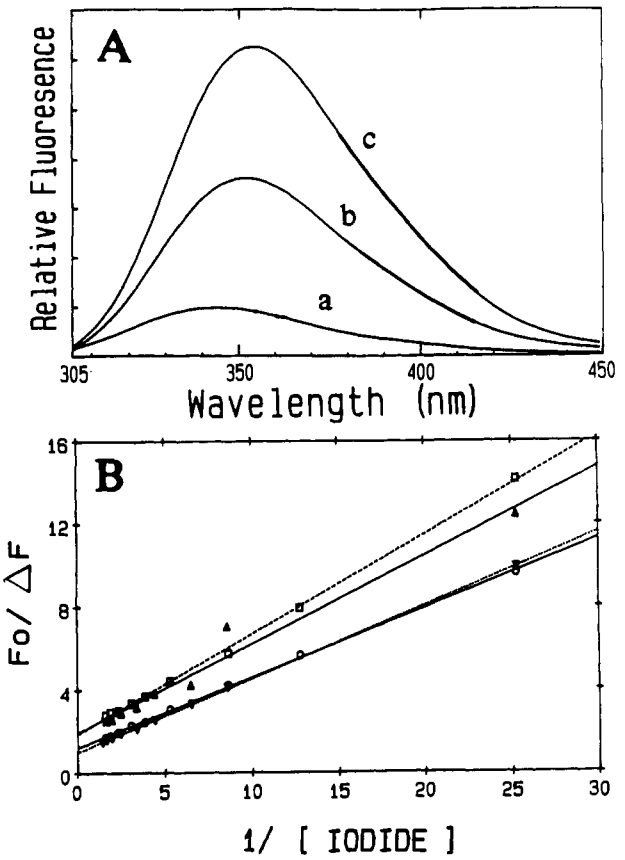

FIGURE 2: (A) Fluorescence spectra of tryptophan residues of GBF in TBS (a), in 4.5 M GdmCl (b), and in 4.5 M GdmCl plus 3 mM dithiothreitol (c). All three spectra were measured at 25 °C at the same protein concentration (10 μM) and the same instrument settings with excitation at 295 nm. Iodide quenching of tryptophan fluorescence of GBF in TBS (□), in 1.0 M GdmCl (▲) in 4.5M GdmCl (○), and in 4.5 M GdmCl after reduction of disulfide bonds with 3 mM DTT (▼). The data were plotted according to Lehrer (1971) and fitted by linear least-squares regression; reciprocal intercepts corresponding to the fraction of fluorescence derived from Trp's that are accessible to iodide were 0.53, 0.53, 0.81, and 1.0, respectively.

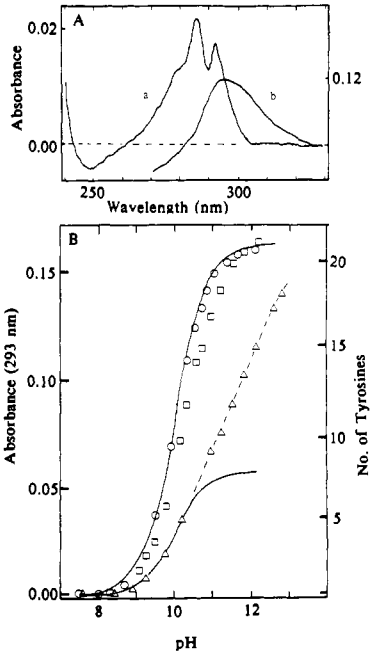

FIGURE 3: (A) Differential UV absorption spectra of GBF induced by 20% glycerol (a), or pH 12 (b). The extent of exposure of tyrosine and tyryptophan residues was estimated from the magnitude of the peaks at 287 and 293 nm in curve a, obtained at a final protein concentration of 7.2 μM. Curve b is an example of the difference spectra obtained at pH 12.0 in TBS during the alkaline titration experiment shown in (B). (B) Alkaline titration of the tyrosine residues in GBF. Small volumes of concentrated KOH were added to the sample cuvette containing GBF (2.9 μM) in TBS (△) or 5 M GdmCl (□), or reduced and carboxymethylated GBF in 5 M GdmCl (○). After determination of the pH and addition of a corresponding volume of buffer to the reference cell to compensate for slight dilution, the difference spectra were recorded as in (A). Data points represent the change in absorbance at 293 nm for tyrosinate residues. The solid curves represent theoretical titration curves corresponding to an ionization of 7 (curve a) and 21 (curve b) tyrosines with a normal pK of 10.

low, having a value of ~0.01. The emission maximum occurs around 341 nm, indicating that much of the fluorescence originates with Trp's that are at least partially exposed to the solvent. Addition of GdmCl to a concentration of 4.5 M caused a 3.5-fold increase in the intensity and shifted the emission maximum to 350 nm, indicating further exposure of Trp's. Cleavage of disulfide bonds by addition of 10 mM dithiothreitol further increased the quantum yield and shifted the wavelength maximum to 353 nm.

Iodide quenching experiments were carried out to ascertain the accessibility of fluorescent Trp's to a nonpenetrating collisional quencher. The data are presented in Figure 2B in the form of a Lehrer plot, the intercept of which is the reciprocal of the fraction of fluorescence derived from Trp's that are accessible to the quencher (Lehrer, 1971). This fraction was 0.53 for the native fragment in TBS and did not change upon addition of 1 M GdmCl. The value increased to 0.81 in the presence of 4.5 M GdmCl. Only after exposure to DTT in the presence of 4.5 M GdmCl did all of the fluorescence become accessible to the iodide, as evidenced by the intercept of 1.0 on the ordinate.

To further characterize the exposure of aromatic residues, solvent perturbation experiments were carried out with glycerol as the spectral perturbant. The differential absorption spectrum of the 42-kDa fragment in TBS vs TBS containing 20% glycerol is shown in Figure 3A, curve a. The positive peaks at 292 and 286 nm are characteristic of Trp and Tyr residues whose environment has been perturbed by this nondenaturing solvent additive. Comparison of the magnitude of the change in extinction to that obtained with model Trp and Tyr derivatives can be used to estimate the degree of exposure (Herskovits, 1967). The results in Figure 3A would be consistent with minimum exposure of 1.8 out of 9 Trp's and 6.1 out of 21 Tyr's, assuming that this many are fully exposed while the rest are completely buried. Alternatively, a greater number of each of these residues could be partially exposed.

In the case of tyrosine, additional information was obtained from alkaline titrations, monitoring the intensity of the phenolate band near 293 nm in the difference spectrum (Figure 3A, curve b) (Donovan, 1969). In the absence of denaturants, the titration curve extends over about 4 pH units (Figure 3B, dashed line); only about 7 out of a possible 21 Tyr's titrate with a normal pK of 10.0 (curve a) (Edelhoch, 1967). This is in contrast to results obtained with the reduced and denatured protein where virtually all of the phenolic groups were fully exposed and titrated with a normal pK (curve b). Note that in GdmCl without reduction of disulfides, there appear to be a few Tyr's that are still shielded from the solvent, titrating with an abnormal pK.

Additional experiments were conducted to determine if binding of gelatin could be detected through a perturbation of the Trp fluorescence. It was noted that the fluorescence intensity was constant within ±3% during titration with gelatin up to a concentration of 1.2 mg/mL (12 μM α chains), over

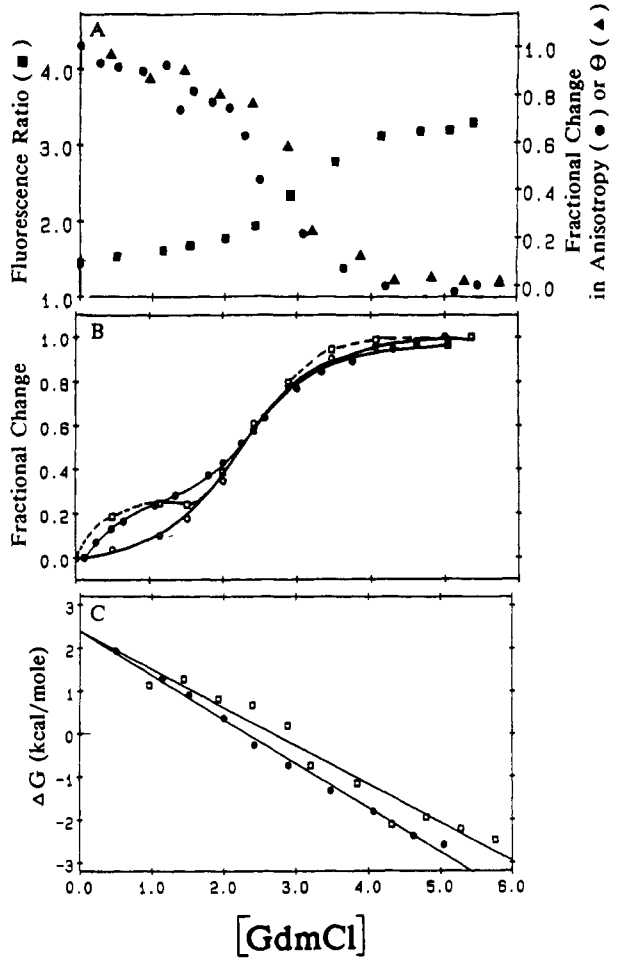

FIGURE 4: (A) GdmCl-induced unfolding transitions in GBF measured by changes in the tryptophan fluorescence intensity ratio (■) and anisotropy (●) and in the molar ellipticity at 220 nm in the far-UV CD spectrum (▲). Measurements were made at 25 °C. The left ordinate represents the change in the fluorescence spectral distribution as expressed by the ratio of the intensity at 350 nm to that at 320 nm with excitation at 295 nm. Anisotropy was measured at 350 nm with excitation at 295 nm. (B) Reversibility of unfolding of GBF after exposure to GdmCl. (○) Titration of native GBF with GdmCl; (□) titration of a sample that had been exposed to 5.4 M GdmCl which was subsequently removed by dialysis; (●) back-titration by sequential dilution with TBS of a sample that initially contained 5 M GdmCl. Folding/unfolding was monitored by the change in spectral distribution as in panel A; data are normalized to facilitate comparison. (C) Plot of ΔG, the free energy of unfolding, vs GdmCl concentration. Fractional changes in the fluorescence ratio (●) and ellipticity (□), from (A), were fit by linear regression and used to determine both the midpoint of the transition ($\Delta G = 0$) and the free energy of unfolding in water, from the intercept on the ordinate.

10 times the dissociation constant (Forastieri & Ingham, 1985). This suggests that Trp is not present in the gelatin-binding site and that binding of gelatin does not produce a conformational change that alters the environment of Trp residues.

Reversible Unfolding in GdmCl. As noted above (Figure 2A), 4.8 M GdmCl caused a large increase in the intensity of GBF fluorescence and shifted the spectral distribution to longer wavelength, presumably because of unfolding of the protein. We therefore followed the possible unfolding transition by monitoring the ratio of the fluorescence intensity at 350 nm to that at 320 nm, a measure of the wavelength shift, as well as the anisotropy at 350 nm, a measure of the rotational freedom of the indole side chains, as a function of GdmCl concentration. As shown in Figure 4A, both parameters exhibited a monophasic transition between 2 and 4 M GdmCl,

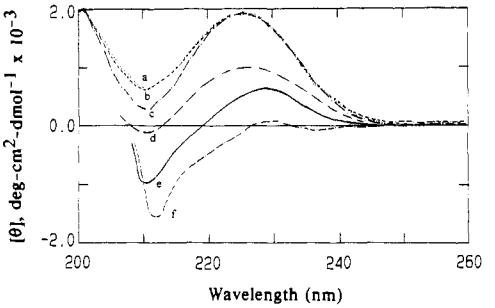

FIGURE 5: Effect of pH, denaturant, and disulfide reduction on the CD spectra of GBF. Spectra represent averages of six to eight scans of a 0.2 mg/mL GBF solution. Curves a–d represent the spectra taken at pH 4.0, 7.4, 10.2, and 12, respectively. The remaining spectra were obtained in 5.4 M GdmCl before (e) and after (f) reduction and carboxymethylation.

the intensity ratio increasing from 1.5 to over 3 and the anisotropy dropping from 0.16 to 0.09. These results are consistent with an unfolding of the fragment such that the Trp residues become exposed to a more polar and less rigid environment. In contrast, no transition was seen with either urea or dimethylformamide at concentrations up to 5 M. Addition of DTT in the presence of 5 M GdmCl caused a further increase in the intensity ratio from 3.1 to 3.8, suggesting in agreement with Figure 1 that disulfide reduction is required for complete exposure of Trp's.

The gelatin-binding fragment, like whole Fn, has a characteristic positive ellipticity band at 225 nm in the CD spectrum whose amplitude is diminished in 5 M GdmCl (Figure 5). The unfolding of GBF was therefore also monitored by changes in this parameter, as a measure of changes in structure. As shown by the triangles in Figure 4A, the concentration dependence of the changes in ellipticity corresponded rather well with changes in tertiary structure as detected by changes in fluorescence parameters. Reduction and alkylation of GBF caused a further decrease in ellipticity at 225 nm, beyond that produced by GdmCl alone (Figure 5).

Figure 4B presents the results of two experiments designed to evaluate the reversibility of the unfolding transition produced by GdmCl. In one case, addition of 5 M GdmCl was followed by successive dilutions, monitoring the change in the 350- to 320-nm intensity ratio as a function of decreasing concentration. Since this ratio is an intensive property, its value should not be affected by the change in protein concentration. In another experiment, GBF was exposed to 5 M GdmCl, dialyzed to remove the denaturant, and then subjected to a second exposure to GdmCl in the form of a titration, also monitoring the intensity ratio. In both cases, the ratio returned to its original value upon removal of the denaturant. However, both the forward and reverse titrations exhibited a slight bulge in the curve between 0 and 1 M GdmCl which was absent in the titration curve of the sample which had not been previously exposed to 5 M GdmCl.

Assuming a two-state transition, the free energy of unfolding was calculated at each GdmCl concentration (Pace, 1986). The results are presented in Figure 4C. Although the midpoint of the transition ($\Delta G = 0$) determined from the CD data occurred at a slightly higher GdmCl concentration (2.7 M) than that determined by the fluorescence data (2.3 M), the two plots have similar slopes and extrapolate to the same free energy of unfolding in water, $\Delta G_{H_2O} = +2.3$ kcal/mol. This is relatively low when compared to values obtained for other globular proteins by similar methods (Pace, 1975).

When a portion of the reversibly denatured sample was applied to gelatin–Sepharose, 97% of the protein bound to the

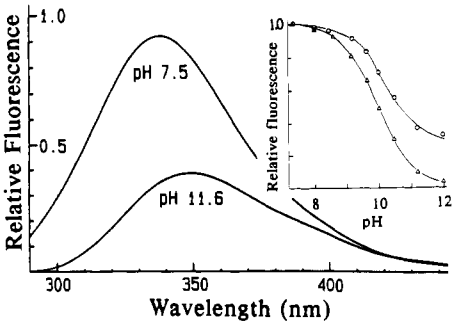

FIGURE 6: Effect of alkaline pH on the emission spectra of GBF, excited at 270 nm. The inset shows the fractional change in the emission intensity at 305 nm (△) and 340 nm (○) as a function of pH in TBS.

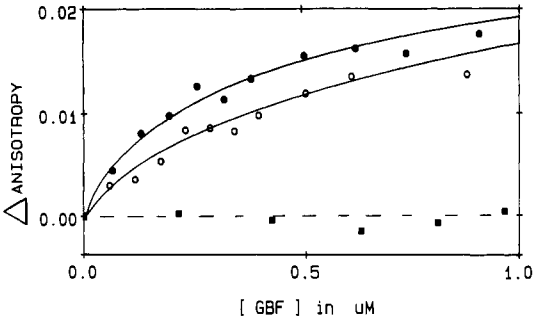

FIGURE 7: Titration of FITC-labeled α-1 chain from rat tail type I collagen in TBS at 25 °C with (●) unmodified GBF, (■) citraconic anhydride treated GBF, and (○) citraconylated GBF that had been incubated at low pH to reverse the modification. The solid curves are theoretical fits corresponding to K_d = 0.3 μM (●) and 0.5 μM (○).

column and was eluted at 0.9 M GdmCl, similar to the original. The dialyzed material was also tested for its ability to form a fluid-phase complex with a fluorescein-labeled α_1 chain of type I collagen, as described elsewhere (Ingham et al., 1988; see also Figure 7 below). The dissociation constant for binding of GBF to the high-affinity site was unaffected by exposure to 5 M GdmCl. Thus, the unfolding transition is completely reversible in terms of function. However, this refolded functionally competent state differed slightly from the native state in that retitration with GdmCl produced a slightly different response at low GdmCl concentrations.

Effects of pH. Titration of GBF with acid caused a gradual 50% increase in the fluorescence intensity between pH 7 and pH 3, with no significant change in spectral distribution and no evidence of a cooperative transition. Modest changes in the intensity alone can be attributed to titration of quenching groups in proximity to Trp's without the need to invoke conformational changes (Longworth, 1971). We therefore examined the CD spectrum as well, finding no significant difference between pH 7.5 and pH 4.0 (Figure 5). Thus, disruption of the interaction between GBF and gelatin by acid pH (Figure 1D) does not appear to result from changes in the conformation of the fragment as monitored by these parameters.

Titration of GBF with NaOH produced the fluorescence changes shown in Figure 6. There was a large decrease in the emission intensity between pH 7.5 and 11.6 which was almost completely reversible, providing the sample was not kept too long at high pH. The magnitude of the quenching was not uniform across the fluorescence spectrum. At 305 nm, the peak of Tyr fluorescence, the quenching was essentially complete whereas at 340 nm, where Trp fluorescence dominates, quenching was only about 60%. The inset in Figure 6 shows that both responses occurred with a midpoint near pH 10, suggesting that they arise from the titration of normal (exposed) Tyr's. In most proteins which contain both Tyr and Trp, the fluorescence spectrum is dominated by the latter (Longworth, 1971). Here, however, a relatively large contribution from the Tyr residues is evident in the 300–330-nm region. The decrease in Tyr fluorescence in the alkaline range can be attributed to ionization of solvent-exposed phenolic protons. Buried Tyr's, if they fluoresce at all, are likely to be quenched by energy transfer to the virtually nonfluorescent tyrosinate ions (Longworth, 1971). Concomitant quenching of Trp can be explained by the same mechanism, without the need to invoke conformational changes.

CD measurements were made in a further effort to detect possible structural changes in GBF when exposed to alkaline pH (Figure 5). Previous reports suggest very little change in the far-ultraviolet CD spectrum of whole Fn between pH 7 and pH 11 (Alexander et al., 1979; Markovic et al., 1983). Similarly, we found the positive ellipticity at 225 nm to be unaffected by raising the pH to 10.2 where the fluorescence transition and elution from gelatin–Sepharose were about half-complete. However, the amplitude of the 225-nm band decreased by about 50% when the pH was raised to 12. Thus, while structural changes may occur at high pH, the changes reflected in the CD spectrum do not correlate with the titration of Tyr residues as monitored by fluorescence or with the pH required for elution from gelatin–Sepharose. The changes in CD, which presumably reflect unfolding of the molecule, provide an explanation for the continued appearance of absorbance at 295 nm in the alkaline titration of native GBF (Figure 3) at pH values well beyond those required to ionize normal (exposed) tyrosines.

Chemical Modifications. The observation that GBF eluted from gelatin–Sepharose at pH 10.2 suggests that the interaction involves Lys or Tyr residues either on gelatin or on GBF. To test the latter possibility, GBF was treated with reagents that selectively alter these residues, and the ability of the resulting derivatives to bind to gelatin–Sepharose was assessed. Treatment with excess citraconic anhydride resulted in the modification of all nine Lys residues. The CD spectrum was unaffected by this treatment, and titration of the modified fragment with GdmCl produced an unfolding transition identical with that of the native fragment. However, the modified fragment did not bind gelatin–Sepharose and, as illustrated in Figure 7, failed to form a fluid-phase complex with FITC-α_1(I) when tested in a previously published anisotropy assay (Ingham et al., 1988). After incubation at pH 5 for 10 h at room temperature to reverse the modification (Habeeb & Atassi, 1970), complete binding to gelatin–Sepharose was recovered, and the anisotropy assay produced a K_d of 0.5 μM, close to that of the original and to the previously published value.

Treatment of GBF with *N*-acetylimidazole resulted in the modification of 6 out of 21 tyrosine residues. The resulting derivative bound quantitatively to gelatin–Sepharose and eluted at the same concentration of GdmCl as the unmodified protein. When the same modification reaction was carried out in the presence of 6 M GdmCl followed by removal of the GdmCl by dialysis, no binding to gelatin–Sepharose occurred. Attempts to quantitate the degree of modification were complicated by background absorbance in the 6 M GdmCl. It is likely that additional tyrosines were modified and that the attached acetyl groups may have interfered with proper refolding of the fragment, an interpretation that is consistent with the fact that binding could be fully recovered by incubating the inactive derivative with NH_2OH to reverse the

modification. An alternative but less likely interpretation is that a key residue at the binding site became accessible only in GdmCl. This would require that the key residue in the native state be accessible to gelatin but not to the reagent.

Treatment of GBF with tetranitromethane resulted in modification of 2.8 Tyr's. Exclusion chromatography revealed that, as is often the case with this reagent (Vincent et al., 1970), substantial polymerization had occurred; only about 25% of the GBF remained monomeric. Nonetheless, the nitrated protein, including the polymers, bound quantitatively to gelatin–Sepharose and actually required a higher concentration of GdmCl for its elution than did the native protein (1.3 M vs 0.9 M). This apparent higher affinity could reflect multipoint attachment of the polymers to the affinity matrix.

To assess the possible involvement of Glu and/or Asp residues in the acid-induced release of GBF from gelatin–Sepharose, GBF was treated with a carbodiimide reagent that reacts preferentially with these residues (see Materials and Methods). Although the extent of the modification reaction was not determined, the treatment resulted in complete loss of the ability to bind to gelatin–Sepharose.

Effect of Disulfide Cleavage. The 42-kDa GBF has 12 disulfide bonds. To evaluate their role in the structure and function of the molecules, different samples were exposed to increasing concentrations of DTT for 2 h at 37 °C and then applied to a gelatin–Sepharose column. A progressive loss in gelatin-binding activity with increasing concentrations of DTT was observed, with 50% loss at about 1 mM. This was accompanied by a time-dependent increase in fluorescence intensity, the extent of which correlated with the loss of function. After several hours in 3 mM DTT, the positive feature at 225 nm in the CD spectrum of the fragment was completely abolished.

Several attempts were made to reverse the structural and functional effects of disulfide cleavage. Slow removal of the reducing agent by dialysis at room temperature failed to regenerate any gelatin-binding activity or positive ellipticity at 225 nm. Analysis by exclusion chromatography revealed that the material had aggregated. GdmCl was then added to a reduced sample, and the concentration of both reagents was gradually lowered by dialysis against a solution whose GdmCl concentration was continuously decreased from 6 to 0.04 M over a 24-h period. While this maneuver prevented aggregation, it did not lead to recovery of measurable activity. Incubation of this monomeric material in 0.5 M GdmCl with thioredoxin, with or without prior exposure to glutathione (Pigiet & Schuster, 1986), also failed to restore activity.

Discussion

In this study, we have examined the structural stability of the 42-kDa gelatin-binding domain of fibronectin in relation to its function. Specifically, we have attempted to ascertain whether conditions sufficient to disrupt noncovalent bonds between the interacting molecules are also sufficient to disrupt intramolecular interactions within the gelatin-binding domain, producing conformational changes detectable by intrinsic fluorescence or circular dichroism. Analytical affinity chromatography with gradient elution was used to determine carefully the concentration of GdmCl, urea, dimethylformamide, acid, and base required to elute GBF from gelatin–Sepharose. The results show that in all cases, the concentrations required to disrupt binding failed to elicit significant changes in conformation. For example, 0.9 M GdmCl was sufficient to elute GBF from the affinity column but caused no significant change in the intensity, anisotropy, or wavelength distribution of Trp fluorescence, no change in the CD spectrum, and no change in the accessibility of Trp residues to iodide ions. The free energy plot in Figure 4C does suggest about a 15% decrease in the barrier to unfolding at the concentration of GdmCl required for elution, and this decreased stability could play a role in elution by this reagent. In general, however, the mechanism of elution seems to involve a direct interference with noncovalent bonds between gelatin and GBF, as opposed to an indirect disruption of tertiary structures within GBF that are important for binding. That such structures are important, however, is revealed by the observation that cleavage of disulfide bonds results in rapid loss of binding coincident with large changes in the fluorescence and CD spectra.

Dimethylformamide is not a particularly strong denaturant. Herskovits et al. (1977) found that concentrations above 5 M were required to unfold several proteins. Thus, the finding that this reagent was more potent than urea or even GdmCl in liberating GBF from gelatin–Sepharose was surprising. The inductive effects of the methyl groups make the nitrogen atom in DMF a better hydrogen-bonding acceptor than those in urea and GdmCl, perhaps accounting for its greater potency. This would be consistent with an earlier hypothesis that hydrogen bonding is important for the interaction between Fn and gelatin (Forastieri & Ingham, 1985). The effects of pH are also consistent with a hydrogen-bonding mechanism. The midpoint of the elution profile at pH 5.2, with no concomitant structural change, suggests the direct involvement of Asp or Glu residues, although histidine also occasionally titrates in this range (Gettins, 1987). Involvement of carboxylate side chains would also be consistent with the observed abolition of binding by chemical modification with the carbodiimide reagent.

On the alkaline side, elution at pH 10.2, again without significant structural changes, suggests that direct involvement of Lys and/or Tyr residues. Gelatin contains very few tyrosines, and a preliminary report by Smith et al. (1986) suggests that lysines on gelatin are not involved. Our chemical modification experiments with GBF implicate Lys and not Tyr in the sensitivity of the binding to alkaline pH. Citraconylation of nine Lys residues on GBF had no detectable effect on its structure but reversibly abolished its binding, not only to immobilized gelatin, but, in a more quantitative assay, also to a purified fluorescein-labeled α_1 chain of type I collagen. By contrast, acetylation of approximately six exposed Tyr residues in the native protein had no effect on binding to immobilized gelatin. Another Tyr-specific reagent, tetranitromethane, caused substantial aggregation of the protein but did not diminish its binding. It is difficult to envision a Tyr residue being sufficiently exposed to interact directly with gelatin while remaining inaccessible to these Tyr-specific reagents. We thus conclude that tyrosine is not involved in the binding and that disruption of binding by alkaline pH involves deprotonation of lysine residues. The previous observation (Forastieri & Ingham, 1983) that low levels of labeling of GBF with fluorescein isothiocyanate did not interfere with binding to gelatin suggests that the amino groups which participate in the binding are not among the most reactive toward this relatively bulky reagent. Additional work is required to identify which lysine residues on GBF are involved in the binding.

Several lines of evidence suggest that approximately one-third of the 21 Tyr residues in native GBF are located at or near the surface of the protein. Differential absorption spectroscopy in mixed nondenaturing solvents has been widely used to gain information about the exposure of aromatic side chains to the solvent (Herskovits, 1967; Donovan, 1969). In

the absence of conformational changes, only those side chains that are significantly exposed will contribute to the observed signal. Our results with glycerol suggest, in agreement with the chemical modification results, that at least six Tyr's are in this category. Similarly, the alkaline titration absorption data indicate that about seven Tyr's ionize with a normal pK of 10, the rest becoming accessible only at higher pH where changes in the CD spectrum indicate unfolding. On the other hand, Tyr fluorescence appeared to be fully quenched with a normal pK near 10.0, suggesting that these same exposed Tyr's are responsible for the bulk of Tyr fluorescence in GBF. This is consistent with the known tendency of buried Tyr's to be quenched by excited-state transfer of phenolic protons to nearby H-bond acceptors (Cowgill, 1976; Ingham et al., 1976; Longworth, 1971). Thus, titration of the exposed Tyr's eliminates the bulk of Tyr fluorescence at a pH value much lower than that required to unfold the protein and expose the buried nonfluorescent Tyr's to the alkaline medium.

In native GBF, about half of the Trp fluorescence comes from residues that are inaccessible to the nonpenetrating quencher iodide. The positions of the eight Trp residues in GBF are highly conserved within the four type I and two type II homologous repeat units in GBF. Five Trp's are either adjacent to (type II) or one residue removed from (type I) a disulfide bond, the remaining three occurring near the ends of three large loops in the type I units (Skorstengaard et al., 1986). Proximity of disulfides is known to reduce fluorescence efficiency (Longworth, 1971; Tsunenaga et al., 1987). This could account for the unusually low quantum yield of Trp fluorescence, even after unfolding in GdmCl, as well as the increase in quantum yield when the disulfides are cleaved. However, at least one of the Trp's must remain buried even in excess GdmCl since complete quenching by iodide required cleavage of disulfides. Such cleavage also caused further changes in the CD spectrum. Thus, it appears that GBF retains some disulfide-stabilized secondary and/or tertiary structure at concentrations of GdmCl beyond that required to complete the observed unfolding transition.

In spite of the occurrence of both type I and type II homologous repeat structures in GBF, we saw no evidence for biphasic unfolding. The data suggest that either (a) the two types of structure have similar stabilities toward GdmCl and cannot be resolved, (b) one type of structure is much more stable than the other and does not unfold under the conditions employed, or (c) all or most of the substructures are intimately associated with each other to form a stable domain that unfolds in a single cooperative transition. The structural changes induced by GdmCl were completely reversed on removal of the denaturant, and functional activity was also recovered. However, subsequent titration with GdmCl produced detectable changes in fluorescence at concentrations lower than required for the native protein, suggesting that at least part of the refolded molecule was slightly less stable than the original.

In summary, the results presented in this paper show that the isolated gelatin-binding domain of human plasma fibronectin undergoes a single cooperative unfolding transition in GdmCl. The concentration of this and other denaturants required to induce unfolding is much greater than that required to disrupt the functional interaction with denatured collagen. Indeed, dimethylformamide, the least denaturing of the compounds tested, was most effective in eluting the domain from gelatin-Sepharose. Modification of lysine residues abolished function without perturbing structure. The only circumstance in which loss of function correlated with changes in structure was reduction of disulfide bonds, which caused irreversible unfolding. Thus, tertiary and secondary structures are important for binding, but binding can be disrupted without perturbing those structures. We envision a rather precisely defined three-dimensional structure in which H-bonding donor and/or acceptor groups are spatially arranged to recognize complementary groups on gelatin. Binding of gelatin does not appear to induce a conformational change in GBF since the fluorescence was not perturbed. Any conformational changes associated with this interaction are more likely to occur in the flexible collagen chain, as evidenced by the increase in anisotropy exhibited by fluorescent labels attached to those chains.

Registry No. L-Lysine, 56-87-1.

References

Agin, P. P., & Gartner, T. K. (1982) *Biochim. Biophys. Acta 716*, 443–445.

Akiyama, S., & Yamada, K. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 59*, 1–57.

Alexander, S., Colonna, G., & Edelhoch, H. (1979) *J. Biol. Chem. 254*, 1501–1505.

Chen, R. F., Edelhoch, H., & Steiner, R. F. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) pp 171–244, Academic, New York.

Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., & Goldberg, G. I. (1988) *J. Biol. Chem. 263*, 6579–6587.

Cool, D. E., Edgell, C.-J. S., Louie, G. V., Zoller, M. J., Brayer, G. D., & MacGillivray, R. T. A. (1985) *J. Biol. Chem. 260*, 13666–13676.

Cowgill, R. W. (1976) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 2, pp 441–486, Dekker, New York.

Dessau, W., Adelmann, B. C., Timpl, R., & Martin, G. R. (1978) *Biochem. J. 169*, 55–59.

Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) pp 138–170, Academic, New York.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Engvall, E., & Ruoslahti, E. (1977) *Int. J. Cancer 20*, 1–5.

Engvall, E., Bell, M. L., & Ruoslahti, E. (1981) *Collagen Relat. Res. 1*, 505–516.

Finazzi-Agro', A., Rotilo, G., Avigliano, L., Guerrieri, P., Bofi, V., & Mondovi, B. (1970) *Biochemistry 9*, 2009–2014.

Forastieri, H., & Ingham, K. C. (1983) *Arch. Biochem. Biophys. 227*, 358–366.

Forastieri, H., & Ingham, K. C. (1985) *J. Biol. Chem. 260*, 10546–10550.

Furcht, L. (1983) *Mol. Cell Biol. 1*, 53–117.

Gettins, P. (1987) *Biochemistry 26*, 1391–1398.

Goding, J. W. (1986) *Monoclonal Antibodies: Principles and Practice*, Chapter 6, Academic, New York.

Habeeb, A. F. S. A. (1967) *Arch. Biochem. Biophys. 119*, 264–270.

Habeeb, A. F. S. A., & Atassi, M. Z. (1970) *Biochemistry 9*, 4939–4944.

Hahn, L., & Yamada, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1160–1163.

Herskovits, T. T. (1967) *Methods Enzymol. 16*, 748–755.

Herskovits, T. T., Behrens, C. F., Siuta, P. B., & Pandolfelli, E. R. (1977) *Biochim. Biophys. Acta 490*, 192–199.

Holland, S. K., Harlos, K., & Blake, C. C. F. (1987) *EMBO J. 6*, 1875–1880.
Hynes, R. (1985) *Annu. Rev. Cell Biol. 1*, 67–90.
Ingham, K. C., Tylenda, C., & Edelhoch, H. (1976) *Arch. Biochem. Biophys. 173*, 680–690.
Ingham, K. C., Brew, S. A., & Miekka, S. I. (1983) *Mol. Immunol. 20*, 287–295.
Ingham, K. C., Brew, S. A., Broekelmann, T. J., & McDonald, J. A. (1984) *J. Biol. Chem. 259*, 11901–11907.
Ingham, K. C., Landwehr, R., & Engel, J. (1985) *Eur. J. Biochem. 148*, 219–224.
Ingham, K. C., Brew, S. A., & Isaacs, B. S. (1988) *J. Biol. Chem. 263*, 4624–4628.
Jilek, F., & Hormann, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 597–603.
Klebe, R. J., Bentley, K. L., Sasser, P. J., & Schoen, R. C. (1980) *Exp. Cell Res. 130*, 111–117.
Kleinman, H. K., McGoodwin, E. B., & Klebe, R. J. (1976) *Biochem. Biophys. Res. Commun. 72*, 426–432.
Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P., & Woolley, D. E. (1978) *J. Biol. Chem. 253*, 5642–5646.
Kornblihtt, A., Umezawa, K., Vibe-Pedersen, K., & Baralle, F. (1985) *EMBO J. 4*, 1755–1759.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Lobel, P., Dahms, N. M., Breitmeyer, J., Chirgwin, J. M., & Kornfeld, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2233–2237.
Longworth, J. W. (1971) in *Excited States of Protein and Nucleic Acids* (Steiner, R., & Weinryb, Eds.) pp 319–484, Plenum, New York.
Markovic, Z., Lustig, A., Engel, J., Richter, H., & Hormann, H. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1795–1804.
Miekka, S. I., Ingham, K. C., & Menache, D. (1982) *Thromb. Res. 27*, 1–14.
Mosher, D. F. (1980) in *Progress in Hemostasis* & Thrombosis (Spaet, T. H., Ed.) pp 111–151, Grune & Stratton, New York.
Myers, B., II, & Glazer, A. N. (1971) *J. Biol. Chem. 246*, 412–419.
Owens, R. J., & Baralle, F. E. (1986) *EMBO J. 5*, 2825–2830.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.
Patel, R. S., Odermatt, E., Schwarzbauer, J. E., & Hynes, R. O. (1987) *EMBO J. 6*, 2565–2572.
Patthy, L., Trexler, M., Vali, Z., Banyai, L., & Varadi, A. (1984) *FEBS Lett. 171*, 131–136.
Pigiet, V. P., & Schuster, B. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7643–7647.
Riordan, J. F., & Vallee, B. L. (1972) *Methods Enzymol. 25*, 500–506.
Simpson, R. T., & Vallee, B. L. (1966) *Biochemistry 5*, 2531–2538.
Skorstengaard, K., Jensen, M., Sahl, P., Petersen, T., & Magnusson, S. (1986) *Eur. J. Biochem. 161*, 441–453.
Smith, R. L., Blackburn, M. N., & Griffin, C. A. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1684.
Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1966) *Biochemistry 5*, 3582–3589.
Teale, F. W. J. (1960) *Biochem. J. 76*, 381–388.
Tsunenaga, M., Goto, Y., Kawata, Y., & Hamaguchi, K. (1987) *Biochemistry 26*, 6044–6051.
Vincent, J. P., Lazdunski, M., & Delaage, M. (1970) *Eur. J. Biochem. 12*, 250–257.
Vuento, M., & Vaheri, A. (1978) *Biochem. J. 175*, 333–336.
Yamada, H., Imoto, T., Fujita, K., Okazaki, K., & Motomura, M. (1981) *Biochemistry 20*, 4836–4842.
Yamada, K. (1983) *Annu. Rev. Biochem. 52*, 761–799.
Zardi, L., Carnemolla, B., Balza, E., Borsi, L., Castellani, P., Rocco, M., & Siri, A. (1985) *Eur. J. Biochem. 146*, 571–579.