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Folding Patterns of Immunoglobulin Molecules Identified by Urea Gradient Electrophoresis*

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The reversible denaturant-induced unfolding of immunoglobulin molecules has been analyzed by transverse urea gradient gel electrophoresis and the effects that urea-induced unfolding exerts on the functional properties associated with their variable region, *i.e.* antigen binding and idiotypic expression, have been determined by Western blot analysis. Results obtained from these experiments indicate that urea-induced unfolding of the immunoglobulin molecule is a highly cooperative reversible process that occurs through a two-state transition with no accumulation of intermediates. The unfolding transition has its midpoint at about 6.5 M urea and appears to be slow on the time scale of electrophoresis. Folding intermediates in rapid equilibrium with the unfolded state as well as molecular forms with different electrophoretic mobility can be detected during refolding reactions. Results from Western blot analysis confirm the highly cooperative reversible urea-induced unfolding of immunoglobulin molecules and demonstrate that the unfolding transition leads to disappearance of both antigen binding and idiotypic expression, whereas the ability to interact with antibodies directed to continuous epitopes of the variable region is preserved. After progressive removal of the denaturing agent, the variable region refolds into structures that regain the functional properties of the native conformation.

The structure of the immunoglobulin (Ig) molecule has been extensively analyzed at both the primary and three-dimensional levels (Alzari *et al.*, 1988; Davies and Metzger, 1983; Marquart and Deisenhofer, 1982). However, the characterization of the folding profiles of these proteins has been hampered by the complexity of their architecture, which consists of a tetrameric structure formed by the association of heavy and light chains. This tetrameric structure includes various domains that associate to form discrete structural/functional regions. The variable (V) region is responsible for antigen binding and for idiotypic expression and is formed by the interaction of the V light and V heavy domains at the amino termini of the chains. The constant region, which includes the remainder of the light and heavy chains, is responsible for effector functions, such as complement fixation and binding to receptors. Single domains are folded into a compact highly conserved globular three-dimensional conformation characterized by a repetitive structural motif termed Ig fold and consisting of β-pleated sheets with antiparallel strands connected by loop regions and arranged in two layers. An intrachain disulfide bond is buried in the interior hydrophobic region between the β-sheets. No α-helical elements are present in the native Ig molecule. Given the complex nature of Ig molecules, only few structural/functional studies have involved the analysis of their folding characteristics (Pantoliano *et al.*, 1991; Buchner *et al.*, 1991; Kawata and Hamaguchi, 1991; Goto *et al.*, 1988; Goto and Hamaguchi, 1982, 1987; Tsunenaga *et al.*, 1987). The majority of these studies have focused on the analysis of the folding properties of isolated Ig chains or Ig fragments. A limited amount of information is currently available on the folding patterns of whole antibody molecules (Buchner *et al.*, 1991). More insight into the folding pathways that determine the native Ig conformation is essential for the general understanding of the antibody molecule structure and for the elucidation of the folding mechanisms leading to the final configurations of multidomain oligomeric proteins. In addition, the study of the Ig folding profiles is of primary importance to fully exploit the recombinant antibody technology. Such technology provides the basis for the manipulation, engineering, and tailoring of Ig molecules to be used for immunotherapeutic and immunodiagnostic applications (Riechmann, 1988a, 1988b; Bird *et al.*, 1988; Horwitz *et al.*, 1988; Skerra and Plucktun, 1988; Better *et al.*, 1988). Specific refolding procedures are necessary for the production of active recombinant whole Ig molecules expressed in *Escherichia coli* and obtained in insoluble form as inclusion bodies (Boss *et al.*, 1984; Cabilly *et al.*, 1984; Kurokawa *et al.*, 1983). The optimization of such procedures requires the analysis of the unfolding/refolding patterns resulting by denaturant exposure/removal and the availability of methods that may assess the homogeneous folded state of the final products.

The understanding of the folding pathway of a protein is based on the identification of the various possible folding intermediates, that is the examination of the conformations which exist between the fully unfolded state and the condensed native form (Jaenicke, 1991; Kim and Baldwin, 1990). While both fully denatured and folded native states are significantly populated at equilibrium, partially folded intermediates are inherently unstable and not present in significant concentrations. Consequently, they are difficult to detect and characterize. One means to examine this problem is to trap folding intermediates. This can be achieved using several methods that include the use of denaturing agents, such as urea or guanidinium chloride (Makhatadze and Privalov, 1992). One of these methods, transverse urea-gradient gel electrophoresis, represents a powerful tool for the qualitative analysis of urea-mediated denaturation and renaturation processes of proteins (Goldenberg and Creighton, 1984; Creighton, 1986). With this technique, unfolding is detected by subjecting proteins to electrophoresis through a slab of polyacrylamide gel containing a continuous linear concentration gradient of urea perpendicular to the direction of migration. Urea-induced conformational transitions that are accompanied by alterations in size are detected as changes in the electrophoretic mobility due to the

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larger hydrodynamic volume of the unfolded protein. Gradual refolding obtained by decreasing concentration of urea results in the progressive restoration of the original mobility. In this study, we have attempted to qualitatively characterize the urea-induced unfolding/refolding transitions of intact IgG molecules and derived $F(ab')_2$, Fab, and Fc fragments using transverse urea gradient gel electrophoresis. Western blot analysis has been used to assess the effects that the unfolding/refolding reactions produce on the functional properties of the V region, *i.e.* antigen binding and idiotypic expression. Results from these studies provide information on the folding profiles of antibody molecules and indicate that transverse urea gradient gel electrophoresis, in conjunction with Western blot analysis, represents a powerful tool to ascertain the presence of correct folding in Ig molecules subjected to denaturation/renaturation processes.

MATERIALS AND METHODS

Mouse Monoclonal Antibodies and Rabbit Polyclonal Reagents—The two murine monoclonal antibodies utilized in this study, G3 and Leu3a (both IgG1, κ) are specific for epitopes expressed on the transmembrane glycoprotein of the human immunodeficiency virus and the human CD4 molecule, respectively. Antibody purification was performed from ascitic fluid using the caprylic acid procedure (McKinney and Parkinson, 1987). Purity was greater than 95% as judged by polyacrylamide gel electrophoresis. $F(ab')_2$, Fab, and Fc fragments derived from the intact G3 molecule were obtained by pepsin and papain digestion following standard procedures (Parham, 1982). Monoclonal antibodies and derived fragments were stored in 0.05 M borate-buffered saline (BBS),[1] pH 8.2. The structural and functional properties of Leu3a have been described previously (Attanasio *et al.*, 1991). The polyclonal anti-isotypic, anti-idiotypic, anti-complementarity determining region (CDR) peptides, and anti-CD4 reagents generated in rabbits have been described in detail elsewhere (Attanasio *et al.*, 1990, 1991, 1993).

Transverse Urea Gradient Gel Electrophoresis—Transverse urea gradient gel electrophoresis was performed using either native proteins or proteins unfolded for 2 h at 25 °C in 8 M urea. Urea-gradient polyacrylamide gels were prepared essentially as described by Creighton (1986) with the following modifications. Tris acetate buffer (0.05 M, pH 8.0) was used to prepare 1.5-mm-thick polyacrylamide gels containing a horizontal linear gradient of 0–8 M urea and a compensatory inverse gradient of 10–7.5% acrylamide. A 5% acrylamide stacking gel, pH 6.8, was also used. For the analysis of proteins previously unfolded in 8 M urea, the stacking gel contained the same concentration of denaturant agent. Proteins were diluted in distilled water with the addition of 10% glycerol, 0.002% bromphenol blue, and for unfolded samples, 8 M urea. Electrophoresis was performed at 40 mA at 4 °C in a 0.05 M Tris glycine buffer, pH 8.0, using a Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Proteins were subjected to electrophoresis at least twice to ascertain reproducibility of results. After completion of electrophoresis, polyacrylamide gels were either silver stained (Blum *et al.*, 1987) or processed for Western blot analysis.

Western Blot Analysis—Proteins were passively transferred to nitrocellulose membranes overnight at 25 °C in presence of 50% methanol and 1% glutaraldehyde. After washing membranes with BBS containing 0.05% of Tween-20 (T-BBS), nonspecific sites were blocked with Blotto (2.5% non-fat dry milk, 2.5% liquid gelatin, 0.05% Tween 20, 0.01% thimerosal, 0.001% antifoam A) for 4 h at 4 °C. Nitrocellulose was then washed with T-BBS and incubated overnight at 4 °C with BBS containing 10% Blotto and one of the following reagents: (i) soluble CD4 and rabbit polyclonal anti-CD4 antibodies, (i) a rabbit anti-idiotypic preparation, or (iii) rabbit anti-CDR peptide antibodies. After washing with T-BBS, membranes were incubated with ^{125}I-protein A (DuPont NEN) in Blotto for 2 h at room temperature, washed extensively with T-BBS, dried, and exposed to x-ray film at −70 °C. Following exposure, membranes were incubated again with a rabbit anti-isotypic preparation, ^{125}I-protein A, dried, and exposed to x-ray film.

RESULTS

To characterize the urea-induced unfolding transitions of intact Ig molecules and respective subunits, including $F(ab')_2$, Fab, and Fc fragments, the various preparations were subjected to transverse urea gradient gel electrophoresis and silver staining. Fig. 1*A* shows silver-stained gels of native or unfolded whole G3 molecule, a mouse monoclonal antibody (IgG1, κ) specific for an epitope expressed on the transmembrane glycoprotein of human immunodeficiency virus. Starting with the native protein, unfolding occurs only at a relatively high denaturant concentration and is observed in an abrupt transition that has its midpoint at about 6.5 M urea. Prior to this transition, there is no indication of any partial unfolding, as demonstrated by the constant mobility of the band of protein across the gel. Consequently, no intermediates in unfolding appear to be populated kinetically and no sequential unfolding of different Ig domains can be observed. Such urea-gradient electrophoresis pattern is indicative of a two-state model of protein folding transitions where only the native and unfolded states are present in significant concentration during the unfolding reaction. However, several bands are observed when the whole Ig molecule is denatured in 8 M urea prior to electrophoresis. One of these bands is identical to that obtained with the native protein, whereas the other bands exhibit a progressively higher electrophoretic mobility and appear to refold only at very low urea concentrations. The bending downward of these bands of unfolded proteins indicates the presence of more compact intermediates in rapid equilibrium with the unfolded state. These results show that, although partially folded intermediates cannot be detected during the unfolding of whole Ig molecules, the refolding reaction occurs through different compact nonnative conformations at urea concentrations below the transition midpoint.

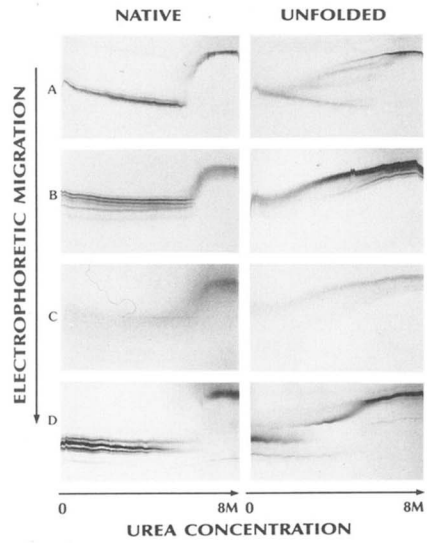

FIG. 1. **Transverse urea gradient gel electrophoresis patterns of folded and unfolded intact G3 or G3 fragments detected by silver staining.** G3 is a mouse monoclonal antibody (IgG1, κ) specific for the transmembrane glycoprotein of the human immunodeficiency virus. *A*, whole Ig molecule; *B*, $F(ab')_2$; *C*, Fab; *D*, Fc. The initially native (*left*) and unfolded (*right*) proteins were electrophoresed at pH 8.0 at 4 °C. The linear gradient of 0–8 M urea was superimposed on an inverse gradient of 10–7.5% of acrylamide.

To further characterize the folding properties of Ig molecules, experiments were performed by using G3-derived $F(ab')_2$ and Fab fragments. These fragments were obtained, respectively, by pepsin and papain digestion of whole G3 molecules. Fig. 1*B* shows the folding-unfolding transitions of $F(ab')_2$ fragments. The native molecule exhibits an unfolding pattern similar to that obtained with the whole G3 molecule, indicating a two-state folding transition. Starting with the unfolded molecule, only the molecular species with higher electrophoretic mobility can be detected when compared with the correspondent profile

[1] The abbreviations used are: BBS, borate-buffered saline; CDR, complementarity determining region; V, variable.

exhibited by the unfolded whole Ig molecule. This band, which shows a progressively higher electrophoretic mobility at lower denaturant concentrations, reaches a migration point indicative of refolding only at 1–2 M urea. A similar unfolding-refolding pattern was obtained by using Fab fragments from the same antibody molecule (Fig. 1*C*). This result indicates that the folding transitions exhibited by $F(ab')_2$ and Fab fragments under these experimental conditions are not affected by the presence of the disulfide bond linking the two H chains nor by the number of subunits or total Ig domains.

The unfolding-refolding pattern of Fc fragments obtained by papain digestion of the G3 molecule is shown in Fig. 1*D*. As for the whole Ig molecule, $F(ab')_2$ and Fab fragments, a two-state transition with no accumulation of intermediates is detected during the unfolding of the native Fc fragments. The refolding transitions exhibited by fully unfolded Fc fragments appear more complex. The unfolded state predominates at high urea concentrations. At intermediate concentrations of denaturant, there is a reversible transition to a more compact state. Following this transition, a fraction of the total proteins maintains a partially unfolded state. An additional discontinuous band of refolded molecules appears at the lowest urea concentrations, indicating a slow folding transition that leads to complete refolding.

To better analyze the folding characteristics of Ig molecules, transverse urea gradient gel electrophoresis in conjunction with Western blot analysis was utilized to investigate the effects of progressive denaturation/renaturation of Ig molecules on the functional properties of the variable region, *i.e.* antigen binding and idiotypic expression. Leu3a, a mouse monoclonal antibody (IgG1, κ) specific for an epitope localized on the V1 region of the human CD4 molecule, was used for this experiment. The structural correlates of antigen binding and idiotypic expression of this monoclonal antibody have been well characterized (Attanasio *et al.*, 1991). The association of heavy and light chain is required for CD4 binding, whereas distinct idiotypes are expressed on isolated chains. An immunodominant idiotype is specifically localized on the light chain and requires the presence of the three-dimensional configuration of this chain for its expression.

Native and urea-denatured forms of Leu3a were subjected to transverse urea gradient gel electrophoresis and passively transferred to nitrocellulose membranes overnight at room temperature in presence of methanol and glutaraldehyde. The bifunctional reagent was included in the transfer buffer to facilitate the detection of association intermediates that might spontaneously refold following transfer to nitrocellulose. Nonspecific binding sites were blocked and, to ascertain the presence of idiotypic expression, a rabbit polyclonal anti-idiotypic preparation was used. To determine presence of antigen binding, membranes were incubated with soluble recombinant CD4, washed, and incubated again with a rabbit polyclonal preparation specific for the human CD4 molecule. Binding of rabbit antibodies was then visualized by adding ^{125}I-protein A. As shown in Fig. 2*A*, anti-idiotypic antibodies are able to recognize the entire continuous band obtained by subjecting to electrophoresis native Leu3a molecules. No binding can be observed above the transition midpoint. The same nitrocellulose membrane was then incubated with rabbit anti-isotypic antibodies, which recognize a variety of linear and conformational determinants localized in several regions of the immunoglobulin molecule. Fig. 2*B* shows that this reagent is able to recognize the native protein as well as the unfolded form. Thus, as expected, at urea concentrations above the transition midpoint the heavy and light chain tertiary configuration responsible for idiotypic expression is lost. Fig. 2*C* shows the binding pattern of the rabbit anti-Id to the unfolded Leu3a molecule subjected

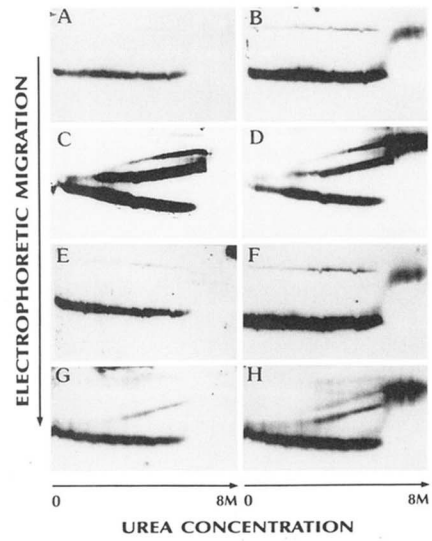

FIG. 2. **Western blot analysis of unfolded and refolded intact Leu3a, a mouse monoclonal antibody (IgG1, κ) that recognizes the human CD4 molecule.** Native (*A* and *E*) or unfolded (*C* and *G*) Leu3a was subjected to transverse urea gradient gel electrophoresis and passively transferred to nitrocellulose membranes. After incubation with various rabbit antibody preparations and ^{125}I-protein A, membranes were exposed to x-ray films at −70 °C. *A* and *C*, nitrocellulose membranes incubated with rabbit anti-idiotypic antibodies; *E* and *G*, nitrocellulose membranes incubated with soluble recombinant CD4 and rabbit anti-CD4 antibodies. Membranes shown in *A, C, E,* and *G* were then probed with rabbit anti-isotypic antibodies, incubated with ^{125}I-protein A, and exposed again to x-ray films (*B, D, F,* and *H*, respectively).

to progressive renaturation through gradient gel electrophoresis. It is evident that this reagent is able not only to recognize the molecular form with fast mobility, but also the partially refolded molecular forms with slow mobility below the transition midpoint at 6.5 M urea. Therefore, these latter forms appear to retain the three-dimensional structure necessary for idiotypic expression. No binding of the rabbit anti-Id is present above the transition midpoint, although the anti-isotypic preparation is able to recognize the full unfolded form of Ig molecule (Fig. 2*D*). The results of Western blot analysis performed to assess the effects of unfolding and refolding of Leu3a on CD4 binding are shown in Fig. 2*E*. Below the transition midpoint, the whole Leu3a molecule fully retains antigen binding ability. No CD4 binding is observed with the fully denatured form, which is recognized by the anti-isotypic reagent (Fig. 2*F*). As shown in Fig. 2*G*, progressive refolding of urea-denatured molecules leads to reappearance of CD4 binding below the transition midpoint. However, only one of the protein bands at higher molecular weight, the one exhibiting the fastest mobility, appears to bind soluble CD4 molecules. This result indicates that the quaternary structure of the Leu3a molecule is not well preserved in the molecular forms exhibiting slower electrophoretic mobility. In both cases, the anti-isotypic preparation recognizes the fully denatured molecular forms. Together, these results demonstrate that both quaternary and tertiary configurations of heavy and light chains of Leu3a are retained at all urea concentrations below the transition midpoint, whereas only secondary structures responsible for the binding of the anti-isotypic reagent are present above the unfolding transition. To confirm this observation, polyclonal rabbit preparations obtained by immunization with synthetic peptides corresponding to the various CDRs of Leu3a were used for additional Western blot analysis. Fig. 3, *A* and *C*, show representative results obtained using rabbit antibodies specific for the first and third CDR of the light chain, respectively. It is evident that these antibody preparations are able to recognize

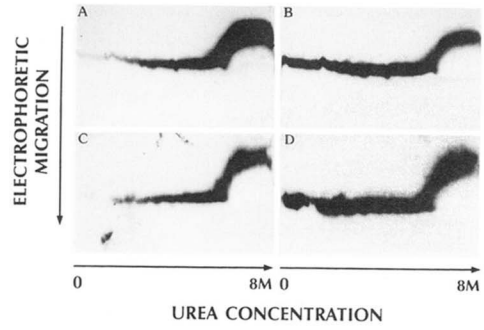

FIG. 3. **Western blot analysis of native Leu3a molecules subjected to transverse urea gradient gel electrophoresis and transferred to nitrocellulose membranes.** *A*, membrane incubated with a rabbit polyclonal preparation generated by immunization with a synthetic peptide corresponding to the first CDR of the light chain of Leu3a; *B*, membrane shown in *A*, incubated again with rabbit anti-isotypic antibodies; *C*, membrane incubated with a rabbit polyclonal preparation generated by immunization with a synthetic peptide corresponding to the third CDR of the light chain of Leu3a; *D*, membrane shown in *C* and incubated again with rabbit anti-isotypic antibodies.

the unfolded form of the Leu3a molecule, confirming the presence of immunoreactive secondary structures at the highest urea concentrations. Since no binding was observed at the lowest urea concentrations, the nitrocellulose membranes were incubated again with the anti-isotypic antibodies. As shown in Fig. 3, *B* and *D*, the anti-isotypic antibodies were able to bind the Leu3a molecule at all urea concentrations. These results indicate that partial denaturation of the antibody molecule is necessary for the exposure of the linear determinant that are recognized by the antipeptide antibodies. However, this denaturation cannot be detected on the basis of changes in electrophoretic mobility. Similar binding patterns were obtained using antipeptide antibodies specific for the second CDR of the light chains and for the second and third CDR of the heavy chain of Leu3a (data not shown). Together, these results show that several different immunoreactive forms occur in the folding pathway of Ig molecules.

DISCUSSION

In this study, we have attempted to gain insight into the urea-induced unfolding profiles of Ig molecules and derived fragments. For this purpose, we have utilized transverse urea-gradient gel electrophoresis in conjunction with Western blot analysis. Results obtained from this study demonstrate that, at pH 8.0 and 4 °C, the urea-induced equilibrium denaturation of whole antibody molecules and enzyme-digested fragments, including $F(ab')_2$, Fab, and Fc fragments is completely reversible. These molecules exhibit a similar two-state unfolding profile in which possible partly folded intermediates are only negligibly populated. The unfolding transition observed in our study has its midpoint at about 6.5 M urea and appears to be slow on the time scale of electrophoresis. Such kinetics of transition can be inferred from the lack of continuity of the protein bands detected by silver staining of molecules in the original native conformation subjected to transverse urea gradient gel electrophoresis. Indeed, several studies have shown that a sharp continuous band generated through the transition zone is typical of a rapid folding equilibrium in comparison to the time of electrophoresis, whereas a smeared or discontinuous band indicates a slow transition (Goldenberg and Creighton, 1984; Creighton, 1986). The two-state behavior appears to be the result of the association of individual Ig domains into folding units. Cooperative interactions reduce the number to a few partially folded intermediates that become populated during the folding process (Murphy *et al.*, 1992). The existence of these highly cooperative units is confirmed by the finding that the urea-induced unfolding transitions of intact Ig molecules, $F(ab')_2$, Fab, and Fc fragments occur with identical patterns and appear therefore independent from the total number of domains and/or disulfide bonds present in the molecules. However, intermediate states can be detected during refolding that show a transition profile different from that observed for the unfolding reactions. This observation is consistent with results obtained in other experimental systems. Even for small monomeric proteins that display two-state behavior during unfolding of the native structure, refolding is frequently complex and involves multiple unfolded forms that refold with different rates (Creighton, 1980). The more frequent detection of intermediates during refolding reactions indicates that the largest energy barrier in the folding transition lies close to the native state and that the rate-determining step in unfolding requires disruption of the cooperatively folded native protein. The difference between the pattern of the unfolding and refolding reactions also confirms that unfolding is slow on the time scale of electrophoresis. The ability of transverse urea gradient gel electrophoresis to identify specific folding profiles that can be the result of minor structural variations is validated by the detection of different refolding properties between fully denatured Fab and Fc fragments. These fragments, although composed of four Ig domains linked by a disulfide bond and therefore structurally homologous, are characterized by stronger and more extensive interdomain interactions that may be responsible for the distinct refolding profiles (Rowe, 1976).

Studies performed by tryptophyl fluorescence to assess the unfolding reaction of single chain Fv Ig fragments expressed in *E. coli* (Pantoliano *et al.*, 1991) indicate that unfolding of single chain Fv occurs through a simple two-state model with no evidence of intermediate species, similar to the unfolding pattern that we have observed by transverse urea-gradient gel electrophoresis. However, the same simple two-state reaction mechanism applies for the refolding transitions of recombinant Fv, whereas our data indicate the presence of folding intermediates during the refolding reactions of whole Ig molecules and derived fragments. These observations on the refolding reactions seem to confirm data obtained by circular dichroism and tryptophyl fluorescence using whole isolated Ig light chains as well as isolated variable and constant domains from the same light chains. Such data demonstrate that folding intermediates that are not detected during unfolding appear in the refolding reactions (Goto *et al.*, 1988; Tsunewaga *et al.*, 1987; Goto and Hamaguchi, 1982). Complete reversibility of unfolding has been observed in all cases.

It is well established that immunochemical methods constitute powerful tools for the analysis of domain folding and subunit assembly (Blond and Goldberg, 1987; Murry-Brelier and Goldberg, 1988). Thus, to better analyze the Ig folding characteristics, whole antibody molecules previously subjected to transverse urea gradient gel electrophoresis were examined by Western blot analysis. Leu3a, a mouse monoclonal antibody specific for the human CD4 molecule, was used for this experiment. The molecular and structural basis of the epitope and idiotypic specificities of Leu3a have been well characterized (Attanasio *et al.*, 1991). Both heavy and light chain are required for antigen binding, whereas different idiotypic specificities are localized on separated and isolated chains. These idiotypic specificities require the presence of three-dimensional configuration of the isolated chains for their expression. Therefore, the ability of soluble CD4 and a specific anti-idiotypic reagent to bind the antibody molecule subjected to progressive unfolding/refolding has been used to identify the presence of its quaternary and tertiary configuration. Results from these experiments show that both soluble CD4 and the anti-idiotypic preparation are able to recognize the entire band of protein that

is present below the transition midpoint. Thus, not only the tertiary configuration of heavy and light chain is not affected by concentration of urea as high as 6.5 M, but also their association in the correct quaternary structure is maintained. CD4 and anti-idiotypic binding cannot be detected above the transition midpoint, demonstrating that the unfolding transition coincides with the disappearance of both quaternary and tertiary molecular interactions. However, Western blot analysis performed with completely denatured proteins subjected to urea-gradient electrophoresis demonstrates that immunoreactivity is completely recovered following gradual renaturation of unfolded molecules. The anti-idiotypic preparation was also able to bind the molecular forms with slower mobility obtained during refolding of the antibody molecule, indicating the presence of tertiary configurations. The lack of detectable antigen binding suggests that quaternary interactions are not present. These forms with slower mobility are characterized by a larger hydrodynamic volume and might correspond to the alternatively folded states of whole Ig molecules observed under acidic conditions and described by Buchner *et al.* (1991). Such alternatively folded states are different from both the denatured and the native state, exhibit alterations in the hydrodynamic properties, and seem to be stabilized by well defined tertiary contacts comparable with those found in native proteins. These characteristics appear indeed similar to those exhibited by the Leu3a forms with slower mobility and further demonstrate the intricate mechanisms involved in protein structure formation.

Additional experiments showed that an anti-isotypic preparation was able to detect the protein band above the transition midpoint, therefore indicating the presence of immunoreactive secondary structures in the fully denatured forms of the antibody molecule. To confirm this finding, Western blot analysis was performed using polyclonal preparations obtained by the immunization of rabbits with synthetic peptides corresponding to various CDRs of Leu3a (Attanasio *et al.*, 1990, 1991, 1993). These preparations recognize linear determinants associated with the Leu3a variable region and are not able to bind the Ig molecule in its native conformation. Partial denaturation, which can be obtained for example by adsorption to solid phase surfaces such as polystyrene wells, is necessary for immunoreactivity. The antipeptide reagents were able to bind the unfolded Ig molecule, indicating the presence of organized secondary structures in the fully denatured form. These data confirm recent experimental results which indicate that proteins fully denatured in urea or guanidine hydrochloride are often very compact, with persistent hydrophobic clustering and considerable residual secondary structure (Dill and Shortle, 1991). Interestingly, the antipeptide antibodies were unable to recognize the protein bands at the lowest urea concentration, suggesting that partial denaturation/unfolding of the antibody molecule is necessary for the binding of the antipeptide reagents. This unfolding is minimal and cannot be detected by changes in the electrophoretic mobility. These data indicate that, in addition to the major unfolding transition present at about 6.5 M urea, smaller modifications in the conformation of the Ig molecule occur at lower urea concentrations and can be detected by immunochemical methods.

Together, results from this study suggest that transverse urea-gradient gel electrophoresis in conjunction with immunochemical techniques is a valid method to compare the configurations of renatured and native proteins and to detect a wide range of conformational changes.

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