Investigation of the Cooperative Structure of Fc Fragments from Myeloma Immunoglobulin G†

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Received October 24, 1997; Revised Manuscript Received January 28, 1998

ABSTRACT: The cooperative structure of Fc fragments prepared from myeloma human IgG1 was studied using scanning microcalorimetry and fluorescence at pH 4.2−8.0. It was shown that the first to be melted are C_H2 domains whose interaction with each other is rather weak, while that with C_H3 domains is strong. Then C_H3 domains which form a single cooperative block are melted. The data for the structure of the Fc fragment in solution agree with the X-ray data according to which the interaction between C_H2 domains is mediated by the carbohydrate moiety while the two C_H3 domains are strongly associated. The presence of intensive C_H2-C_H3 interaction is a distinctive feature of the state of the Fc fragment in the given pH region as compared to that at pH <4.1 [Tischenko, V. M., et al. (1982) *Eur. J. Biochem. 126,* 517−521; Ryazantsev, S., et al. (1990) *Eur. J. Biochem. 190,* 393−399]. First, cis interactions greatly increase the free energy of the native structure stabilization in C_H2 domains. Second, they decrease this energy for C_H3 domains when compared to the state of the latter at pH 3.8 or within the Fc′ fragment (the dimer of C_H3 domains). The temperature and enthalpy of melting of C_H2 domains coincide in all the samples studied despite heterogeneity of the carbohydrate moiety. Thus, it may be postulated that the conservative part of C_H2 domains makes a cardinal contribution to the interaction of these domains with the carbohydrate moiety.

In addition to the ability to bind antigens, immunoglobulins have a number of other functions, the so-called effector functions the most part of which depends on the Fc[1] fragment (*1*). At present, there are definite ideas on the structure of the Fc fragment obtained first of all from X-ray data (*2*, *3*). It has been shown that the main peculiarity of this structure is connected with the unusual mutual arrangement of C_H2 domains. This difference is explained by the presence in C_H2 domains of a hydrocarbon covalently coupled to a certain amino acid residue and by the presence of an S−S bond between heavy chains in the hinge region (*4, 5*). Appreciable success has been achieved not only in the distribution of definite biological functions between certain domains but also in identification of individual amino acid residues included in active centers (*1*). At the same time, some important questions on peculiarities of the structural arrangement of individual domains, in the first place of C_H2 domains, have not been finally clarified. This is due to the fact that a small number of Fc fragments that have been studied by the X-ray method and a relatively low resolution are available at present.

The thermodynamic data characterizing the stability of both individual domains and interdomain interactions are also scarce. Only estimates of such parameters as the association constant and the free energy of interaction between two H chains (*6*) as well as between isolated domains of the Fc fragment (*7*, *8*) are available. Calculations of the free energy of stabilization of C_H2 and C_H3 domains have also been reported, but they were obtained from processing experimental data with the use of a number of a priori assumptions which per se should be corroborated experimentally (*9*).

The experimental data obtained by direct measurements of several important physical parameters are used to describe thermodynamically the Fc fragment. Scanning microcalorimetry used in the studies made it possible, first, to determine such a key value as heat capacity and then calculate all other thermodynamic functions (*10*). Earlier, a similar approach was applied to study IgGs and their fragments at acidic pHs (*11*). The obtained thermodiagrams showed that, in certain immunoglobulins, in particular in IgG1, the Fab and Fc subunits are quasi-independent subsystems within a whole molecule. It was also demonstrated that this approach can be applied in some cases to investigation of the Fab and Fc fragments per se (*11*−*13*). The use of a more adequate calorimetric technique, a wider combination of structural and thermodynamic methods, and

† This work was supported by the International Science and Technology Centre (Moscow) (Grant 091) and the European Community Concerted Action for Arthritis and Carbohydrate Research.

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[1] Abbreviations IgG, immunoglobulin G; Van, Sem, and Mur: abbreviations of family names of patients; Fc, papain cleavage fragment from IgG containing two C_H2 and two C_H3 domains and part of the hinge region (Fc is the C-terminal half of the heavy chain with the inter-heavy chain disulfide bond intact); Fc′, trypsin cleavage fragment from Fc containing two C_H3 domains; pFc′, pepsin cleavage fragment from Fc containing two C_H3 domains; C_H2 and C_H3 domains, regions comprising around 110 amino acid residues with an "immunoglobulin fold" structure; T_m, melting temperature, corresponding to the maximum C_p for the heat absorption peak; ΔG, free energy of stabilization of the native structure of domains; FITC, fluoresceinyl 5-isothiocyanate; GuHCl, guanidine hydrochloride.

S0006-2960(97)02647-0 CCC: $15.00
Published on Web 03/31/1998

the variety of fragments studied allowed us to analyze this problem in a wider region, including the neutral region where immunoglobulins reveal their basic functions.

MATERIALS AND METHODS

Isolation, Purification of Human Myeloma IgG, and Preparation of Fc and Fc′ Fragments. IgG1 myeloma proteins Van, Sem, and Mur were isolated from the serum of patients with myelomatosis, and Fc and Fc′ fragments were generated according to standard procedures (*14*−*17*). Protein purity was controlled by SDS−PAGE, on 10 or 15% cross-linked gels, under reducing or nonreducing conditions according to ref *18*.

Preparation of C_H2 Domains. C_H2 domains were obtained from the Fc fragment of IgG1 Sem, for the most part as described earlier (*7*). However, we have incubated the Fc fragment prior to trypsin hydrolysis at pH 2.8, and not at pH 2.5.

Preparation of Antisera. Antisera to C_H2 domains and to Fc, pFc′, and Fc′ fragments were prepared by immunizing goats with purified antigens (with or without the fluorescent label).

Preparation of Fc Fragment Derivatives. The FITC−Fc fragment was prepared by treating the Fc fragment in borate-buffered saline [BBS, 200 mM $NaBO_3$, and 150 mM NaCl (pH 9.1)] with FITC for 12 h at 4 °C, the protein/label ratio being 3/1. As a result of the reaction, in 40−50% of all labeled IgG, the label is in the Fc fragment; i.e., Fc fragments rather than Fab fragments are preferentially modified. However, within the Fc fragment, C_H2 domains (60−70%) are preferentially modified. Upon immunization by labeled C_H2 domains (or by pFc′ fragments), antibodies are formed to both antigen determinants of only the protein and the label and their joint determinants. The latter were isolated as a fraction that did not bind during affinity chromatography on columns with immobilized proteins (C_H2 domains or pFc′ fragments) or with the immobilized label. However, they do bind to labeled Fc fragment or its labeled subfragments. Such immobilized antibodies were used to obtain selectively labeled Fc fragments. To obtain two fractions of Fc fragments in which different domains are modified, we used not only corresponding antibodies but also elution with appropriate labeled subfragments. To prepare two fractions of the Fc fragment in which different domains are modified, in addition to corresponding antibodies, we used elution caused by appropriate labeled subfragments. The fractions obtained were passed through the column containing antibodies to the other labeled subfragment. Thus, Fc fragments in which both domains were labeled were removed. The concentration of bound FITC was determined from the OD at 495 nm using an extinction coefficient which corresponded to the appropriate ratio of OD(495)/OD(470) from the data of Mercola et al. (*19*).

Calorimetry Experiments. Scanning calorimetry experiments were performed using a computerized version of the DASM-4A microcalorimeter (*10*). The protein concentrations were between 1 and 2.0 mg/mL for the Fc fragment and 0.5 and 9.0 mg/mL for the pFc′ fragment. The calorimetric measurements were carried out using 10 mM glycine, acetate, or phosphate buffers. Gel filtration on an ultragel ACA-34 column equilibrated with a corresponding buffer was used prior to the calorimetric measurements of the samples.

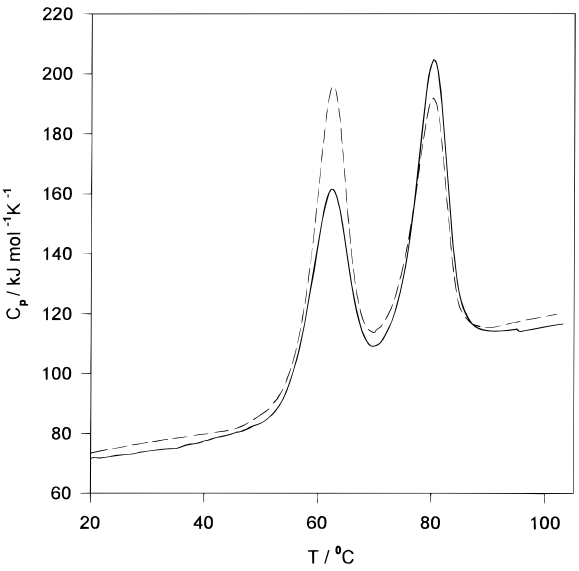

FIGURE 1: Temperature dependences of molar partial heat capacity of Fc fragments of human IgG1 Van at pH 3.8 with 10 mM glycine buffer (solid line) and at pH 4.6 with 10 mM acetate buffer (dashed line).

Analysis of Scanning Calorimetry Data. The partial heat capacity of the protein was calculated from the calorimetric data as described previously (*10*). The partial specific volume was 0.73 mL/g. The observed excess heat capacity function and the heat capacity change upon denaturation were calculated according to ref *20*. This function was deconvoluted into simple constituents corresponding to two-state transitions using the recurrent procedure suggested by Freire and Biltonen (*21*) with some modifications to the algorithm (*10*). The free energy of stabilization of the native structure of domains was calculated as described elsewhere (*22*).

Fluorescence Measurements. Fluorescence measurements were made on a MPF-44A device (Perkin-Elmer). To exclude heat quenching effects, the dependence of both $I(\lambda_1)$ on $I(\lambda_2)$ and $1/Q$ on T/η was determined. The real dependences of the extent of completeness of the transitions obtained by the two methods coincide.

RESULTS AND DISCUSSION

Figure 1 shows molar values of the partial heat capacity of the papain Fc fragment of IgG1 Van at two different pH values and the heating rate of 1 K/min. Identical values were also obtained upon melting of the papain Fc fragment of IgG1 Sem and the Fc fragment of Mur. At neutral pH (4.2−8.0) as opposed to acidic pH (2.4−3.8), in all thermodiagrams the first heat absorption peak is always greater than the second one in both its amplitude and area. The main thermodynamic parameters for separate heat absorption peaks are given in Table 1. Upon melting of relatively complicated protein molecules such as the Fc fragment, sharply pronounced heat absorption peaks on the calorimetric curve are explained by the existence in the studied macromolecule of isolated discrete structural domains. Within the molecule of the Fc fragment, first of all, C_H2 and C_H3 domains should be ascribed to such structures. Thus, the first question arising on analysis of melting curves of the Fc fragment is the

Table 1: Thermodynamic Parameters of Melting of Fc and Fc′ Fragments from Different Myeloma IgG

		temperature (°C)		enthalpy (kJ mol^{-1})	
fragment	pH	first peak	second peak	first peak	second peak
Fc(Van)	3.8	63.0[a]; 63.8[b]	80.0[a]; 80.9[b]	470	718
Fc(Van)	4.2	54.6[a]; 54.1[b]	73.7[a]; 74.2[b]	668	525
Fc(Van)	4.2	55.0[a]; —[b]	73.6[a]; —[b]	661	519
Fc(Mur)	4.2	54.9[a]; —[b]	74.1[a]; —[b]	675	531
Fc(Sem)	4.2	54.2[a]; —[b]	73.4[a]; —[b]	663	519
Fc(Van)	4.6	63.5[a]; 64.0[b]	81.1[a]; 81.4[b]	811	596
Fc(Van)	5.5	66.1[a]; 66.5[b]	82.2[a]; 81.5[b]	861	638
Fc(Van)	8.0	70.8[a]; 72.1[b]	83.3[a]; 82.9[b]	899	693
pFc′(Van)	4.6	—, —	81.3[a]; 74.6[b]	—	542
Fc′(Van)	4.6	—, —	80.7[a]; 74.0[b]	—	537

[a] Scanning microcalorimetry data. [b] Optical data.

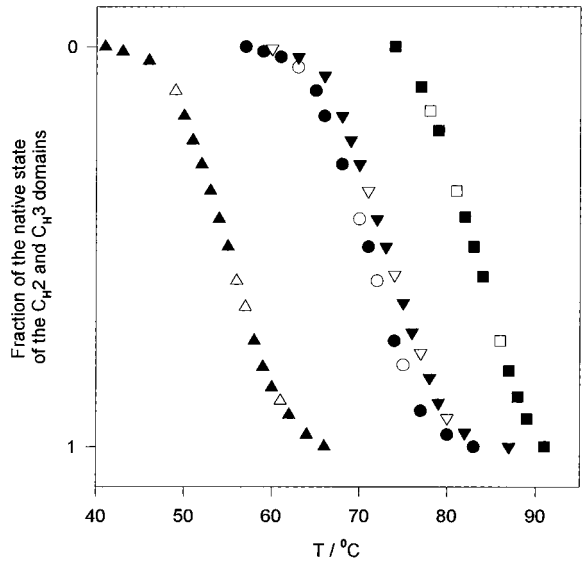

FIGURE 2: Diminution of the fraction of the native state of C_H2 (△ and ▽) and C_H3 domains (○ and □) by increasing the temperature at pH 4.2 (△ and ○) and 8.0 (▽ and □). The curves were calculated from optical (filled symbols) and calorimetric (open symbols) data.

question on the sequence of melting of these domains. The usual approach to the solution of this problem consists of first, studying smaller fragments obtained from a large molecule. The following circumstance should be taken into account. Domains can interact quite intensively within an intact molecule, and therefore, isolated fragments can have greatly varying thermodynamic parameters of melting. This refers first of all to the less thermostable domain since within an intact molecule it is additionally stabilized (or destabilized) by interdomain interactions and its melting is accompanied by destruction of these interactions. Inasmuch as both the C_H2 and C_H3 domains have a β-structural conformation and a similar amino acid composition, two preparations of the Fc fragment have been obtained using the method described in Materials and Methods in which the fluorescent label is present in either the C_H2 or C_H3 domain. This has provided grounds for parallel studies of the domain stability upon melting of the Fc fragment using a joint analysis of the calorimetric and spectral data (*20*).

Figure 2 represents data that are evidence for the changes in the structure of the Fc fragment at pH 4.2 and 8.0 caused by temperature. These changes were recorded with the fluorescent label present only in the C_H2 domain or only in the C_H3 domain. Figures 1 and 2 as well as the data listed in Table 1 demonstrate that the transition temperatures determined using fluorescence labels coincide with the maxima of the first and second peaks on the heat absorption curves. This allows us to conclude that the first peak on the calorimetric curves at pH from 4.2 to 8.0 always denotes melting of C_H2 domains, while the second peak denotes melting of C_H3 domains.

The conclusions are verified by studying subfragments of the Fc fragment with calorimetric and spectral techniques. Moreover, they contain important additional information. The calorimetric studies of the subfragments make it possible to determine their intrinsic thermodynamic parameters without the contribution of interdomain interactions and, consequently, provide grounds for a preliminary evaluation of these interactions. In our case, such subfragments are Fc′ fragments (dimers of C_H3 domains) and C_H2 domains. We have succeeded in obtaining C_H2 domains only from IgG1 Sem.

An attempt was made to study this fragment calorimetrically. However, upon melting of C_H2 domains as both a monomer and a dimer (*7*) due to aggregation effects, no conclusions were made even on the stability of these domains.

Therefore, in working with the fragments, we had to use only the experimental data obtained from the studies of heat denaturation of different Fc′ fragments. However, as follows from the data on measuring thermal stability given in Table 1, there are certain difficulties in analyzing the melting curves of these fragments. The melting temperatures determined from spectral and calorimetric measurements differ significantly. Such a result could be expected since the Fc′ fragment is a dimer formed by C_H3 domains, having no covalent bonds. Thus heat denaturation of the Fc′ fragment may be accompanied by its dissociation into separate polypeptide chains; i.e., the order of the reaction may change. An important feature of changing the reaction order is the dependence of the transition temperature on the concentration of the substance studied. Thus, it is not surprising that the most significant differences in determining this parameter are obtained in the fluorometric (the concentration of the pFc′ fragment being 0.2 mg/mL) and microcalorimetric (the concentration of the pFc′ fragment being 9 mg/mL) experiments.

The dependence of the transition temperature on the protein concentration was observed earlier when studying melting of rabbit pFc′ at pH 2.2−3.5 (*11*). Similar effects were also observed in the calorimetric experiments with human pFc′ fragments at pH 3.5−5.5 (*17*). This is seen from the data (Figure 3a and Table 1) obtained upon melting of the Fc′ fragments. A clearly pronounced dependence not only of the melting temperature but also of the enthalpy of this process on the concentration of the studied preparation is observed (Figure 3). This is direct experimental evidence that the order of the reaction has changed. Thus, if within the Fc fragment the denaturation of C_H3 domains is the reaction of the first order, a much higher order of the reaction should be postulated upon denaturation of the Fc′ fragment. In general coupling between unfolding and dissociation processes, $N_n \leftrightarrow nU$, where n is the number of associated molecules of the native domain N and U is its denatured form, equilibrium thermodynamics predicts the following relationship between the total domain concentration c and the unfolding temperature T_m

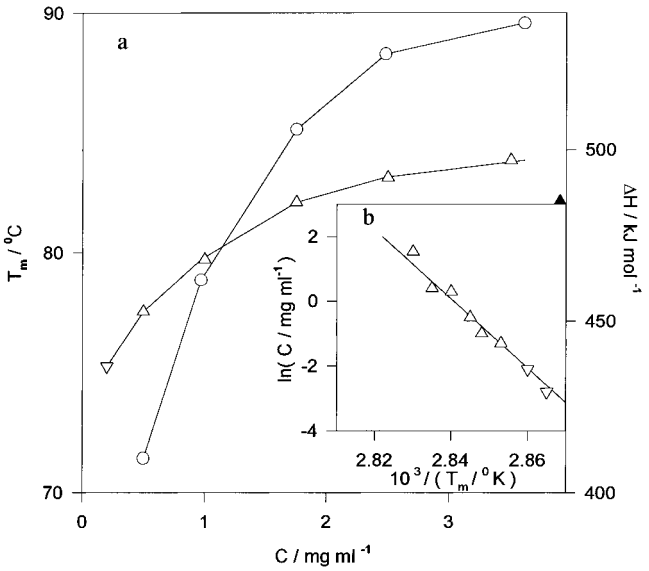

FIGURE 3: (a) Dependence of temperature (△) and enthalpy (○) of melting of the pFc′ fragment on protein concentration according to the scanning microcalorimentry data at pH 4.6. ▽ is the temperature of melting of the pFc′ fragment according to the optical data at pH 4.6. The filled symbol shows the temperature of melting of C_H3 domains within the Fc fragment at pH 4.6. (b) Plot of C vs $1/T$ for the pFc′ fragment at pH 4.6. △ and ▽ are temperatures of melting of the pFc′ fragment determined from the calorimetric and optical data, respectively.

$$\ln c \sim -n\Delta H/(n-1)RT_m$$

where ΔH stands for the unfolding enthalpy per monomer (*23*). The plot of ln c versus $1/T_m$ for the dimer gives a straight line with a slope egual to $-2\Delta H/R$ (Figure 3b). The ΔH value obtained from the slope is 530 kJ mol^{-1} at pH 4.2, close to the calorimetric enthalpy, 542 kJ mol^{-1}. As well, the data listed in Table 2 show that there is good coincidence of the calorimetric enthalpy determined experimentally and the effective enthalpy calculated from the van't Hoff equation for a biomolecular reaction. This demonstrates that denaturation of the Fc′ fragment is indeed a biomolecular reaction.

Therefore, it is impossible to make a direct comparison of the thermodynamic parameters of melting of C_H3 domains within the Fc and Fc′ fragments at an arbitrary concentration of the Fc′ fragment. However, an increase in the protein concentration induces an increase in the thermodynamic parameters of melting of the Fc′ fragment. The temperature and enthalpy of melting of the pFc′ fragment increase to the temperature and enthalpy of melting of C_H3 domains within the Fc fragment (Table 1). These data are a strong argument in favor of the fact that the second heat absorption peak appears due to melting of C_H3 domains. It is natural that the two heat absorption peaks characterizing heat denaturation of C_H3 domains within different fragments still have different shapes.

At first glance, only calorimetric data on Fc′ fragments are sufficient for unambiguous interpretation of melting curves of the Fc fragment. Indeed, at pH 4.2 and 4.6, the denaturation enthalpy and temperature determined by various methods coincide at the second heat absorption peak and Fc′ fragments. However, at pH 5.5−8.0, the melting enthalpy of some Fc′ fragments is much higher than that at the second heat absorption peak (Table 1). As will be shown, this is explained by the fact that the structure of Fc′ fragments can differ under definite conditions from the structure of C_H3 domains within the Fc fragment (*24*). Therefore, for interpretation of the melting curves of Fc fragments at pH 6.0−8.0, we used the data obtained in fluorescence studies (Figure 2 and Table 1).

Thus, proceeding from the thermodynamic data, we can make an unambiguous conclusion on the order of melting of individual elements of the Fc fragment of IgG1. Let us analyze in what way the thermodynamic parameters obtained from the melting curves correlate with the data on the three-dimensional structure of the protein studied. The calorimetric enthalpy of the second heat absorption peak corresponding to the melting of a pair of C_H3 domains is equal to the effective enthalpy (Table 2). This means that a pair of given domains forms a single cooperative block. Let us analyze what structural prejudices exist for the formation of a single cooperative block of two domains. A quite definite conclusion can be made from the analysis of different ways of domain packing in IgGs. It is known that the packing of a pair of C_H3 domains is similar to that of a pair of C_H1-C_L domains in the Fab fragment (*4*). As shown earlier, constant domains form a single cooperative block within the Fab fragment and an intact molecule of IgG and in an isolated form under a wide range of conditions (*11*, *13*).

In addition, a simple analysis of the three-dimensional structure of the Fc fragment revealed by X-ray studies shows that C_H3 domains are closely associated by different types of interactions, including hydrophobic interactions; i.e., a new complex is formed from the two domains (*2*, *4*). This complex has characteristics of a globular protein with the usual tertiary structure. Therefore, it is not surprising that two C_H3 domains form a single cooperative system whose melting proceeds via two states.

On the contrary, the first heat absorption peak corresponding to the melting of C_H2 domains is characterized by a systematic excess of the calorimetric enthalpy over the effective one (Table 2). Such an excess can be, first, a consequence of the fact that in the analyzed range of temperatures at least two cooperative blocks undergo melting. Second, the widening of the peak can be a trivial consequence of different thermal stabilities of C_H2 domains caused by heterogeneity of the hydrocarbon component. The results of the analysis show that the Fc fragment obtained from myeloma immunoglobulin contains more than 10 hydrocarbon components varying in their structure (*25*−*28*). At the same time, the thermodynamic parameters characterizing the first heat absorption peak, as well as its shape, do not change from preparation to preparation in all the Fc fragments studied. This is a strong argument in favor of the fact that the widening of the first peak observed on the heat absorption curve is not connected with different structures of the hydrocarbon components in C_H2 domains and reflects distortions in the melting of a pair of such domains according to the two-state mechanism. Evidently, the actual structural situation is that though hydrocarbons themselves have greatly differing structures; nevertheless, all of them have a more conservative core part. It is namely this conservative part that provides the main number of hydrocarbon contacts with the protein template (*2*−*4*).

The computer analysis of the excess heat absorption curves showed that the first heat absorption peak can be decomposed

Table 2: Thermodynamic Parameters of Melting of C_H2 and C_H3 Domains with Fc and pFc′ Fragments from Diffferent Myeloma IgG[a]

fragment	pH	calorimetric/effective ratio: first peak	calorimetric/effective ratio: second peak	result of deconvolution of the first peak (kJ mol $^{-1}$)	ΔG_N (kJ mol^{-1}) at 25 °C for a pair of domains: C_H2 domains	ΔG_N (kJ mol^{-1}) at 25 °C for a pair of domains: C_H3 domains
Fc(Van)	3.8	1.18	1.03	255 + 213 (470)	25.9	75.8
Fc(Van)	4.2	1.15	0.96	357 + 309 (668)	45.3	48.7
Fc(Van)	4.6	1.17	1.01	452 + 370 (811)		
Fc(Van)	5.5	1.23	0.98	476 + 391 (861)		
Fc(Van)	8.0	1.28	1.05	489 + 420 (899)		
Fc(Sem)	8.0	1.25	1.05	487 + 423 (894)		
Fc(Mur)	8.0	1.29	1.03	496 + 426 (892)	66.1	52.4

[a] The change in heat capacity upon denaturation is determined using the procedure described in ref 20 and is 11 and 14 kJ mol^{-1} K^{-1} for C_H2 at pH 3.8 and 8.0, respectively, and 12.2 kJ mol−1 K−1 at pH 3.8 and 8.0 for C_H3 domains.

in two transitions whose enthalpies are given in Table 2. It is seen that the enthalpy of the first transition always exceeds that of the second transition. The results could be interpreted as melting of two similar domains whose interaction is not intensive, at least much less than in the case of a pair of C_H3 domains. Melting out of the first domain is also accompanied by disruption of interdomain interactions which is reflected in the high enthalpy value of the first transition. It should be noted that for a correct analysis of excess heat absorption curves experments at different pHs are required. Only the existence of a logistic relation between the parameters of melting of C_H2 domains at pH 4.2−8.0 (a systematic excess of the enthalpy of the first elementary transition over the enthalpy of the second one, a smooth growth of the enthalpy of elementary transitions with increase in thermal stability) allows us to consider the possibility that such deconvolution is correct (*10*, *29*).

To analyze to what extent the thermodynamic data correlate with the structural ones, let us once again examine the three-dimensional model of the Fc fragment obtained using X-rays (*2*, *4*). According to these data, the position of C_H2 domains in IgG is unique which is explained by specific glycosylation with Asp297. The presence of hydrocarbons on the contacting surface of each of these domains hinders their association into dimers, characteristic of other pairs of domains. Therefore, the interaction between C_H2 domains proceeds only through the hydrocarbon components per se and is realized via a limited number of contacts. This number of interdomain contacts is insufficient for the domains to form a single cooperative block, and that is why they melt as separate weakly interacting structures.

Only weak interactions are observed between C_H2 domains, but the enthalpy of their melting at pH 4.2−8.0 exceeds systematically the enthalpy of melting of C_H3 domains. This is explained by the circumstance that under such conditions there are strong cis interactions between C_H2 and C_H3 domains (*2*, *3*). These interactions are mediated by 17 amino acid residues pertaining to C_H2 and C_H3 domains that form a contact surface of about 400 Å^2. It is just these interactions that provide additional enthalpy. It is shown that, after incubation at pH 2.8, there appears to be a possibility of achieving selective proteolysis of the peptide links between these domains (*7*, *30*, *31*; see also Materials and Methods), and the intensity of the first heat absorption peak at pH 4.2−8.0 decreases drastically due to the decline of interactions between C_H2 and C_H3 domains. This results in enhancement of domain mobility and decompactization of the Fc fragment which is demonstrated by polarization fluorescence and high rate sedimentation, respectively, characteristic of the fragment at low pH (*32*, *33*). Thus, the melting curves also acquire the shape characteristic of low pH (*11*). The Fc fragment was studied at different pHs because one of the tasks was to investigate the influence of pH on its coopreative structure. As seen from Figure 1 and Tables 1 and 2, a change in the pH range from 4.2 to 8.0 results in a smooth decrease of the thermal stability and enthalpy of domain melting. At pH ≤3.8, the ratio between the enthalpies of domain melting changes qualitatively, which in combination with other data are evidence for a conformation transition (weakening of the interactions between C_H2 and C_H3). X-ray studies showed that the state of some domains of the Fc fragment affects the state of other domains (*24*). However, due to a relatively low resolution of the structure of the Fc fragment of human IgG [there are no data on the structure of the human pFc′ fragment and the structure of the guinea pig pFc′ fragment is known at a far lesser resolution of 3.1 Å (*34*)], it is difficult to make even a qualitative assay of whether such an interaction results in stabilization or destabilization of any domain. The calculation of the free energy ΔG of the native structure stabilization from the calorimetric data allows us to assert that dimerization of C_H3 domains strongly stabilizes them compared to highly homologous uncoupled C_H2 domains when at pH 3.8 cis interactions are weakened. On one hand, the existence of cis interactions at pH 8.0 stabilizes C_H2 domains but, on the other hand, decreases the energy of C_H3 domains (Table 2). In ref *9*, the free energy ΔG_{GuHCl} of C_H2 and C_H3 domains was determined in guanidine hydrochloride for the Fc fragment with alkylated interchain disulfide bonds. Recalculation of this energy for water ΔG_{H_2O} is done as follows:

$$\Delta G_{GuHCl} = \Delta G_{H_2O} - \Delta nRT \ln(1 + Ka)$$

where Δn is the difference in the number of binding sites for GuHCl between the denatured and native states, K is the average binding constant of the sites, and a is the mean ion activity of GuHCl. Each of the three parameters is determined with significant error. Therefore, it is not surprising that in ref *9* the free energies of C_H2 and C_H3 domains practically coincide.

Thus, interpretation of the obtained thermodynamic data is in part based on the X-ray data and agrees with them. However, they enlarge essentially our knowledge on the Fc

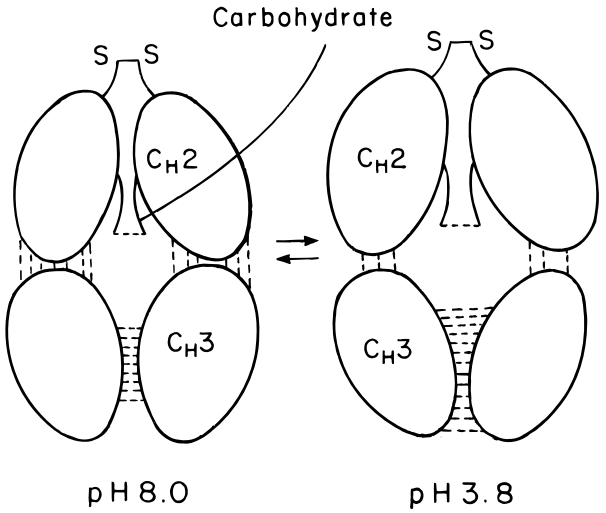

FIGURE 4: Scheme of interdomain interactions in the Fc fragment in acidic and neutral pH. Line density corresponds to the intensity of interactions.

fragment. First, they describe a protein in solution. Second, these data characterize the protein at different pH and temperatures. The most important is that they make it possible to calculate the energy of interdomain interactions and its change at various pHs. This information cannot be obtained from structural data. It should be noted that strong interdomain interactions result in quite different consequences. Dimerization of C_H3 domains increases the free energy of stabilization of their structure. Their interactions with C_H2 domains at pH 8.0 lead to a drop in this energy (Figure 4). Definitely, such a loss in C_H3 domains is compensated excessively by the growing energy of C_H2 domains and the increasing general energy of stabilization of the structure of the Fc fragment. The destabilizing effect of domains was demonstrated in troponin C and calmodulin (*35*), when curves of melting of intact proteins and their fragments were analyzed. To make such a conclusion in this paper, it was sufficient to analyze thermodynamic parameters of domains of the intact fragment at different pH ranges. As will be shown below, the same conclusion can be drawn for the Fc fragment from the data on subfragments (V. M. Tischenko, manuscript in preparation).

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BI972647A