A Study of the Interactions Between an IgG-Binding Domain Based on the B Domain of Staphylococcal Protein A and Rabbit IgG

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Abstract

The nonantigenic interaction between a recombinant immunoglobulin G (IgG)-binding protein based on the B domain of Protein A from *Staphylococcus aureus* (termed SpA^1) and the Fc fragment of rabbit IgG has been investigated. The contribution to binding of four putative hydrogen bond contacts between SpA^1 and IgG-Fc were examined by the individual substitution of the residues in SpA^1 involved in these interactions by others unable to form hydrogen bonds. It was found that the most important of the hydrogen bonds involved Tyr 18 which, when replaced by Phe, resulted in a twofold decrease in IgG-binding affinity. The residues of SpA^1 proposed to make close, mainly hydrophobic, contacts with Fc were replaced by residues with potential electrostatic charge to establish the importance of the hydrophobic interaction in the complex. The IgG-binding affinities of the mutant proteins were compared to the wild-type protein by a competitive enzyme-linked immunosorbant assay. The replacement of individual hydrophobic residues by His generated a number of novel IgG-binding proteins with reduced binding affinity at pH 5.0 but which maintained strong binding affinities at pH 8.0. The elution profile of human IgG_1-Fc (Fc fragment of human IgG_1) from a column made from an immobilized two-domain mutant protein shows that the complex dissociates at a higher pH relative to that of the non-mutated protein thus offering favorable elution characteristics.

Index Entries: Protein A; immunoglobulins; mutagenesis; affinity chromatography; ELISA.

1. Introduction

Many pathogenic micro-organisms display proteins on their cell surface which can bind to various plasma proteins such as human serum albumin, proteinase inhibitors and to immunoglobulins in a nonantigenic manner *(1)*. This specific interaction with immunoglobulins (Ig) does not elicit an immune response by the host against the bacterium. Examples of these Ig-binding proteins are protein A from *Staphylococcus aureus* and protein G from groups C and G *Streptococci (2)* which interact predominantly with the CH_2-CH_3 interface of IgG. The ability to bind to these plasma proteins is thought to help the bacteria evade the hosts defence mechanism *(1)*.

Protein A has five homologous IgG-binding domains (termed E, D, A, B, and C) *(3,4)*, situated in the amino terminal region, each of approx 58 amino acids. The carboxyl terminal region is covalently bound to the peptidoglycan of the cell wall and the cell membrane of *S. aureus (4)*. Protein A interacts with the Fc region of human, rabbit, pig, and guinea pig IgG with high affinity and specificity *(5)*. It also shows weak reactivity toward the Fab region of IgA, IgE, and IgM *(6)*, and this binding site has been mapped more precisely for human Igs, to the heavy-chain-variable region of molecules bearing the VHIII gene segments *(7)*. It has been proposed that the Fab binding to protein A is mediated by the D domain *(8)*.

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The structure of the B domain of protein A bound to human Fc has been determined by X-ray crystallography at 2.8Å resolution *(9)*. This showed that the major part of domain B is composed of two antiparallel α-helices (Helix 1, Gln 13–Leu 21 and Helix 2, Glu 29–Asp 40) with the rest of the polypeptide chain being folded irregularly. The interactions in the complex were reported to be mainly hydrophobic, with four putative hydrogen bonds contributing to the stability of the complex. Later, the 2-dimensional ^{1}H-NMR of free domain B revealed an additional helical region (Ser 45–Ala 58) *(10)* and subsequent 3-dimensional ^{1}H-^{15}N-NMR studies of the Fc-bound domain B complex led to the conclusion that the tri-helical bundle is retained in the complex (*see* **Fig. 1**) *(11)*. Helices 2 and 3 are antiparallel to each other interacting with each other the most intimately, forming a hydrophobic core, whereas the long axis of Helix 1 is tilted at an angle of ~30° relative to the other helices.

Here we report studies that have examined the protein-protein interactions formed in the complex between IgG-binding proteins based on the B domain of protein A from *S. aureus* (called SpA^1 and SpA^2 for proteins with one or two sequential binding domains, respectively (for full details, *see* **ref. *12***) and the Fc region of rabbit IgG. The B domain contacts three regions of human Fc. Region 1 involves residues 252–254, region 2 is formed from residues 309–312, and region 3 includes residues 433–436 of Fc. All of these regions are conserved in rabbit IgG. The crystallographic studies of Deisenhofer *(9)* implicated a contribution to the stability of the complex from four hydrogen bonds formed between Gln 13, Asn 15, Tyr 18, and Asn 32 of SpA and Fc. Site-directed mutagenesis has been used to evaluate the contribution of these hydrogen bonds to the stability of the complex with rabbit IgG. Other replacements of hydrophobic residues in SpA by His have been made to examine the contribution of hydrophobic contacts to the stability of the SpA-Fc complex. Protein A is a versatile immunological tool because of its strong interaction and wide binding specificity with the Fc domain of a large number of Igs and has been used

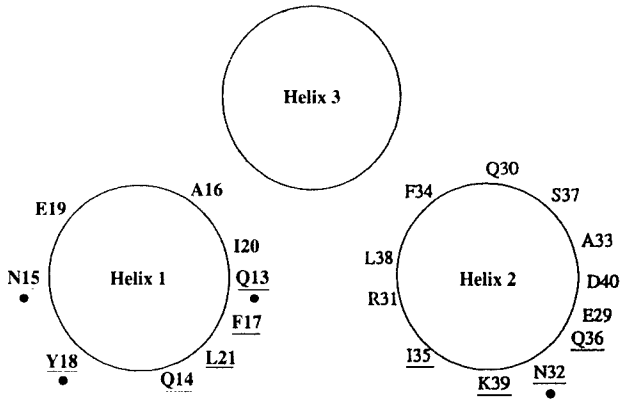

Fig. 1. A helical wheel representation of the amino acid residues in helix 1 and helix 2 of the B domain of protein A. The underlined residues are those that are shown to contact Fc in the crystal structure of Deisenhofer *(9)*, and those marked by a closed circle are those that are predicted from the same data to participate in hydrogen bonds with Fc.

to purify antibodies and their fragments *(6)*. The principal problem with using protein A in this application is the acidic pH required to disrupt the binding complex. One of the goals of our research is to engineer new proteins based on SpA^1 that allow the use of more favorable elution conditions when used as affinity ligands.

2. Materials and Methods

2.1. Chemicals

Chemicals were obtained from Sigma (Poole, Dorset, UK) and BDH (Poole, Dorset, UK). Rabbit IgG-HRP was purchased from Serotec (Oxford, UK). The triazine-activated agarose was purchased from Affinity Chromatography (Isle of Man, UK).

2.2. Bacterial Strains, Plasmids, Mutagenesis, and Cell Growth

Escherichia coli strain JM103 was used as a bacterial host in all experiments. The plasmid and phage vectors used were pKK223-3 and phage M13mp18). Bacteria were routinely grown and the protein purified as described in *(12)*. The construction of the gene fusion plasmid pSpA^1 and pSpA^2 are described elsewhere (termed pCSpA$_B$-1 and pCSpA$_B$-2 in **ref. *13***). The plasmid pSpA^1 was constructed by digesting pSpA^2 with Mlu I,

which removed a 174bp DNA fragment coding for the first IgG-binding domain. The plasmid was then religated to generate pSpA[1]. These plasmids were then used as templates for mutagenesis studies. The *Eco*R I–*Pst*I fragment encoding the gene SpA[2] was subcloned into bacteriophage M13mp18. Site-directed mutagenesis was performed in both domains of SpA[2] simultaneously, as described in ***(13)***. The oligonucleotides used were synthesised on an Applied Biosystems 381A DNA synthesizer. The sequences of the mismatch primer oligonucleotides are shown below.

Q13A5' gaa cgc gtt ctg c**gc** ttc ttt gtt 3'
Q14H5' gta gaa cgc gtt **g**tg ctg ttc ttt 3'
N15A5'c gta gaa cgc **cgc** ctg ctg ttc ttt 3'
N15H5' c gta gaa cgc gt**g** ctg ctg ttc ttt 3'
F17H5' taa gat ctc gta g**tg** cgc gtt gtc gtc 3'
L21H5' gtt cgg cag atg **gtg** gat ctc gta gaa 3'
N32H5' g aat gaa agc gt**g** acg ctg ttc ttc 3'
K39H5' gct cgg gtc gtc **gtg** cag aga ctg a 3'

The mutated base(s) in each codon is shown in boldface in each case. The nucleotide sequences of the mutant genes were verified by DNA sequencing of the single-stranded M13mp18 using a Sequenase kit obtained from US Biochemical Corporation. The mutated gene was excised from M13mp18 by *Eco*RI–*Pst*I digestion and ligated into the expression vector pKK223-3. The single-domain recombinant proteins are termed SpA[1], and the double-domain proteins SpA[2] with the mutation indicated in brackets.

2.3. Competitive ELISA

The wells of a microtiter plate were coated with a constant amount of SpA[2] (115 ng/well) (except for lane 1 which serves as a blank control) at 37°C for 2 h. The plates were then washed four times with 20 m*M* bis-tris-propane tween (BTPT) buffer at the desired pH. 100 µL of BTPT buffer was added to each well, and 50 µL of the mutant protein of known concentration (0.2 mg/mL) was added to lane 2 and serial dilutions of the mutant protein made across the plate (except for lane 12, which serves as a control for maximal IgG binding by the immobilised SpA[2]). Then 100 µL of a rabbit antimouse IgG-horseradish peroxidase (HRP) conjugate diluted 2500-fold in BTPT buffer at the desired pH was added to each well. This was then incubated for 30 min. The plate was then washed four times with BTPT buffer at the desired pH and finally with BTPT buffer at pH 8.0 to ensure all wells are under the same conditions for the substrate reaction. 200 µL of the substrate solution was then added to each well (0.4 mg/mL o-phenylenediamine, 0.1% v/v H_2O_2 in 0.1 *M* citrate/phosphate buffer). The reaction was stopped after 20 min by the addition of 50 µL of 12.5% sulfuric acid, and the absorbance measured at 490 nm on a Dynatech MR5000 automated plate reader.

2.4. Protein Concentration

For routine estimations the Bicinchoninic acid method ***(14)*** was used. Alternatively, the Molar Extinction Coefficients at 280 nm were used. For wild-type SpA[1] and SpA[2], these are 10240/M/cm and 11520/M/cm, respectively. For the mutants with Tyr 18 Phe or Tyr 18,76 Phe replacements the Molar Extinction Coefficients at 280 nm are 8960/M/cm and 10240/M/cm, respectively.

2.5. Circular Dichroism

Circular Dichroism studies were carried out on a Jasco J-720 spectropolarimeter using 0.1-cm light path cells and 0.1 mg/mL of each protein in 20 m*M* potassium phosphate buffer pH 8.0 at 25°C. The units are given as mean residue ellipticity (deg.cm^2/dmol).

2.6. Immobilization of the Protein

The proteins were immobilized onto triazine-activated agarose as recommended by the manufacturers at a concentration of approx 3 mg/mL moist gel. Column chromatography was performed at room temperature using the following procedure. Columns of immobilized protein (2 × 1 cm) were equilibrated with 20 m*M* phosphate buffer pH 8.0 at a flow rate of 0.2 mL/min and 1 mg of human IgG_1-Fc dissolved in equilibration buffer was loaded on to the column. The column was then washed with the equilibration buffer for 40 min to remove unbound material. The bound human IgG_1-Fc was then eluted stepwise firstly

by changing the pH to 5.0 with 0.5 *M* acetate buffer, pH 5.0, at a new flow rate of 0.4 mL/min for 20 min and then secondly by a linear pH gradient from pH 5.0 to pH 3.0 starting with 0.5 *M* acetate buffer, pH 5.0, and ending with 0.5 *M* acetate buffer, pH 3.0. 0.6-mL fractions were collected and the protein content of each fraction measured by reading the OD_{280nm}. The pH of each fraction was also measured using a micro pH electrode. The columns of immobilized SpA^2 proteins were stored in 20 m*M* phosphate buffer, pH 8.0, containing 0.02% sodium azide at 4°C.

3. Results

The first aim of this investigation was to determine the contribution made by the four putative hydrogen bonds to the stability of the complex formed between each domain of SpA^2 and rabbit IgG. The residues involved in the putative hydrogen bonds are shown in **Fig. 1** and listed in **Table 1**, and the contribution of these hydrogen bonds to the binding interaction was determined by replacing each of the residues of SpA^2 by a residue that cannot participate in hydrogen bond formation. The removal of the hydrogen bond involving Tyr 18, which donates the hydrogen atom from its hydroxyl group to the carbonyl group of the peptide backbone between residues 432 and 433 of Fc, was achieved by the replacement of Tyr 18 by Phe. The other three residues per domain of SpA^2 that form hydrogen bonds were replaced by Ala, which cannot participate in hydrogen bond formation. The effect of these mutations on the IgG-binding affinities of two domain proteins was determined by competitive ELISA and the results are summarized in **Table 2**. The Kd_{app} value is calculated from the concentration of mutant protein in free solution, which causes a 50% inhibition of IgG-HRP binding to immobilized SpA^2 protein. It was found that replacing Gln 13 by Ala, Asn 15 by Ala or His, and Asn 32 by His in each domain makes little difference to the Kd_{app} values (*see* **Table 2**). The most significant change noted was for the Tyr 18 to Phe replacement in each domain resulting in a two fold decrease in binding affinity compared with native SpA^2.

The second aim of this study was to investigate the contribution made by some hydrophobic contacts to the stability of the complex between each domain of SpA^2 and rabbit IgG. The residues of SpA that make hydrophobic contacts with human Fc are Gln 13 (which also makes a hydrogen bond with Ser 254 of Fc), Gln 14 (which makes hydrophobic contacts with Leu 251 and Ile 253 of Fc), Phe 17 (which also makes close contacts with Ile 253 of Fc), Asn 15 and Asn 32 (involved in intermolecular hydrogen bonds with Fc as discussed earlier), Leu 21 (makes hydrophobic contacts with the main chain groups of Gln 311 and the imidazole ring of His 435 of Fc) and Lys 39 (involved in hydrophobic interactions with His 310, Ile 233, and Leu 309 of Fc). The same residues in the second domain of a two-domain construct are numbered +58 relative to those in the first domain (e.g., Tyr 18,73). These residues in each domain were individually substituted by His residues. It was proposed that the presence of a positive electrostatic charge at low pH would disrupt the hydrophobic interactions formed in the complex and may therefore reduce the IgG-binding affinity. Any change in this affinity was examined by competitive ELISA performed at pH 8.0 (the pH optimum for SpA binding to Fc) and at pH 5.0 (a pH at which the His residues should be predominantly positively charged).

Data shown in **Table 3** confirm previous observations ***(12)*** that the single-domain construct (SpA^1) binds IgG approx fourfold weaker than the two domain protein, at pH 8.0. In the mutant protein SpA^1 (Phe17His) the binding affinity for IgG at pH 8.0 is more than 100 fold weaker than nonmutated SpA^1 and was too weak to be determined at pH 5.0. The affinity for rabbit IgG of SpA^1 (Leu21His) is fivefold weaker than the wild-type protein at pH 8.0 (Kd_{app} = 206 n*M*) and decreases approx 25 fold more at pH 5.0 (Kd_{app} > 5000 n*M*). The two domain protein SpA^2 shows only a twofold reduction in the IgG-binding affinity at pH 5.0 compared with pH 8.0 showing that its binding interaction with IgG is still relatively strong at the lower pH. At pH 8.0 the binding affinities of two-domain mutant proteins for the complex with rabbit IgG are similar to that of native SpA^2.

Table 1
The Residues Involved in the Four Putative Hydrogen Bonds Formed in the Complex Between the B Domain of Protein A and Human Fc

SpA		Fc		
Residue	Donor/acceptor	Residue	Donor/acceptor	Distance (Å)
Gln 13	Donor	Ser 254	Acceptor	3.18
Asn 15	Donor	Asn 434	Acceptor	2.96
Tyr 18	Donor	Leu 432	Acceptor	3.09
Asn 32	Acceptor	Gln 311	Donor	2.86

Table 2
The Kd_{app} Values (n*M*) for the Complex with Rabbit IgG Formed by Nonmutated and Mutated SpA^2 Proteins at pH 8.0

Protein	Kd_{app} (n*M*) +/– Std. Dev. (*n* = 3)
SpA^2	10 ± 4
SpA^2 (Gln 13,71 Ala)	14 ± 4
SpA^2 (Asn 15,73 Ala)	14 ± 2
SpA^2 (Asn 15,73 His)	9 ± 4
SpA^2 (Tyr 18,76 Phe)	20 ± 2
SpA^2 (Asn 32,90 His)	11 ± 5

Table 3
The Kd_{app} (n*M*) of SpA^2 and His mutants of SpA^2 at pH 8.0 or pH 5.0

	Kd_{app} (n*M*) +/– Std. Dev. (*n* = 3)	
Protein	pH 8.0	pH 5.0
SpA^1	40 ± 5	ND
SpA^1 (Leu 21 His)	206 ± 31	>5568
SpA^1 (Phe 17 His)	>4000	ND
SpA^2	10 ± 4	22 ± 7
SpA^2 (Leu 21,79 His)	52 ± 10	1005 ± 12
SpA^2 (Asn 15,73 His)	9 ± 3.6	39 ± 7
SpA^2 (Gln 14,72 His)	26 ± 12	80 ± 14
SpA^2 (Asn 32,90 His)	11 ± 5	53 ± 20
SpA^2 (Lys 39,97 His)	17 ± 1	41 ± 5

[a]ND, not determined.

In all cases, however, there is a marked reduction in the binding affinity of those mutants bearing His replacements at pH 5.0 relative to pH 8.0. The most notable of changes between these two pH values is that given by the SpA^2 (Leu 21,79 His). **Figure 2** shows the result of a competitive ELISA performed at pH 8.0 and at pH 5.0 for this mutant and SpA^2. Importantly, the Kd_{app} of SpA^2 (Leu 21,79 His) at pH 8.0 is only 52 n*M* (**Table 3**) which is still indicative of tight binding and only fivefold higher than that for the nonmutated domain. However, there is an approx 25-fold increase in its Kd_{app} at pH 5.0 relative to that at pH 8.0 compared with only a two- to threefold increase shown by the nonmutated SpA^2. Smaller reductions in IgG-binding affinity at pH 5.0 relative to that found at pH 8.0 were found with the other His proteins. SpA^2(Gln 14,72 His), SpA^2 (Asn 15,73 His), SpA^2 (Asn 32,90 His), or SpA^2 (Lys 39,97 His) all bind similarly to SpA^2 at pH 8.0 with binding affinities reduced by factors of only 2.6-, 1.0-, 1.1-, and 1.7-fold, respectively. At pH 5.0 their Kd_{app} values are increased in all cases to between 40 and 80 n*M*, a two- to fourfold increase compared to SpA^2 at the same pH. Therefore the change in pH has a much more significant effect on the complex formed between IgG and the mutated proteins than for the complex between IgG and the nonmutated protein.

All of the mutant proteins used in these experiments were subjected to spectroscopic examination by circular dichroism (CD) to examine the effects, if any, of the mutation on the folding of the protein. **Figure 3** gives typical CD spectra (for details *see* figure caption) obtained in the far UV region of the spectrum that arise from the contributions from the peptide backbone of the proteins. All of the mutants studied here have almost identical spectra, suggesting that no distortion of the protein folding had occurred and therefore differences in binding affinities have arisen from local rather than global effects of the mutations.

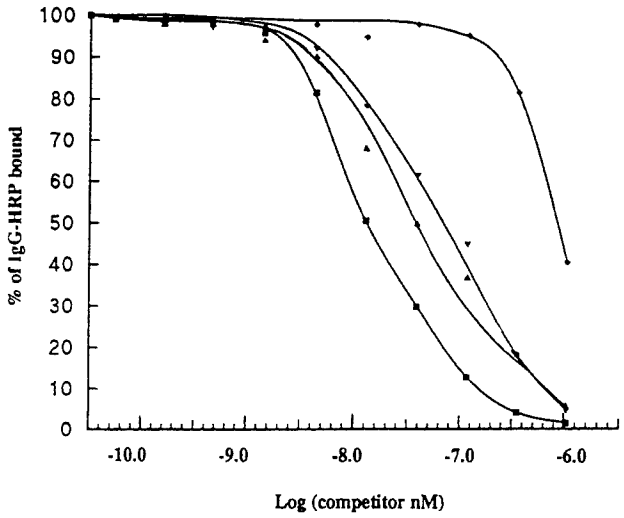

Fig. 2. A competitive ELISA performed to compare the IgG-binding affinity of SpA^2 and SpA^2(Leu 21,79 His) at pH 8.0 and at pH 5.0. The curves show the absorbance at 490nm when rabbit IgG-HRP is bound to SpA^2 at pH 8.0 (■) and pH 5.0 (▲) or SpA^2 (Leu 21,79 His) at pH 8.0 (▼) or pH 5.0 (◆). The points are the average of two readings.

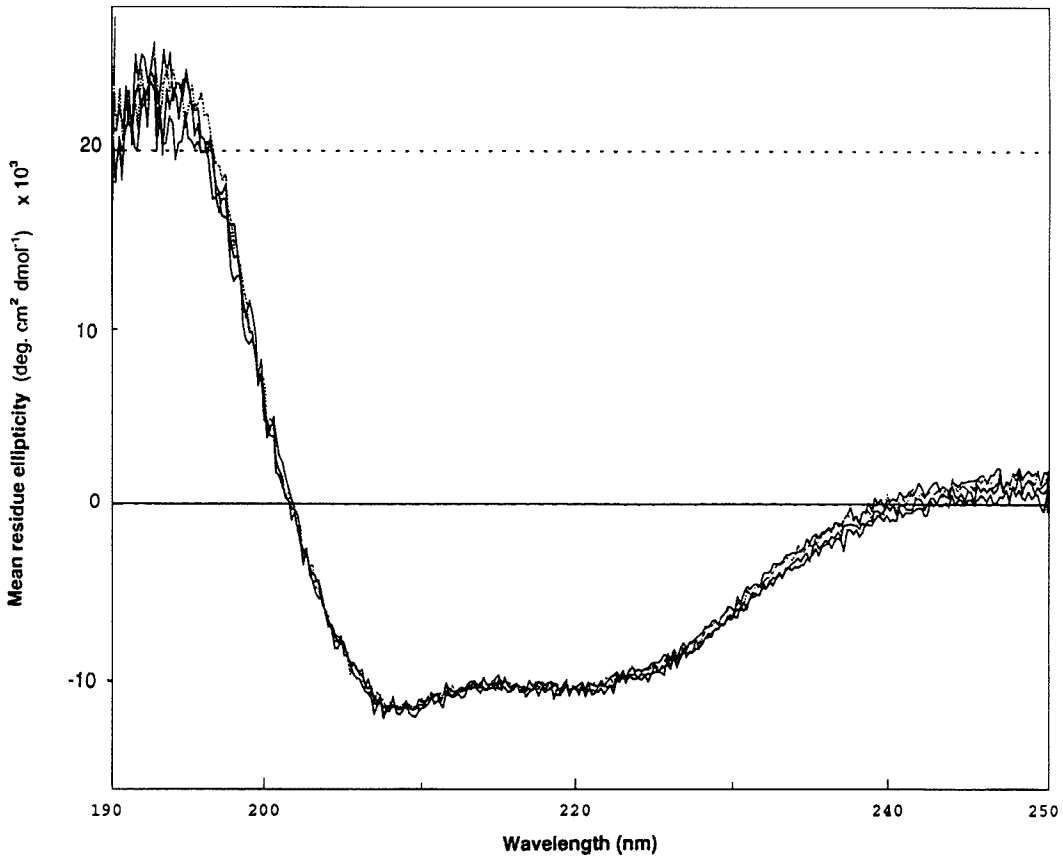

Fig. 3. The Far UV CD spectra of SpA^2 proteins. The figure demonstrates the almost identical spectra given by different mutants of SpA^2. Six spectra are overlaid. These are SpA^2 or SpA^2 bearing the mutations Lys 39,97 His/Asn 15,73 His/Gln 13,71 Ala/Gln 14,72 His or Leu 21,79 His. The other proteins described in this paper gave similar spectra. Units are in mean residue ellipticity using an average residue weight of 110. For experimental details *see* **Methods**.

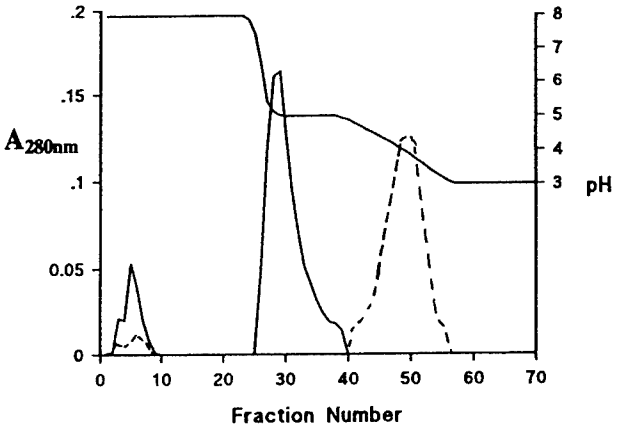

Fig. 4. The elution profile of IgG_1-Fc from a column of SpA^2 or SpA^2 (Leu 21,79 His) immobilised onto triazine-activated agarose. The elution of approx 1 mg of IgG_1-Fc from 2 × 1 cm columns of SpA^2 *(dashed line)* or SpA^2 (Leu 21,79 His) *(solid line)* proteins. The eluted protein was detected by its absorbance at 280 nm. *See* **Subheading 3.** for details of the gradient buffers.

The practical applications of one of these mutated proteins in affinity chromatography is shown in **Fig. 4**. Human IgG-Fc was applied at pH 8.0 to columns of nonmutated SpA^2 and SpA^2 (Leu 21,79 His) bound to agarose. The nonbound protein was washed off in the same buffer and then a pH gradient was used to elute the bound material from each column. It is clear from the elution profiles shown in the figure that all of the bound protein elutes at pH 5.0 from the column made from SpA^2(Leu 21,79 His), whereas a lower pH (3.75) was required to elute the protein from the column containing SpA^2.

4. Discussion

The X-ray crystallographic studies of Deisenhofer *(9)* suggest that the forces involved in stabilizing the SpA-Fc complex are mainly hydrophobic with four hydrogen bonds. The aim of this investigation was to determine the contribution of these forces to the binding interaction. It was found that Gln 13, Asn 15, and Asn 32 from SpA form weak, if any, hydrogen bonds with Fc whereas the hydrogen bond involving Tyr 18 is more significant. The change in binding energies per binding domain of each of the mutations (relative to wild-type SpA^2) were calculated using the equation.

$$\Delta G = RTIn\ [Kd_{app}\ SpA^2 / Kd_{app}\ SpA^2 mutant]$$

where ΔG is the difference in binding energy on formation of a complex of SpA^2 and a SpA^2 mutant domain with rabbit IgG , R is the gas constant and T is the absolute temperature .

The mutation SpA^2 (Tyr 18, 76Phe) resulted in a change in binding energy compared to SpA^2 of 4.5 kJ/mol. This is similar to the values obtained from studies of tyrosyl-tRNA synthetase which showed that the presence of an unpaired hydrogen bond donor or acceptor in a protein-ligand complex weakens the binding energy by 2.1–18.8 kJ/mol (0.5–4.5 kcal/mol) *(15)*. An individual hydrogen bond is not a great determinant of biological specificity since the presence of a single unpaired uncharged hydrogen bond donor or acceptor in a complex only increases the dissociation factor by a factor of 2–20 *(15)*. The investigations described here show that the hydrogen bonds formed in the SpA^2-rabbit Fc complex are very weak and are more likely to be important for molecular recognition than contributing significantly to stability of the complex.

The contribution of the hydrophobic interactions to the SpA^2-Fc complex were also studied by replacement of key residues thought to make close hydrophobic contacts with Fc by His. This strategy generated a number of IgG-binding proteins with reduced binding affinities at pH 5.0 relative to the those for wild-type proteins under the same conditions. It was found that the replacement of Leu 21 by His led to increased dissociation of the complex with Fc at pH 5.0 in both of the protein constructs studied (SpA^1 and SpA^2). In the crystal structure of the complex with human Fc *(9)* the side chain of Leu 21 is close to the Fc making hydrophobic contacts with main chain Gln 311 and the imidazole ring of His 435. It also makes an intramolecular contact with Ile 35 of helix 2 *(16)*. Therefore, it is possible that the replacement of Leu 21 in SpA by His leads to a charge-charge repulsion with His 435 of Fc at approx pH 5.0 leading to dissociation of the complex.

The application of these His mutants to the affinity chromatographic purification of IgG has been examined. The elution of human IgG_1-Fc occurs at pH 5.0 from a column made from immobilized SpA^2(Leu 21, 79 His), which is more than 1-pH unit higher than the pH required to elute IgG_1-Fc from a column of immobilized wild-type protein.

In these studies, a number of residues of SpA^1 and SpA^2 have been individually replaced by His to examine the contribution of hydrophobic forces to the stability of the complex. These studies have resulted in the generation of protein A variants that have reduced binding affinities at pH 5.0 relative to those of the nonmutated protein and these offer improved binding characteristics for use in affinity chromatography.

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