Observation and Characterization of the Interaction between a Single Immunoglobulin Binding Domain of Protein L and Two Equivalents of Human κ Light Chains*

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Protein L is a multidomain cell wall bound protein found in ~10% of the Peptostreptococcus isolates (1) and contains a series of repeated domains, some of which are able to bind to immunoglobulins without initiating an immune response. Expression of this protein has been correlated to the virulence of these opportunist pathogenic bacteria (1) that are found in the gastrointestinal and urogenital tracts (2). Its presence has been found to cause cellular responses such as histamine release from basophils and mast cells (3) presumably by cross-linking IgE molecules bound to surface Ig receptors.

1H NMR spectroscopy of a single Ig-binding domain of protein L (isolated from strain 312, PpL312) revealed that PpL312 is a rigid structure consisting of a β-sheet, formed by two pairs of antiparallel β-strands, lying on top of a single α-helical section (4), with a flexible N terminus (5). Further NMR studies (6) led to the proposal that the binding site of PpL312 for κ-chain involves residues from the second β-strand and from the loop between the third β-strand and the α-helix and located the binding site for PpL312 on the κ-chain to the second β-strand and the two β-strands located on the outer surface of the framework region of the Vκ domain (7).

Parallel studies on the binding interaction of a single Ig-binding domain of protein L from strain 3316 (PpL3316) found that it forms a high affinity complex with κ-chains with a Kd of 112 nM (8, 9). The x-ray crystallographic structure of this domain has been determined in complex with the human antibody fragment, Fab 2A2, and revealed that two Fab 2A2 fragments can in fact bind to sites on opposite faces of PpL3316 (10). Site 1 of PpL3316 is characterized by Beckingham et al. (9) and equivalent to the site studied on PpL312 using NMR by Wikström et al. (5). Site 2 of PpL3316, involving part of the helix and strands 3 and 4, was identified for the first time by the crystallographic study (10). This site has not yet been characterized although preliminary studies suggested that the affinity of site 2 is lower than that of site 1 (10).

By use of a program of site directed mutagenesis we have been able to derive the relative binding affinities of sites 1 and 2 and propose a mechanism by which PpL3316 binds κ light chains that is consistent with published data. The hydroxyl group of Tyr53a is important for the formation of a high affinity complex at site 1. Previous enzyme-linked immunosorbent assay experiments (11) have shown that nitration of Tyr53a or its mutation to Phe dramatically increases the Kd of the κ-chain-PpL3316 interaction. Another important residue at site 1 is Leu57. No binding of the κ-chain used in these studies can be detected at site 1 of the mutant Y53F/L57H by fluorimetry or isothermal titration calorimetry, and this mutant is thus ideal for measuring the affinity at site 2.

Important residues for binding at site 2 are Asp55 and Ala66. The mutation D55A eliminates a salt bridge and dramatically weakens or eliminates binding (12), and conversely, the mutation A66W is predicted to cause a steric clash removing binding at site 2. This is born out by fluorimetry experiments reported here and therefore the A66W PpL3316 is suitable for determining the affinity of site 1.

The fluorescence properties of tryptophan residues are highly sensitive to their immediate environment (13), and changes in the fluorescence emission from Trp residues have been used to study protein folding and unfolding events, pro-
tein conformational changes, and to monitor binding interactions between proteins and various ligands (14). We have therefore introduced unique Trp residues into PpL3316 to monitor the binding reaction with κ-chain. Previous fluorescence studies have used F39W PpL3316 to report the binding interaction between κ-chain and PpL3316. However, upon introduction of secondary mutations into the F39W PpL3316 construct, expression levels and stability of the protein are compromised. Therefore the I34W PpL3316 mutant, which has a Trp residue located toward the N terminus of β-strand 2, has been selected to study binding at the site 1 interface (see Fig. 1a); this construct is tolerant to the introduction of further mutations. The mutant Y64W PpL3316 (Trp residue on β-strand 3 and close to residues Thr65, Ala66, Asp67, and Leu68, all of which are involved in site 2 contacts (10)) was made to monitor binding at site 2 (see Fig. 1b).

**EXPERIMENTAL PROCEDURES**

Phosphate buffers and other reagents were all of analytical grade and purchased from BDH. The human IgG and κ-chain for fluorescence experiments were generous gifts from Professor Martin Glennie, Southampton General Hospital, UK.

**Mutagenesis**—The mutated PpL3316 constructs, I34W, Y64W, and Y64W/F53, were produced through the Kunkel method (15) of site-directed mutagenesis. The I34W/Y64F, I34W/Y53F/L57H, Y64W/F53/L57H, A66W, and I34W/A66W PpL3316 mutant proteins were made through two-sided splicing by overlap extension PCR mutagenesis (16). The presence of each mutation was confirmed by DNA sequencing and the mutant proteins were prepared in the same manner as the wild-type (wt) protein (8).

**Fluorescence Measurements**—Fluorescence emission spectral measurements were made on a Hitachi F-2000 fluorimeter, with the temperature of the cell being maintained at 15 °C by circulating water. All solutions were prepared in filtered 20 mM potassium phosphate buffer, pH 8.0. Scans of the emission spectra were made using excitation and emission band passes of 5 nm. Emission spectra were measured using excitation wavelengths of 280 and 295 nm, with a scan rate of 60 nm/min.

**Stopped-flow Fluorescence Measurements**—An Applied Photophysics SX.17MV stopped-flow spectrophotometer fitted with a 2-ml syringe was used to study pre-equilibrium fluorescence changes upon mixing of PpL3316 with κ-chain. The temperature was maintained at 15 °C (unless otherwise stated) using a Neslab RTE-111 circulating water bath. Measurements were made over various time courses between 0.2 and 2000 s, collecting 1000 data points with oversampling activated. Excitation wavelengths of 280 or 295 nm were selected by a monochromator and fluorescence emission above 335 nm was selected using a cut-off filter. All solutions were prepared in filtered and degassed 20 mM potassium phosphate buffer, pH 8.0. Each reaction was carried out in triplicate and the data averaged and analyzed using the manufacturer’s software and single or double exponential curve fitting algorithms.

For the determination of the rate constants for the association and dissociation of the complex, the concentration of κ-chain was kept at 1.5 μM, and the concentrations of the PpL3316 mutants were varied between 10 and 50 μM. Alternatively, in some experiments the concentration of PpL3316 was kept constant at 1.5 μM, and the concentration of κ-chain was varied.

To study the rate of dissociation of the complexes formed between PpL3316 mutants and κ-chain, a solution containing a preformed complex (3 μM κ-chain and 3 μM mutant PpL3316) was rapidly diluted by an equal volume of the non-fluorescent (above 335 nm) wt PpL3316 (30 μM). All concentrations quoted are final concentrations after 1:1 v/v mixing.

**Sequential Mixing Stopped-flow Fluorimetry**—To identify any intermediate complexes in the formation of the equilibrium complex between the κ-chain and e-chain, the stopped-flow fluorimeter was reconfigured to facilitate sequential mixing. Initially 6 μM Trp-labeled PpL3316 (i.e., I34W or Y64W PpL3316) was mixed with an equal volume of 6 μM κ-chain. After a delay to allow complex formation (50 ms to 50 s), the 3 μM Trp-labeled PpL3316, 3 μM κ-chain solution was mixed with an equal volume of 30 μM wt PpL3316, in 20 mM potassium phosphate buffer, pH 8.0. Heat changes associated with the dilution of aliquots of 380 μM κ-chain into 20 mM potassium phosphate buffer, pH 8.0. The enthalpy profile for the control titration into buffer was subtracted from the experimental data, with the resulting binding data being analyzed using the manufacturer’s software.

To further resolve the binding interactions of κ-chain at sites 1 and 2 of PpL3316, mutant PpL3316 constructs with site 2 or site 1 eliminated (Y64W and Y64W/Y53F, respectively) were titrated into κ-chain (28.3 and 25 μM, respectively). As above, binding data from these titrations were analyzed using the manufacturer’s software.

**Circular Dichroism**—CD measurements were made using a Jasco J-720 CD spectrometer thermostated at 15 °C. The spectra between 195 and 295 nm were measured at a scan rate of 50 nm/min using a 1-mm path length. The spectra were scanned in triplicate, with a response time of 4 s, slit width of 500 μm, bandwidth of 1.0 nm, and resolution of 0.2 nm. Spectral corrections and calculations were carried out using the manufacturer’s software, and the units are given in mean residue ellipticity.

**RESULTS AND DISCUSSION**

Changes in the environment of the Trp reporter groups were examined by comparing the emission spectra of 1.5 μM κ-chain and 1.5 μM of each of the PpL3316 mutants, in free solution and complex, using excitation wavelengths of 280 and 295 nm. The spectra showed that Trp64 and Trp34 have wavelengths of maximum emission at 335 and 345 nm, respectively, indicating that Trp64 is in a more hydrophobic environment than Trp34. The latter points downwards from β-strand 2 into the solvent and away from the center of the domain, whereas the side chain of Trp64 is surrounded by side chains from the α-helix and β-strand 2 (see Fig. 1). No detectable shift in position of maximum emission is noted for the Y64W PpL3316 κ-chain complex; however, a 5-nm blue shift is seen with the I34W PpL3316 κ-chain complex suggesting that Trp34 becomes less solvent exposed on complex formation. Details of the spectral characteristics of the various PpL3316 κ-chain complexes, and the same proteins in free solution are given in Tables I and II. The data show that both the I34W and Y64W PpL3316 domains exhibit fluorescence intensity changes upon complex formation with κ-chain. The intensity of Trp34 decreases significantly whether excited at 280 or 295 nm, whereas that of Trp64 increases slightly when excited at 295 nm but decreases when excited at 280 nm.

The respective distances of these residues from Tyr53 are ~10 Å and 15 Å, respectively, well within the critical distance
for efficient Förster fluorescence resonance energy transfer (FRET), and Trp<sup>64</sup> and Trp<sup>34</sup> are almost certainly excited due to resonance energy transfer from this residue. Previous studies have shown the fluorescence of Tyr<sup>53</sup> is quenched upon complex formation with κ-chain (9), and therefore the component of Trp<sup>64</sup> or Trp<sup>34</sup> fluorescence arising due to FRET from Tyr<sup>53</sup> (when excited at 280 nm) will also decrease. The changes in intensity, and the location of the two Trp groups, 34 and 64, close to site 1 and in site 2, respectively, support the concept of κ-chain binding at both sites.

It should be noted that no fluorescence intensity changes (excited by light at 295 nm) occur when κ-chain is mixed with the I34W/Y53F/L57H or Y64W/A66W PpL<sub>3316</sub> proteins (data not shown) suggesting that binding does not take place at site 1 or site 2 of these mutants, respectively. However, a decrease in fluorescence intensity at 335 nm or above does occur when κ-chain binds to Y64W/A66W PpL<sub>3316</sub> and an excitation wavelength of 280 nm is employed. This is almost certainly due to decreased FRET from Tyr<sup>53</sup> to Trp<sup>64</sup> when κ-chain occupies site 1 (see above).

The largest change in fluorescence intensity occurs when Y64W/Y53F PpL<sub>3316</sub> forms a complex with κ-chain (Δex = 280 nm or 295 nm). The Y53F mutation (site 1) has a clear effect on the fluorescence properties of Y64W in site 2. This could be due to a direct spectral perturbation (although the expected effect would be a decrease in fluorescence intensity from Y64W when excited at 280 nm, see above) or more probably a consequence of the decrease in affinity at site 1 caused by this mutation (9).

Under the conditions of the above experiments there is only sufficient κ-chain to form a 1:1 mol:mol complex with PpL<sub>3316</sub>. As a result, sites 1 and 2 are both competing for the available κ-chain, with the amount bound to each site being dependent upon the relative affinities of the sites. Therefore, any mutation (e.g. Y53F) that causes a decrease in the amount of κ-chain bound at site 1 will lead to a concomitant increase in κ-chain available to bind to site 2 and thus have an effect on the magnitude of the signal change from either Trp<sup>34</sup> or Trp<sup>64</sup>. Pre-equilibrium Binding Studies—Stopped-flow fluorescence techniques were used to study the binding reactions between mutant PpL<sub>3316</sub> proteins and κ-chain. They illustrated that the observed fluorescence changes that occur for the Y64W/Y53F, Y64W, I34W/Y53F, and I34W mutants seen above are in fact the net results of more complex changes (Fig. 2).

The single mutation Y64W produces a domain that shows large and complex changes in fluorescence when mixed with κ-chains. When excited at 280 nm a rapid increase in fluorescence intensity is followed by a slow and larger decrease. The binding of I34W to κ-chain under these conditions also appears to be a two-step process, both steps being accompanied by a decrease in fluorescence intensity. Biphasic kinetics such as these have been observed previously and occur when the mutant F39W PpL<sub>3316</sub> is mixed with κ-chain (9). The unidirectional, biphasic fluorescence intensity changes observed in those studies are consistent with a system in which the rapid fluorescence change corresponds to the formation of an encounter complex that subsequently undergoes a slower local rearrangement from a low affinity complex to a high affinity equilibrium complex. However, this model cannot explain the reaction progress curves that show more complex bidirectional fluorescence changes. In view of the recent crystallographic results that detail the existence of a second, albeit much weaker, binding site for κ-chain on PpL<sub>3316</sub>, we can now reconcile these data with a two-site binding model. This model not only explains the complex fluorescence changes described above but also allows the K<sub>d</sub> for the two binding sites to be determined.

In this model κ-chains bind independently to sites 1 (1) and 2 (2) on PpL<sup>2</sup>. The overall K<sub>d</sub> for each site will depend upon the ratios of their dissociation (k<sub>−1</sub> and k<sub>−2</sub>) and association rate constants (k<sub>1</sub> and k<sub>2</sub>), respectively. Thus if the two sites have different K<sub>d</sub> values for their respective complexes with κ-chains and the rates of association of κ-chain to the two sites are similar, then the rates of dissociation from these sites must be different. Such a phenomenon (17) will explain the biphasic fluorescence changes described above. For example, consider

**Table I**

| PpL construct | Wavelength of emission maximum (nm) | Fluorescence changes (ΔFluorescence) |
|---------------|------------------------------------|-------------------------------------|
| Y64W          | 335                                | -8.6                                |
| Y64W/Y53F     | 335                                | +18.6                               |
| I34W          | 345                                | -16.9                               |
| I34W/Y53F     | 345                                | -6.8                                |

**Table II**

| PpL<sub>3316</sub> construct | Wavelength of emission maximum (nm) | Fluorescence changes (ΔFluorescence) |
|------------------------------|------------------------------------|-------------------------------------|
| Y64W            | 335                                | +11.6                               |
| Y64W/Y53F       | 335                                | +49.9                                |
| I34W            | 345                                | -5.9                                |
| I34W/Y53F       | 345                                | -1.7                                |
the situation when PpL$_{9316}$ is present in excess and the rates of association to each site are similar. Under these conditions the κ-chain will initially bind to both sites 1 and 2. However, if site 2 has a higher dissociation rate than site 1 the occupancy of site 2 decreases and the κ-chain is redistributed in favor of site 1. Thus a reporter group in site 2, e.g. Trp$^{64}$, may show an initial fluorescence increase, as it becomes less solvent exposed due to the binding of κ-chain, followed by a decrease in fluorescence as the κ-chain is redistributed in favor of site 1. Similarly, the site 1 reporter group P39W shows a biphasic change on complex formation. In this case an initial rapid increase in fluorescence intensity, due to binding of free κ-chain to this site, followed by a slower increase, due to the further saturation of available site 1 a consequence of κ-chain being released from site 2. This is in accord with previous data (9). An alternative reporter group in site 1, I34W, which lies on the opposite side of β strand 2 and points away from the helix, interestingly shows a biphasic decrease in fluorescence on formation of a complex with κ-chain.

If the binding of κ-chain to each site is independent of the presence of κ-chain at the other site, then rates $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ will be the same as rates $k_4$, $k_{-4}$, $k_5$, and $k_{-5}$ respectively. However, in the presence of excess PpL$_{9316}$, the complex involving one PpL$_{9316}$ domain and two κ-chains ($\kappa^1$PpL$^2$κ, shown boxed in Scheme 1) will not be present in significant amounts. This model is supported by the dramatic effects of the additional mutation Y53F on the fluorescence properties and the reaction progress curves observed on complex formation. It virtually eliminates the second, slow decrease in fluorescence intensity shown by Trp$^{64}$ (Fig. 2) and is almost certainly attributable to the decreased affinity of site 1, a result of the Y53F replacement (9). Thus if the affinity of site 1 is decreased to, or below that, of site 2, then under the conditions used, more κ-chain will remain bound to site 2, and the fluorescence intensity at equilibrium from Trp$^{64}$ will be higher. This conclusion is supported by experiments using the mutant I34W/Y53F. This protein gives a reaction progress curve that appears to have a single-phase fluorescence change with a decreased amplitude compared with that observed for the binding of the single mutant I34W to κ-chain. These observations are consistent with the idea that the Y53F mutation reduces the affinity of site 1 close to that of site 2.

Finally, I34W/Y53F/L57H PpL$_{9316}$ containing the binding site mutations Y53F/L57H with the site 1 reporter Trp$^{34}$ shows no fluorescence change when mixed with κ-chain. This is because binding at site 1 has been substantially reduced/eliminated, and any significant remaining κ-chain binding is due to site 2. This is supported by the results gained from experiments using the same site mutations but with the Y64W reporter (Y64W/Y53F/L57H PpL$_{9316}$). This domain binds κ-chain resulting in the largest increase in fluorescence intensity (see Fig. 2). Assuming that the spectral response of the Trp$^{64}$ reporter group, excited by light at 295 nm, is not affected directly by the substitutions on the opposite side of the domain, these data suggest that the fluorescence intensity of Y64W PpL$_{9316}$ at equilibrium reflects the occupancy of site 2 only. Thus the large fluorescence change given by Y64W/Y53F/L57H and the absence of any fluorescence change for the same reaction with I34W/Y53F/L57H support the proposal that all of the bound κ-chain is located at site 2.

If the binding model described in the above scheme is correct then one would expect to see different reaction progress curves when the experiment is repeated with increasing concentrations of κ-chain. Eventually there will be sufficient κ-chain to occupy both binding sites to give the species $\kappa^1$PpL$^2$κ, and thus the reaction progress curves will become monophasic. Such an experiment is described in Fig. 3, which displays three reaction curves in which 1.5 μM Y64W PpL$_{9316}$ is mixed with 1.8, 9.9, and 28.8 μM κ-chain (c) in 20 mM potassium phosphate buffer, pH 8.0 at 15 °C. Concentrations quoted are after mixing.

The reaction was initiated by mixing 1.5 μM Y64W PpL$_{9316}$ with 1.8 μM κ-chain (a), 9.9 μM κ-chain (b), and 28.8 μM κ-chain (c) in 20 mM potassium phosphate buffer, pH 8.0 at 15 °C. Concentrations quoted are after mixing.

**Do the Mutations Alter the Structure of the PpL Domain?**—Circular dichroism studies (see Fig. 4) have shown that wt, L57H, Y53F, and Y53F/L57H domains have identical far UV CD spectra suggesting that the substitutions have not disturbed the secondary structures of the proteins. The presence of Trp residues in position 34 or 64 leads to an increase in ellipticity between 205 and 210 nm that is almost certainly due to spectral contributions from the aromatic side chains rather than disruption of the secondary folding of the protein. Furthermore, the structure of Y64W obtained by x-ray studies shows no significant backbone differences to that of the wt PpL$_{9316}$ (18). Further mutations, e.g. Y53F, L57H, or Y53F/
L57H made to the I34W or Y64W domains, caused no further spectral changes again indicating that they do not disrupt the folding of PpL3316.

Does the Binding Reaction Involve a Change in Structure of the Complex?—The determination of the x-ray crystallographic structures of the complexes between wt PpL3316 and a human Fab (10) and Y64W PpL3316 and a mouse Fab (18) have shown that the secondary structure of the free PpL3316 is the same as that of PpL3316 in the complex. Furthermore, the far-UV CD spectrum of wt PpL3316 is the same in the absence or presence of an equimolar concentration of κ-chain (see inset to Fig. 4) suggesting that there is no change in secondary structure on complex formation.

Determination of $K_d$ for Complexes at Sites 1 and 2—By determining the rates $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ for κ-chain binding to sites 1 and 2 of PpL3316, respectively, the $K_d$ of the complex at each site can be determined. In experiments using domains with both sites functioning then the overall observed rate of association, $k_o$, will be a combination of the association rates for each site ($k_1 + k_2$, see Fig. 5). Thus to determine the association rate to each site individually it was necessary to eliminate the alternative site in each case. This was achieved by the mutations Y53F/L57H and A66W on the I34W or Y64W templates. These remove binding capacity at sites 1 and 2, respectively.

$ka$ can be calculated from the slope of a plot of the observed rate, $k_{app}$, against the concentration of the variable ligand (PpL3316). Such data using various PpL3316 mutants are given in Table III. In the absence of κ-chain binding at site 1 the reaction of κ-chain with site 2 of the Y64W/Y53F/L57H PpL3316 mutant is a simple bimolecular binding process and the observed rate of reaction $k_{app} = k_2[\text{Y64W/Y53F/L57H PpL3316}] + k_{-2}$ (see Fig. 5). The variation of $k_{app}$ with varying concentrations of Y64W/Y53F/L57H PpL3316 is shown in the inset to Fig. 5, and the value of $k_2$ is $0.15 \pm 0.02 \mu M^{-1} s^{-1}$ (Table III). Similarly experiments show that $k_1$ for binding at site 1 (using the mutant I34W/A66W) is $0.25 \pm 0.01 \mu M^{-1} s^{-1}$ at 25 °C. The sum of these two association rate constants ($0.40 \mu M^{-1} s^{-1}$) is very close to that determined for domains in which binding occurs to both sites simultaneously, as described above, e.g. Y64W ($0.35 \pm 0.03 \mu M^{-1} s^{-1}$) or I34W ($0.33 \pm 0.02 \mu M^{-1} s^{-1}$).
To corroborate these calculations we used domains with the Trp34 reporter and determined $k_1$ and $k_{-1}$ for the reaction by rapidly mixing a constant amount of the PpL3316 domain with various excess concentrations of $\kappa$-chain, the latter always in large excess so that pseudo-first order conditions can be achieved. The observed rate of reaction $k_{app}$ will depend upon the concentration of $\kappa$-chain, and a plot of $k_{app}$ against $\kappa$-chain concentration yielded a line of slope $k_1$, in this case 0.23 ± 0.04 $\mu$M$^{-1}$s$^{-1}$ (data not shown), which is in close agreement with the values determined by other methods (see Table III).

**Determination of the Rates of Dissociation of the Complexes**

Although estimates of the dissociation rates $k_{-1}$ or $k_{-2}$ can be obtained from the intercept on the ordinate of the plot shown in the inset to Fig. 5, these are prone to large errors when their values are so low (19). Therefore rates of dissociation of the complexes between PpL3316 domains and $\kappa$-chain were measured as described under “Experimental Procedures,” and the data are summarized in Fig. 6, a–c, and Table III. The dissociation of the $\kappa$-chain from the complex at site 1 of I34W PpL3316 gives rise to a slow, single-phase fluorescence increase, with a rate of 0.013 ± 0.001 s$^{-1}$. The rate of dissociation of the

**Table III**

| PpL construct | $k_a$ | Site 1 | Site 2 |
|---------------|------|-------|-------|
|               | $k_1$ | $k_{-1}$ | $K_d$ | $k_2$ | $k_{-2}$ | $K_d$ |
| Y64W          | 35 ± 3 | 20 ± 1 | 38 | 15 ± 2 | 560 ± 20 | 37 |
| Y64W/Y53F     | 33 ± 6 | 18 ± 2 | ND | 15 ± 2 | 410 ± 20 | 28 |
| Y64W/Y53F/L57H| 15 ± 2 | No detectable binding | 15 ± 2 | 500 ± 20 | 34 ± 7 |
| I34W          | 33 ± 2 | 18 ± 1 | 68 | 15 ± 2 | ND | ND |
| I34W/Y53F     | 33 ± 3 | 18 ± 1 | 4400 | 15 ± 2 | ND | ND |
| I34W/A66W     | 25 ± 1 | 12 ± 1 | 48 ± 8 | No detectable binding | |

*Values of $k_1$ calculated by subtraction of $k_2$ (determined for the Y64W/Y53F/L57H-$\kappa$-chain interaction) from $k_a$ (the combined rates of $k_1$ and $k_2$).  

*The value of $k_2$ determined from experiments using Y64W/Y53F/L57H PpL in which binding at site 1 has been eliminated.
When the dissociation experiment with the Y64W PpL3316κ-chain complex was repeated using an excitation wavelength of 280 nm, only the initial fluorescence decrease was apparent (Fig. 6c). We can conclude from this that the initial fluorescence decrease is due to changes in the environment of Trp64 as the κ-chain dissociates from site 2. The slower fluorescence change noted when the excitation wavelength is 280 nm arises from increased FRET from Tyr53 as the complex at site 1 dissociates (removing quenching from Tyr53). Therefore it is possible to simultaneously observe dissociation at sites 1 and 2. Using the values of the rates of association ($k_a$) and dissociation ($k_d$) of the Y64WY53F/L57H PpL3316κ-chain complex from the above experiments the $K_d$ at site 2 is 3.4 ± 0.70 μM (Table III).

**Sequential Mixing Stopped-flow Fluorimetry**—In the original single site binding model shown below (9), it was proposed that two structurally different forms of the PpL3316κ-chain complex exist.

\[
\text{PpL} + \kappa \rightleftharpoons \text{PpL} \cdot \kappa \rightleftharpoons \text{PpL} \text{-chain complex}.
\]

In this scheme $k_a$ and $k_d$ represent the rates of association and dissociation, respectively, of the encounter complex PpLκ, and $k_r$ and $k_r$ represent the forward and reverse rates of the structural rearrangement, respectively.

In the proposed model (9) the transition from PpLκ to [PpL·κ]⁺ was suggested to increase the affinity by ~30-fold. Thus under equilibrium conditions, the majority of the PpL and κ-chain would be present in the form of the high affinity complex. Also dissociation of the encounter complex occurs rapidly, while dissociation of the equilibrium complex is rate limited by $k_r$ (9). Therefore upon dissociation of the complex at equilibrium most of the observed fluorescence change would occur with the rate $k_r$. However, if dissociation of the PpLκ-chain complex is invoked shortly after the mixing of PpL and κ-chain, before the encounter complex has undergone the structural rearrangement, the observed rate would be $k_{diss}$, that of the dissociation of the encounter complex. When both encounter and equilibrium complexes are present, the observed dissociation profile would be expected to be biphasic, the amplitude of each phase being dependent on the relative abundance of each complex.

However, sequential-mixing stopped-flow studies on the dissociation of the I34W PpL3316κ-chain complex reveal a single-phase fluorescence increase upon dissociation. Regardless of the incubation time prior to initiation of dissociation, a rate constant of ~0.01 s⁻¹ was observed. Therefore, the formation of the I34W PpL3316κ-chain complex does not appear to occur by a two step process involving a structural rearrangement.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry was used to verify the $K_d$ values obtained for κ-chain binding to sites 1 and 2 of PpL3316 determined using the fluorimetric methods above. To attempt to resolve binding to sites 1 and 2, κ-chain was titrated into wt PpL3316. As a result of the very high concentration of κ-chain required when used as the titrant the data required significant correction for the contribution to the enthalpy profile due to κ-chain dilution. The $K_d$ values for sites 1 and 2 were estimated to be 21.1 ± 10.21 nM and 7.5 ± 3.1 μM, respectively (see Table IV). The enthalpic contributions to binding at sites 1 and site 2 were −54.3 ± 0.7 kJ/mol and −28.1 ± 6.2 kJ/mol, respectively, and the entropic changes were −41.4 and 0.7 J/mol/degree, respectively.

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**Table III.**

| Site | $K_d$ (μM) | $k_a$ (s⁻¹) | $k_d$ (s⁻¹) | $k_r$ (s⁻¹) |
|------|-----------|-------------|-------------|-------------|
| 1    | 0.001     | 41.4        | 1           | 54.3        |
| 2    | 0.70      | 1           | 3.4         | 0.70        |

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**Fig. 6.** Fluorescence intensity changes observed upon the dissociation of PpL3316κ-chain complexes at pH 8.0 and 15 °C. a, emission at wavelengths greater than 335 nm, using an excitation wavelength of 280 nm, observed upon the dissociation of the I34W PpL3316κ-chain and Y64W PpL3316κ-chain complexes. b, emission at wavelengths greater than 335 nm, using an excitation wavelength of 280 nm, upon dissociation of the I34WY53F PpL3316κ-chain, Y64W/Y53F, PpL5316κ-chain, and Y64W/Y53F/L57H PpL3316κ-chain complexes. c, emission at wavelengths greater than 335 nm, using an excitation wavelength of 295 nm, upon dissociation of the Y64W PpL3316κ-chain complex.
However, to accurately determine these values for each site, mutants in which either site 1 or site 2 had been eliminated were used. Titration of Y64W/Y53F/L57H PpL3316 into PpL3316 allows the binding at site 2 to be measured. Clear enthalpy profiles are obtained (see Fig. 7), while titration of Y64W/Y53F/L57H PpL3316 into buffer shows insignificant enthalpy changes. Analysis of the heat changes of the titrations fitted a one site binding model and indicated a $K_d$ value of 4.6 ± 0.8 μM, an enthalpy change of −21.3 ± 1.2 kJ/mol and an entropic change of 28.4 ± 5.6 J/mol/degree.

A66W PpL3316 was similarly used to study binding to site 1 in the absence of binding at site 2. The A66W titration into κ-chain gives an enthalpy profile that fits a one site binding model with a $K_d$ equal to 37.5 ± 7.3 nM, an enthalpy change of −40.2 ± 0.3 kJ/mol and an entropic change of 28.2 ± 6.2 J/mol/degree.

**CONCLUSIONS**

The experiments described above show conclusively that a PpL3316 domain has two binding sites for κ-chain of different affinities. The placement of unique Trp residues in either of the two binding sites has facilitated the binding constants to be determined and a model developed that explains the complex fluorescence signal changes noted previously. Estimates of $K_d$...
by such techniques as enzyme-linked immunosorbent assay will reflect the presence of both sites. Thus we have resolved the issue by the production of mutants that have binding at one site or the other eliminated under these experimental conditions.

Fig. 2 shows that the final level of fluorescence (λex = 295 nm) from the complex formed by mixing a 1:1 mol: mol ratio of Y64W PpL3316 and κ-chain is indicative of the relative occupancy of site 2. Thus if all of the bound κ-chain is at site 1, then there is little fluorescence increase at equilibrium from the Trp64 and vice versa. A domain with two sites of equal affinity would show equilibrium fluorescence intensity half way between the two extremes described above. We estimate that for the Y64W PpL3316 κ-chain complex, greater than 95% of the κ-chain is bound to site 1, and this is reflected by an ~2% net increase in the fluorescence intensity at equilibrium. The residual fluorescence intensity at equilibrium given by Y64W/κ-chain from site 1 due to the loss of the hydrogen bond formed between the hydroxyl group and the peptide carbonyl group of Thr30. This leads to a decrease in affinity of at least 90 fold to yield a Kd of 4.42 μM, higher than in a previous report (9). However, it is now clear from the studies described in this paper that the affinity (3.2 μM) measured in (9) was for κ-chain binding that was occurring at the then unreported weaker binding site at site 2.

To date, the binding of wt PpL3316 to two different κ-chains has been studied. These are a human κ1-chain (κL1) described here and the other a human Fab (2A2 (10)). Additionally the binding of mutated PpL3316 domains to a murine κ9 (Fab 19D9D6) has been studied (12). All of these confirm the presence of two binding sites per domain although the elimination of site 2 for the murine κ9 (Fab 19D9D6) has been demonstrated for the mutant D55A (12). Binding at the second site is not easily detected due to the presence of the much higher affinity site 1. However, this problem is eliminated when site 1 is either weakened or abrogated and thus by use of suitable mutants we have in these studies been able to measure the affinity of site 2 for κ-light chains.

Because the binding interactions at sites 1 and 2 involve backbone and different side chain contacts between PpL3316 and κ-chain, it is probable that the relative affinities of each site for κ-chains may depend upon the subclass or origin of the κ-chain. This may well offer an advantage to the bacterium, allowing it to maximize potential binding capacity.

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