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KINETICS OF DIMERIZATION OF THE BENCE-JONES PROTEIN AU

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The dimerization reactions of complete Bence-Jones protein Au (VC-Au) and of its variable fragment (V-Au) were compared in 0.2 M (ionic strength) sodium phosphate buffer, pH 6.8 at 20°C. The dimerization constant for VC-Au (6.6×10^4 M^{-1}) was slightly smaller than a previously published value for the fragment (1.1×10^5 M^{-1}). The reaction enthalpies were positive for both processes. Temperature jump experiments exhibited two kinetic phases. The relaxation time of the fast phase as well as its concentration dependence and amplitude were almost identical for VC-Au and V-Au. Only small differences were observed in the slow phase. These close similarities between the reactions of the two proteins demonstrate that dimerization occurs mainly via interactions between the variable domains and that the constant domains interfere very little. From the observation of two relaxation times it follows that the dimerization mechanism for both VC-Au and V–Au must include at least three reacting species. Mechanisms with an isomerization between monomers in two conformational states and a single dimer species are excluded by the data. Alternative mechanisms with a single monomeric species but isomerization between dimers give a rather unsatisfactory fit. A good fit can be obtained if it is assumed that both monomers and dimers can exist in two states. Rate constants of the association and dissociation steps are of the order of 10^7 M^{-1} s^{-1} and 10^2 s^{-1}. Isomerization rate constants are in the range of 10 s^{-1}.

1. Introduction

Immunoglobulin molecules consist of several domains and noncovalent interactions between these domains are considered to be important for their structure and function [1,2]. In the present report, the results of a study on the dimerization kinetics of the alkylated (carbamidomethylated) Bence-Jones protein Au (VC-Au) are presented. These results combined with previous [3] and new results on the dimerization of the variable fragment of the same protein (V-Au) provide information about the interactions between homologous domains of light (L) chains.

Bence-Jones protein Au belongs to the κ_1 subgroup immunoglobulin L chain. The amino acid composition and partial amino acid sequence have been published [4] and the X-ray structure of V-Au was solved [5].

2. Materials and methods

Bence-Jones protein Au was purified first by a DEAE-cellulose column (2.5 × 30 cm) (eluted with 0.17 M Tris-HCl buffer, pH 7.5 at 30-35 ml/hr). Fractions were concentrated by means of ultrafiltration to about fifteen millilitres. The concentrated solutions were applied to a Sephadex G-75 column (2.5 × 100 cm), equilibrated with sodium phosphate buffer, pH 6.9, ionic strength = 0.1 M, containing 0.5 g/100 ml sodium azide and eluted with the same buffer at a flow rate of 15 ml/hr. The purification with the Sephadex column was repeated at least three times. Purified samples showed a single band on SDS-polyacrylamide gel electrophoresis in the presence of mercaptoethanol. When the electrophoresis was performed in the absence of reducing agents, this band was rather faint and a new slower migrating strong band appeared, probably due to the formation of disulfide linked dimers. In order to prevent this reaction

the material was reduced and alkylated. About five mg of the protein were dissolved in about one ml of 0.4 M Tris-HCl buffer, pH 8.0 and dithioerythritol was added to a final concentration of 0.2 M. The solution was incubated at 30–35°C for 50–60 min and subsequently cooled to 0°C for 10 min. Iodoacetamide was added in 10% excess over dithioerythritol and the mixture was kept at 0°C for 20 min. This solution was applied to a Sephadex column G-10 which was equilibrated with 0.02 M sodium acetate buffer, pH 5 and eluted with the same buffer. Fractions were collected and dialyzed against distilled water or desalted with a Sephadex G-10 column equilibrated with HCl, pH 4. Finally solutions were concentrated by ultrafiltration and lyophilized. Purity was checked by SDS-acrylamide gel electrophoresis in the absence of reducing agents. Most preparations showed a single band corresponding to the monomer. In some preparations an additional faint band at a place between the monomer and the dimer was found, which, however, had no effect on the kinetic behavior.

Phosphate buffer, pH 6.8 (0.050 M KH_2PO_4, 0.050 M Na_2HPO_4, ionic strength = 0.2 M was used throughout. The total protein concentration C_p is defined as $C_p = C_M + 2C_D$, where C_M and C_D represent molar concentrations of monomer and dimer. The concentration of the protein was calculated from the absorbance at 278 nm using a molar absorption coefficient per monomer unit of 3.3×10^4 M^{-1} cm^{-1}. The effect of dimerization reaction was negligible at this wavelength.

The sedimentation equilibrium measurements were performed with a Beckman Spinco Model E analytical ultracentrifuge equipped with a scanner. Different rotor speeds were used ranging from 15 000 to 24 000 rev/min at 20°C. The column height was 3 mm. The weight average molecular weight M_w was calculated from the following equation where nonideality of the solution was neglected.

$$M_W = (2 - \alpha)M_r = [2RT/(1 - \bar{v}\rho)\omega^2](\mathrm{d} \ln C_p/\mathrm{d}r^2). \quad (1)$$

Here $M_r = 2.4 \times 10^4$ denotes the molecular weight of the monomer, α is the degree of dissociation, r is the distance from the rotor axis, ω is the angular velocity, ρ is the density of the solution, $\bar{v}$ is the partial specific volume of the protein. R is the gas constant and T is the absolute temperature. A value of $\bar{v} = 0.73$ ml/g was calculated from the amino acid composition. Difference spectra were measured with a Cary 118 spectrophotometer. Cells of 1, 2, 5, 10, 20 mm pathlength were employed for the dilution difference spectra. Mismatching with respect to cell length was smaller than 1%. Difference spectra obtained between different temperatures were corrected for volume changes. The temperature was measured with a thermistor, which was placed in the cell. For the recording of difference spectra the total absorbance was less than 0.4 at 235 nm. Temperature-jump experiments were carried out with an apparatus constructed in the Department of Biophysical Chemistry, Biocenter of the University of Basel by Dr. G. Hänisch. The temperature was raised from 16°C to about 21°C in 10 μsec or less. The observed amplitude for a single kinetic phase ranged from 0.0003 to 0.013 expressed as absorbance/cm pathlength. Kinetic data were digitized by a Datalab DL 905 transient recorder and processed by a PDP 11-40 computer (Digital Equipment Co.). Three to five data points were averaged for the slow kinetic phase and six to ten points for the fast phase. This procedure improved the signal-to-noise ratio greatly and enabled us to obtain more reliable data than in the previous study [3]. Also, measurements with solutions more concentrated than 2×10^{-5} M [3] were possible in the present study. This was also important since VC-Au has a 1.5 times larger absorbance coefficient than V-Au. The variable fragment V-Au was prepared and purified as described before [3].

The calculation of the theoretical dependencies of relaxation times on concentration as well as the concentration dependencies of the amplitudes were performed by standard procedures [6]. The numerical calculations and evaluation of rate constants by fitting procedures were carried out with a FACOM 230-60 computer at the Computation Center of Nagoya University. Details of the calculation procedures may be obtained from the authors on request.

3. Results

3.1. Dimerization equilibrium

Sedimentation equilibrium experiments were performed at various total concentrations. The weight

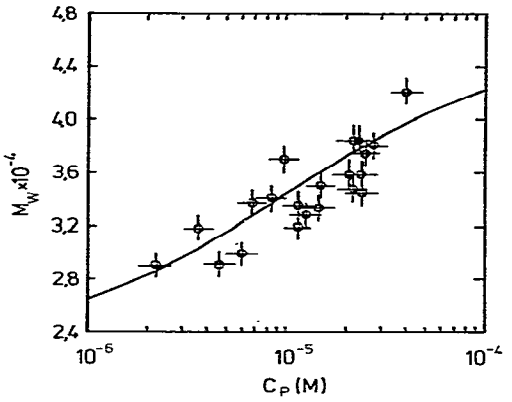

Fig. 1. Weight-average molecular weight (M_W) as a function of the total protein concentration C_p at 20°C in 0.2 M sodium phosphate buffer, pH 6.8. The curve is drawn assuming a monomer-dimer equilibrium with a dimerization constant of 6.6×10^4 M^{-1}.

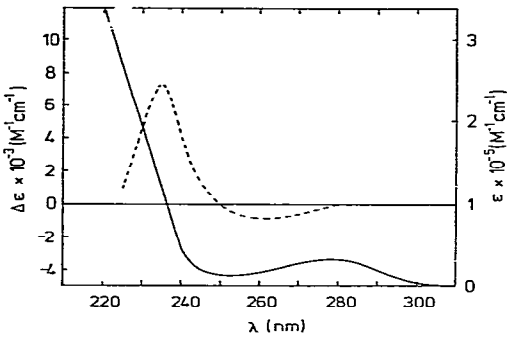

Fig. 2. Dilution difference spectra at 20°C in 0.2 M sodium phosphate buffer, pH 6.8 (- - - -). Concentrations and light path: 3.36×10^5 M and 1 mm in the sample cell, and 1.68×10^{-6} M and 20 mm in the reference cell. $\Delta\epsilon = \epsilon$ (sample) $- \epsilon$ (reference). Absorption spectra of the sample solution refer to the ordinate on the right-hand side.

average molecular weight M_W was evaluated from the middle region of the ultracentrifuge cell in order to avoid errors characteristic to the region near the bottom and the meniscus. The concentration dependence of M_W is shown in fig. 1. For a monomer-dimer equilibrium the dimerization constant K is

$$K = (1 - \alpha)/2C_p\alpha^2 \qquad (2)$$

Experimental points are best fitted by a curve calculated by eqs. (1) and (2) with a value of $K = 6.6 \times 10^4$ M^{-1}.

The difference spectra between a concentrated solution ($C_p = 3.36 \times 10^{-5}$ M) in the sample cell against a 20 fold diluted solution in the reference beam are shown in fig. 2. Only a main difference band centered at 235 nm could be reliably measured. Temperature difference spectra were similar to the dilution difference spectra. When the temperature was increased in the sample cell, the difference band at 235 nm increased indicating a positive enthalpy of dimerization.

3.2. Temperature-jump kinetics

All experiments were performed at a wavelength of 235 nm at the peak maximum of the temperature difference spectra. When the temperature was raised, a very fast unresolved increase of absorbance was followed by a further increase which occurred in two well separated kinetic phases. The total amplitude P_t, including the unresolved phase, was in agreement with static temperature difference spectra. At a concentration of 4.2×10^{-6} M, $\Delta\epsilon = \epsilon_{21°} - \epsilon_{16°}$ was about 1.9×10^3 M^{-1} cm^{-1} in the static measurements, whereas at the same concentration P_t/C_p was about 1.7×10^3 M^{-1} cm^{-1} in the temperature-jump experiments. This agreement is considered to be good for such measurements and there is no indication that a slower phase remained undetected.

In fig. 3 the relaxation times of the fast (τ_F) and the slow (τ_S) kinetic phases are plotted against the total protein concentration C_p. In fig. 4 amplitudes of the kinetic phases, $\Delta\epsilon_F$ and $\Delta\epsilon_S$, and of the unresolved fast change $\Delta\epsilon_0$ are given as functions of C_p. The $\Delta\epsilon$-values are the observed absorbance changes divided by C_p. Table 1 summarizes the directly measured data and the qualitative features of the dimerization reactions for VC-Au and for V—Au.

3.3. Analysis of the relaxation data

Since two relaxation times are observed the reaction mechanism must include at least three species. The two relaxation times are separated sufficiently well

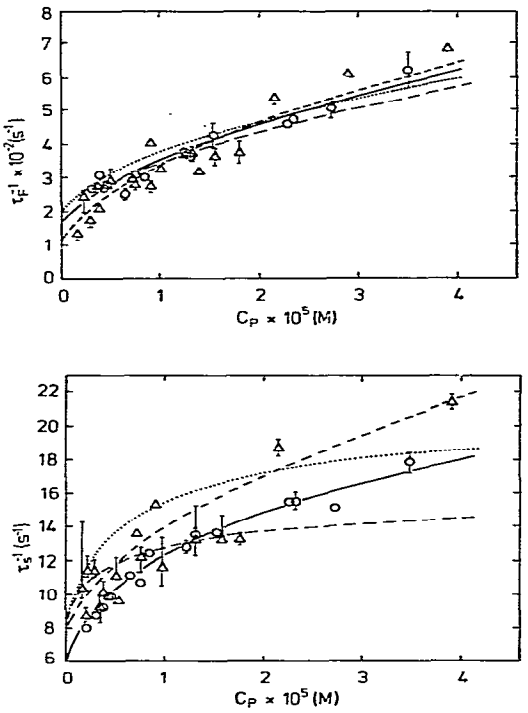

Fig. 3. Concentration dependence of the fast and slow relaxation times τ_F and τ_S for VC-Au (○) and V-Au (△) in 0.2 M sodium phosphate buffer, pH 6.8 at 21°C. Theoretical curves were calculated for the simple mechanism (. . . . .) and the extended mechanism (- - - - -) for V-Au and for the simple mechanism (- . - . -) and the extended mechanism for VC-Au (———). The curves were obtained by a simultaneous best fit to both τ_S and τ_F with the parameters listed in table 2.

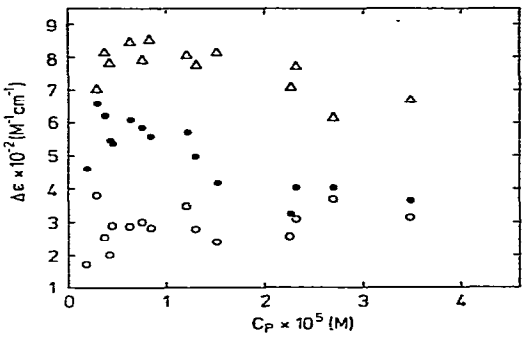

Fig. 4. Concentration dependence of amplitudes. (△): unresolved fast change ($\Delta\epsilon_0$), (○): the fast relaxation phase ($\Delta\epsilon_F$), (●): the slow relaxation phase ($\Delta\epsilon_S$).

that in a first approximation the fast phase may be considered to be uncoupled from the slow step. It follows from the increase of τ_F^{-1} with increasing concentration that an elementary step responsible for the fast phase must be bimolecular. Consequently earlier kinetic data of the dimerization of the variable fragment V-Au were analyzed in terms of a mechanism in which the formation of a dimer D′ is followed by an isomerization step

$$2\,M \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} D' \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} D'' \qquad (3)$$

With the approximation that the first monomer dimer equilibrium occurs on a much shorter time scale than the isomerization step ($k_{21} \gg k_{23}$) the reciprocal relaxation times read [6]

$$\tau_F^{-1} = 4k_{12}C_M + k_{21} \qquad (4a)$$

and

$$\tau_S^{-1} = k_{23}\frac{4k_{12}C_M}{4k_{12}C_M + k_{21}} + k_{32}. \qquad (4b)$$

The equilibrium concentration of monomers is given by

$$C_M = \frac{1}{4K}(\sqrt{1 + 8KC_p} - 1). \qquad (5)$$

It is apparent that τ_F^{-1} increases with increasing C_p since C_M increases according to eq. (5). The reciprocal slow relaxation time τ_S^{-1} also increases with C_p until the fast pre-equilibrium 2 M ⇌ D′ is saturated when it becomes independent of concentration $\tau_S^{-1} = k_{23} + k_{32}$. The amplitude of the fast phase is expected to increase with increasing concentration when C_p is well below K^{-1}. As long as the main spectral signal is provided by the first step, the amplitude of the slow phase is expected to decrease with increasing concentration in the same concentration range in which the 2 M ⇌ D′ equilibrium is not completely shifted towards D′.

Mechanism (3) can therefore describe all experimentally observed concentration dependencies in a qualitative way and provide a satisfactory fit to earlier, less accurate experimental relaxation data for V-Au which were limited to a smaller concentration

Table 1

		VC-Au	V-Au
Dimerization constant (M^{-1})		$(6.6 \pm 1.4) \times 10^4$	1.3×10^5
Dilution difference spectra		similar	
	$\Delta\epsilon_{dim}$ ($=\epsilon_D - 2\epsilon_M$) ($M^{-1}$ cm^{-1})	-3.2×10^4	3.1×10^4
Temperature difference spectra		similar	similar
	$\Delta\epsilon_{235}$ (16.0°→21.0°C) (M^{-1} cm^{-1})	2×10^3 ($C_p = 4.24 \times 10^{-6}$ M)	9×10^2 ($C_p = 7.73 \times 10^{-6}$ M)
Relaxation time	Fast phase	identical	
	Slow phase	slower	faster
Amplitude	fast unresolved phase	$\Delta\epsilon_0 = 650-850$ M^{-1} cm^{-1}	$\Delta\epsilon = 400-500$ M^{-1} cm^{-1}
	fast phase	identical, $\Delta\epsilon_F \sim 300$ M^{-1} cm^{-1} no significant concentration dependence	
	slow phase	$\Delta\epsilon_S = 350-660$ M^{-1} cm^{-1}	$\Delta\epsilon_S = 100-300$ M^{-1} cm^{-1}
		decreases with C_p	

range than the present data. The quantitative fit to the more complete new data for both V-Au and VC-Au is rather unsatisfactory. The fitted curves are shown in figs. 3a and 3b and the corresponding data are summarized in table 2.

An improvement of the quantitative agreement between the theoretical and experimental concentration dependence of the relaxation times can be obtained by completing mechanism (3) with the reasonable assumption that the monomers also exist in two states M′ and M″ which correspond to the states of the dimer D′ and D″.

$$\begin{array}{ccc} 2\,\mathbf{M'} & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & \mathbf{D'} \\ k_{14} \Downarrow \; k_{41} & & k_{23} \Downarrow \; k_{32} \\ 2\,\mathbf{M''} & \underset{k_{34}}{\overset{k_{43}}{\rightleftharpoons}} & \mathbf{D''} \end{array} \qquad (6)$$

Mechanism (3), which is printed in bold type in this scheme, certainly provides the main pathway, since the alternative simplified mechanism $2\,M' \rightleftharpoons 2\,M'' \rightleftharpoons D''$ cannot account for the observed dependencies even in a qualitative way.

This extended mechanism predicts three relaxation times, two of which can, however, be so close that they are not distinguished experimentally. Only with the assumption that two slow relaxation times coincide, a good agreement between the theoretical and experimental relaxation times and their concentration dependencies is obtained (see figs. 3a and 3b and parameters in table 2). To reduce the number of additionally introduced parameters it was assumed that the equilibrium constant $K_M = M''/M'$ is 1.

Because of the large number of parameters such as absorption coefficients and enthalpies of reaction of the individual steps no attempt was made to provide a quantitative fit of the amplitudes. It was, however, verified that such a fit can be obtained for both mechanism (3) and (6) with the corresponding set of rate constants and reasonable values for the amplitude specific parameters.

Table 2
Rate and equilibrium constants of the dimerization of intact Bence-Jones protein (VC-Au) and for the variable fragment (V-Au) according to the simplified mechanism eq. (3) and the extended mechanism eq. (6)

	VC-Au		V-Au	
	simple mechanism	extended mechanism	simple mechanism	extended mechanism
k_{12} $(M^{-1}\ s^{-1})$	8×10^6	2×10^7	10^7	2×10^7
k_{43} $(M^{-1}\ s^{-1})$	–	7×10^5	–	1×10^6
k_{21} (s^{-1})	1.6×10^2	1.8×10^2	1.8×10^2	1.1×10^2
k_{34} (s^{-1})	–	4	–	5
k_{14} (s^{-1})	–	3	–	4
k_{41} (s^{-1})	–	3	–	4
k_{23} (s^{-1})	7	7	16	5
k_{32} (s^{-1})	10	4	8	4
$K_D = k_{23}/k_{32}$	0.7	1.75	2	1.25
$K_M = k_{14}/k_{41}$	–	1	–	1
$K\ (M^{-1})$	8×10^4	6.6×10^4	1.5×10^5	1.2×10^5

The rate constants as well as $K_D = [D'']/[D']$ were obtained from a best fit of the theoretical functions predicted by the corresponding model to the experimental concentration dependencies of the two relaxation times. The fitted curves which correspond to the set of parameters in one of the columns of the table are shown in figs. 3a and 3b. For the extended mechanism with two monomeric species $K_M = [M'']/[M'] = 1$ was assumed.

4. Discussion

The main result of the present study is the demonstration of a striking similarity between the dimerization reactions of complete Bence-Jones protein (VC-Au) and a fragment (V-Au) in which the constant domain is missing. The similarity is apparent already from a comparison of the qualitative features and directly measurable quantities (table 1).

The dilution difference spectra are similar in shape and magnitude for both systems suggesting that the same chromophores are involved and that these are located at the variable domain. For both systems only about 50% of the change in absorbance at 235 nm reflect the dimerization reaction. The kinetically unresolved part of the change is probably due to the effect of temperature on chromophore mobility and solvation. The amplitudes of the fast phase are identical and only the amplitude of the slow phase is significantly larger for VC-Au than for V-Au. Tryptophan 96 is probably responsible for most of these spectral changes. This residue is located in the contact area between the protomers of dimers in crystallized V-Au [5]. Spectroscopically the main difference band at 235 nm cannot be unambiguously assigned to a tryptophan. A difference band at 290 nm was however explained by the reduced exposure of a tryptophan to the aqueous surrounding in the V-Au dimer [3]. Further support for this assignment is lent by the observation that only very small kinetically resolved changes were found for Bence-Jones protein Rei. The variable part of this protein is very similar to Au in its structure [7] and equilibrium constant of dimerization (J. Engel and H. Maeda, unpublished results) but it lacks tryptophan 96.

The fast phase of the dimerization kinetics has not only an identical amplitude but also the corresponding relaxation times and their concentration dependencies are identical. Small but significant differences are apparent between the relaxation times of the slow phase. With regard to the detailed mechanism of the dimerization reaction ti follows from the analysis of the kinetic data that the main pathway is a bimolecular reaction of monomers followed by a monomolecular isomerization of the initially formed dimer (part of the mechanism printed in bold face in eq. (6)). It is

noteworthy that this mechanism was distinguished from other possible model mechanisms on the basis of its ability to explain the correct concentration dependencies of the amplitudes. The alternative mechanism $2M' \rightleftharpoons 2M'' \rightleftharpoons D''$ could explain the concentration dependencies of relaxation times even better but yielded qualitatively wrong amplitude dependencies. This again demonstrates the advantage of even rather qualitative amplitude data which are often ignored in kinetic works. The isomerization reaction is probably a conformational rearrangement of the protomers in the dimer. It leads to an equilibrium between the two states of dimers which is not far from 1 : 1. Suggestive evidence for a conformational change comes from the circular dichroism spectra of V-Au (H. Maeda, unpublished work). The spectra consisted of essentially three bands in a wavelength region between 210 and 250 nm, a negative band around 217 nm, a positive one at 224 nm and a negative one around 235 nm. Mean residue ellipticities of the two bands around 217 nm and 224 nm did change with concentration, whereas that of the band at 235 nm remained unchanged. Since the band around 217 nm includes contributions from peptide chromophore, some change in secondary structure with concentration is likely.

In the framework of the simple mechanism $2M \rightleftharpoons D' \rightleftharpoons D''$ the fast reaction of monomers is essentially uncoupled from the isomerization step since it occurs at a much faster time scale than the $D' \rightleftharpoons D''$ reaction ($k_{21} \gg k_{23}$). The identity of the fast phase in the reactions of VC-Au and V-Au therefore suggests that the first encounter of monomers occurs in the variable region. This step contributes most of the stabilizing energy of dimerization, the values of which are identical for V-Au and VC-Au. It follows that the contacts between the constant domains in the dimer of VC-Au contribute little if any stabilizing energy. This conclusion is suggested already by the similar overall equilibrium constants of dimerization for V-Au and VC-Au as derived by sedimentation equilibrium. As these constants contain contributions of both steps, an unambiguous conclusion could be reached only by kinetic analysis. VC-Au shows a somewhat different second phase. This may be explained by a slight influence of the constant domains on the isomerization reaction.

A more accurate analysis of the data indicates that the simple mechanism $2M \rightleftharpoons D' \rightleftharpoons D'$ should perhaps be extended by the assumption of a second isomerization equilibrium between monomers $M' \rightleftharpoons M''$ (eq. (6)). A better quantitative fit of the concentration dependencies of the relaxation times may be achieved by the increased number of parameters of the extended mechanism, but the reaction $2M \rightleftharpoons D' \rightleftharpoons D''$ remains the main pathway.

The V and C domains of VC-Au are connected by extended peptide chains which are called switch regions [1]. Some contacts are observed between the globular V and C domains of the same chain in crystallized Bence-Jones protein dimers in addition to the extended contacts between the V-domains of different chains and the C-domains of different chains [5,7]. Contacts between domains of the same and of different chains are sometimes called trans and cis contacts respectively [2]. In solution the switch region is extremely susceptible to proteolytic attack and to hydrolysis [1]. The flexible connections probably allow for a large number of quickly variable orientations of the V-domain relative to the C-domain in the VC-monomer. This explains the independence of the association between V-domains (fast phase of the kinetics) on the presence or absence of the C-part. The association is not diffusion controlled but occurs with a rate constant of about $10^7\ M^{-1}\ s^{-1}$. A difference between the diffusion constant of V-Au and VC-Au is therefore not expected to influence the rate.

The isomerization reaction of dimers and perhaps also monomers is found for both V-Au and VC-Au and is therefore a property of the V-domain. The data suggest that the isomerization is slightly altered by the C-domain. This influence probably occurs at the level of the dimeric state via the known contacts between V and C domains of the same chain in the dimers. The weak cis interactions are detectable in the association studies because the total energy involved in dimerisation is rather small. They probably escaped detection in denaturation studies [8] because of their small contribution to the overall stabilizing energy, whereas the strong trans interaction in F_{ab}-fragments were easily demonstrated by the denaturation technique [14].

The data demonstrate that there are essentially no interactions between the C-domains in dimers of VC-Au. This is probably different for the interactions between light (L) and heavy chains (H) [9] or for the

VC-fragment of H chains (Fd-fragment) with L-chains [9,10]. Interaction between these complementary chains is much stronger than self-association between like chains [11]. The enthalpy of interaction is negative [9,12] as contrasted by the positive enthalpy found for VC-Au or V-Au dimerization. Rate constants for the association of L-chains with Fd-fragments or H-chains were reported to be of the order of 10^2–10^4 M^{-1} s^{-1} [1,9,13,17] as compared to the rate constants of about 10^7 M^{-1} s^{-1} found for the fast dimerization step of V-Au and VC-Au. The smaller rate constants and the negative enthalpy indicate the formation of more specific interactions between the complementary domains of L and H-chains. The species specificity of the interaction between L-chains was found to be high [15]. Hybrid formation between L and H chains derived from IgG molecules of different species is however possible although the autologous combination is again prefered [16,17]. The interactions between complementary C_L and C_H1 domains probably account for the stability of heterologous H-L pairs.

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