Biochem. J. (1999) **340**, 193–199 (Printed in Great Britain)

Interactions between a single immunoglobulin-binding domain of protein L from *Peptostreptococcus magnus* and a human κ light chain

Jennifer A. BECKINGHAM*, Stephen P. BOTTOMLEY*, Roger HINTON†, Brian J. SUTTON‡ and Michael G. GORE*[1]

*Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton, Hants. SO16 7PX, U.K., †Centre for Applied Microbiology and Research, Porton, Salisbury, Wilts. SP4 0JG, U.K., and ‡The Randall Institute, King's College London, 26–29 Drury Lane, London WC2B 5RL, U.K.

The placement of a tryptophan residue into a single Ig-binding-domain of protein L from *Peptostreptococcus magnus* has been used to examine the binding interactions between the binding domain and κ light chains (κ-chains). The fluorescence intensity of the mutant domain increases on the formation of a complex with κ-chains. This has been used to determine the K_d of the complex under a range of conditions by using both pre-equilibrium and equilibrium methods. The K_d values determined for the complex with κ-chains at a number of different pH values are very close to those obtained with the wild-type domain, indicating that the mutation has not substantially affected its binding properties. Examination of the reaction between the mutant domain and κ-chains by stopped-flow fluorescence shows that complex formation takes place by two discrete, sequential processes. A fast bimolecular reaction, with a rate constant of 8.3×10^5 $M^{-1} \cdot s^{-1}$ (at pH 8.0 and 25 °C), is followed by a slow unimolecular process with a rate (1.45 s^{-1}) that is independent of the concentration of the reactants. This suggests that a conformational change occurs after the initial encounter complex is formed. The dissociation of the complex at equilibrium occurs in a single process of rate 0.095 s^{-1} at pH 8.0 and 25 °C. Stopped-flow CD studies show that a slow decrease in ellipticity at 275 nm occurs with a rate of 1.3 s^{-1} when wild-type protein binds to κ-chains, suggesting that the conformational transition might involve a change in environment around one or more tyrosine residues.

Key words: dissociation constant, fluorescence, mutagenesis, stopped-flow kinetics, tryptophan.

INTRODUCTION

Protein L is a multidomain cell-wall protein isolated from *Peptostreptococcus magnus* [1]. It consists of up to five repeating Ig-binding domains, depending on the strain of bacterium from which the protein is isolated. Each binding domain is able to bind to the framework region of the variable domain of κ light chains (κ-chains) without disrupting the function of the antigen-binding site. Other bacterial cell-wall Ig-binding proteins include Protein A from *Staphylococcus aureus* [2] and Protein G from groups C and G streptococci [3]. Both of these proteins have multiple repeat domains and bind predominantly to the interface between C_H2 and C_H3 domains of human γ (IgG) heavy chains and also, for Protein A, μ and α heavy chains [4,5]. However, both can also bind weakly to Fab fragments. Protein A interacts with the variable domain of the heavy chain of Ig molecules from the V_HIII gene family [6], whereas protein G binds to C_H1, the constant domain of the heavy γ chain [7]. The function of these proteins *in vivo* is not clear but their ability to bind non-antigenically to Ig as well as other blood proteins such as albumin and protease inhibitors suggest that they help the bacteria to evade the host's immune system [5]. Only 10% of *P. magnus* strains express protein L; however, it has been correlated with the virulence of the bacteria [8]. Patella et al. [9] have also shown that the presence of protein L stimulates the release of histamine from basophils and mast cells, presumably by interaction with the κ-chain of IgE.

The structure of a single Ig-binding domain of protein L (PpL) from strain 312 has been resolved by ^{1}H NMR [10,11]. It reveals a domain consisting of a four-stranded β-sheet and a central α-helix. The solution structure of its complex with κ-chain has also been studied by high-field NMR [12]. These studies show that the residues that show the largest signal differences in the PpL_{312} domain compared with the bound state in the complex are concentrated along the second β-strand and the loop between the helix and third β-strand, implying that these are the contact regions. ^{15}N NMR relaxation measurements suggest that this structure is very rigid with significant flexibility only in the N-terminal region, outside the folded core of the domain [13].

Initial binding studies [1], with immobilization techniques, suggested an association constant (K_a) for protein L to κ-chain of 1.5×10^9 M^{-1}, indicative of a tightly bound complex. This is approx. one-seventh of that found for the reaction with IgG containing κ-chains ($K_a = 10^{10}$ M^{-1}). The K_a for a single Ig-binding domain with κ-chain is 1.6×10^7 M^{-1}, which is approx. 1/100 of that determined when the multi-domain protein L is used [14].

Here we give a detailed study of the κ-chain-binding characteristics of a single Ig-binding domain from protein L isolated from *P. magnus* strain 3316 [15]. This protein contains 78 amino acid residues with high sequence similarity to the domain from strain 312 [14]. Nearly all of the residues of the single domain from the strain 312 implicated by the NMR studies as participating in binding to κ-chains [12] (Figure 1) are also present in

Abbreviations used: κ-chain, κ light chain; PpL, single Ig-binding domain from protein L; F39W, single Ig-binding domain from protein L with Phe^{39} → Trp replacement.

[1] To whom correspondence should be addressed (e-mail M.G.Gore@soton.ac.uk).

© 1999 Biochemical Society

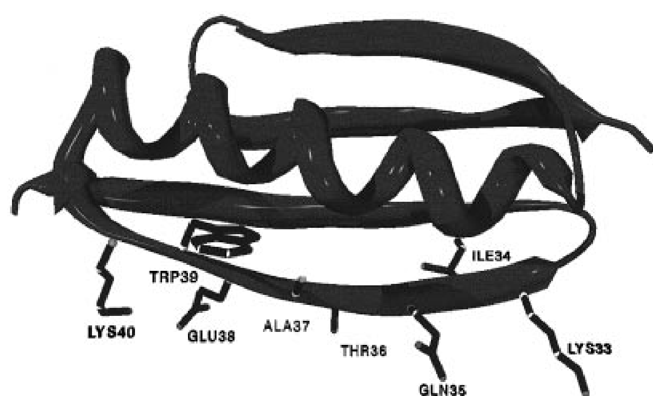

Figure 1 Ribbon diagram of PpL

The figure shows the positions of residues Gln[35], Thr[36], Ala[37], Glu[38], Trp[39] and Lys[40], which have been implicated by NMR studies in the binding interaction [12].

the domain from strain 3316 [15]. The two exceptions are an Ala → Val replacement at position 25 and a Lys → Ala replacement at position 58 (using the numbering system of [15], which is +3 compared with the numbering system of [12]).

We have used site-directed mutagenesis to introduce a unique tryptophan residue so that fluorescence spectroscopy can be employed to characterize the interactions of PpL with free κ-chain and human IgG by pre-equilibrium and equilibrium fluorescence spectroscopy. The substitution made in PpL_{3316} was Phe[39] → Trp (this variant will be referred to hereafter as F39W), positioned near the carboxy end of β-strand 2 among other residues (Gln[35], Thr[36], Ala[37], Glu[38], Lys[40] and Gly[41]) that have been implicated by NMR studies [12] as being involved in the binding interactions with κ-chain (see Figure 1).

MATERIALS AND METHODS

Materials

Phosphate buffers and other reagents were all of analytical grade and purchased from BDH. The immunochemical reagents for the ELISA were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). The human IgG and κ_1-chain for fluorescence experiments were a gift from Dr. Martin Glennie (Southampton General Hospital, Southampton, Hants., U.K.).

Sample preparation

The cloning, expression and purification of the PpL binding domain have all been described elsewhere [15].

Mutagenesis

Site-directed mutagenesis was performed with the mismatch primer method of Kunkel [16]. The mutagenic primer oligonucleotide used to make F39W was (5′-AAATGTTCCTTT-**CCA**TTCTGCTGTTTG-3′), in which the mismatch codon is shown in bold. The sequence of the mutated DNA gene was confirmed by automated DNA sequencing on a LiCoR 4000 DNA sequencer.

Fluorescence measurements

All equilibrium fluorescence measurements were made with a Hitachi F-2000 fluorescence spectrometer, with the temperature maintained at 25 °C by a thermally jacketed cuvette holder. Scans of the emission spectra were made with excitation and emission slit widths of 10 nm. An excitation wavelength of 280 nm and a scan rate of 10 nm/min were used. All measurements were corrected for the inner filter effect by the equation:

$$F_{corr} = F_{obs}\exp(0.5A_{ex} + 0.5A_{em})$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the observed fluorescence intensity, A_{ex} is the absorbance at the excitation wavelength and A_{em} the absorbance at the emission wavelength, both measured in a cell with a 1 cm light path [17].

Fluorescence titrations

Titrations were performed by the stepwise addition of small volumes (2.5–5 μl) of a stock solution of the wild type or F39W (typically 50–100 μM) into 1 ml of κ-chain (usually between 0.8 and 1.5 μM). Readings were taken over a 20 s period after allowing the solution to reach equilibrium. Each titration was repeated three times and the fluorescence intensities were corrected for the inner filter effect (see above) and dilution. The buffer used was 20 mM potassium phosphate at the required pH. All titrations were performed at 25 °C unless stated otherwise. The excitation wavelength was 280 nm and the emission wavelength was 305 nm for wild-type PpL whereas the emission wavelength was 325 nm for F39W. The results given are means ± S.D.

Analysis of saturation curves

The titration curves were analysed by fitting the data to the equation:

$$K_d/(1-\alpha) = [P]_0/\alpha - [B]_0$$

where K_d is the dissociation constant, α is the fractional saturation of PpL sites on κ-chain, $[P]_0$ is the total concentration of PpL and $[B]_0$ is the total molarity of binding sites available for PpL.

Competitive ELISA

This was based on the protocol detailed in [15] except that increasing concentrations of F39W were added to each row of wells at the same time as human IgG containing κ-chains. Thereafter the procedure was the same as in [15]. The concentration of F39W that caused a decrease of 50 % in A_{400} was used to estimate its K_d.

Stopped-flow measurements

All stopped-flow measurements were made with an Applied Photophysics SX.17MV stopped-flow spectrophotometer fitted with 2 ml syringes [mixing ratio 1:1 (v/v)]. Measurements were made over a 0.5, 5 or 50 s period with 1000 data points. An excitation wavelength of 280 nm was selected by a monochromator and the fluorescence emission above 335 nm was measured with a suitable cut-off filter. All solutions were prepared in 20 mM potassium phosphate buffer and adjusted to the required pH at the stated temperature. All concentrations quoted are final concentrations after mixing. The reactions were performed in triplicate and analysed by the manufacturer's software by using double-exponential and single-exponential curve-fitting algorithms. In experiments to determine the rate constant for the association of the complex, the κ-chain concentration was kept constant at 3 μM and the concentration of F39W was varied between 20 and 40 μM. The use of a minimum of a 7-fold excess

© 1999 Biochemical Society

of F39W allowed the reactions to be analysed as pseudo-first-order processes with the relationship:

$$F_t = F_0 e^{-kt} + C$$

where F_0 and F_t are the fluorescence intensities at time 0 and time t respectively, k is the apparent first-order rate constant for the reaction and C is a constant. However, the reaction progress curves were also analysed by a double-exponential equation:

$$F(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C$$

in which $F(t)$ is the fluorescence at time t, A_1 and A_2 are the amplitudes of the different phases of the change in fluorescence, k_1 and k_2 are the rate constants for the first and second phase respectively and C is a constant.

Determination of the rate of dissociation of the complex

Dissociation reactions were performed by mixing a solution containing 3 μM F39W and 3 μM κ-chain with 30 μM wild-type PpL. Native PpL is devoid of Trp residues and therefore has a very low fluorescence emission at 335 nm; owing to its high concentration it binds very rapidly to κ-chain released from its complex with F39W.

CD spectroscopy

CD measurements were made on a JASCO J-720 CD spectrometer. The spectra in the far-UV range (200–250 nm) were measured at a scan rate of 20 nm/min, with a 1 mm pathlength. The spectra were scanned in triplicate, with a protein concentration of 16 μM, a response time of 4 s, a slit width of 500 μm, a bandwidth of 1.0 nm and a resolution of 0.2 nm. The spectra in the near-UV range (250–300 nm) were determined at a scan rate of 20 nm/min with a 2 mm pathlength. The spectra of 100 μM solutions of wild-type PpL, κ-chain and complex were measured in triplicate with a slit width of 500 μm, a response time of 8 s, a bandwidth of 1.0 nm and a resolution of 0.2 nm. Spectral corrections and calculations were performed with the manufacturer's software.

Stopped-flow CD

Pre-equilibrium CD studies were performed with a rapid mixing device from Hi-Tech Scientific (Salisbury, Wilts., U.K.). Reactions were initiated by the rapid mixing of each protein at 100 μM. To achieve these concentrations, a mixing ratio of 6:1 (v/v) was used with 700 μM κ-chain in the smaller syringe and 117 μM PpL in the larger syringe. The reaction was measured over a 10 s period at 275 nm in a cuvette with a pathlength of 4 mm and a response time of 32 ms. Each trace shown is an accumulation of 20 reactions.

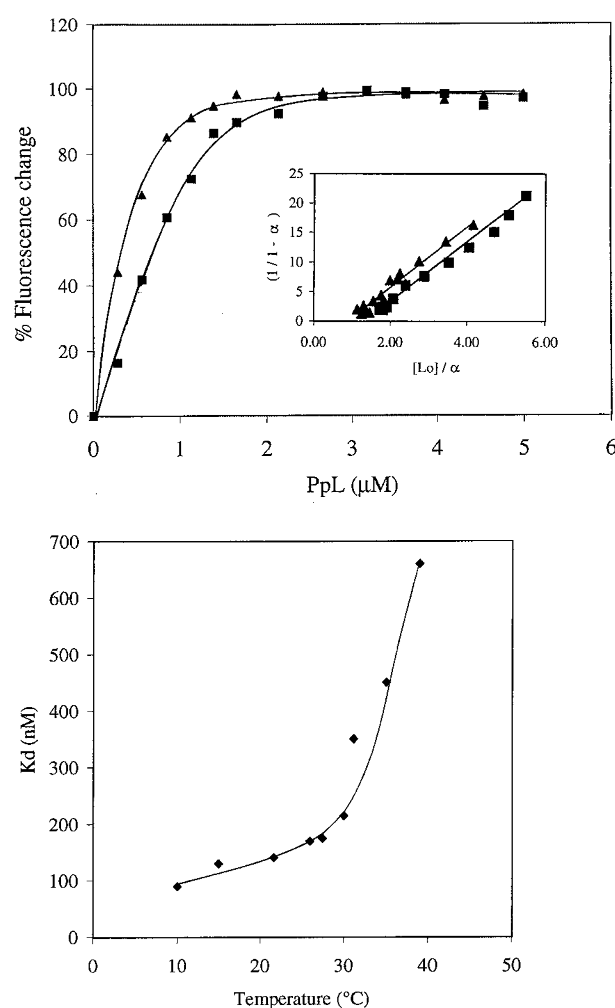

Figure 2 Fluorimetric determination of the stability of the complex formed between κ-chain and PpL

Upper panel: determination of the K_d for the complex formed between PpL and κ-chain. PpL (50 μM) was titrated into 1 ml of 0.8 μM κ-chain in 20 mM potassium phosphate buffer, pH 8.0 at 25 °C, and the fluorescence intensity at 305 nm (▲) was monitored. Similar titrations of PpL into buffer alone were performed and the fluorescence values obtained were subtracted from those obtained with κ-chain to generate the saturation curve shown. For convenience the signal change has been inverted. F39W (stock solution 50 μM) was titrated into 1.5 μM κ-chain in 20 mM potassium phosphate buffer, pH 8.0 at 25 °C, and into the same buffer alone; in each case the fluorescence intensity at 325 nm (■) was monitored. The results were treated as above. The inset shows the graphical determination of the stoichiometry of binding and the K_d for each titration (see the Materials and methods section). Lower panel: K_d for the binding reaction between F39W and κ-chain at various temperatures. The conditions used were as given in the legend to the upper panel.

RESULTS AND DISCUSSION

There are no naturally occurring Trp residues in PpL [15] and therefore its fluorescence properties are limited to the relatively weak emission from its three Tyr sidechains that emit maximally at 302 nm. The fluorescence emission spectrum of the κ-chain used in these experiments shows only a very weak fluorescence at wavelengths near 326 nm despite the chain containing one Trp residue. When equimolar concentrations of the two proteins are mixed the fluorescence intensities obtained over the wavelength range 290–350 nm are approx. 20 % lower (with a minimum at 302 nm) than the sum of the intensities from the two individual proteins. This suggests that one or more of the Tyr residues in the proteins are subject to quenching effects when the proteins form the complex. The apparent quenching of fluorescence on formation of a binary complex between the two proteins was used in titration experiments (Figure 2) to estimate the stoichiometry of binding and the K_d for the equilibrium (note that for convenience the sign of the signal change has been inverted). These titration experiments show that the two proteins combine in the ratio of κ-chain to PpL of 1.0:(1.03 $\pm$ 0.08), and that K_d (see the Materials and methods section) is 132 $\pm$ 3 nM ($n = 3$) at pH 8.0. The K_d is higher than that (62.5 nM) obtained by Kastern et al. [14] with the use of ELISA techniques. The discrepancy might be the result of the different methods used or be due to protein sequence differences between the PpL from the

© 1999 Biochemical Society

Table 1 Effect of pH on K_d at equilibrium determined by fluorescence titration experiments

Results are means ± S.D. ($n = 3$) obtained from wholly independent experiments. Abbreviation: n.d., not determined.

	K_d (μM)	
pH	PpL	F39W
5.0	0.43 ± 0.03	n.d.
6.0	0.30 ± 0.02	0.38 ± 0.07
7.0	0.27 ± 0.02	0.31 ± 0.06
8.0	0.13 ± 0.03	0.16 ± 0.03
9.0	0.48 ± 0.01	0.52 ± 0.06

two strains of *P. magnus* used in the experiments. These include two residues (Ala[25] → Val and Lys[58] → Ala in strains 312 and 3612 respectively) located in the proposed binding site for κ-chain on PpL.

The fluorescence emission from excited Trp residues is usually much higher than that from Tyr residues; it has been shown to be sensitive to the residues' immediate environment and to alter in a characteristic way when the local environment changes [17]. Therefore to shift the fluorescence emission from PpL to a longer wavelength to facilitate further experiments, and to increase sensitivity, a mutant was made by the replacement of Phe[39] close to the proposed binding site [12] by Trp. The fluorescence emission maximum of F39W PpL is at 336 nm when in free solution; this is blue-shifted to 325 nm in the complex with κ-chain, typical of a Trp residue moving into a more hydrophobic environment [17]. These spectral changes could arise because Trp[39] might be located at a position that is directly involved in the binding interactions. NMR studies [12] showed that spectral changes from residues in a homologous domain (PpL_{312}) occur when a complex with κ-chain is formed, and in particular that resonances attributable to Phe[39] undergo chemical shifts. However, it is also possible that the presence of κ-chain in the complex could create a more hydrophobic environment around the Trp residue, resulting in fluorescence signal changes without the residue's direct involvement in binding.

The formation of the complex containing F39W and κ-chain was accompanied by an increase of 55 % in the emission intensity at 325 nm, in contrast with the 20 % quenching of fluorescence noted for the same reaction when PpL was used. This change in fluorescence emission intensity had a peak at 325 nm and was used to examine the nature of the complex formed between F39W and κ-chain in terms of its stoichiometry and K_d.

The saturation curve shown in Figure 2 is the result of subtracting the increase in fluorescence intensity due to increasing concentrations of F39W (determined by the addition of F39W to buffer) from the fluorescence intensity observed when F39W was added to κ-chain. The results were analysed to yield a K_d of 161 ± 27 nM ($n = 3$) with a κ-chain-to-F39W stoichiometry of 1.0:(0.91 ± 0.12) at pH 8.0. The K_d for the complex containing either wild-type or F39W with κ-chain was sensitive to pH and increased on either side of pH 8.0, with the largest increase being between pH 8.0 and 9.0 (Table 1). The similar values of K_d for the interactions with κ-chain and the same sensitivities to changes in pH suggest that the mutation did not affect the nature of the binding interaction and did not cause large structural changes in the domain. This conclusion was further supported by far-UV CD studies (results not shown). The K_d for the reaction was also very dependent on temperature: at pH 8.0 K_d was approx. 80 nM at 10 °C and rose to 620 nM at 37 °C (Figure 2, lower panel).

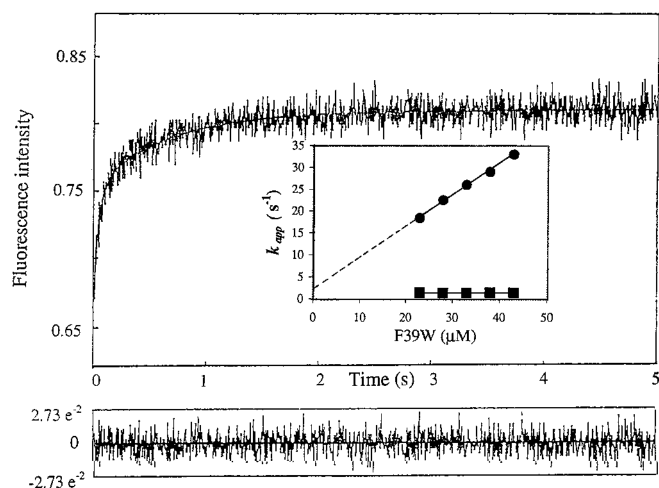

Figure 3 Progress curve for the reaction between F39W and κ-chain

F39W (30 μM) was mixed with an equal volume of 3 μM κ-chain (final concentrations) in 20 mM potassium phosphate buffer, pH 8.0 at 25 °C. The progress curve shows the two-phase fluorescence change observed over the first 5 s of the reaction. The plot underneath shows the residuals of the fit of the data to a double-exponential equation. The inset shows the linear dependence of the rate of the first phase of the change in fluorescence, k_{app}, on F39W concentration (●) and yields values of k_{-1} (intercept on y-axis) of 1.96 s^{-1} and k_1 (slope) of 0.83×10^6 $M^{-1} \cdot s^{-1}$. The inset also shows the effect of the concentration of F39W on the rate of the second phase of the change in fluorescence, k_2 (■).

Pre-equilibrium binding studies

The increase in fluorescence intensity that occurs on mixing F39W with κ-chain was used in pre-equilibrium stopped-flow fluorescence experiments to determine the rates of association [k_1 ($M^{-1} \cdot s^{-1}$)] and dissociation [k_{-1} (s^{-1})] of the complex. From these values K_d could be determined by using the relationship:

$$k_{-1}/k_1 = K_d \quad (1)$$

By the use of excess concentrations of F39W (a minimum of 7-fold molar excess) first-order reaction conditions could be approached and the reaction progress curves analysed as described in the Materials and methods section.

A typical reaction progress curve relating the change in fluorescence intensity observed after the mixing of 3 μM κ-chain and 30 μM F39W is shown in Figure 3. The curve shows an initial fast increase in fluorescence intensity with a half-time of approx. 26 ms followed by a further slow increase with a half-time of approx. 0.5 s. The complete curve could only be fitted to a double-exponential equation (see the Materials and methods section) with apparent first-order rates of 22.5 and 1.45 s^{-1}. The rate of the initial phase was found to be linearly dependent on the concentration of F39W, which is typical of a simple bimolecular interaction. The plot of k_{app}, the apparent rate of reaction, against the protein concentration used allowed k_{ass} and k_{diss} to be determined (Figure 3, inset). At pH 8.0 k_{ass} was 8.3×10^5 $M^{-1} \cdot s^{-1}$ and k_{diss} was 1.96 s^{-1} (Table 2), resulting in an estimated K_d of 2.4 μM, 16.5-fold higher than the K_d determined at equilibrium. This suggests that the initial fast increase in fluorescence might

© 1999 Biochemical Society

Table 2 Effect of pH on k_1, k_{-1}, k_2 and k_{-2} for the reaction between F39W and κ-chain

The values of K_d (pre) (the K_d for the encounter complex before equilibrium has been attained) and K_d (eq) (the K_d for the complex at equilibrium determined from titration experiments) have been calculated from k_1, k_{-1}, k_2 and k_{-2}. Results are means ± S.D. ($n = 3$) obtained from wholly independent experiments.

pH	k_1 ($\mu M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	K_d (pre) (μM)	K_d (eq) (μM)
6.0	0.58 ± 0.05	7.7 ± 1.9	1.33 ± 0.02	0.06 ± 0.0004	12.8 ± 0.8	0.63
7.0	0.60 ± 0.02	5.7 ± 1.7	1.36 ± 0.04	0.055 ± 0.0006	9.5 ± 0.8	0.37
8.0	0.83 ± 0.02	1.9 ± 0.06	1.45 ± 0.03	0.095 ± 0.0003	2.3 ± 0.1	0.14
9.0	0.49 ± 0.11	7.3 ± 1.1	2.0 ± 0.08	0.10 ± 0.0005	14.0 ± 1.8	0.67

be due to the formation of an encounter complex that subsequently rearranges to result in an increase in affinity. The slow increase in fluorescence might arise from the equilibrium being pulled to the right by the formation of a higher-affinity complex at equilibrium. Such a binding model is represented as:

$$F + \kappa \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F\kappa \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F\kappa^* \quad (2)$$

in which an initial interaction occurs between F39W (F) and κ-chain (κ) to give an encounter complex (Fκ) which gives rise to an increase in fluorescence. One (or both) proteins then undergoes a structural change to give the final high affinity complex (Fκ*).

In this scheme k_1 and k_{-1} are the rate constants k_{ass} and k_{diss} respectively for the formation and dissociation of the initial encounter complex. k_2 is the forward rate constant of the structural rearrangement detected by the slow second phase of fluorescence change seen in Figure 3, and k_{-2} is the rate constant of this conformational change in the reverse direction. If this model is correct then k_2 should be independent of the concentration of F39W used. This was subsequently shown by the determination of k_2 in reactions in which the concentration of F39W was varied (see inset to Figure 3). Furthermore, given that K_d at pH 8.0 is 2.4 μM and that k_2 is 1.45 s^{-1}, then from the equation [18]:

$$K_d = (k_{-1}k_{-2}/k_1k_2)[1/(1+k_{-2}/k_2)] \quad (3)$$

k_{-2} should be approx. 0.1 s^{-1} if the K_d of the complex at equilibrium at pH 8.0 is to be 1/16.5 of that determined from the values of k_1 and k_{-1}.

Determination of k_{-2}

The complex formed between equimolar concentrations (3 μM) of F39W and κ-chain was rapidly mixed with 30 μM wild-type PpL. The presence of the PpL increased the extent of the dissociation between F39W and κ-chain by binding rapidly to κ-chain released from complex with F39W. From the values of k_1 given in Table 2 the calculated rate of binding of 30 μM PpL to κ-chain would be approx. 24 s^{-1}, approx. 200-fold faster than the expected value of k_{-2} (see Table 2). A single-exponential decrease in fluorescence intensity back to that of unbound F39W and κ-chain (corrected for the small contribution made by the added PpL) was observed with a rate of 0.095 s^{-1} (results not shown). No perturbation of the fluorescence intensity at 335 nm or above occurs when PpL and κ-chain form a complex. The rate of this reaction was independent of the free concentration of PpL and F39W present and therefore seems to be rate-limited by something other than the simple dissociation of the complex [19]. This is probably the rate of the reverse direction of the proposed conformational change that occurs after formation of the binary complex.

Interaction of F39W with human IgG

Similar experiments were also performed with human IgG_1 (containing κ-chains) to test the possibility that the biphasic binding behaviour seen above was a feature of isolated κ-chains only. Fluorescence titration experiments to determine the K_d for the complex formed betwen IgG_1 and F39W proved difficult because the IgG_1 itself had a substantial protein fluorescence intensity. Therefore the approximate K_d for this complex (138 nM) was first established by a competitive ELISA protocol described in the Materials and methods section (results not shown). This value of K_d compares well with that obtained for the PpL–κ-chain complex (180 nM) with the same technique. Then the kinetic constants for the formation of the F39W–IgG_1 complex were determined by stopped-flow fluorescence experiments as described above. Once again there was an increase in fluorescence intensity that took place in two phases on mixing the proteins. Data obtained from the initial rate of change of fluorescence gave a value of k_1 of 5.4×10^5 $M^{-1} \cdot s^{-1}$ and a value of k_{-1} of 1.12 s^{-1}, yielding a K_d for the encounter complex of 2.1 μM at pH 8.0 and 25 °C. k_2 and k_{-2} were higher at 2.97 and 0.20 s^{-1} respectively. By using these values a K_d at equilibrium of 140 nM was calculated. The observation of a biphasic binding process eliminates the possibility that one of the binding phases noted between F39W and κ-chain was due to the dissociation of any κ-chain dimers after the initial complex between F39W and one of the κ-chains in a dimer. k_1 and k_{-1} for the formation and dissociation of the encounter complex between F39W and IgG_1 were both lower than the equivalent rate constants for the reaction with κ-chain, although k_2 and k_{-2} were higher. Dorrington and Smith [20] and Edmundson et al. [21] have shown that the conformation and the quaternary structure of κ-chain are different when it is in a complex with the heavy chain rather than in free solution (κ_2), which might explain the differences in the rates determined above.

Effect of temperature on the kinetic parameters k_1, k_{-1}, k_2 and k_{-2} and calculation of the activation energies of the binding reactions

The titration experiments described above showed that temperature had a large effect on the equilibrium value of K_d at pH 8.0. To identify which of the reaction rates were giving rise to the overall effect, k_1, k_{-1}, k_2 and k_{-2} were determined at different temperatures. To achieve this, F39W was reacted with 3 μM κ-chain (final concentration) at pH 8.0 over a temperature range of 15–40 °C and k_1, k_{-1} and k_2 were determined as above. The E_a of the dissociation process of the complex at equilibrium (possibly the reverse of the structural rearrangement) was calculated from values of k_{-2} that were determined by rapidly mixing the preformed F39W–κ-chain complex with 30 μM PpL, as described previously, over the same range of temperatures. The results in Figure 4 show that, whereas k_1 changes from 5.2×10^5 to 1.4×10^6 $M^{-1} \cdot s^{-1}$ (2.7-fold) between 23 and 39 °C, k_{-1} increases from 1.03 to 8.7 s^{-1} (8.5-fold) over the same temperature range. Using these values of k_1, the E_a for the formation of the encounter complex was 50.2 kJ/mol, which is lower than the 93.2 kJ/mol determined for k_{-1}, the rate of dissociation of the pre-equilibrium complex (Figure 5). The E_a for the proposed forward structural rearrangement was also high at 84.9 kJ/mol (calculated from the temperature dependence of k_2) as was that

© 1999 Biochemical Society

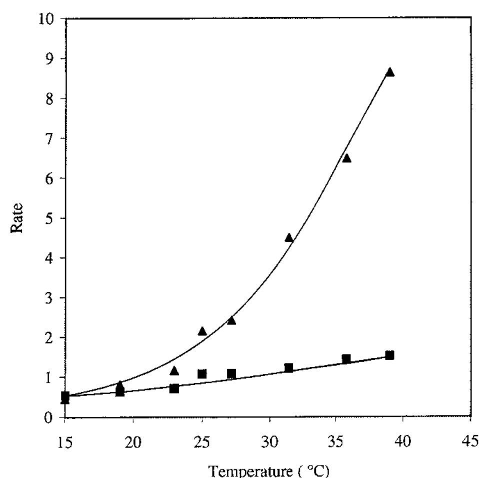

Figure 4 Effect of temperature on k_1 and k_{-1}

The plot shows the values of k_1 ($\mu M^{-1} \cdot s^{-1}$) (■) and k_{-1} (s^{-1}) (▲) at various temperatures.

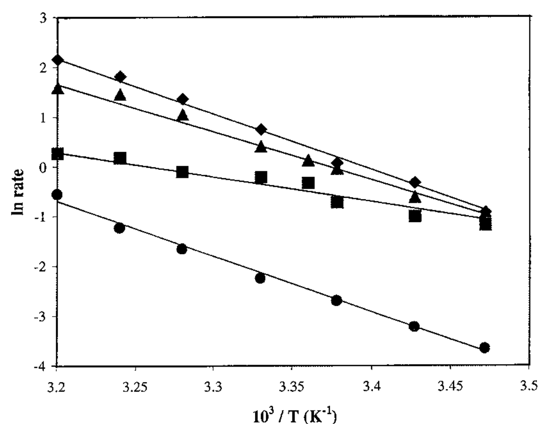

Figure 5 Arrhenius plots for the reactions describing the formation and dissociation of the complex between F39W and κ-chain

Reactions between 30 μM F39W and 3 μM κ-chain were performed between 15 and 40 °C. The natural logarithms of k_1 ($\mu M^{-1} s^{-1}$) (the rate of formation of the encounter complex; ■), k_{-1} (s^{-1}) (◆) and k_2 (s^{-1}) (the forward rate of the proposed structural change; ▲) were plotted against the reciprocal of the temperature (K). The results for k_{-2} were determined by measuring the rate of the reaction observed when preformed F39W–κ-chain complex (see the text) was mixed with an excess of PpL over the same temperature range (▼).

for the dissociation of the complex at equilibrium (102.8 kJ/mol, calculated from values of k_{-2}), which might correspond to the reverse of the structural transition.

CD spectropolarimetry

CD spectropolarimetry experiments were performed to test whether the formation of the encounter complex between wild-type PpL and κ-chain, or the structural rearrangement that follows initial complex formation, gave rise to spectral changes at far-UV or near-UV wavelengths of the spectrum. Between 190 and 250 nm (CD from the peptide backbone) there were no differences between the spectrum of the wild-type domain and that of the F39W mutant, further suggesting that the mutation does not disturb the overall folding of the protein. CD experiments over this wavelength range also showed that there were no differences between the spectrum of the complex and the sum of the spectra of the wild-type PpL and κ-chain used (results not shown), suggesting that no secondary structural changes occur when these proteins interact. However, at wavelengths between 250 and 300 nm (CD arising from the aromatic amino acid side chains) the results were more informative. The spectrum of PpL displayed several clear spectral details. At 258 and 265 nm there were peaks of positive ellipticity corresponding to contributions from Phe residues and a broad peak between 270 and 285 nm with a maximum at 278 nm that almost certainly arose from the three Tyr residues in PpL ([15]; results not shown). The spectrum of κ-chain followed the same pattern observed in earlier experiments with Bence Jones κ-chains [22,23]. On mixing PpL with κ-chain, the spectrum obtained was different from that expected from the sum of the two component spectra. There was a decrease in ellipticity at all wavelengths between 260 and 285 nm, with a maximum difference at 278 nm with respect to the sum of the individual components. This suggests that the environment around one or more Tyr residues was changing as the proteins interacted. It might be significant that NMR studies [12] showed that the resonances of Tyr^{53} are shifted on formation of the complex with κ-chain.

No time-dependent changes in CD at 275 nm occurred when PpL or κ-chain were mixed with buffer alone. However, experiments with a rapid-mixing device (see the Materials and methods section) gave reaction progress curves that revealed a slow decrease in ellipticity at 275 nm over a 5–10 s period after the initial complex was formed by mixing PpL and κ-chain (final concentrations 100 μM). Analysis of the reaction progress curve gave a rate of change of ellipticity of 1.3 s^{-1}, which is close to the value of k_2 determined fluorimetrically for the reaction between the F39W mutant and κ-chain (1.4 s^{-1}). This suggests that the conformational change to form a higher affinity complex involves a change of environment around one or more tyrosine residues after the proteins have their initial contacts, and also demonstrates that the wild-type domain binds to κ-chain in two phases.

Electrostatic nature of the interaction

The contribution of electrostatic interactions to the two binding phases was studied by repeating the stopped-flow experiments in the pH range 6.0–9.0. k_1 and k_{-1} and the calculated K_d values (pre-equilibrium and equilibrium) over this pH range are shown in Table 2. The variation due to changing pH is found predominantly in the values of k_{-1}, with the largest differences between pH 8.0 and 9.0. The most significant change in k_1 is also between pH 8.0 and 9.0, suggesting that one or more ionizable side chains have a role in the initial contacts between the proteins. A small increase in the rate of k_2 from 1.33 s^{-1} at pH 6.0 to 2.0 s^{-1} at pH 9.0 is observed for the reaction of the F39W mutant with κ-chain. The effect of pH on k_{-2} shows that above pH 7.0 k_{-2} increases, suggesting the involvement of a residue with a high pK in the dissociation reaction. If the latter rate is the reversal of the structural transition, then a group with a high pK might have an important role in this process. Of the residues in PpL_{312} suggested by NMR experiments [12] to make contact with κ-chain, Lys^{40}, Tyr^{53}, Lys^{58}, Lys^{59} and Lys^{78} might provide ionizable side chains with high pK values. Initial NMR experiments with isotopically labelled κ-chain [24] suggest that the binding site for PpL on κ-chain involves the second β-strand (Ala^{19}, Ile^{21} and Cys^{23}) and the two β-strands located on the outer surface of the framework region of the V_L domain (Leu^{11} and Leu^{73}). Of these, only Cys^{23} offers a side chain with a high

© 1999 Biochemical Society

pK. However, a full determination of the structure of the complex has not yet been achieved, making absolute identification of participating residues impossible.

Conclusion

The use of fluorescence studies with naturally occurring Trp residues as intrinsic reporter groups to report ionization events, ligand binding or protein conformational changes is well established [25,26]. More recently such fluorescence studies have been facilitated by the use of site-directed mutagenesis to place fluorescent Trp residues into specific locations in proteins so that similar experimentation and, in particular, protein folding events [27,28] can be performed on proteins that lack naturally occurring reporter groups. The success of such a venture depends on the location and type of residue chosen for the substitution. In the studies outlined here a conservative replacement, that of Phe^{39} by Trp, was used in a single domain of PpL_{3316} at a point near to or in the proposed binding site for κ-chain. The increase in quantum yield from the protein and the shift of the fluorescence emission away from the endogenous Tyr residues (302 nm) to that of the Trp residue (327 nm) has made it possible to study the interaction of PpL_{3316} with IgG and κ-chain by both equilibrium and pre-equilibrium techniques. The technique is a direct and sensitive method for the determination of the K_d for the complex and does not require the immobilization of either component. The value of K_d for the complex formed between the mutant and κ-chain (160 nM, compared with 130 nM for the wild-type domain at pH 8.0) suggests that the larger indole ring can be readily accommodated in the complex. The studies have shown that the binding reaction is not a single process and involves some conformational relaxation after an initial encounter complex is formed.

We are indebted to The Wellcome Trust for grant 039117/93/Z providing the stopped-flow/CD instruments, and to the BBSRC for the fellowship of S.P.B. and the studentship of J.A.B. (also sponsored by CAMR, Porton). We are indebted to Nick Housden for the preparation of the figures.

Received 17 November 1998/18 February 1999; accepted 3 March 1999

REFERENCES

1 Åkerström, B. and Björck, L. (1989) J. Biol. Chem. **264**, 19740–19746
2 Sjöquist, J., Movitz, J., Johansson, I. B. and Hjelm, H. (1972) Eur. J. Biochem. **30**, 190–194
3 Björck, L. and Kronvall, G. (1984) J. Immunol. **133**, 969–974
4 Langone, J. J. (1982) Adv. Immunol. **32**, 157–252
5 Goward, C., Scawen, M., Murphy, J. and Atkinson, T. (1990) Trends Biochem. Sci. **18**, 136–140
6 Sasso, E. H., Silverman, G. J. and Mannik, M. (1991) J. Immunol. **147**, 1877–1883
7 Derrick, J. P. and Wigley, D. B. (1992) Nature (London) **359**, 752–754
8 Kastern, W., Holst, E., Nielson, E., Sjöbring, U. and Björck, L. (1990) Infect. Immun. **58**, 1217–1222
9 Patella, V., Casolara, V., Björck, L. and Marone, G. (1990) J. Immunol. **145**, 3054–3061
10 Wikström, M., Sjöbring, U., Kastern, W., Björck, L., Drakenberg, T. and Forsén, S. (1993) Biochemistry **32**, 3381–3386
11 Wikström, M., Drakenberg, T., Forsén, S., Sjöbring, U. and Björck, L. (1994) Biochemistry **33**, 14011–14017
12 Wikström, M., Sjöbring, U., Drakenberg, T., Forsén, S. and Björck, L. (1995) J. Mol. Biol. **250**, 128–133
13 Wikström, M., Forsén, S. and Drakenberg, T. (1996) Eur. J. Biochem. **235**, 543–548
14 Kastern, W., Sjöbring, U. and Björck, L. (1992) J. Biol. Chem. **267**, 12820–12825
15 Bottomley, S., Beckingham, J., Murphy, J., Atkinson, M., Atkinson, T., Hinton, R. and Gore, M. (1995) Bioseparation **5**, 359–367
16 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
17 Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York
18 Chaiyen, P., Brissette, P., Ballou, D. P. and Massey, V. (1997) Biochemistry **36**, 2612–2621
19 Benson, S. W. (1960) The Foundation of Chemical Kinetics, McGraw-Hill, New York
20 Dorrington, K. J. and Smith, B. R. (1972) Biochim. Biophys. Acta **263**, 70–81
21 Edmundson, A. B., Ely, K. R. and Abula, E. E. (1978) Contemporary Topics in Molecular Immunology, vol. 7, pp. 95–118, Plenum Press, New York
22 AIkeda, K., Hamaguchi, K. and Migita, S. (1968) J. Biochem. (Tokyo) **63**, 654–660
23 Ghose, A. (1972) Biochim. Biophys. Acta **278**, 337–343
24 Enokizono, J., Wikström, M., Sjöbring, U., Björck, L., Forsén, S., Arata, Y., Kato, K. and Schimada, I. (1997) J. Mol. Biol. **270**, 8–13
25 Ashby, B., Wootton, J. C. and Fincham, J. R. S. (1974) Biochem. J. **143**, 317–329
26 Gore, M. G., Greasley, P. J., McAllister, G. and Ragan, C. I. (1993) Biochem. J. **236**, 811–815
27 Bottomley, S. P., Popplewell, A. G., Scawen, M., Wan, T., Sutton, B. J. and Gore, M. G. (1994) Prot. Eng. **7**, 1463–1470
28 Thumser, A. E. A. and Wilton, D. C. (1994) Biochem. J. **300**, 827–833

© 1999 Biochemical Society