Perturbation of the antigen-binding site and staphylococcal Protein A-binding site of IgG before significant changes in global conformation during denaturation: an equilibrium study

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Although conformational perturbation of the active sites of many enzymes has been reported to precede global molecular conformational changes [Tsou (1993) *Science* **262**, 380–381], little effort has been made to compare the susceptibility of the ligand-binding site of proteins and the protein molecules as a whole to perturbation by denaturants. Immunoglobulin is chosen in this study to address this problem. It is found that the variable and constant regions (Fv and Fc) of a monoclonal antibody of an IgG subclass against adenylate kinase lose their abilities to

bind antigen and staphylococcal Protein A after treatment with guanidinium chloride concentrations considerably lower than those required to change the global conformation of the antibody as a whole, as detected by fluorescence and second-derivative UV absorption spectroscopy. These results indicate that both ligand-binding sites of the antibody concerned are more fragile than the molecule as a whole and that the Fv and Fc regions of the antibody molecule unfold sequentially during denaturation.

INTRODUCTION

Many enzymes had been reported to be inactivated by guanidinium chloride (GdmCl), urea or heat before gross molecular conformational changes could be detected by conventional physicochemical methods [1–5] (for a review, see [5]). It has therefore been proposed that the active sites of enzymes are much more prone to perturbation during denaturation than the rest of the molecule [6]. However, the susceptibility to denaturants of the ligand-binding sites of proteins as compared with the global structure of the molecule has been little explored and it remains to be ascertained whether the functional sites of non-enzyme proteins are likewise more susceptible to perturbation than the molecules as a whole. Plasma fibronectin loses its agglutination activity at a lower temperature than that required to induce conformational changes as detected by its immuno-reactivity [7]. The inhibition of choline transport of the carrier protein in erythrocytes by *n*-alkanols involves the unfolding of a small but essential part of the molecule [8]. The interaction of the storage protein legumin from *Vicia faba* to form micelles was perturbed at urea concentrations lower than those required for global conformational changes of the molecule [9].

Immunoglobulin is a Y-shaped molecule containing two ligand-binding sites, one for its specific antigen at the N-terminal variable region (Fv) and the other for staphylococcal Protein A (SpA) at the C-terminal CH2 and CH3 hinge region (Fc) [10]. In this investigation, changes in ligand-binding abilities of both the Fv and Fc regions of immunoglobulin are compared with global molecular conformational changes during denaturation in GdmCl of different concentrations.

MATERIALS AND METHODS

Reagents

The monoclonal antibody McAb3D3 of the IgG subclass was raised against adenylate kinase (EC 2.7.4.3), and purified as

described previously [11]. Adenylate kinase was isolated from rabbit muscle [12]. Horseradish peroxidase-labelled SpA and goat anti-mouse IgG conjugate were purchased from Bio-Rad. Tetramethyl benzidine (TMB) was used as the substrate for horseradish peroxidase. GdmCl was an ultrapure product of Schwartz/Mann. Biotinamidocaproate *N*-hydroxysuccinimide ester (BNHS) and ExtrAvidin Peroxidase were purchased from Sigma. Avidin was the product of Serva. Other reagents were local products of analytical grade.

Measurement of second-derivative absorption spectra

Antibody at a final concentration of 175 µg/ml was incubated with different concentrations of GdmCl overnight at 4 °C. The second-derivative spectra were scanned at 20 °C using a Beckman DU-7500 spectrophotometer. The relative changes in the peak-to-trough distance between 287 and 283 nm of the second-derivative spectra in different concentrations of GdmCl were taken to represent exposure of the aromatic residues [13].

Measurement of fluorescence

Antibody at a final concentration of 16 µg/ml was incubated with different concentrations of GdmCl overnight at 4 °C. The intrinsic fluorescence emission spectra (excitation wavelength 280 nm) between 300 and 400 nm were recorded at 20 °C with a Perkin-Elmer MPF-66 fluorescence spectrophotometer. The bandwidths for both excitation and emission were 10 nm. The shifts of maximum emission wavelength from native state were taken to represent the relative changes in fluorescence for denatured antibodies.

Determination of Fc-binding activity

Binding of antibody McAb3D3 to horseradish peroxidase-labelled SpA was taken as a measure of the binding activity of

Abbreviations used: Fc, constant region of IgG (CH2 and CH3 domains); Fv, variable region of IgG (VH and VL domains); GdmCl, guanidinium chloride; PBST, PBS/Tween; SpA, staphylococcal Protein A; TMB, tetramethyl benzidine; BNHS, biotinamidocaproate *N*-hydroxysuccinimide ester.

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the Fc region. The antibodies (McAb3D3) were first coated on to the wells of microplates by overnight incubation in PBS at 4 °C. Wells were then further treated with 5 mg/ml gelatin, after which GdmCl of different concentrations was added to wells in triplicate. The supernatants were decanted after 1 h of incubation and the wells thoroughly washed with PBST (0.5 % Tween 20 in PBS) to remove denaturants. Labelled SpA was then added and incubated for 1 h. After being rinsed three times with PBST, TMB solutions were added. Reactions were stopped by the addition of 100 μ l of 2 M H₂SO₄. A_{450} of the TMB solution was measured on a Bio-Rad 3550 microplate reader and found to be proportional to the amount of antibody applied per well over the range 0–0.1 μ g per well. The amount of antibody that retained binding ability after treatment with GdmCl of different concentrations was quantified by comparison with standard curves.

Determination of Fv-binding activity

Biotinylation of adenylate kinase

Biotinylated adenylate kinase was prepared by the method of Bonnard et al. [14]. The biotinylation reagent, BNHS, was dissolved in dimethylformamide to a final concentration of 1 mg/ml as a stock solution. One volume of biotinylation reagent stock solution was mixed with 30 vol. of adenylate kinase (0.5–1.0 mg/ml in 0.1 M Na₂HCO₃ buffer, pH 9.0) and incubated for 3 h. After overnight dialysis against 500 vol. of PBS at 4 °C, an equal volume of glycerol was added to the dialysed solution, which was stored at –20 °C.

Determination of antibody-binding activity of biotinylated adenylate kinase

Avidin (0.5 μ g/100 μ l/well) was adsorbed on to the microplates and the wells then blocked with 5 mg/ml gelatin. Biotinylated adenylate kinase of different concentrations was added to each well and incubated for 1 h. After three rinses with PBST, antibodies (McAb3D3) were added to each well and incubated for 1 h. After three similar rinses, 100 μ l of horseradish peroxidase-labelled goat anti-mouse IgG conjugate (1:3000 dilution) was added to each well, and incubated for 1 h. After rinsing, 100 μ l of TMB solution was added. Reactions were stopped by the addition of 100 μ l of 2 M H₂SO₄. A_{450} was recorded on a Bio-Rad 3550 microplate reader and shown to be linear with the amount of biotinylated adenylate kinase over the range 0–50 μ g/ml.

Determination of Fv-binding activity

Binding of antibody (McAb3D3) to biotin-labelled adenylate kinase was taken as a measure of the binding activity of the Fv region. The antibodies were first coated on to the microplates. The remaining non-specific binding sites on the wells were then blocked with 5 % gelatin. GdmCl of different concentrations was then added to the wells in triplicate, which were incubated for 1.5 h, and then rinsed with PBST five times to remove the denaturant. Biotinylated adenylate kinase (100 μ l) was added. After three rinses, 100 μ l of ExtrAvidin Peroxidase (1:500 dilution) was added and the mixture incubated for 30 min. After three rinses with PBST, 100 μ l of TMB/H₂O₂ solution was added and the reaction stopped after 10 min with 100 μ l of 2 M H₂SO₄. The change in A_{450} was recorded on a Bio-Rad 3550 microplate reader and shown to be linear with immunoglobulin over the range 0–0.12 μ g/well.

RESULTS

Fluorescence emission spectra of native and denatured immunoglobulin

To characterize the overall conformational changes in immunoglobulin in different concentrations of GdmCl, intrinsic fluorescence and second-derivative absorption spectra were measured. The denatured protein shows increased emission intensity together with a shift in the emission maximum from 330 to 350 nm, indicating a change in the microenvironment of the aromatic residues of the immunoglobulin molecule with an excitation wavelength of 280 nm (results not shown).

Second-derivative spectra of native and denatured immunoglobulin

The difference in peak-to-trough distance between 287 and 283 nm represents a change in the microenvironment of tyrosine residues (results not shown).

Conformational changes in immunoglobulin in different concentrations of GdmCl

The relative changes in the shift in emission maximum of the intrinsic fluorescence spectra and the peak-to-trough distance of the second-derivative spectra of immunoglobulin in GdmCl of different concentrations are shown in Figure 1. The maximum emission wavelength of native immunoglobulin at 330 nm is shifted gradually to longer wavelengths during denaturation, reaching 350 nm in 5 M GdmCl. Little shift in maximum emission wavelength can be observed in GdmCl concentrations lower than 1.5 M, and significant shifts take place between 1.5 and 2.8 M, with the concentration of GdmCl required to bring a 50 % shift in emission maximum at approx. 2.3 M. The second-derivative spectra of immunoglobulin do not change in GdmCl concentra-

Figure 1 Relative changes in fluorescence, second-derivative spectra and antigen-binding activities of the Fc and Fv parts of McAb3D3 in GdmCl (GuHCl) of different concentrations

Curve 1, relative shift of fluorescence emission maximum wavelength; curve 2, relative values of peak-to-trough distance between 287 and 283 nm of second-derivative absorption spectra; curve 3, relative activity of Fc region of McAb3D3; curve 4, relative activity of Fv region of McAb3D3.

tions lower than 1 M. The peak-to-trough distance changes sharply between 2.2 and 3.1 M GdmCl, with a transition point at 2.7 M. The immunoglobulin is unfolded almost completely in 3.5 M GdmCl. The fluorescence and second-derivative spectra monitor the structural environment of the aromatic residues in the molecule, the latter being more sensitive to micro-environmental changes in tyrosine residues and the former more sensitive to those of tryptophan residues.

Change in activity of Fc region of McAb3D3 in GdmCl

The Fc part of an immunoglobulin comprises the two C-terminal constant regions of the heavy chains that interact with the complement system and with specific receptors on the surface of a variety of cells. Part of the polypeptide chain of SpA is a series of four highly homologous regions [15] each of which is able to bind to the Fc part of IgG from various species. The structure of the complex formed by human Fc fragment and fragment B of Protein A has been determined to high resolution by Deisenhofer [10]. In this study, changes in binding of the Fc region of McAb3D3 to SpA in different concentrations of GdmCl were followed by monitoring the binding to horseradish peroxidase-labelled SpA. The amount of functionally reactive antibody remaining after treatment with GdmCl of different concentrations was quantified by comparison with standard curves obtained in the absence of GdmCl. Figure 1 curve 3 shows the percentage of Fc region that remains capable of binding to SpA after denaturation. It is clear that the SpA-binding ability of Fc is affected by GdmCl at relatively low concentrations. The concentration of GdmCl required to inactivate the Fc part of immunoglobulin by 50% is about 1.0 M, and the ability of the Fc region to bind to SpA is completely destroyed at GdmCl concentrations higher than 2 M.

Change in the activity of the Fv part of McAb3D3 in GdmCl

The Fv part of an immunoglobulin contains its specific antigen-binding site. Several structures of the antigen-bound Fab fragment have been determined [16]. For measurement of the binding ability of McAb3D3 to adenylate kinase as its antigen by indirect ELISA, biotinylated adenylate kinase was prepared and tested by ELISA to ensure the immunoreactivity of the conjugates (see the Materials and methods section). The results show that the binding of biotinylated adenylate kinase to McAb3D3 is proportional to the amount of biotinylated adenylate kinase. In this study, changes in binding of the Fv region of McAb3D3 to adenylate kinase after treatment with different concentrations of GdmCl were followed by monitoring binding to biotinylated adenylate kinase. Figure 1 curve 4 shows the relative percentage of Fv region that retains its ability to bind to adenylate kinase. It is evident that the Fv region loses its ability to bind to adenylate kinase by 50% at a GdmCl concentration of 0.18 M, which is much lower than that required to bring about conformational changes in both the Fc region (Figure 1 curve 3) and the molecule as a whole, as detected by fluorescence and second-derivative spectra (Figure 1 curves 1 and 2). Although it could be objected that some refolding of the denatured antibody might have occurred with partial recovery of antigen-binding activity after GdmCl was washed out, if some re-activation had occurred, this could only result in, if anything, an overestimation of the antigen-binding activity remaining, making the difference in GdmCl concentration required to bring about conformational changes and inactivation of the antibody even greater.

DISCUSSION

Sequential conformational changes during unfolding

The two-state model has been the dominant theory of protein folding for several decades. This is based on the experimental results of identical relative changes in conformation as measured by different methods during the folding–unfolding transitions [17]. In recent years, technical progress in both kinetic and equilibrium studies of protein folding and unfolding has revealed the existence of relatively stable intermediates [18]. Several folding intermediates of Fab and Fab_{alk} have been recognized by Lilie et al. [19], and the denaturation kinetics of its antigen-binding activity for both Fab and Fab_{alk} have also been compared [20]. These authors found that the rate of loss of its antigen-binding activity, as measured by ELISA, is much faster than that of its secondary structure, as measured by CD, and concluded that the correct quaternary structure of Fab disappeared first, followed by a loss of secondary structure. In the present study, the activities of the Fv and Fc regions of immunoglobulin are separately compared with global conformational changes of the molecule in GdmCl of different concentrations. The transition points of the overall conformational change in immunoglobulin McAb3D3 with increasing concentrations of GdmCl differ significantly as detected by second-derivative spectroscopy and by fluorescence. The difference suggests that the unfolding of immunoglobulin does not follow an ‘all or none’ model. Moreover, the Fc and Fv regions are inactivated at much lower GdmCl concentrations, 1.0 and 0.18 M respectively, than required for the overall conformational change, suggesting that the perturbation of the antigen-binding site and SpA-binding site precedes overall changes in conformation during GdmCl denaturation. With increasing GdmCl concentrations, the antigen-binding site is affected first, followed by the SpA-binding site, and the overall conformational change takes place last. This indicates a non-parallel sequential unfolding between sub-domains of the immunoglobulin molecule.

The Fv and Fc regions are more fragile than the immunoglobulin molecule as a whole

Although previous studies in this laboratory have shown that the active sites of many enzymes are more fragile than the molecules as a whole [6], little effort has been made to compare the susceptibility of the ligand-binding site of proteins and the overall structure of protein molecules. From the results presented above, it appears that, like many enzymes, the ligand-binding sites of immunoglobulin are also more fragile than the molecule as a whole. The substrate-binding sites of many enzymes are situated in the cleft region between domains [21], whereas in immunoglobulins, antigens bind to the interfaces composed of the N-terminal domains of the light and heavy chains, and the SpA-binding site consists of residues from the CH2 and CH3 domains of immunoglobulin heavy chain [8]. The resemblance of ligand-binding sites of enzyme and non-enzyme proteins is further illustrated in the structure of the catalytic sites of abzymes by the transformation of the non-catalytic binding site of immunoglobulin to the catalytic site of an enzyme without marked alterations in other regions of the same molecule [22].

The conformation of the protein macromolecule is held together by a large number of weak bonds either of different nature or of similar nature but with different environments and consequently different in stability; protein unfolding is of necessity a complicated process. At low concentrations of some denaturants, the disruption of some particularly susceptible

bonds, essential to keep the enzyme or protein in an active state, inactivates the enzyme without conspicuous structural changes. Results presented in the present study indicate that the unfolding of a non-enzyme protein, immunoglobulin, is likewise a sequential or stepwise process, and its Fv and Fc regions are more easily perturbed than the molecule as a whole.

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