

# Studies on a single immunoglobulin-binding domain of protein L from *Peptostreptococcus magnus*: the role of tyrosine-53 in the reaction with human IgG

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Chemical modification experiments with tetranitromethane (TNM) have been used to investigate the role of tyrosine residues in the formation of the complex between PpL (the single Ig-binding domain of protein L, isolated from *P. magnus* strain 3316) and the kappa light chain ( $\kappa$ -chain). Reaction of PpL with TNM causes the modification of 1.9 equiv. of tyrosine (Tyr<sup>51</sup> and Tyr<sup>53</sup>) and results in an approx. 140-fold decrease in affinity for human IgG. Similar experiments with mutated PpL proteins suggest that nitration predominantly inactivates the protein by modification of Tyr<sup>53</sup>. Reduction of the nitrotyrosine groups to aminotyrosine by incubation with sodium hydrosulphite does not restore high affinity for IgG. Modification of  $\kappa$ -chain by TNM resulted in the nitration of  $3.1 \pm 0.09$  tyrosine residues. When the PpL- $\kappa$ -chain complex was incubated with TNM,

$4.1 \pm 0.04$  tyrosine residues were nitrated, indicating that one tyrosine residue previously modified by the reagent was protected from TNM when the proteins are in complex with each other. The  $K_d$  for the equilibrium between PpL, human IgG and their complex has been shown by ELISA to be  $112 \pm 20$  nM. A similar value ( $153 \pm 33$  nM) was obtained for the complex formed between IgG and the Tyr<sup>64</sup> → Trp mutant (Y64W). However, the  $K_d$  values for the equilibria involving the PpL mutants Y53F and Y53F,Y64W were found to be  $3.2 \pm 0.2$  and  $4.6 \pm 1$   $\mu$ M respectively. These suggest that the phenol group of Tyr<sup>53</sup> in PpL is important to the stability of the PpL- $\kappa$ -chain complex.

**Key words:** circular dichroism, fluorescence, stopped-flow, tetranitromethane.

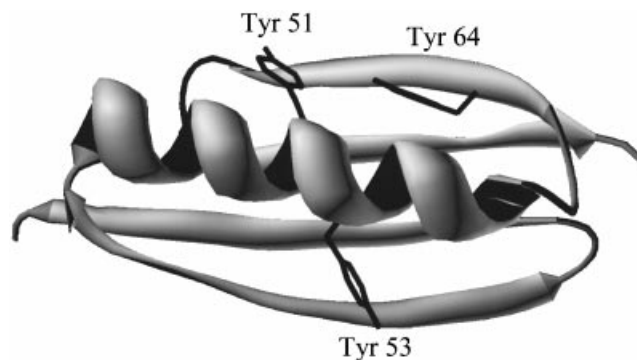
## INTRODUCTION

Protein L is a multi-domain cell-wall-bound protein found in approx. 10% of *Peptostreptococcus magnus* strains; its presence has been correlated to the virulence of the bacteria [1]. Like Protein A from *Staphylococcus aureus* [2] and Protein G from groups C and G *Streptococci* [3], it contains several Ig-binding domains. Proteins A and G bind to the C<sub>H</sub>2-C<sub>H</sub>3 interface of the Fc fragment of some classes of Ig, predominantly IgG [4,5]; however, the Ig-binding domains of protein L bind exclusively to the framework region of the V<sub>L</sub> domain of kappa light chains ( $\kappa$ -chains) [6,7]. Each of these proteins has been used to purify immunoglobulins from a wide selection of species. Protein L has been shown to purify  $\kappa$ -chain-containing Fv and Fab fragments prepared from monoclonal antibodies and recombinant DNA systems [6]. It offers an advantage over proteins A and G as it binds to  $\kappa$ -chains regardless of heavy-chain subclass and can therefore bind up to 60% of antibodies in a human serum sample [8].

The global fold of an Ig-binding domain of protein L from *Peptostreptococcus magnus* strain 312 (PpL<sub>312</sub>) has been determined by NMR [9,10] and a similar domain (PpL) from strain 3316 by X-ray crystallography (T. Wan and B. J. Sutton, personal communication). Both structural determinations showed that a domain consists of a four-stranded  $\beta$ -sheet held by a central  $\alpha$ -helix (Figure 1). The domains are stabilized by hydrophobic cores and several hydrogen bonds, with a  $T_m$  of approx. 75 °C [9,11]. NMR studies performed on PpL<sub>312</sub> by Wikström et al. [12] indicated that the residues involved in the interaction between it and the  $\kappa$ -chain were concentrated along the second  $\beta$ -strand, the C-terminal end of the  $\alpha$ -helix and the loop between the  $\alpha$ -helix and

third  $\beta$ -strand. The sequences of the PpL<sub>312</sub> and PpL<sub>3316</sub> domains described above share 60% similarity, with almost identical secondary and tertiary structures ([10], and B. J. Sutton and T. Wan, personal communication). NMR studies on PpL<sub>312</sub> [12] have shown that the backbone amide signal from Tyr<sup>127</sup>, equivalent to residue Tyr<sup>53</sup> in PpL<sub>3316</sub>, is shifted on interaction between PpL<sub>312</sub> and  $\kappa$ -chain.

PpL contains three native tyrosine residues: Tyr<sup>51</sup> and Tyr<sup>53</sup>, located on opposite sides of the helix, and Tyr<sup>64</sup>, located on  $\beta$ -strand 3 (Figure 1). Initial fluorescence studies of a mutant in



**Figure 1** Ribbon diagram of PpL

The positions of tyrosine residues 51, 53 and 64 are shown; the numbering system used is from [20].

Abbreviations used: PpL, single Ig-binding domain of protein L, isolated from *P. magnus* strain 3316; PpL<sub>312</sub>, single Ig-binding domain of protein L, isolated from *P. magnus* strain 312; TNM, tetranitromethane.

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which Phe<sup>39</sup> was replaced by tryptophan to generate the PpL mutant F39W, led to the proposal that binding to  $\kappa$ -chain takes place in two steps [13]. Stopped-flow kinetic data suggest that an initial encounter complex is rapidly formed between the two proteins and that this is followed by a slow conformational rearrangement that results in the high-affinity complex observed at equilibrium [14].

To define the role of tyrosine residues in the interaction between PpL and the  $\kappa$ -chain we have performed studies using a combination of spectroscopy, site-directed mutagenesis and chemical modification. The proteins have been subjected to chemical modification by the reagent tetranitromethane (TNM) and the consequences of the modification on complex formation have been examined. TNM is a reasonably specific modification reagent that primarily targets tyrosine and cysteine residues between pH 7.5 and 8.0 [15]. Because PpL does not contain any cysteine residues the reaction is predominantly with tyrosine side chains. The nitrotyrosines are formed by a free-radical mechanism [16] and have an increased absorbance at 430 nm, although the fluorescence properties of tyrosine residues are lost. TNM has also been widely used as tool for studying the role of tyrosine residues in protein complex formation [17–19].

## MATERIALS AND METHODS

### Materials

All buffers were of analytical grade and were obtained from Merck Ltd (Poole, Dorset, U.K.). The TNM, ELISA reagents and antibodies were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). The G25-PD10 columns were purchased from Amersham Pharmacia Biotech. The  $\kappa_1$ -chain was a gift from Professor M. Glennie (Tenovus Laboratories, Southampton General Hospital, Southampton, Hants., U.K.).

### Protein purification and determination of protein concentration

The purification procedure for PpL and F39W PpL has been described elsewhere [20]. The PpL mutants Y64W and Y53F were expressed and purified in the same manner as wild-type PpL. The protein concentration of TNM-modified PpL was determined by the bicinchoninic acid assay, with bovine serum albumin as a standard [21]. The concentrations of solutions of unmodified protein were estimated from their absorbances at 280 nm with the following values: PpL,  $\epsilon_{280}$  3840 M<sup>-1</sup>·cm<sup>-1</sup>; Y64W,  $\epsilon_{280}$  8250 M<sup>-1</sup>·cm<sup>-1</sup>; Y53F,  $\epsilon_{280}$  2560 M<sup>-1</sup>·cm<sup>-1</sup>; Y53F, Y64W,  $\epsilon_{280}$  6970 M<sup>-1</sup>·cm<sup>-1</sup>.

### Mutagenesis

The three PpL mutants Y64W, Y53F and Y53F, Y64W were constructed by the methods of Kunkel [22] and were made with PpL DNA as the template. The mismatch primer used for Y64W PpL was 5'-TAATGCTGCTGTCCATTCGCCATTTAC-3' and for Y53F PpL was 5'-TAATAAGTCTGCAAATCTGTAA-GCTTC-3' (both anti-sense strand). The double mutant Y53F, Y64W was made by mutating residue 53 on the Y64W template DNA. The mismatched codons are shown in bold. The mutations were confirmed by DNA sequencing with the Sequenase 2.2 DNA sequencing kit (United States Biochemical Corporation).

### Reaction of PpL with TNM

Typically, 100–250  $\mu$ M protein in 20 mM potassium phosphate buffer, pH 8.0, was reacted with a 10-fold molar excess of TNM at 20 °C for 22 h. For the analysis of the rate of modification,

aliquots of the PpL mixture were removed at various intervals up to 22 h. The reactions were stopped by rapid gel filtration with a G25-PD10 column. The protein concentration was again determined with the bicinchoninic acid assay and quantitative analysis for the extent of nitration was made with a sample's  $A_{430}$ , taking the molar absorption coefficient as 4100 M<sup>-1</sup>·cm<sup>-1</sup> [15]. Modification of the complex was performed in the same manner with equimolar concentrations of PpL and  $\kappa$ -chain (each 100  $\mu$ M), which had been incubated for 1 h at room temperature before the addition of TNM. The figures quoted are means for three experiments.

The reduction of nitrotyrosine to aminotyrosine was performed by incubating the nitrated protein with a 10-fold molar excess of sodium hydrosulphite at room temperature. The reaction was stopped as described above and the extent of the reduction was analysed by  $A_{288}$ , taking the molar absorption coefficient as 2800 M<sup>-1</sup>·cm<sup>-1</sup> [15].

### Determination of the binding capacity of modified proteins by ELISAs

Sodium carbonate/bicarbonate buffer, pH 9.5 (200  $\mu$ l), was added to each of the wells of a 96-well microtitre plate; 150  $\mu$ l of a Y64W PpL solution (0.1 mg/ml) was added to lane two and mixed, leaving lane 1 as a control. A 150  $\mu$ l sample from lane 2 was then transferred to lane 3; this serial dilution was continued across the plate. The plate was then incubated for 2 h at 37 °C, followed by three washes with PBST [PBS containing 0.15% (v/v) Tween 20]. A 200  $\mu$ l sample of a 1:250 dilution of human IgG was added to each well and incubated for 1 h. After a further three washes with PBST a 1:1250 dilution of goat anti-(human Fc) specific IgG conjugated with horseradish peroxidase was added to each well and incubated for 1 h. After a further three washes with PBST, the substrate [0.4 mg/ml *o*-phenylenediamine/0.01% (v/v) H<sub>2</sub>O<sub>2</sub> in citrate/phosphate buffer] was added and the plate was incubated for 20 min at room temperature. The reaction was stopped by the addition of 12.5% (v/v) H<sub>2</sub>SO<sub>4</sub>. The amount of product formed was determined from  $A_{490}$  with a Dynatech MR5000 automated plate reader.

To determine the relative binding capacity of PpL at various stages of modification by TNM, the following changes were made. A 150  $\mu$ l portion of the 100 mM sodium carbonate/bicarbonate buffer was added to three wells followed by 15  $\mu$ g of the protein sample being analysed in 50  $\mu$ l of 20 mM phosphate buffer, pH 7.4. This was incubated at 37 °C for 2 h and then the method was continued as described above. Unlabelled protein was used as a control to determine the maximal binding of the protein under study. The percentage loss of binding capacity was calculated at the midpoint of the ELISA curve given by the control protein.

### Competitive ELISA techniques

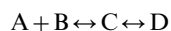
Competitive ELISAs were used to determine the  $K_d$  of the interactions between human IgG and mutated or nitrated PpL. Lanes 2–12 of a 96-well microtitre plate were coated with 200  $\mu$ l per well of a 0.04 mg/ml solution of wild-type PpL dissolved in sodium carbonate/bicarbonate buffer, pH 9.5, at 37 °C for 3 h. Lane 1 was not coated with PpL and served as a negative control. The plate was washed three times with PBST. PBST (150  $\mu$ l) was added to each well, followed by 50  $\mu$ l of 2.27 mg/ml unmodified PpL to wells A2–C2, wild-type PpL to wells D2–E2 and TNM-modified PpL to wells F2–H2. A 50  $\mu$ l sample was then removed from lane 2 and added to lane 3, mixed; this serial dilution was continued across the plate to lane 11. Lane 12 was left as a positive control. A 100  $\mu$ l sample of a 1:250 dilution of human

IgG was added to each well and the mixture was incubated for 45 min. The plate was then washed three times with PBST before the addition of 200  $\mu$ l of a 1:1250 dilution of horseradish-peroxidase-conjugated goat anti-(human IgG) to each well. Again the plate was washed three times with PBST before the addition of 200  $\mu$ l of substrate [0.5 mg/ml 2,2'-azinodi(3-ethylbenzothiazolinesulphonic acid)/0.03% (v/v)  $H_2O_2$  in 70 mM citrate/phosphate buffer, pH 4.2] to each well. After 20 min the amount of product formed was determined from  $A_{410}$  with a Dynatech MR5000 automated plate reader. Data given are means for two or three experiments (see the Results section).

The  $K_d$  values for mutated or modified PpL were calculated from the concentrations of the competitor protein required to inhibit by 50% the binding of IgG.

### Stopped-flow measurements

Stopped-flow measurements were made as generally described in [14] with an Applied Photophysics SX.17MV stopped-flow fluorimeter fitted with 2 ml syringes thermostatically controlled at 20 °C. An excitation wavelength of 280 nm was selected with a monochromator and the tyrosine emission was monitored with a suitable cut-off filter (305 nm). Concentrations quoted are final concentrations after 1:1 (v/v) mixing. The data were analysed by single-exponential and double-exponential algorithms supplied by the manufacturer. A global analysis algorithm (Pro-kineticist; Applied Photophysics Ltd.) was used to model the binding reaction to a fully reversible two-stage binding process in which a second-order binding reaction was followed by a first-order reaction of the type:



where A and B are the reactants and C is a binary initial complex that rearranges to form D.

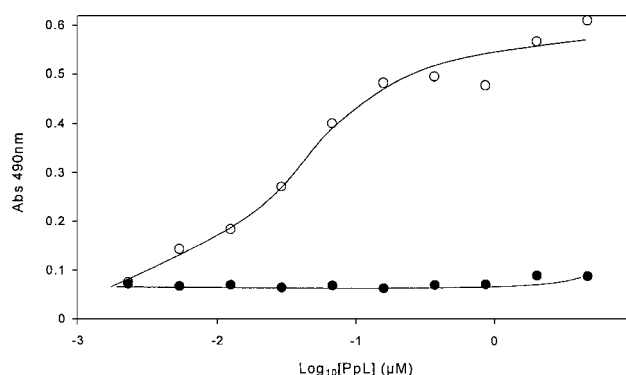
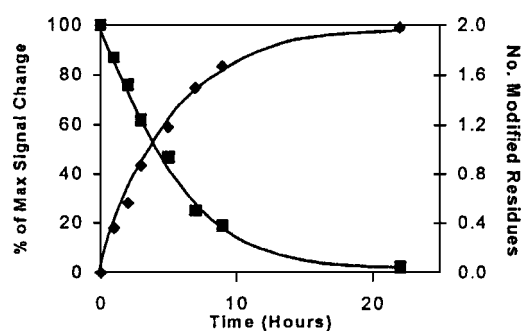
### CD spectropolarimetry

CD measurements were made on a JASCO J-720 CD spectrometer with procedures described in [14]. Spectral corrections and calculations were performed with the manufacturer's software. The results are expressed as molar ellipticity with units of  $deg \cdot cm^2 \cdot dmol^{-1}$ .

## RESULTS

### Modification of PpL

Table 1 and Figure 2 (upper panel) show that  $1.9 \pm 0.06$  equiv. of the three native tyrosine residues of wild-type PpL were modified



**Figure 2** Decrease in binding affinity caused by the modification of Y64W PpL by TNM

Upper panel: the rate of modification of tyrosine residues of PpL by TNM (◆) and the corresponding change in binding capacity of the modified protein (■). Binding capacity was determined by ELISA experiments and is defined here as the percentage of the  $A_{490}$  obtained with modified protein relative to that obtained with unmodified protein. Lower panel: the results of a standard ELISA experiment in which the binding of human IgG to unmodified (○) and modified (●) Y64W PpL proteins was measured.

over a period of 22 h when incubated with TNM. No further modification was observed either by extending the incubation time or increasing the excess of TNM. Figure 2 (upper panel) also illustrates the loss of binding capacity of PpL for IgG (see the Materials and methods section) that occurs as the PpL is nitrated and shows that it decreased to an almost undetectable level when approx. 2 equiv. of tyrosine had been modified. Figure 2 also summarizes the results from standard ELISAs performed with the Y64W mutant PpL domain and Y64W PpL modified by reaction with TNM. It is clear from this figure that

**Table 1** Number of equivalents of tyrosine modified by TNM in various PpL proteins,  $\kappa$ -chain or complexes and the effect on  $K_d$  for the complexes formed with IgG

Results are means  $\pm$  S.D. for three experiments. Abbreviation: n.d., not determined.

Protein species	$K_d$ for complex containing non-nitrated domain and IgG	Equivalents of modified tyrosine	$K_d$ for complex containing nitrated domain and IgG
Wild-type PpL	$112 \pm 20$ nM	$1.9 \pm 0.06$	$15.2 \pm 3$ $\mu$ M
Y64W PpL	$153 \pm 33$ nM	$1.9 \pm 0.04$	$\sim 30$ $\mu$ M
Y53F PpL	$3.2 \pm 0.2$ $\mu$ M	$1.1 \pm 0.21$	n.d.
Y53F,Y64W PpL	$4.6 \pm 1$ $\mu$ M	$1.0 \pm 0.14$	$23 \pm 6$ $\mu$ M
$\kappa$ -chain	—	$3.1 \pm 0.09$	—
PpL- $\kappa$ -chain complex	—	$4.1 \pm 0.04$	—
Y53F PpL- $\kappa$ -chain complex	—	$3.9 \pm 0.1$	—

the nitration of this mutant protein also led to a substantial loss of binding affinity for human IgG and resulted in the nitration of approx. 2 equiv. of tyrosine ( $1.9 \pm 0.04$ ). This shows that the removal of Tyr<sup>64</sup> by mutagenesis did not affect the number of residues susceptible to modification, suggesting that the two nitratable residues are Tyr<sup>51</sup> and Tyr<sup>53</sup>, which lie on opposite sides of the  $\alpha$ -helix (see Figure 1). Reduction of the nitrate groups to amino groups was achieved by the addition of sodium hydrosulphite as described in [15]. However, there was no change in the binding capacity of the nitrated Y64W PpL after this procedure.

### Nitration of the PpL- $\kappa$ -chain complex

Some of the tyrosine residues in PpL or  $\kappa$ -chains susceptible to modification in the free protein might be protected from TNM after formation of the PpL- $\kappa$ -chain complex. To check this, equimolar amounts (100  $\mu$ M) of each protein were incubated for 1 h, to allow the complex to form, before the addition of 1 mM TNM under the same conditions as before. Analysis showed that  $4.1 \pm 0.04$  tyrosine residues had been modified after 22 h. When  $\kappa$ -chain alone was incubated with TNM,  $3.1 \pm 0.1$  equiv. of tyrosine on the  $\kappa$ -chain became nitrated over a period of 22 h. This suggested that there was a loss of one available tyrosine residue for reaction with TNM when the proteins were in a complex.

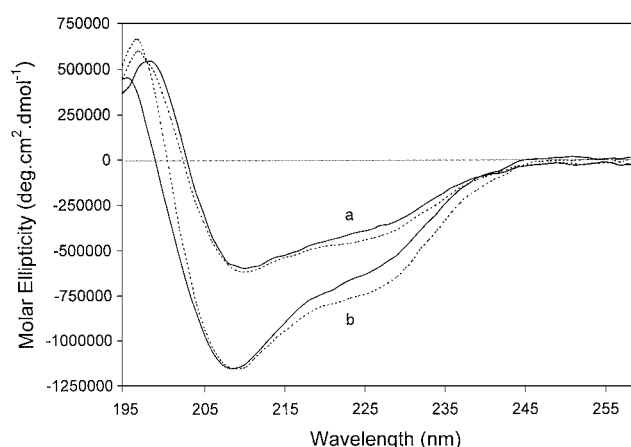
The NMR data published by Wikström et al. [12] showed that the <sup>1</sup>H-<sup>15</sup>N heteronuclear single-quantum coherence NMR signal of the Tyr<sup>127</sup> backbone amide in PpL<sub>312</sub>, equivalent to Tyr<sup>53</sup> in PpL, was significantly shifted when PpL<sub>312</sub> was complexed with the V<sub>1</sub> domain of the  $\kappa$ -chain. No such change in the backbone amide signal of Tyr<sup>125</sup> (Tyr<sup>51</sup> in PpL) was observed, suggesting that this residue was not involved in interactions with  $\kappa$ -chain. Tyr<sup>53</sup> was therefore targeted for mutagenesis experiments and was replaced by Phe to form Y53F PpL.

Only  $1.1 \pm 0.21$  equiv. of tyrosine were nitrated when Y53F PpL was incubated with TNM, suggesting that Tyr<sup>53</sup> was one of the residues in PpL modified by the reagent. A similar stoichiometry of reaction ( $1.0 \pm 0.14$ ) was found in experiments with the doubly mutated Y53F,Y64W protein, confirming that Tyr<sup>51</sup> was also nitrated. A solution of the complex Y53F PpL- $\kappa$ -chain (made by incubating 100  $\mu$ M Y53F PpL with 100  $\mu$ M  $\kappa$ -chain) was also reacted with TNM; once again, approx. four Tyr residues ( $3.9 \pm 0.1$ ) were modified. This result strongly suggests that Tyr<sup>53</sup> was the residue protected from TNM when the proteins were in a complex.

### Determination of the $K_d$ values for the complexes formed between $\kappa$ -chain and nitrated or non-nitrated PpL proteins

The  $K_d$  values of the complexes formed between IgG and non-nitrated or nitrated mutants of PpL were determined by a competitive ELISA protocol described in the Materials and methods section; they are summarized in Table 1. The  $K_d$  for the complex formed between non-nitrated Y64W PpL and IgG,  $153 \pm 33$  nM, was close to that for the non-mutated PpL ( $112 \pm 20$  nM), showing that the Y64W substitution had little effect on the binding interaction with  $\kappa$ -chain. Nitration of PpL almost totally removed IgG binding, with an approx. 140-fold increase in  $K_d$  ( $15.2 \pm 3$   $\mu$ M). Similarly, nitration of Y64W PpL caused an approx. 200-fold increase in  $K_d$  (estimated to be approx. 30  $\mu$ M).

The complex formed between non-nitrated Y53F and IgG ( $3.2 \pm 0.2$   $\mu$ M) had a much higher  $K_d$  than that for native PpL; that formed between the double mutant Y53F,Y64W and IgG



**Figure 3** CD spectra of modified and unmodified PpL

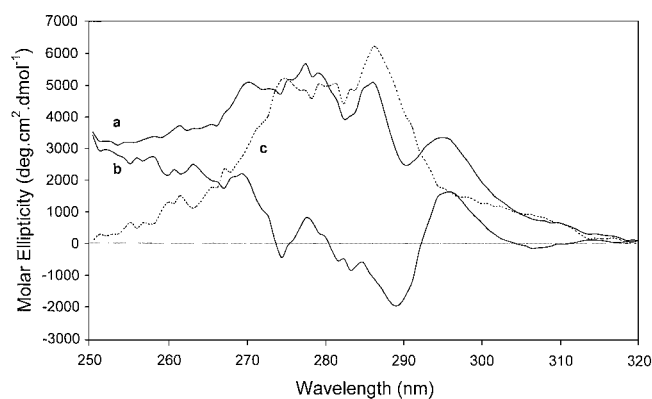
Far-UV CD spectra of 16.5  $\mu$ M samples of (traces a) unmodified Y64W PpL (solid line) and Y53F, Y64W PpL (dotted line) and (traces b) the same proteins modified by TNM.

was similarly raised to  $4.6 \pm 1$   $\mu$ M under these conditions. These results show that the replacement of Tyr<sup>53</sup> by phenylalanine caused a marked decrease in binding affinity for IgG. When nitrated, the Y53F,Y64W domain exhibited an approx. 5-fold increase in  $K_d$  to  $23 \pm 6$   $\mu$ M, showing that the nitration of Tyr<sup>51</sup> led to a relatively small decrease in affinity for IgG.

### Observation of the formation of complexes by CD spectropolarimetry

Figure 3 shows the effect of nitration on the CD spectra of Y64W and Y53F,Y64W PpL domains between 195 and 260 nm. NMR data show that PpL has only one helix lying  $11^\circ$  relative to the two anti-parallel  $\beta$ -sheets (see Figure 1) and the spectra all show a minimum at 208 nm typical of proteins that contain  $\alpha$ -helical elements. However, the CD contribution from the high  $\beta$ -sheet content of the domains decreases the amplitude of the minimum at 222 nm (also associated with  $\alpha$ -helices) to a shoulder. The spectra of both proteins show significant ellipticity changes at wavelengths between 195 and 245 nm on nitration. These changes are almost certainly due to the nitration of Tyr<sup>51</sup> because the spectral changes from the wild-type and the Y53F,Y64W proteins are very similar and only Tyr<sup>51</sup> is available for reaction in the Y53F,Y64W protein. However, structural changes due to nitration of Tyr<sup>53</sup> cannot be excluded.

Figure 4 shows the difference in molar ellipticity between the summed spectra of wild-type PpL and  $\kappa$ -chain and that of their complex between 250 and 320 nm (curve a). This difference spectrum shows the complex to have decreased ellipticity at all wavelengths between 250 and 320 nm, wavelengths normally associated with aromatic side chains. The figure also displays the equivalent difference spectrum obtained in similar experiments with Y53F PpL (curve b). In this case there is a significantly decreased difference between the spectra of the non-complexed and complexed proteins at these wavelengths, indicating that the large signal change seen in experiments with the wild-type protein most probably arises from Tyr<sup>53</sup>. The net difference in ellipticity on complex formation arising from the Y53F replacement is given by curve c (curve a – curve b) in Figure 4. This clearly shows that the CD spectral changes shown when the Y53F or wild-type PpL binds to  $\kappa$ -chain are localized to wavelengths of approx.



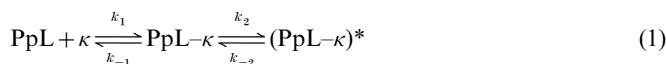
**Figure 4** Spectral changes between 250 and 320 nm on the formation of complexes between Y53F or wild-type PpL with  $\kappa$ -chain

The difference in molar ellipticity between the summed spectra of wild-type or Y53F PpL and  $\kappa$ -chain and that of their complex (curves a and b respectively) is shown. The net difference in molar ellipticity on complex formation arising from the Y53F replacement, i.e. curve a—curve b, is also shown (curve c).

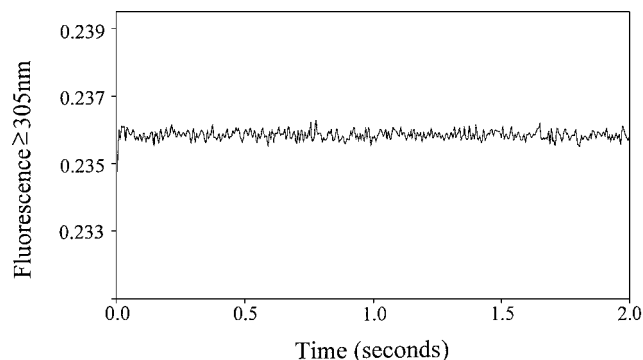
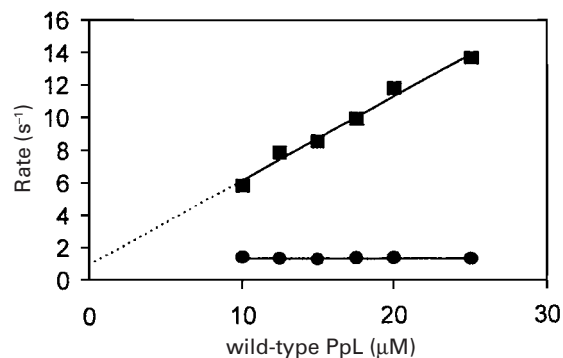
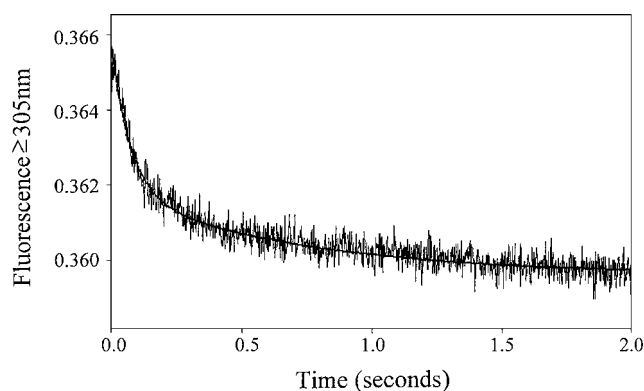
280 nm with peaks at 276, 280 and 286 nm, normally attributable (in the absence of tryptophan residues) to tyrosine contributions.

#### Observation of the formation of the wild-type PpL- $\kappa$ -chain complex by stopped-flow fluorimetry

The interaction between wild-type PpL and  $\kappa$ -chain can be detected by a net decrease in fluorescence intensity at 302 nm, indicative of a perturbation of the fluorescence emission from a tyrosine residue [14]. This change in fluorescence intensity was investigated by stopped-flow fluorimetry; Figure 5 (top panel) shows a typical reaction progress curve obtained when 25  $\mu$ M PpL was reacted with 1.5  $\mu$ M  $\kappa$ -chain in phosphate buffer, pH 8.0, at 20 °C. The fluorescence intensity decreased in two phases: the first with a pseudo-first-order rate of 11.7 s<sup>-1</sup> and the second with a rate of 1.2 s<sup>-1</sup>. The observation of a two-step binding process, the rapid formation of an encounter complex followed by a conformational change, is consistent with previous studies performed with mutated PpL domains bearing a tryptophan reporter group [14]. The proposed reaction can be described by:



in which (PpL- $\kappa$ )\* is a conformationally different form of PpL- $\kappa$ . This reaction starts with the formation of a relatively low-affinity intermediate complex [described by the forward ( $k_1$ ) and reverse ( $k_{-1}$ ) rates of the fast initial process] that relaxes into a high-affinity complex as a conformational change takes place. Figure 5 (middle panel) describes the relationship between the concentration of the variable ligand used (PpL) and the rates of the observed fluorescence changes ( $k_{1,obs}$ ) for the formation of PpL- $\kappa$ . Also shown is a plot of the dependence of the observed rate of the conformational change in the forward direction ( $k_{2,obs}$ ) on PpL concentration. The value of  $k_{1,obs}$  increases linearly with the concentration of PpL used, as would be expected for a bimolecular reaction. From these plots  $k_1$  and  $k_{-1}$  can be shown to be  $5.15 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$  and  $0.94 \text{ s}^{-1}$  respectively and  $k_{2,obs}$  can be estimated to be approx.  $1.2 \text{ s}^{-1}$ . The pre-equilibrium  $K_d$  was calculated as 1.8  $\mu$ M from these values of  $k_1$  and  $k_{-1}$  under these



**Figure 5** Stopped-flow fluorescence analysis of the reaction between wild-type PpL and  $\kappa$ -chain

Top panel: a typical reaction progress curve obtained when 1.5  $\mu$ M  $\kappa$ -chain was reacted with 25  $\mu$ M wild-type PpL in 20 mM potassium phosphate buffer, pH 8.0, at 20 °C. Details are given in the Materials and methods section. The line represents the global analysis fit to the average of three experimental curves with the values  $k_1 = 5.15 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ ,  $k_{-1} = 0.94 \text{ s}^{-1}$ ,  $k_2 = 1.33 \text{ s}^{-1}$  and  $k_{-2} = 0.068 \text{ s}^{-1}$ . Middle panel: the relationship between the concentration of PpL used and the observed rate of the initial step of the reaction (■) and the second step of the reaction (●). Bottom panel: the lack of fluorescence change when Y53F PpL was reacted with  $\kappa$ -chain under the same conditions as above. The fluorescence intensity of the Y53F mutant was 65% of that of the wild-type protein owing to the replacement of the tyrosine residue by phenylalanine.

conditions.  $K_d$  for the reaction at equilibrium can be calculated from:

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

The rate  $k_{2,obs}$  is equal to the sum of  $k_2$  and  $k_{-2}$  and changes with the concentration of the PpL used, as described by:

$$k_{2,obs} = k_{-2} + k_2 \{ [PpL] / ([PpL] + K_d) \} \quad (3)$$

in which [PpL] is the concentration of ligand used and  $K_d$  is the dissociation constant for the initial complex formed between PpL and  $\kappa$ -chains. At high [PpL] (relative to the  $K_d$  of the initial complex formed) eqn (3) simplifies to:

$$k_{2,obs} = k_{-2} + k_2 \quad (4)$$

The value of  $k_2$  is therefore derived from eqn (3) or eqn (4) by experimental determination of  $k_{2,obs}$ . However, in this case no value for  $k_{-2}$  could be measured experimentally owing to the high stability of the complex at equilibrium and therefore a global analysis algorithm was used to model the system with the determined values of  $k_1$  and  $k_{-1}$  and an estimated value of  $k_2$ . If  $k_2$  is significantly greater than  $k_{-2}$  (as was the case here because the pre-equilibrium  $K_d$  was approx. 14-fold the equilibrium  $K_d$  for the PpL- $\kappa$ -chain complex determined elsewhere [14]), then  $k_{2,obs}$  approaches  $k_2$ . Therefore the value of  $k_{2,obs}$  at high concentrations of PpL was used as an estimate of  $k_2$  for global analysis. The curves (average of three) were fitted to yield calculated values of all four rate constants. These were  $k_1 = 5.15 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ ,  $k_{-1} = 0.94 \text{ s}^{-1}$ ,  $k_2 = 1.33 \text{ s}^{-1}$  and  $k_{-2} = 0.068 \text{ s}^{-1}$ . As  $k_2 \gg k_{-2}$  then eqn (2) simplifies to:

$$K_d \text{ at equilibrium} = (k_{-1}k_{-2}) / (k_1k_2) \quad (5)$$

and yields a value of 91 nM. This is close to the value ( $132 \pm 3 \text{ nM}$ ) determined by fluorescence methods [14] and shows that the conformational change increases the affinity of the complex approx. 20-fold under these conditions.

Stopped-flow experiments with the Y53F mutant (Figure 5, bottom panel) showed that no transient change in fluorescence emission occurred on mixing with  $\kappa$ -chain, further supporting the proposal that Tyr<sup>53</sup> was the source of spectral changes on complex formation. Examination of the fluorescence emission spectrum of the Y53F mutant in free solution showed that the replacement of the tyrosine residue by phenylalanine resulted in a 35% decrease in fluorescence intensity at 302 nm and that no spectral differences were evident at equilibrium (results not shown) when in complex with  $\kappa$ -chain.

## DISCUSSION

The results presented here show that only two of the three tyrosine residues in PpL can be significantly nitrated by TNM. The loss of binding affinity for  $\kappa$ -chain reaches a maximum when both residues have been modified. This indicates either that both residues are important in the binding reaction or that the two tyrosines are equally exposed and reactive to the reagent but the modification of only one of them is responsible for the loss of binding affinity. Because experiments with the mutant Y64W PpL yielded the same result it can be concluded that the two modified tyrosine residues lie in positions 51 and 53, which are located on opposite faces of the  $\alpha$ -helix. Examination of the structure of PpL shows that the side chains of both residues are available for approach by the reagent. Tyr<sup>51</sup> faces towards the third  $\beta$ -strand, away from the proposed binding site for  $\kappa$ -chains, whereas the side chain of Tyr<sup>53</sup> faces towards the second  $\beta$ -strand and directly towards the proposed binding site. The nitration experiments with mutated proteins showed that the modification of two residues in the Y64W protein leads to an increase in the  $K_d$  for the binding reaction from 153 nM to more than 30  $\mu\text{M}$ , whereas the nitration of Tyr<sup>51</sup>, present in the mutant Y53F, Y64W, only leads to an increase in  $K_d$  from 4.6  $\mu\text{M}$  to

$23 \pm 6 \mu\text{M}$ . Thus the primary cause of loss of affinity is most probably the nitration of Tyr<sup>53</sup>.

The loss of binding observed on the nitration of the tyrosine residues in PpL could be due to a number of factors. First, Tyr<sup>53</sup> might have a direct role in the binding with  $\kappa$ -chain. Secondly, the nitration of tyrosine residues results in a decrease in the  $pK_a$  of the phenol group from approx. 10.5 to 6.8. Thirdly, the bulky nitrate group might cause steric hindrance to the approach of the  $\kappa$ -chain (although a decrease in the size of the nitrate group to an amino group failed to restore higher binding affinity to the modified protein). Lastly, the possibility that secondary structural changes lead to a loss of binding cannot be excluded. The CD experiments suggest that the loss of binding activity due to the nitration of Tyr<sup>51</sup> is most probably due to gross changes in secondary structure rather than a loss of key binding interactions.

The replacement of the Tyr<sup>53</sup> residue by phenylalanine and the resulting 28-fold increase in  $K_d$  for the complex with  $\kappa$ -chain indicates that this residue does have a key role in the stability of the complex. Calculations based on the change in  $K_d$  on replacement of this residue by phenylalanine suggest a loss of binding energy of 8 kJ/mol, which is similar to that associated with hydrogen-bond formation [23]. Therefore the decrease in affinity shown by this mutant might well indicate a role of the hydroxy group in a hydrogen-bond interaction with the  $\kappa$ -chain.

Recent NMR studies by Enokizono et al. [7] suggest that no tyrosine residues on the  $\kappa$ -chain are involved in the formation of the PpL<sub>312</sub>- $\kappa$ -chain complex; this is largely supported by experiments performed with nitrated  $\kappa$ -chain. Despite the fact that three tyrosine residues are accessible to and can be modified by the reagent, the  $K_d$  for the complex with a mutant PpL (with the same binding characteristics as the wild-type protein) is only 4-fold higher [24] than for the same complex formed with non-nitrated  $\kappa$ -chain.

Previous spectroscopic and NMR studies showed that the signal from the backbone amide of Tyr<sup>127</sup> (equivalent to Tyr<sup>53</sup>) broadened on formation of the complex with the  $V_L$  domain of  $\kappa$ -chains, suggesting that it is directly involved in the interaction. This is supported by the CD experiments described above on wild-type PpL and the Y53F PpL mutant, which show that a decrease in ellipticity at wavelengths associated with tyrosine side chains does not occur when Tyr<sup>53</sup> is replaced by Phe<sup>53</sup>. Furthermore, a decrease in fluorescence intensity that occurs when PpL binds to  $\kappa$ -chain does not occur when the Y53F mutant is used to make the complex. Therefore the fluorescence changes that accompany the two-step binding reaction between PpL and  $\kappa$ -chain must also arise from perturbation of the environment around Tyr<sup>53</sup>. It is possible that Tyr<sup>53</sup> reports the formation of the initial encounter complex, PpL- $\kappa$ , and that as the conformational change pulls the equilibrium to the right [see eqn (1)] more PpL becomes complexed with  $\kappa$ -chain. Thus the rate of change in fluorescence intensity that accompanies the second step in the binding reaction is limited by  $k_2$ .

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