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A 'MOLTEN GLOBULE'-LIKE UNFOLDING INTERMEDIATE OF A FOUR DOMAIN PROTEIN, THE Fc FRAGMENT OF THE IgG MOLECULE

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SUMMARY: The Fc fragment of human IgG1 can be trapped in a stable intermediate state during thermal denaturation. In this conformation the molecule is compact with a native-like secondary structure, however, the tertiary structure is perturbed as revealed by intrinsic fluorescence measurements, the near-UV CD spectra and by mapping of antigenic sites with monoclonal antibodies. Similar phenomena were recently described for a few globular proteins of small size, and termed 'the molten globule' state. Our observation is a unique example of this phenomenon for a four domain protein.

It is clear, that the cooperative all or none mechanism to describe protein unfolding has no general validity (1), and recent studies have revealed other mechanisms that go through stable or metastable intermediates (1-6). Detection and characterization of unfolding intermediates is important for understanding protein self-organization and stability. The biphasic heat absorbtion curves of the Fc fragment of immunoglobulins called our attention to this protein for further investigation (7,8).

The Fc fragment is composed of the four C terminal domains of the two identical heavy chains of IgG (9,10). These domains have an almost identical folding pattern, and they are arranged pairwise, connected by covalent /-S-S-/ and noncovalent forces. The Fc fragment carries several specific binding functions of IgG, and it is considered as a quasi independent part of the molecule. The native conformation and binding functions of Fc are retained even when it is isolated as a fragment (11).

We observed that a stable intermediate form (I) can be obtained after the human Fc fragment has been heated through its first transition. In an attempt to characterize the unfolding pathway of human Fc and to elucidate the factors stabilizing the ordered structure we studied the I form with a variety of physico-chemical techniques.

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MATERIALS AND METHODS

Human myeloma IgG1 (Bal) was isolated as described previously (12). The Fc fragment of IgG1 was prepared by papain digestion and purified as described by Porter (13). The homogeneity of preparation was checked by SDS polyacrylamide gel electrophoresis. Protein concentration was determined using the same extinction coefficient $E^{1\%}_{280}$=13.8 as for IgG. Experiments were carried out in 100 mM acetate buffer in the pH range between 4.0 and 5.5.
Calorimetric measurements were performed using a DASM-4 differential scanning calorimeter at a heating rate of 1°C/min. Solution concentrations of 0.5 to 1.5 mg/ml were employed. CD spectra were recorded on a Jasco 40C dichrograph using standard procedures. Sedimentation velocity experiments were performed in a MOM /Hungarian Optical Works/ 3170/b analytical ultracentrifuge at 20°C according to (12). Fluorescence intensity and anisotropy were measured at an excitation wavelength of 280nm and an emission wavelength of 340nm in an Applied Photophysics SP3 instrument as described in (14).
In affinity chromatographic experiments the Fc was absorbed on and eluted from a SpA-Sepharose CL-4B column (15). Ion exchange chromatography was performed on a Mono S column with a Pharmacia FPLC system. The elution buffer was 100 mM acetate, pH 5.0, and a 0-1 M/20min NaCl gradient was used at a 1 ml/min flow rate.
Solid phase competitive RIA was carried out according to (16) using monoclonal antibodies specific for the CH2 domain /kindly provided by Dr. G.Sármay, Department of Immunology, L.Eötvös University, Göd/ and for the CH3 domain /OB7/ and the CH2-CH3 contact region /8a4/. These latter antibodies were kindly provided by Dr. Roy Jefferis and characterized in (17).

RESULTS

Differential scanning calorimetry: Thermal denaturation profiles of the Fc fragment of human IgG obtained by differential scanning calorimetry exhibit two well separated transitions above pH 4. The T_m values and the transition enthalpies of both transitions are pH dependent, where T_m is the temperature at which the heat capacity function exhibits a local maximum. At pH 4.5, the first transition at T_m=58°C is accompanied by an enthalpy change of ΔH=530 kJ/M. The second transition at T_m=76°C is characterized by a ΔH value of 490 kJ/M. During the second transition some irreversible aggregation occurs which may contribute to the observed thermodynamic effect. Entropy changes calculated from the $\Delta H - T_m \Delta S = 0$ equilibrium relation represent the lower limits for the real transition entropies because the entropy change of an irreversible process is necessarily larger than that of the corresponding reversible one. These values are 1.6 kJ/M/deg and 1.4 kJ/M/deg for the first and second transitions, respectively.

Sequential heating experiments show (Fig.1) that the transitions are separate, apparently irreversible processes. Thus, after the protein has been heated through the first transition to 64°C and cooled down, the second transition is the only one observed upon rescanning. In a subsequent scan neither transition appears.

In the simplest model, the calorimetric profile of Fc reflects a two-step denaturation of the folded fragment, that can be described by the following

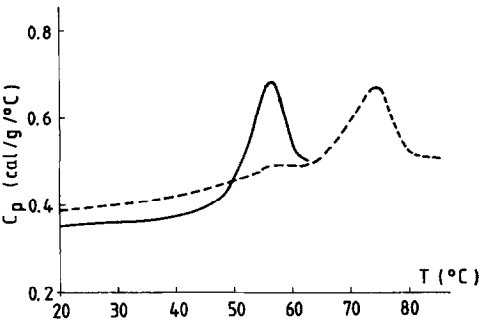

Fig.1. Temperature dependence of partial heat capacity of the Fc fragment of human IgG at pH 4.5 in 100 mM acetate buffer.
—— first heating up to 64°C; --- second heating

reaction scheme:

$$N \dashrightarrow I \dashrightarrow D$$

where N denotes the folded state, D is the heat-denatured state which is formed on heating through both transitions, and I is an intermediate state which is formed as a result of the first transition. This intermediate form of Fc is stable even at room temperature for a long time, at least for several days. The properties of this intermediate state were further investigated by a variety of physico-chemical techniques in order to characterize its physical nature.

Ultracentrifugation: Sedimentation velocity experiments show that the I form is as compactly folded as the native conformation. Sedimentation coefficients characteristic of the overall shape of the entire macromolecule are virtually identical both for the intermediate, $s_{20,w}=4.1\pm0.1$ S, and for the native state, $s_{20,w}=4.1\pm0.1$ S. These experiments do not show any evidence for aggregate formation in the intermediate state.

CD measurements: The far-UV CD spectrum from 200 to 250nm contains information about the amount of regular structural elements, i.e. α-helix and β-structure in proteins, while the near-UV region CD from 250 to 310nm depends on the environment and clustering of the aromatic side chains.

All Fc domains are folded in a similar way into a pair of β-pleated sheets (9). The far-UV CD spectrum of such a structure is characterized by a single negative trough at 220nm (18). The CD spectra of the N and I forms in the amide region are very similar, suggesting that the secondary structure is largely unaltered in the I form (Fig.2a).

Significant differences arise in the aromatic region of the circular dichroic spectra, however (Fig.2b). The ellipticity of the I form is decreased by about 30% in the 250-310nm region, and the shape of the spectrum is also slightly distorted. These results show that the asymmetric environments and the

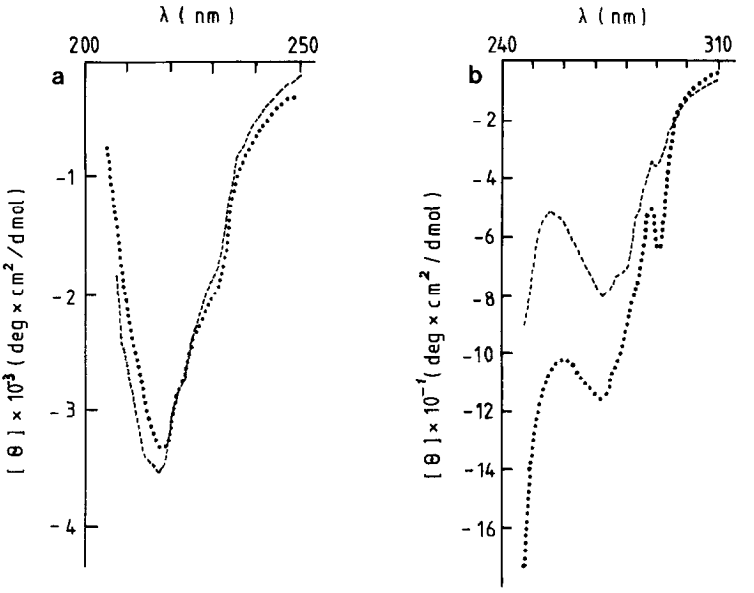

Fig.2. Circular dichroic spectra of the two forms of the Fc fragment in 100 mM acetate buffer, pH 5.0 at 25°C.
····· native (N) state; ---- intermediate (I) state.
a.) far-UV region 250-200 nm
b.) near-UV region 310-250 nm.

specific tertiary structure around aromatic side chains have been substantially perturbed in the intermediate state as compared to the native state.

Fluorescence measurements: Two apparently conflicting results were obtained from the intrinsic fluorescence measurements on the N and I forms. The value of the steady state fluorescence anisotropy in state I was the same as in the state N, thus, A=0.13 in both states. This confirms the ultracentrifuge and CD evidence that the intermediate form of Fc was not unfolded, by showing that the fast intramolecular movements of the aromatic side chains on the ns time scale were not less restricted than in the native conformation.

In contrast, the intensity of the tryptophan fluorescence in the I state was almost twice that of the N state. As Trp fluorescence is critically dependent on specific interactions between the tryptophan and its environment, the largely unquenched emission of fluorescence in the intermediate state indicated that the tertiary structures around Trp residues had been significantly disturbed.

Binding studies: To detect rearrangements of hydrophobic patches and clusters of charged residues on the surface of the Fc molecule brought about by the N → I transition, SpA-Sepharose affinity chromatographic and FPLC Mono S ion exchange chromatographic experiments were performed.

Protein A (SpA) is a major cell wall component of most strains of Staphylococcus aureus which binds tightly to the Fc region of IgG. X-ray diff-

Table 1

Apparent dissociation constants for the binding of various monoclonal antibodies to the corresponding domains of Fc

The specificity of the monoclonal IgG	$K_d(M)^*$	
	N	I
CH2	$2\ 10^{-8}$	$8\ 10^{-8}$
CH2-CH3	$0.8\ 10^{-8}$	$3\ 10^{-8}$
CH3	$1\ 10^{-8}$	$5\ 10^{-8}$

* $K_d(M)$ values were estimated from the inflexion points of RIA inhibition curves.

N - denotes Fc in native state

I - denotes Fc in intermediate state.

raction studies have revealed that the SpA binding site is localized at the interface of the CH2-CH3 domains and involves residues from two large hydrophobic patches (9). The abilities to bind SpA-Sepharose of both the N and I forms of Fc were compared, and the affinity chromatography profiles were found to be essentially the same. Similar results were obtained by FPLC on Mono S ion exchange column that is, the elution profiles were also indistinguishable.

While SpA affinity chromatography and ion exchange chromatography look at gross structural features, binding of antibodies to specific surface regions can reveal slight differences in the arrangements of surface residues. The binding of monoclonal antibodies specific for the CH3 or CH2 domains or for their contact region was studied by competitive RIA, and the data are summarized in Table 1. In all cases the antibodies exhibited significantly reduced affinities, with an increase of about 5 fold in the apparent dissociation constants towards the I form relative to the native conformation. These observations suggest that subtle changes in the native structure of both domain pairs occured, while dramatic conformational rearrangements or partial unfolding of the chain did not take place.

DISCUSSION

Although intermediate states generally are regarded as partially unfolded states of the main chain, the I form of the human Fc fragment which has been trapped during the thermal unfolding process has many properties in common with the native state. Our studies demonstrate that the I form is compact and has a secondary structure similar to the native one. No significant difference has been found in the fast rotational movements of aromatic side chains taking place on the ns time scale. In addition, the arrangements of hydrophobic patches and the distribution of charged residues is essentially the same in both forms on the surface of the Fc macromolecule.

The I form, however, has a higher entropy and a smaller internal binding energy than the native state. The changes in these thermodynamic quantities suggest the elimination of some kind of organized intramolecular interactions and an increase of flexibility and confromational fluctuations. Because our fluorescence anisotropy measurements cannot detect any change in fast intramolecular movements, we conclude that slow conformational fluctuations of the tertiary structure have increased, resulting in the disturbance and randomization of the specific tertiary structure. This conclusion is supported by the results of the near-UV CD, intrinsic fluorescence intensity and competitive RIA experiments.

Our experiments suggest that the I form of the human Fc fragment is the equivalent of the 'molten globule' state observed for some smaller globular proteins (19-22). Although the domains of Fc are supposed to be independent with respect to their folding and stability, it is interesting that the two domain pairs attain the 'molten globule' state as a whole, as revealed by our binding studies with monoclonal antibodies. The very similar fold of the Fc domains may account for this phenomenon (9).

Based on theoretical calculations it has been proposed that the increase of slow conformational fluctuations in the 'molten globule' state is due to a slight increase of the molecular volume leading to a sharp decrease of Van der Waals intramolecular interactions (23). According to this model, the loss of binding energy is compensated by an increase of conformational entropy, i.e. internal motion is liberated, resulting in a local minimum of Gibbs free energy different from the native state. The molecular basis of the relative stability of the 'molten globule' state is not completely clear. Cis-trans isomerization of proline residues and/or disulphide exchange might contribute to the stabilization of the polypeptide chain in this metastable conformation. Our preliminary results suggest, that for large multidomain proteins such as the Fc not only one, but several minima may exist corresponding to distinct 'molten globule' states.

The existence of the 'molten globule' state for a number of different proteins suggests tnat this state may play a significant role in protein self-organization. Indeed, a native-like intermediate has been shown to accumulate on the refolding pathway of several proteins (2-6,24-26) and this intermediate was demonstrated to be identical to the 'molten globule' state for carbonic anhydrase B and α-lactalbumin (24-26). During refolding of the constant fragment of the IgG light chain, formation of an intermediate with a folded conformation similar to the native protein has been established (27,28). As IgG domains have a very similar folded structure (9,10), this intermediate may represent a 'molten globule' state similar to that which we have studied here.

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