Domain Characteristics of the Carboxyl-Terminal Fragment 206–316 of Thermolysin: Immunochemical Studies†

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ABSTRACT: The extent of nativeness of the stable conformation of the thermolysin fragment containing the carboxyl-terminal third of the protein (from residues 206 to 316, denoted fragment FII) was examined by its immunogenic and antigenic characteristics. Antisera elicited in rabbits by either intact thermolysin or fragment FII were fractionated serially on two affinity columns, containing either the isolated fragment or intact protein. Both sera gave rise to substantial antibody populations which recognized the fragment FII region in native thermolysin. The relative affinities of these specific antibodies for isolated fragment FII and intact thermolysin were evaluated by radioimmunoassay, by assessing the relative extents of competition by these for binding of either ^{14}C-labeled thermolysin or ^{14}C-labeled fragment FII to each antibody population. Competition by fragment FII was substantial, though generally weaker than that for intact thermolysin, for antibody binding of both labeled antigens. The data demonstrate that the stable structure of fragment FII as observed spectroscopically likely is one which possesses conformational features similar to those of this region in intact thermolysin, but with perhaps less conformational rigidity. The results support the view that the region of thermolysin composed primarily of residues 206–316 is a conformational domain of the intact protein and that isolated fragment FII retains domain-like characteristics of stable and native-like conformation.

It has been found previously (Vita et al., 1979) that the carboxyl-terminal cyanogen bromide fragment of thermolysin, called fragment FII[1] and containing residues 206–316, retains substantial conformational stability in solution. The approximately 45% α-helical content of the fragment as measured by far-ultraviolet circular dichroism corresponds to a similar percentage of α helix in the 206–316 region of intact thermolysin, as judged by X-ray crystallographic analysis of the protein (Colman et al., 1972). These data argue that fragment FII can attain a stable, helix-rich structure independently from the rest of the thermolysin molecule. Further, since this fragment can elicit antibodies that recognize native but not denatured thermolysin (Vita et al., 1979), at least some of the fragment FII molecules, but an unknown proportion, could be defined as having a native-like structure bearing conformation-related antigenic determinants. These results have led to a hypothesis that fragment FII could represent an isolatable conformational domain of thermolysin. However, a quantitative estimate of the extent of antigenic nativeness could not be assessed based on immunodiffusion data alone. Thus, a definitive conclusion could not be made as to how closely related the stable structure attained by fragment FII is to the structure attained by the 206–316 region in the native protein.

In the present study, we have evaluated the extent of stability of native-like conformation in fragment FII from the immunogenic and antigenic properties of this fragment. Previous studies have shown that the extent of conformational relatedness of various protein species at the level of their antigenic determinants can be assessed by comparing the relative affinities of these species for particular antibody populations (Atassi & Saplin, 1968; Atassi & Singhal, 1970a,b; Prager & Wilson, 1971; Sachs et al., 1972a; Arnon, 1973; Crumpton, 1974; Furie et al., 1974; Lazdunski et al., 1975; Lazdunski, 1976; Reichlin & Noble, 1977; Atassi, 1979; Tai et al., 1980). In the thermolysin fragment case, we have isolated substantial amounts of the specific antibody populations, anti-Th(FII) and anti-FII(Th), from antisera to thermolysin and fragment FII, respectively. These antibody preparations both recognize the region 206–316 in the intact protein. Radioimmunoassays have been used to show a substantial similarity in the amounts of fragment FII and intact protein needed for comparable competition of ^{14}C-labeled antigen binding to antibodies in the two purified subpopulations. These data argue that fragment FII molecules possessing native-like conformational features likely represent at least a large percentage of total fragment FII in solution. Correlation of these immunochemical data with previously described spectroscopic properties (Vita et al., 1979) has been used to propose how domain-like characteristics of the FII fragment may reflect folding and conformational properties of the intact protein.

Experimental Procedures

Materials. Thermolysin from *Bacillus thermoproteolyticus* (*Rokko*) was obtained from Calbiochem (San Diego, CA) as a lyophilized product containing about 20% calcium acetate and 10% sodium acetate. The enzyme was purified by affinity chromatography using an affinity matrix of Gly-D-Phe bound to Sepharose via a 9-atom spacer (Pangburn et al., 1973; Walsh et al., 1974). The purified enzyme was stored at −20 °C in 0.1 M Tris-HCl buffer, pH 7.2, containing 10 mM

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hepes buffer, 30 mM Hepes, 0.12 M NaCl, 0.02 M $CaCl_2$, and 0.04% NaN_3, pH 7.4; fragment FII, C-terminal thermolysin fragment corresponding to sequence 206–316; [^{14}C]thermolysin and ^{14}C-labeled fragment FII, labeled species obtained by carbamoylation with K^{14}CNO; anti-Th(FII), antibody subpopulation specific for the 206–316 region of thermolysin, obtained from thermolysin antiserum by serial fractionation on Sepharose–thermolysin and then Sepharose–fragment FII; anti-FII(Th), antibody subpopulation specific for the 206–316 region of thermolysin, obtained from fragment FII antiserum by serial fractionation on Sepharose–fragment FII and then Sepharose–thermolysin.

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$CaCl_2$. Fragment FII was obtained by cyanogen bromide cleavage of thermolysin and was purified by gel filtration as described previously (Vita et al., 1979). Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. (Kankekee, IL), and *Staphylococcus aureus* Cowan I strain (Pansorbin) was from Calbiochem. Goat anti-rabbit whole serum was obtained from Miles-Yeda Ltd. (Rehovot, Israel). Tris was from Fluka AG (Basel, Switzerland); $CaCl_2 \cdot 2H_2O$, *o*-phenanthroline, and Gdn·HCl were from C. Erba (Milan, Italy); $K^{14}CNO$ was from Amersham/Searle (Arlington Heights, IL); Sepharose 4B was from Pharmacia (Uppsala, Sweden).

Rabbit anti-thermolysin and anti-fragment FII sera were obtained as described previously (Vita et al., 1979).

Preparation of Thermolysin and Fragment FII Covalently Bound to Sepharose. Thermolysin and fragment FII were coupled to cyanogen bromide activated Sepharose, which was prepared by using the method of March et al. (1974). A 10-mL bed volume of the activated Sepharose was mixed gently, at 4 °C for 2 days, with 30 mg of purified thermolysin dissolved in 20 mL of 0.1 M Hepes buffer (pH 7) containing 10 mM $CaCl_2$. The resin then was washed sequentially with distilled water, 0.2 M sodium acetate buffer (pH 5.8) containing 0.5 M NaCl and 20 mM $CaCl_2$, 0.1 M Tris-HCl buffer (pH 9.0) containing 0.5 M NaCl and 20 mM $CaCl_2$, and finally Hepes buffer (pH 7.4).

A solution of 20 mg of fragment FII in 17 mL of 0.2 M Hepes buffer, pH 7.0, was added to 10 mL of settled activated Sepharose and gently stirred at 4 °C for 2 days. The reacted resin was washed sequentially with distilled H_2O, 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl, 0.1 M Tris-HCl buffer (pH 10.0) containing 0.5 M NaCl, 0.1 M Tris-HCl buffer (pH 7.4), and finally Hepes buffer.

About 2.5 mg of thermolysin or 2 mg of fragment FII was bound covalently per mL of the respective resin, as determined by amino acid analysis of acid-hydrolyzed samples. In the thermolysin case, the covalently bound enzyme retained full catalytic activity as measured with furylacryloylglycyl-L-leucinamide (Pangburn et al., 1973).

Preparation of ^{14}C-Labeled Thermolysin and Fragment FII. Both thermolysin and fragment FII were carbamoylated by using the cyanate method (Stark & Smyth, 1963; Stark, 1967) under conditions favoring α-amino group reaction and minimizing derivatization of ϵ-amino groups. Commercial thermolysin (6 mg) was dissolved in 0.7 mL of a mixture of 20% dimethyl sulfoxide and 80% 0.1 M Hepes buffer, pH 8.0, containing 20 mM $CaCl_2$. The solution was used to dissolve 0.4 mg of dry $K^{14}CNO$ (54 mCi/mmol) and was incubated overnight at 37 °C. The carbamoylated protein was separated from free cyanate by gel filtration on a Sephadex G-25 column (1.5 × 5 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$. ^{14}C-Labeled, active thermolysin was purified by affinity chromatography as described under Materials. The specific radioactivity of [^{14}C]thermolysin was 6.3×10^3 cpm/μg, as determined by quantitative amino acid analysis.

Lyophilized fragment FII (2.5 mg) was dissolved in 0.5 mL of 0.1 M Hepes buffer, pH 7.0, containing 6 M urea and the solution used to dissolve 0.4 mg of dry $K^{14}CNO$ (54 mCi/mmol). The solution was incubated overnight at 25 °C and then was applied to a Sephadex G-50 SF column (0.7 × 47 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.2. Fractions of 1 mL were collected and assayed for radioactivity. The first peak was pooled, lyophilized, and rechromatographed on the same column. The specific radioactivity of ^{14}C-labeled fragment FII was 17.5×10^3 cpm/μg, as determined by quantitative amino acid analysis.

Purification of Antibody Subpopulations. For isolation of antibody populations specifically directed against the region 206–316 of thermolysin, antisera were fractionated by a series of affinity chromatographic steps. Anti-thermolysin serum (10 mL) was applied directly to a column (1.0 × 9.0 cm) of Sepharose–thermolysin and eluted with Hepes buffer to wash off nonbinding material, 1 M Gdn·HCl in Hepes buffer to remove weakly bound protein, and then 4 M Gdn·HCl in Hepes buffer. Proteins eluted with 4 M Gdn·HCl were dialyzed immediately into Hepes buffer, applied to a column (0.7 × 8.0 cm) of Sepharose–fragment FII, and eluted as before. Antibodies eluted with 4 M Gdn·HCl [denoted anti-Th(FII)] were dialyzed into Hepes buffer and, if necessary, concentrated by ultrafiltration using an Amicon PM-10 membrane. Anti-fragment FII serum (6 mL) was fractionated analogously on the two columns but in reverse order, first on Sepharose–fragment FII and then on Sepharose–thermolysin. These purified antibodies [denoted anti-FII(Th)] were dialyzed and concentrated analogously by using an Amicon PM-10 membrane. All chromatographic and dialysis steps were carried out at 4 °C.

The concentrations of fractionated antibody solutions were determined by absorbance using an $A_{280nm}^{0.1\%} = 1.4$.

Immunoelectrophoresis. Antisera and fractionated antibody samples (5 μL) were analyzed by immunoelectrophoresis in agar gel (1.2% in 0.1 M sodium glycinate buffer, pH 8.6). Samples were run for 2 h at 120 V and then reacted with goat anti-rabbit whole serum for 24 h. Gel plates were photographed without staining.

Titration of Labeled Antigens with Antibody. The binding of [^{14}C]thermolysin and ^{14}C-labeled fragment FII to anti-Th(FII) and anti-FII(Th) was examined by precipitation of the antigen–antibody complex with Cowan cells (Pansorbin). Labeled antigen [20 μL of [^{14}C]thermolysin (4000 cpm) or 10 μL of ^{14}C-labeled fragment FII (5000 cpm)] was incubated in microfuge tubes (Beckman) for 1 h at 25 °C, with increasing amounts of anti-Th(FII) or anti-FII(Th) in the presence of 2% bovine serum albumin and Hepes buffer to bring the final volume to 150 μL. A 150-μL aliquot of a suspension (9% v/v) of Cowan cells in Hepes buffer was added. After a 1-h incubation at 25 °C, the mixture was centrifuged in a Beckman Model 152 microfuge. The precipitate was resuspended and washed once with 150 μL of cold Hepes buffer. Both the supernatants (pooled) and the precipitates (resuspended in Hepes buffer) were mixed, separately, with 10 mL of scintillation fluid (Aquasol; New England Nuclear) and measured for radioisotope content in a Searle Mark III scintillation counter. All assays were carried out in duplicate; controls without antibodies were included.

Competition Radioimmunoassays. Competition assays were carried out as described for the titration assays above, except that unlabeled thermolysin and fragment FII were used to compete with the labeled antigen ([^{14}C]thermolysin or ^{14}C-labeled fragment FII) for binding to a fixed amount of anti-Th(FII) or anti-FII(Th). A fixed volume of purified antibody solution was used which was sufficient (as determined by titration) to bind about 50% of the maximum precipitable counts of the labeled antigens. Bovine serum albumin (2%) and 3 mM *o*-phenanthroline were included in the assay mixture, the latter to inhibit the proteolytic activity of thermolysin (Latt et al., 1969). Controls included samples without competitors (binding controls) and samples without antibodies (nonspecific precipitation controls). All assays were done in

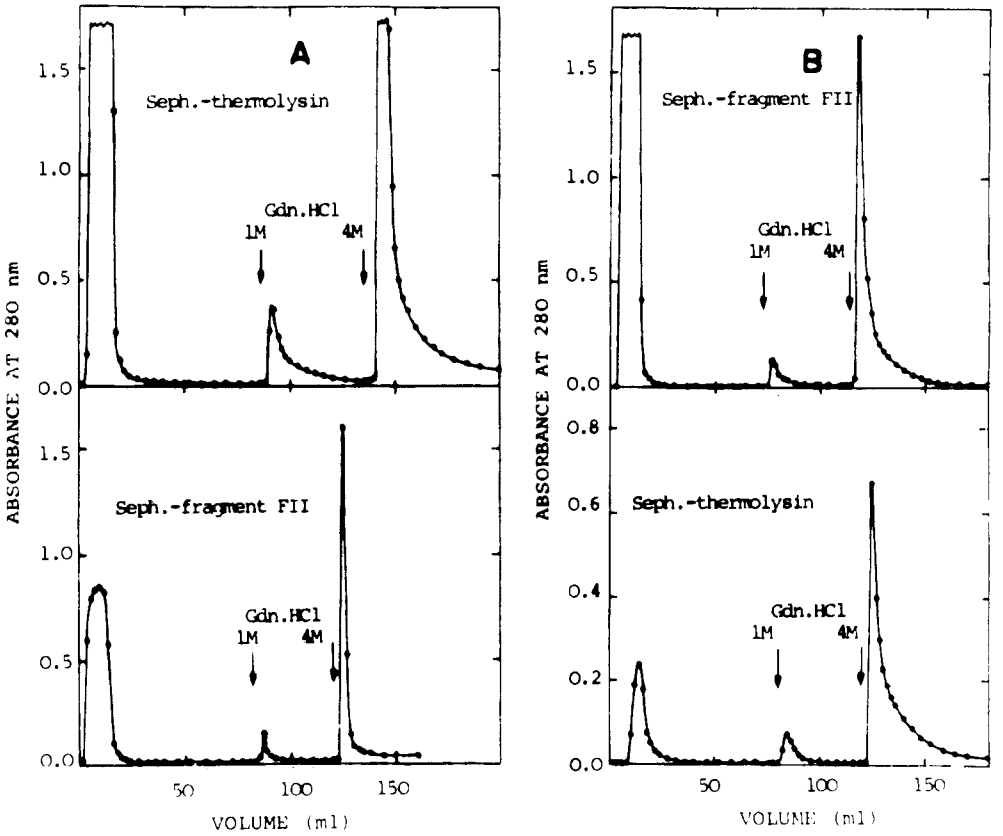

FIGURE 1: Purification of (A) anti-Th(FII) and (B) anti-FII(Th) by affinity chromatography, with elution initially by Hepes buffer, pH 7.4, and then by 1 M Gdn·HCl in Hepes buffer, pH 7.4 ("1 M Gdn·HCl"), followed by 4 M Gdn·HCl in Hepes buffer, pH 7.4 ("4 M Gdn·HCl"). Further details are given in the text.

duplicate and at room temperature, with an incubation of 1 h for binding before centrifugation. Both precipitate and supernatant were counted. The percent of either [^{14}C]thermolysin or ^{14}C-labeled fragment FII bound to antibody was calculated relative to the maximum amount of labeled antigen bound to the antibody present.

Results

Isolation of Specific Antibodies. Antibodies specific for fragment FII were obtained by serial affinity chromatography (Figure 1). From 10 mL of thermolysin antiserum, 40 mL containing 16.6 mg of specific antibody was obtained after elution on thermolysin–Sepharose (Figure 1A, top). Elution of an aliquot of these anti-thermolysin antibodies on Sepharose–fragment FII yielded anti-Th(FII) (the peak eluted with 4 M Gdn·HCl) as about 35% of the total anti-thermolysin fraction (Figure 1A, bottom). This yield can be related to the fact that fragment FII represents about one-third of the thermolysin primary structure.

From 6 mL of fragment FII antiserum, 5.5 mg of anti-FII in 14 mL was obtained after Sepharose–fragment FII chromatography (Figure 1B, top). A large portion (about 70%) of anti-fragment FII antibodies, denoted anti-FII(Th), was adsorbed specifically to Sepharose–thermolysin. A significant (about 30%) portion of anti-FII does not bind to thermolysin–Sepharose and is largely ignored in subsequent analyses. However, since the majority (about 70%) of anti-FII does recognize native thermolysin, the use of this latter subpopulation as a reflection of the nature of isolated fragment FII is not considered unreasonably biased.

Immunoelectrophoresis analyses were carried out for the products of successive purification of both anti-thermolysin and anti-fragment FII rabbit sera. Goat anti-rabbit serum was used as a precipitating agent. Whereas antisera showed, as expected, precipitin lines corresponding to several types of serum proteins, anti-thermolysin, anti-fragment FII, anti-Th(FII) and anti-FII(Th) all showed a single line corresponding to immunoglobulin G.

Both purified antibody subpopulations, anti-Th(FII) and anti-FII(Th), did not form precipitin bands with either fragment FII or thermolysin when analyzed by immunodiffusion using the Ouchterlony technique (Ouchterlony, 1968). This is in contrast to the occurrence of precipitation for thermolysin antisera with thermolysin and fragment FII and for fragment FII antisera with fragment FII. Whether the lack of precipitation with anti-Th(FII) and anti-FII(Th) reflects the presence of a limited number of antigenic determinants in the 206–316 region that are specific for the antibody subpopulations isolated (Marrack et al., 1951), increased dilution of the purified antibodies or a combination of these factors cannot be defined from the present data. In general, a failure of anti-fragment antibody populations to form precipitates with their respective antigens has been observed often (Furie et al., 1974; Sachs, 1974).

Binding Studies. Interactions of anti-Th(FII) and anti-FII(Th) with thermolysin and fragment FII were evaluated by measuring the ability of these antigens to compete with [^{14}C]thermolysin and ^{14}C-labeled fragment FII for antibody binding in radioimmunoassays. Sufficient antibody was added to either labeled antigen to bind about half of the maximum precipitable counts of the labeled antigen used. The results of the competition radioimmunoassay experiments are illustrated in Figures 2 and 3. As shown in Figure 2 native thermolysin inhibits 50% of the binding of [^{14}C]thermolysin to anti-Th(FII) or anti-FII(Th) when added in approximately 1:1 molar ratios with respect to labeled antigen. This indicates that the labeling procedure does not affect the antigenic integrity of the protein. On the basis of the analogous results

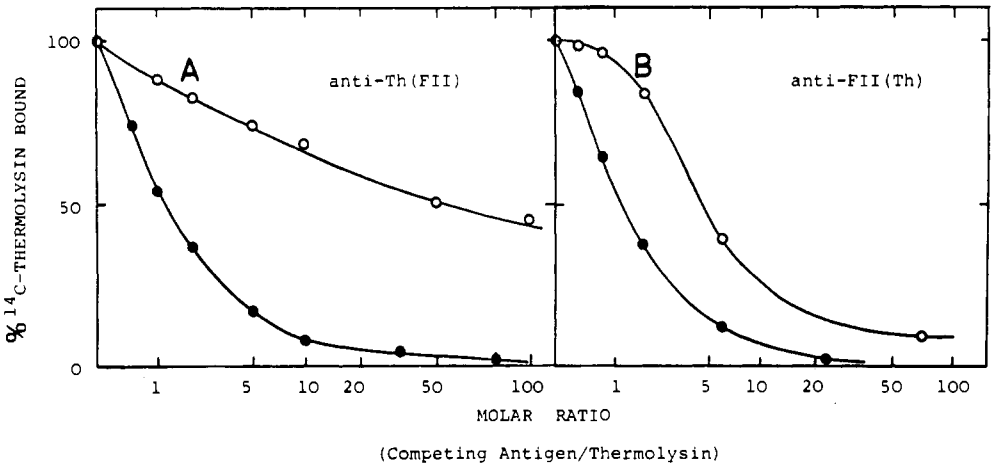

FIGURE 2: Competition binding profiles of [^{14}C]thermolysin for anti-Th(FII) (A) and anti-FII(Th) (B) in the presence of unlabeled thermolysin (●) and unlabeled fragment FII (○). Binding in the absence of competitor corresponds to 100% [^{14}C]thermolysin bound. [^{14}C]Thermolysin was at 0.2 mM, anti-Th(FII) at 21 μg/mL, and anti-FII(Th) at 31 μg/mL.

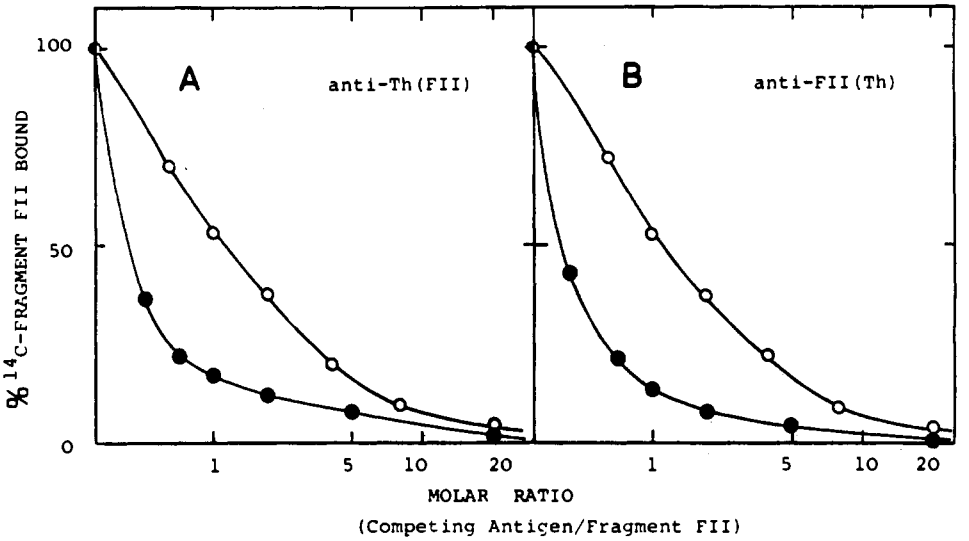

FIGURE 3: Competition binding profiles of ^{14}C-labeled fragment FII for anti-Th(FII) (A) and anti-FII(Th) (B) in the presence of unlabeled fragment FII (○) and unlabeled thermolysin (●). Binding in the absence of competitor corresponds to 100% ^{14}C-labeled fragment FII bound. ^{14}C-Labeled fragment was added at 0.7 mM, anti-Th(FII) at 47 μg/mL, and anti-FII(Th) at 52 μg/mL.

shown in Figure 3A with fragment FII vs. labeled fragment FII, it can be concluded that the immunochemical integrity of the fragment also is preserved upon carbamoylation.

Comparison of the extents of competition by fragment FII and thermolysin as shown in Figures 2 and 3 indicates that, with one notable exception (see below), the fragment is quantitatively nearly as effective as the native protein. Thus, only approximately a 5-fold increase is required in the concentration of fragment FII over that of native thermolysin to achieve about equal extents of competition for binding of ^{14}C-labeled fragment FII to anti-FII(Th) or anti-Th(FII) or for binding of [^{14}C]thermolysin to anti-FII(Th). Since, in all these cases, it is likely that the affinity constants of isolated fragment and native protein are closely similar, the results affirm that the fragment FII possesses antigenic determinants which are stable to a degree similar to that of analogous determinants in the 206–316 region in native thermolysin. Nonetheless, given the generally greater competitiveness of thermolysin over fragment FII, it is likely that there is some destabilization of structure of antigenic determinants preserved in isolated fragment FII.

The data of Figure 2A do show a distinct reduction of competitiveness in fragment FII for native thermolysin in assays with [^{14}C]thermolysin and anti-Th(FII). A reasonable interpretation of these data is that, while some determinants of fragment FII may be relatively intact and stable, leading to about 50–60% competition of [^{14}C]thermolysin binding, this fragment may have substantially lost (in terms of conformational stability) one or more determinants that are present in the 206–316 region of native thermolysin. Thus, fragment FII would not be able to interact effectively with one subclass of anti-Th(FII), leading to a leveling off of competition at about 40–50% bound labeled antigen (Figure 2A). Under the assumption that the particular subclass of anti-Th(FII) for which binding to [^{14}C]thermolysin is competed effectively by FII is about 60% of the total antibody, the 50% level of competition requires about a 5-fold greater concentration than that of native thermolysin required for similar competition. The antigenic determinants specific for the latter subclass of antibodies likely are the same as those expressed in the competition data of Figures 3 and 2B.

Discussion

Significant effort has been made to assess the use of conformationally specific antibodies as specific reporters for structural features of proteins (Atassi & Saplin, 1968; Prager & Wilson, 1971; Sachs et al., 1972a; Arnon, 1973; Crumpton, 1973; Furie et al., 1974; Lazdunski et al., 1975; Lazdunski,

1976; Reichlin & Noble, 1977; Tai et al., 1980). Specific antibody populations can be obtained from protein antisera by fractionation on affinity columns containing immobilized native protein. Subsequent fractionation of the total protein antibodies on columns containing immobilized protein fragments can lead to subpopulations of the total which recognize only one part of the protein (Omenn et al., 1970; Sachs et al., 1972a,b; Furie et al., 1974, 1975, 1978; Young et al., 1975; Lazdunski, 1976; Curd et al., 1976; Benjamin & Teale, 1978; Chavez & Scheraga, 1979; Dean & Schechter, 1979; Lou et al., 1979). A central and testable assumption in the use of such affinity-purified antibody populations and subpopulations for detecting conformational events is that these antibodies recognize native protein (as assumed from the affinity chromatographic purification) with much higher affinity than denatured forms. When this is so, detection of native-like conformation in particular parts of a protein is a realistic goal. The quantitative use of specific antibodies can be confused by potential uncertainty as to how much of a conformational unit in fact is needed for antibody recognition. It is likely, for example, that antibodies recognize limited, exposed regions of globular protein molecules, such as corners and loops. Thus, only local conformational nativeness may be ascertainable with assurance. Nonetheless, antibodies preferring proteins and derivatives of native conformation have been helpful in detecting protein folding events (Anfinsen & Scheraga, 1975; Teale & Benjamin, 1976, 1977; Chavez & Scheraga, 1977, 1979, 1980; Chavez & Benjamin, 1978), the capacity for protein fragments to attain native-like conformation at least transitorily (Atassi, 1967; Sachs et al., 1972a,b, 1974; Celada et al., 1974; Furie et al., 1974; Habeeb & Atassi, 1976; Harrell et al., 1977), and conformational relationships between proteins (Prager & Wilson, 1971; Lazdunski et al., 1975; Moir & Paulus, 1977; Lindstrom et al., 1979).

In the present study, the antibody subpopulations anti-Th(FII) and anti-FII(Th) have been used as probes for the presence of native-like structure in isolated fragment FII. The antisera used as the source of these subpopulations have been found previously to interact with intact thermolysin but only much more weakly if at all with the denatured protein (Vita et al., 1979). Thus, strong interactions of both anti-Th(FII) and anti-FII(Th) with fragment FII can be assumed as a first approximation to be due to the presence in the fragment of native-like conformational features corresponding to many of the antigenic (and immunogenic) determinants. For the two antibody subpopulations tested here, the amount of fragment FII needed to compete for antigen binding generally was within an order of magnitude (only about 5-fold higher) of the amount of thermolysin needed for comparable competition. And, with one exception, all antibody binding competed by thermolysin is competed also by fragment FII. The one exception is the competition of binding of [^{14}C]thermolysin to anti-Th(FII). With the latter, fragment FII does not appear to compete effectively for binding to a major subset of antibodies comprising about 40–50% of the total anti-Th(FII) population (Figure 2A). This phenomenon has been discussed formally (Berzofsky & Schechter, 1981).

The results of the competition assays are consistent with the view that fragment FII can achieve a stable structure which is related to that of the 206–316 region in intact thermolysin, but somewhat more flexible than the latter and perhaps missing at least one antigenic determinant altogether. Considering the level of competition effectiveness often seen for polypeptide species interpreted as folded, or as having a propensity for folding (Atassi & Saplin, 1968; Sachs et al., 1972a, 1974;

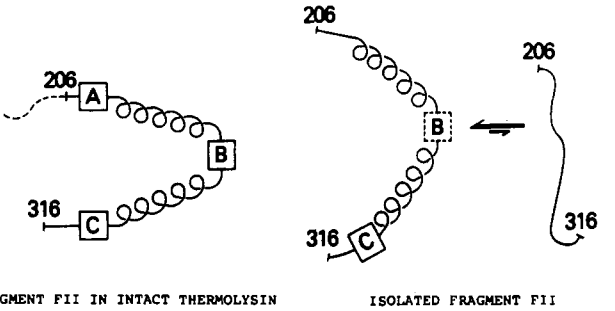

FIGURE 4: Schematic representation of the conformations of the sequence 206–316 in native thermolysin and in the isolated fragment FII.

Habeeb & Atassi, 1976; Harrell et al., 1977; Benjamin & Teale, 1978; Furie et al., 1978), the retention of competitiveness in fragment FII at concentrations within an order of magnitude of those required for thermolysin is a good indication that the competing native-like conformation is a relatively stable one. Since fragment FII has been found previously by spectroscopic analysis to form a stable, helix-rich conformation (Vita et al., 1979), it is likely that it is this structure which is competing in the radioimmunoassays. It seems reasonable to consider the structure of isolated fragment FII to be somewhat more open than that of the 206–316 region in intact thermolysin, as schematically illustrated in Figure 4. This view would account, in determinants B and C, for the generally substantial though somewhat weaker competitiveness of fragment FII vs. protein (Figures 2B and 3) and, in determinant A, for the loss of competitiveness to one subclass of anti-Th(FII) antibodies (Figure 2A). Since the extent of completeness of folding of fragment FII into an open, native-like structure cannot be defined with certainty, Figure 4 depicts the possibility of totally unfolded forms of the fragment in solution. However, it is worth emphasizing that the equilibrium is expected to strongly favor the folded form. This is predictable from spectroscopic data (Vita et al., 1979) showing that very little nonhelical, unfolded form of fragment FII exists under the solution conditions of the radioimmunoassays.

Taking the immunochemical (this study) and spectroscopic data (Vita et al., 1979) together, it is reasonable to conclude that isolated fragment FII is able to refold to a stable, native-like structure. The fragment therein can be defined as an isolated protein domain. This definition connotes that, in the thermolysin molecule, the region including fragment FII is likely to be a "hyperstable" part of the protein. Recently, Rashin (1981) has predicted from surface calculations based on the X-ray crystallographic structure of thermolysin (Colman et al., 1972) that sequence 203–316 is capable of independent folding in the protein molecule. This prediction correlates well with what we have observed experimentally.

The results of the present study can be related to domain concepts of protein structure and folding (Wetlaufer, 1973; Liljas & Rossmann, 1974; Levitt & Chothia, 1976; Schulz & Schirmer, 1979). Visual examinaton of the three-dimensional structures of many proteins leads to recognition of structural domains, that is, areas which are compact subzones of the overall globular protein (Wetlaufer, 1973). In the thermolysin case, the fragment FII is part of but not an entire structural domain. Visually, the protein appears to be bilobal, with the C-terminal half comprising residues 158–316 (Colman et al., 1972). While the significance of the presence of fragment FII as part of the visualized C-terminal structural domain has not been defined, it is not unlikely that the FII sequence is a major promoter of stable conformation in this region.

Further, a variety of folding studies (Levinthal, 1966; Lewis et al., 1971; Anfinsen, 1972; Wetlaufer & Ristow, 1973; Creighton, 1974; Robson & Pain, 1976; Acharya & Taniuchi, 1977; Scheraga, 1980; Baldwin & Creighton, 1980) lead to the view that protein folding may occur through a multiple but probably limited number of pathways through limited random search. Especially for large proteins, this search may be directed at least in part by folding domains (Lindstrom et al., 1979), regions that fold more readily than other regions and therein define pathways for folding. While a role for the 206–316 region in dictating stable conformation can be concluded reasonably based on present experimental data, a role for this region in directing thermolysin folding is an attractive but yet untested idea.

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References

Acharya, A. S., & Taniuchi, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2362–2366.

Anfinsen, C. B. (1972) *Biochem. J. 128*, 737–749.

Anfinsen, C. B., & Scheraga, H. (1975) *Adv. Protein Chem. 29*, 205–299.

Arnon, R. (1973) in *The Antigens* (Sela, M., Ed.) Vol. 1, pp 89–159, Academic Press, New York.

Atassi, M. Z. (1967) *Biochem. J. 103*, 29–35.

Atassi, M. Z. (1979) *CRC Crit. Rev. Biochem. 7*, 337–400.

Atassi, M. Z., & Saplin, B. J. (1968) *Biochemistry 7*, 688–698.

Atassi, M. Z., & Singhal, R. P. (1970a) *Biochemistry 9*, 3854–3861.

Atassi, M. Z., & Singhal, R. P. (1970b) *Biochemistry 9*, 4252–4259.

Baldwin, R. L., & Creighton, T. E. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 217–260, Elsevier/North-Holland Biomedical Press, Amsterdam and New York.

Benjamin, D. C., & Teale, J. M. (1978) *J. Biol. Chem. 253*, 8087–8092.

Berzofsky, J. A., & Schechter, A. N. (1981) *Mol. Immunol. 18*, 751–763.

Celada, F., Ullmann, A., & Monod, J. (1974) *Biochemistry 13*, 5543–5547.

Chavez, L. G., & Scheraga, H. A. (1977) *Biochemistry 16*, 1849–1856.

Chavez, L. G., & Benjamin, D. C. (1978) *J. Biol. Chem. 253*, 8081–8086.

Chavez, L. G., & Scheraga, H. A. (1979) *Biochemistry 18*, 4386–4395.

Chavez, L. G., & Scheraga, H. A. (1980) *Biochemistry 19*, 996–1004.

Colman, P. M., Jansonius, J. N., & Matthews, B. W. (1972) *J. Mol. Biol. 70*, 701–724.

Creighton, T. E. (1974) *J. Mol. Biol. 87*, 603–624.

Crumpton, M. J. (1974) in *The Antigens* (Sela, M., Ed.) Vol. 2, pp 1–78, Academic Press, New York.

Curd, J. G., Ludwig, D., & Schechter, A. N. (1976) *J. Biol. Chem. 251*, 1283–1289.

Dean, J., & Schechter, A. N. (1979) *J. Biol. Chem. 254*, 9185–9193.

Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1974) *Biochemistry 13*, 1561–1566.

Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1975) *J. Mol. Biol. 92*, 497–506.

Furie, B., Provost, K. L., Blanchard, R. A., & Furie, B. C. (1978) *J. Biol. Chem. 253*, 8990–8987.

Habeeb, A. F. S. A., & Atassi, M. Z. (1976) *J. Biol. Chem. 251*, 4616–4621.

Harrell, J. G. R., Smith, J. A., & Leach, S. J. (1977) *Biochemistry 16*, 175–185.

Latt, S. A., Holmcuist, B., & Vallee, B. L. (1969) *Biochem. Biophys. Res. Commun. 37*, 333–339.

Lazdunski, C. J. (1976) *Trends Biochem. Sci. (Pers. Ed.)*, 231–233.

Lazdunski, C. J., Pages, J.-M., & Louvard, D. (1975) *J. Mol. Biol. 97*, 309–335.

Levinthal, C. (1966) *Sci. Am. 214*, 45–52.

Levitt, M., & Chothia, C. (1976) *Nature (London) 261*, 552–558.

Lewis, P. N., Momamy, F. A., & Scheraga, H. A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2293–2297.

Liljas, A., & Rossmann, M. G. (1974) *Annu. Rev. Biochem. 43*, 475–507.

Lindstrom, J., Walter (Nave), B., & Einarson, B. (1979) *Biochemistry 18*, 4470–4480.

Lou, H. K., Rosenberg, J. S., Beeler, D. L., & Rosenberg, R. D. (1979) *J. Biol. Chem. 254*, 8751–8761.

March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.

Marrack, J. R., Hoch, H., & Johns, R. G. S. (1951) *Br. J. Exp. Pathol. 32*, 212–230.

Moir, D., & Paulus, H. (1977) *J. Biol. Chem. 252*, 4655–4661.

Omenn, G. S., Ontjes, D. A., & Anfinsen, C. B. (1970) *Nature (London) 225*, 189–190.

Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis*, pp 32–47, Humphrey Scientific Publishers, Ann Arbor, MI.

Pangburn, M. K., Burstein, Y., Morgan, P. M., Walsh, K. A., & Neurath, H. (1973) *Biochem. Biophys. Res. Commun. 54*, 371–379.

Prager, E. M., & Wilson, A. C. (1971) *J. Biol. Chem. 246*, 5978–5988.

Rashin, A. A. (1981) *Nature (London) 291*, 85–87.

Reichlin, M., & Noble, R. W. (1977) in *Immunochemistry of Proteins* (Atassi, M. Z., Ed.) Vol. 2, pp 311–352, Plenum Press, New York.

Robson, B., & Pain, R. H. (1976) *Biochem. J. 155*, 331–344.

Sachs, D. H. (1974) *Current Topics in Biochemistry* (Anfinsen, C. B., & Schechter, A. N., Eds.) pp 73–107, Academic Press, New York.

Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972a) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790–3794.

Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972b) *J. Immunol. 109*, 1300–1310.

Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1974) *Nature (London) 251*, 242–244.

Scheraga, H. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 261–288, Elsevier/North-Holland Biomedical Press, Amsterdam and New York.

Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure*, Chapter 5.3, Springer, New York.

Stark, G. R. (1967) *Methods Enzymol. 11*, 125–138.

Stark, G. R., & Smyth, D. G. (1963) *J. Biol. Chem. 238*, 214–226.

Tai, M. M., Furie, B. C., & Furie, B. (1980) *J. Biol. Chem. 255*, 2790–2795.

Teale, J. M., & Benjamin, D. C. (1976) *J. Biol. Chem. 251*, 4603–4608.

Teale, J. M., & Benjamin, D. C. (1977) J. Biol. Chem. 252, 4521-4526.
Vita, C., Fontana, A., Seeman, J. R., & Chaiken, I. M. (1979) Biochemistry 18, 3023-3031.
Walsh, K. A., Burstein, Y., & Pangburn, M. K. (1974) Methods Enzymol. 34, 435-440.
Wetlaufer, D. B. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 697-701.
Wetlaufer, D. B., & Ristow, S. (1973) Annu. Rev. Biochem. 42, 135-158.
Young, N. S., Curd, J. G., Eastlake, A., Urie, B., & Schechter, A. N. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4759-4763.

Ligation and Quaternary Structure Induced Changes in the Heme Pocket of Hemoglobin: A Transient Resonance Raman Study†

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ABSTRACT: The extent to which ligation and quaternary structure modify the heme–heme pocket configuration is determined by generating and analyzing transient resonance Raman spectra from various photolyzed and partially photolyzed hemoglobins (Hb). From small frequency shifts in Raman band I ($\sim$1355 cm^{-1}) it is determined that ligation induces a configurational change about the heme. The extent to which ligation modifies the heme pocket is influenced by the quaternary structure. With respect to the structural parameter responsible for variations in the π^* orbital electron density of the porphyrin, the degree of alteration of the heme pocket configuration relative to deoxy-Hb(T) follows the sequence: liganded Hb(R) > liganded Hb(R) + IHP > liganded Hb(T) [α chain > β chain] > deoxy-Hb(R). This progression of configurations also forms a sequence with respect to the "retentiveness" of the heme pocket as reflected in the ligand dynamics associated with geminate recombination. The results indicate that the heme–heme pocket of the R-state Hb's, relative to those of the T-state species, favors ligand retention in a dynamic, as well as thermodynamic, sense. The analysis of these and other related data implicates a ligation and quaternary structure modulated electronic and/or electrostatic interaction between the π system of the porphyrin and the surrounding heme pocket as the basis for this variation in ligand dynamics as well as for the energetics of cooperativity.

The binding of ligands to the hemes in Hb[1] is a highly localized perturbation that initiates a sequence of propagating structural events that culminates in a change in quaternary structure from the low-affinity deoxy T state to the high-affinity liganded R state (Perutz, 1970). Although these two equilibrium species have been well characterized, both the mechanism by which ligation triggers structural destabilization and the structural basis for the dynamics remain as yet undetermined. Moreover, these questions are interdependent—the dynamics being influenced by ligation-sensitive structures surrounding the heme. Understandably, it is of fundamental interest to probe those ligation-dependent interactions that affect the stability of the heme environment (heme pocket) and to establish the relationship between structural variations of this environment and kinetic phenomena.

In the equilibrium structures of Hb the functionally relevant energies associated with the coupling of ligation and quaternary structure need not be localized near the heme. However, in metastable species, generated immediately after ligation or deligation, these energies must be manifested, at least transiently at the interface between the binding site and the surrounding protein. Hence, appropriate time-resolved studies of these metastable species would enhance the likelihood of detecting these hitherto unidentified physiologically important heme–protein interactions. Photolysis of liganded Hb's has been used extensively to prepare nonequilibrium populations of Hb. In turn, transient absorption spectroscopy has yielded extensive data on the kinetics of these nonequilibrium species. Such studies (Shank et al., 1976; Noe et al., 1978; Reynolds et al., 1981; Greene et al., 1978; Chernoff et al., 1980) have shown that photodissociation and the subsequent recovery to the ground electronic state of deoxyheme occur on a picosecond time scale. However, transient absorption studies (Chernoff et al., 1980; Sawicki & Gibson, 1974; Lindquist et al., 1980) also reveal differences between the spectrum of the stable T-state deoxy-Hb and that of the photolyzed species. In all likelihood, differences in spectra observed on these time scales originate from environmental differences about the porphyrin chromophore. Absorption spectroscopy, unfortunately, cannot be used to determine the structural origins of these interactions because of the numerous potential contributions to a porphyrin absorption spectrum. In contast, resonance Raman spectroscopy [for a detailed review, see Spiro (1981) and Asher (1981)] provides a more suitable probe of localized structure.

It has been established by Raman difference spectroscopy (Shelnutt et al., 1979a,b; Rousseau et al., 1981) that resonance Raman scattering is well suited as a probe of heme–protein interactions. Raman band I, which occurs at $\sim$1355 cm^{-1} for deoxyhemes and in the 1370s for liganded ferrous hemes, has been shown to display a sensitivity in its frequency (for deoxyheme) to variations in the π^*-electron distribution originating from perturbations occurring either through the iron

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[1] Abbreviations: Hb, hemoglobin; Mb, myoglobin; HbCO, carboxyhemoglobin; HbNO, nitrosylhemoglobin; HbO_2, oxyhemoglobin; Hb(x), hemoglobin with the x (R or T) quaternary structure; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol.

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