Antigen–antibody interactions: Elucidation of the epitope and strain-specificity of a monoclonal antibody directed against the pilin protein adherence binding domain of *Pseudomonas aeruginosa* strain K

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Abstract

The C-terminal region of *Pseudomonas aeruginosa* strain K (PAK) pilin comprises both an epitope for the strain-specific monoclonal antibody PK99H, which blocks pilus-mediated adherence, and the adherence binding domain for buccal and tracheal epithelial cells. The PK99H epitope was located in sequence 134–140 (Asp-Glu-Gln-Phe-Ile-Pro-Lys) by using a single alanine replacement analysis on the 17-residue synthetic peptide corresponding to the PAK C-terminal sequence 128–144. Indeed, a 7-residue peptide corresponding to this sequence was shown to have a similar binding affinity to that of the native conformationally constrained (disulfide bridged) 17-residue peptide. This epitope was found to contain two critical residues (Phe¹³⁷ and Lys¹⁴⁰) and one nonessential residue (Gln¹³⁶). Interestingly, the peptide, Phe-Ile-Pro-Lys, which constitutes the four most important side chains for antibody binding did not bind to PK99H. It was of interest to investigate the structural basis of the strain-specificity of PK99H utilizing naturally occurring pilin sequences. Therefore, all different residues found in the sequence corresponding to the PK99H epitope of the four other strains (PAO, CD4, K122-4, and KB7) were substituted one at a time in the PAK sequence and the changes in binding affinity of these analogs to the antibody PK99H were determined by competitive ELISA. The strain-specificity of PK99H for strains PAO, K122-4, and KB7 can be explained by the accumulated sequence changes in these strains, and at least two amino acid changes were required to explain the strain-specificity of PK99H. Similarly, cross-reactivity of PK99H with CD4 can be explained by the fact that there was only one side chain responsible for decreasing binding affinity compared to the PAK sequence.

Keywords: antigen–antibody interaction; epitope; pilin; *Pseudomonas aeruginosa*; strain-specificity; synthetic peptide

Pseudomonas aeruginosa is a rod-shaped, gram-negative bacterium and a prominent pathogen in patients with their natural host-defense mechanism disrupted (Bodey et al., 1983). In hospitals, nosocomial infections due to

this opportunistic microorganism are prevalent (Cross et al., 1983) and constitute one of the major causes of mortality among burned, cystic fibrosis, and cancer patients (Pitcher-Wilmott et al., 1982; Bodey et al., 1983).

Several *P. aeruginosa* virulence factors have been described (Pitt, 1986; Woods et al., 1988). The critical role of bacterial adherence to the epithelial cell surfaces in *P. aeruginosa* pathogenesis has been well established (Johanson et al., 1979, 1980; Woods et al., 1980; Todd et al., 1989). Three distinct adherence mechanisms have been described to date: pilus-mediated adherence (Woods et al., 1980; Doig et al., 1988), alginate-mediated adherence (Ramphal & Pier, 1985; Doig et al., 1987), and exoen-

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; I_{50} , concentration of peptide required to produce 50% inhibition in competitive ELISA; PBS, phosphate-buffered saline; tBoc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid.

zyme S-mediated bacterial binding (Lingwood et al., 1991). The adherence of *P. aeruginosa* to the respiratory epithelium through its polar pili has been considered as the first step for initial colonization (Pier, 1985; Irvin et al., 1990).

The *P. aeruginosa* pilus is composed of a monomeric subunit termed pilin, and pilins from several *P. aeruginosa* strains have been sequenced (Sastry et al., 1985; Pasloske et al., 1988; Paranchych et al., 1990). Previous studies using protein fragments and synthetic peptides of *P. aeruginosa* strain K (PAK) pilin have shown that the C-terminal region of PAK pilin contains an epithelial cell binding domain (Paranchych et al., 1986; Irvin et al., 1989). Monoclonal antibodies directed against the PAK pilus have been produced (Doig et al., 1990), and one of them, PK99H, was found to bind specifically to the C-terminal region of PAK pilin and block adherence to buccal and tracheal epithelial cells (Doig et al., 1990).

Doig and coworkers (1990) have also shown that PK99H was strain specific, and that among the different strains tested it only recognized PAK pili. A competitive inhibition binding study using both oxidized and reduced 17-residue synthetic peptides at the C-terminal region demonstrated that the disulfide bridge in this region is not critical for PK99H binding: that is, the reduced peptide can fold into the PK99H binding site. Thus, the epitope for PK99H was mapped by systematically synthesizing a series of alanine-substituted analogs as shown by Geysen et al. (1984) and Hodges et al. (1988) in studying antibody-antigen interactions. In addition, the nature of the strain-specificity of PK99H was studied by comparing the binding affinity of peptide analogs of the PK99H epitope containing substitutions observed in this region of other *P. aeruginosa* pili.

Results

Monoclonal antibody PK99H has been shown to be strain-specific in that it binds to the pilus of *P. aeruginosa* strain K (PAK) and does not bind to the heterologous strain O (PAO). It also binds to the C-terminal region of PAK pilin within the sequence 121–144 (Doig et al., 1990). We have defined an antibody as strain-specific if its binding affinity for another strain is decreased by at least 1,000-fold. The relative binding affinity of the PAK and PAO peptides (AcPAK(128–144)OH and AcPAO(128–144)OH; Table 1) to PK99H was assessed by means of their ability to inhibit the binding of PK99H to PAK pili. As indicated in Figure 1, these two peptides had different binding affinities to PK99H in the competitive ELISA assay. The apparent association constants (K_a) were calculated from the I_{50} values as described by Nieto et al. (1984). The I_{50} values (peptide concentration required for 50% inhibition of PK99H binding to PAK pili) for both peptides showed that a 1,500-fold higher concentration of PAO peptide was required to attain the same in-

Table 1. Amino acid sequences of the C-terminal regions from residues 128 to 144 of five different *Pseudomonas aeruginosa* strains

Strain	Sequence ^a															
	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143
PAK	K	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	S
PAO	(A)	C	(K)	S	(T)	Q	D	(P)	(M)	F	(T)	P	K	G	C	(D)
CD4	(T)	C	T	S	(T)	Q	(E)	(E)	(M)	F	I	P	K	G	C	(N)
K122-4	(A)	C	T	S	(N)	(A)	D	(N)	(K)	(Y)	(L)	P	K	(T)	C	(Q)
KB7	(S)	C	(A)	(T)	(T)	(V)	D	(A)	(K)	F	(R)	P	(N)	G	C	(T)

^a The sequence of the five *P. aeruginosa* strains (PAK, PAO, CD4, K122-4, and KB-7) were taken from Paranchych et al. (1990). The circled residues denote sequence differences from strain PAK.

hibitory effect as that of PAK peptide (K_a 's of PAK and PAO peptides were $4.00 \times 10^6 \text{ M}^{-1}$ and $2.63 \times 10^3 \text{ M}^{-1}$, respectively; Table 2). The loss of binding free energy, $\Delta(\Delta G)$, of PAO peptide was 4.32 kcal/mol compared with PAK peptide.

PK99H epitope

In order to locate the PK99H epitope, residues (excluding the two cysteines) in the PAK sequence (128–144) were substituted with alanine to prepare single Ala-substituted analogs (Fig. 2). The effects of these substitutions on PK99H binding were examined. If a single alanine-substituted peptide showed a decrease in its ability to inhibit the binding of PK99H to PAK pili, the side chain

Fig. 1. Competitive ELISA profiles of both AcPAK(128–144)OH and AcPAO(128–144)OH synthetic peptides showing the competitive inhibition of monoclonal antibody PK99H binding to PAK pili by AcPAK(128–144)OH (○) and AcPAO(128–144)OH (●). The amino acid sequences of the two peptides are listed in Table 1. The ELISA wells were coated with PAK pili (see Materials and methods).

Table 2. Thermodynamic analysis of the binding of synthetic peptides from various strains of *Pseudomonas aeruginosa* pilins to monoclonal antibody PK99H

Peptide name ^a	K_a^b (M ⁻¹)	K_N/K_S^c ratio	$\Delta(\Delta G)^d$ (kcal/mol)
AcPAK(128–144)OH	4.00×10^6	1	
AcPAO(128–144)OH	2.63×10^3	1,500	4.32
AcPAK(134–140)NH ₂	2.22×10^6	1	
AcPAO(134–140)NH ₂	2.00×10^2	11,000	5.50
AcCD4(134–140)NH ₂	1.18×10^5	19	1.73
AcKB7(134–140)NH ₂	7.14×10^2	3,100	4.75
AcK122-4(134–140)NH ₂	$<1.00 \times 10^2$	>22,000	>5.91

^a The peptide sequences are listed in Table 1.

^b K_a is the apparent association constant of monoclonal antibody PK99H for the corresponding peptide analog, which is calculated by the formula $K_a = 1/I_{50}$ (Nieto et al., 1984).

^c Where K_N and K_S stand for the apparent association constants (K_a) of the PAK peptides (K_N) (i.e., AcPAK(128–144)OH or AcPAK(134–140)NH₂) and peptides of other strains (K_S).

^d $\Delta(\Delta G)$ is the loss of binding free energy of the synthetic peptide analogs to monoclonal antibody PK99H as compared with the native PAK peptide. It is calculated as described by Bhattacharyya and Brewer (1988): $\Delta(\Delta G) = RT \ln(K_N/K_S)$.

substituted was considered to be important for PK99H binding and contributed to the epitope.

The I_{50} value of each peptide analog was compared with the I_{50} value of the native peptide (I_{50}^0), and was represented graphically by $\log I_{50} - \log I_{50}^0$. A positive value indicated a loss of binding affinity of the peptide analog. In Figure 2, the importance of the intrachain disulfide bond in PAK peptide for PK99H binding was also assessed. The I_{50} value of the reduced native peptide, AcPAK(128–144)OH, is similar to that of the oxidized peptide (2.2×10^5 pM and 2.5×10^5 pM, respectively; data not shown), which agreed well with previous studies (Doig et al., 1990). In addition, the absence of the disulfide bridge does not affect the determination of the PK99H epitope, since the reduced and oxidized peptide analogs showed similar binding profiles. The only difference observed between reduced and oxidized peptides was a general decrease in the magnitude of the $\log I_{50} - \log I_{50}^0$ values for the reduced peptides (Fig. 2). This Ala replacement analysis suggested that the minimum peptide sequence for maximum binding to PK99H was located in the linear sequence 134–140. Of this 7-residue sequence (region boxed in Fig. 2), six side chains were found to be important for PK99H binding (Asp¹³⁴, Glu¹³⁵, Phe¹³⁷, Ile¹³⁸, Pro¹³⁹, and Lys¹⁴⁰) and showed decreases in binding affinity from 10- to 4,000-fold. These results suggested that a 7-residue peptide representing the epitope could bind to PK99H.

To assess whether the minimum peptide sequence required for antibody binding was the 7-residue epitope or a peptide shorter than 7 residues, peptides of various lengths were synthesized (Fig. 3). These peptides varied

in length from 4 to 8 residues and constituted all or part of the binding epitope as predicted from Figure 2. Even though the 4 residues ¹³⁷Phe-Ile-Pro-Lys¹⁴⁰ (FIPK) represented the 4 most important residues in the epitope with decreases in binding affinity ranging from 76- to 4,000-fold for the single alanine-substituted 17-residue oxidized peptides (Fig. 2), it was demonstrated in Figure 3 that this sequence alone (FIPK) was not sufficient to compete with PAK pili binding to PK99H. The peptide FIPK lost its ability to compete with PAK pili for PK99H binding (>10,000-fold decrease) as compared with the native AcPAK(128–144)OH peptide. As the peptide sequence of the core peptide FIPK was extended toward the C-terminus, binding affinity to PK99H remained poor (7,600-fold decrease). On the other hand, extension toward the N-terminus resulted in increased binding affinity. When Gln¹³⁶ was added (QFIPK), an enhancement in binding affinity by at least 33-fold was observed. A further 20-fold enhancement was observed as the Glu¹³⁵ was added (EQFIPK) and at least 8-fold as Asp¹³⁴ was added to obtain the maximum affinity for PK99H. Thus, the PK99H epitope was again located to the sequence 134–140 (DEQFIPK). Interestingly, there is no significant change in binding affinity for this 7-residue peptide as compared with the native oxidized 17-residue AcPAK(128–144)OH peptide. Within any linear epitope there is also the minimum sequence required to exhibit antibody binding (within 1,000-fold in binding affinity compared to the epitope peptide). This sequence consists of residues 136–140 compared to the epitope sequence 134–140 (Fig. 3).

Importance of individual side chains in PK99H epitope

The importance of individual side chains in the epitope was assessed by single alanine substitutions in the 7-residue and 17-residue peptides. Figure 4 shows representative competitive binding curves of the competitive ELISA from two of the single alanine-substituted peptide analogs, together with the native epitope peptide. The change in the inhibition of the alanine-substituted peptide can be reflected by a corresponding shift of the binding curve. The results from Figures 2 and 5 suggested that the 7-residue peptide analogs have similar antibody binding characteristics to the 17-residue peptide analogs. In addition, it further confirmed that the disulfide bridge is not important to PK99H binding. Though the disulfide bond in the pilin protein controls the conformation of this region in the protein, the residues between the two cysteines involved in the disulfide bond can still adapt to the binding pocket of PK99H as easily as the nonconstrained linear 7-residue peptide.

We have defined the side chains within the epitope arbitrarily as one of three types: critical, important, and nonessential to antibody binding. A critical side chain is one that on substitution by alanine (in a synthetic peptide

Fig. 2. Effects of single alanine substitution on the binding of AcPAK(128-144)OH to monoclonal antibody PK99H as determined by competitive ELISA. On the legend of the ordinate, the amino acid sequence of the peptide is listed from N- (top) to C- (bottom) terminus, and the position of alanine substitution in each peptide analog is denoted. OH → NH₂ denotes peptide analog with an amide group substituted at its C-terminus. The K_N/K_S ratio (K_N and K_S represent the apparent association constant [K_a] of the oxidized native and substituted peptides, respectively) indicates the relative loss of binding affinity to PK99H with respect to that of the native PAK peptide. A value greater than 1.0 denotes an x -fold decrease in binding affinity, whereas a value less than 1.0 denotes an x -fold enhancement in binding. I_{50} is the concentration of the peptide analogs required for 50% inhibition of PK99H binding to PAK pili. I_{50}^0 is the I_{50} of the native AcPAK(128-144)OH peptide in either the oxidized or reduced state. A positive value of $\log I_{50} - \log I_{50}^0$ indicates a loss of binding of the peptide analog to PK99H as compared with the native peptide. The solid bars denote the results of oxidized peptides (disulfide bridge between cysteines at positions 129 and 142 [Table I]), and the hatched bars are the results of the reduced peptides (cysteine residues at 129 and 142). The boxed sequence defines the linear epitope for PK99H. The ELISA wells were coated with PAK pili (see Materials and methods).

representing the epitope only) decreases binding affinity greater than 1,000-fold as compared to the native sequence. On the other hand, if the decrease in binding affinity is less than 3-fold, the side chain is considered as nonessential. Side chains whose contribution falls between these two extremes are defined as important.

Charged side chains

There are three charged residues in the epitope (Asp¹³⁴, Glu¹³⁵, and Lys¹⁴⁰). The importance of the negative charges on Asp¹³⁴ and Glu¹³⁵ to antibody binding was investigated by the isosteric substitutions of Asn¹³⁴ and

Fig. 3. Determination of the binding epitope for monoclonal antibody PK99H. Six truncated peptides with lengths ranging from residues 133 to 144 were synthesized. The change in binding affinity of each peptide analog to PK99H was assessed by comparing their I_{50} values with that of the native AcPAK(128-144)OH peptide. The boxed sequence defines the linear epitope for PK99H. All peptides are N^α -acetylated at the N-terminus and have an amide group at the C-terminus except ¹³⁷FIPKGCSK¹⁴⁴, which has an α -carboxyl group at the C-terminus. The ELISA wells were coated with PAK pili (see Materials and methods).

Fig. 4. Competitive ELISA profiles of some of the peptide analogs of the PK99H epitope showing the competitive inhibition of PK99H binding to PAK pili by AcPAK(134-140)NH₂ (□), (Ala¹³⁹)AcPAK(134-140)NH₂ (■), and (Ala¹³⁵)AcPAK(134-140)NH₂ (▲). The amino acid sequences of the peptides can be found in the legend of Figure 5. The ELISA wells were coated with PAK pili (see Materials and methods).

Gln¹³⁵, respectively. Each individual negative charge was not critical for binding because the peptides NEQFIPK and DQQFIPK showed a 4-fold decrease and a 10-fold enhancement in binding affinity, respectively (Fig. 5). However, a negative charge at either position could still play a role in binding. The isosteric side-chain substitutions were compared to the Ala substitutions. In both cases, an enhancement in binding affinity was observed (Asn¹³⁴ showed a 2-fold enhancement in binding affinity

compared to the Ala¹³⁴ analog; Gln¹³⁵ showed a 29-fold enhancement in binding affinity compared to the Ala¹³⁵ analog). This suggests that hydrogen bonding may be more important for binding than the ionic interactions with the antibody binding site at these positions. In contrast, the positively charged residue, Lys¹⁴⁰, is one of the two critical residues in the epitope. Substitution (Figs. 5, 6) and deletion (Fig. 3) of this residue had a tremendous effect on binding affinity (Lys¹⁴⁰ → Ala, 1,100-fold decrease; Lys¹⁴⁰ → Asn, 2,000-fold decrease; AcPAK(133-139)NH₂ compared to AcPAK(134-140)NH₂, >10,000-fold decrease). Shi et al. (1984) have reported the importance of a lysine residue at the C-terminus of a peptide antigen in antibody binding. Hence, Lys¹⁴⁰ may contribute an electrostatic attraction that increases the rate of antibody-antigen complex formation by facilitating the stability of an initial complex (Geysen et al., 1987b).

Hydrophobic side chains

Phe¹³⁷ and Ile¹³⁸ are the two major hydrophobic residues found in the epitope along with Pro¹³⁹. Substitution of Pro¹³⁹ with Ala resulted in 22-fold and 76-fold decreases in binding affinity for the 7- and 17-residue peptides, respectively (Figs. 2, 5). By comparison, substitution of Ile¹³⁸ with Ala showed 190-fold and 600-fold decreases in binding affinity for the 7- and 17-residue peptides, respectively. Substitution of Phe¹³⁷ with Ala caused 8,900-fold and 4,000-fold decreases in binding affinity for the 7- and 17-residue peptides, respectively, making it a critical side chain. The importance of these side chains can be related to their relative hydrophobicity

Fig. 5. Effects of single amino acid substitution of the epitope to monoclonal antibody PK99H (boxed). Single alanine-substituted PAK peptide analogs corresponding to the binding epitope (residue 134-140) of PK99H were synthesized. The charge effects of residues Asp¹³⁴ and Glu¹³⁵ were examined by substituting these two residues with their uncharged isosteric counterparts, Asn and Gln, respectively. I_{50}^0 is the I_{50} value of the native peptide sequence corresponding to the PK99H epitope, i.e., AcPAK(134-140)NH₂. All peptides are N^α-acetylated at the N-terminus and have an amide group at the C-terminus. The ELISA wells were coated with PAK pili (see Materials and methods).

Fig. 6. Comparison of the binding affinity of synthetic peptide analogs from five *Pseudomonas aeruginosa* strains with sequence confined to the PK99H epitope of PAK pilin (boxed). The native peptide sequence of each strain is indicated by the name of the strain on the legend of the ordinate. Circled residues denote different residues as compared with the native PAK sequence. All peptides are N^α -acetylated at the N-terminus and have an amide group at the C-terminus. The ELISA wells were coated with PAK pili (see Materials and methods).

ties (Phe > Ile > Pro) (Parker et al., 1986). Thus, it seems that hydrophobic interactions could be one of the major forces that determine the affinity of the antibody. Interestingly, on either side of the hydrophobic residues are charged residues that could also be important in ensuring that these hydrophobic residues are surface exposed and available for antibody (receptor) binding (Hodges et al., 1988).

Importance of position 136 to PK99H binding

The PAK sequence contains Gln at position 136, and the side chain was judged nonessential based upon the Ala substitution in the 7- and 17-residue peptides. Actually, there was a small enhancement of binding affinity in the case of PAK epitope sequence 134–140 (5-fold; Fig. 5) and in the reduced sequence 128–144 (Fig. 2). However, in the case of oxidized peptide (sequence 128–144), there was a very small decrease in binding affinity (1.6-fold; Fig. 2). Interestingly, substitution with amino acids other than Ala at this position in the PAK sequence can dramatically affect binding to PK99H. The Gln¹³⁶ to Lys mutation found in strains K122-4 and KB7 decrease binding affinity by 950-fold to PK99H (Fig. 6). It is possible that Gln¹³⁶ could be positioned in the binding interface with PK99H but the side chain's contribution to binding affinity is small. Thus, removal of the side chain by a smaller residue such as Ala would have little effect on binding affinity. On the other hand, the presence of Lys at this position, which is larger and carries a full positive charge, would seriously disrupt the binding site.

Interestingly, Met¹³⁶, which is much closer in molecular size to Gln and is a neutral amino acid, caused a much

smaller disruption in binding affinity (14-fold decrease). It is also possible that the Gln residue at this position is important for controlling conformation of the folded peptide in the antibody binding site and itself is not part of the binding interface. NMR studies to determine the structure of the native sequence bound to this monoclonal antibody should distinguish between these two possibilities.

Enhancement of peptide binding to PK99H

As shown in Figures 2, 5, and 6, the binding affinity of peptides to PK99H could be enhanced as compared to the native sequence. For the 17-residue peptides, whether in their oxidized or reduced forms, there were small enhancements of binding affinity for residues substituted outside the epitope of PK99H (up to 4.8-fold). Similarly, substitutions of certain residues inside the epitope showed enhancements (Asp¹³⁴ to Glu, Glu¹³⁵ to Gln, and Gln¹³⁶ to Ala resulted in 5-fold, 10-fold, and 5-fold increases in binding affinity, respectively; Fig. 5). These results confirm previous studies that have demonstrated that even though an antibody is generated to a certain sequence, the antibody is not absolute in its specificity, and changes in sequence can enhance as well as decrease binding affinity (Imanishi & Mäkelä, 1973; Reichlin & Noble, 1974). It has been well documented that large enhancements in binding can be achieved for both peptide substrates and inhibitors of enzymes, so it is not unreasonable to expect amino acid substitutions to enhance binding affinity of peptides to monoclonal antibodies. In order to test whether these enhancements were additive, a synthetic peptide containing the above three substitutions was pre-

pared (EQAFIPK). This peptide also showed enhanced binding affinity to PK99H as compared to the native PAK sequence, but the magnitude of the enhancement was not additive (about 2.5-fold rather than 20-fold if the effect was additive; Fig. 5).

Strain-specificity of PK99H

The sequences of several *P. aeruginosa* strains (Paranchych et al., 1990) of heterologous pilus types are shown in Table 1. The 7-residue peptides (residues 134–140) containing the sequences of the strains PAO, CD4, KB7, and K122-4 were synthesized. Two of these strains, PAO (purified pili and whole cell extract) and K122-4 (whole cell extract), were tested previously and were not recognized by PK99H (Doig et al., 1990). As we compared the sequences of these four strains in the sequence 134–140 corresponding to the PK99H epitope (Table 1), PAO has three different residues as compared with PAK sequence, CD4 has two, K122-4 has four, and KB7 has four. Indeed, as shown in Table 2, three of them, PAO, KB7, and K122-4, demonstrated strain-specificity of PK99H with decreases in binding affinity from 3,100- to >22,000-fold. PK99H does react with CD4 where only a 19-fold decrease in affinity was observed. The loss of binding free energy, $\Delta(\Delta G)$, of such peptides varied from 1.73 kcal/mol (CD4) to greater than 5.91 kcal/mol (K122-4) with respect to the native 7-residue peptide AcPAK(134–140)NH₂ (Table 2).

To understand which side chains were accounting for strain-specificity in strains PAO, KB7, and K122-4 or the decrease in binding affinity of strain CD4 to PK99H, all different residues found in these strains were substituted one at a time in the PAK sequence (Fig. 6). In the case of the PAO sequence, the affinity decreased greater than 10,000-fold for binding to PK99H. However, no single substitution of the PAO residues in the PAK sequence was able to account for this specificity (Fig. 6). The PAO residues Pro¹³⁵, Met¹³⁶, and Thr¹³⁸ as single mutants of the PAK sequence decreased affinity by 7.4-, 14-, and 79-fold, respectively. Interestingly, the three double mutants decreased affinity in more than an additive manner. In fact, only one of the double mutants (Pro¹³⁵Met¹³⁶) was able to account for the decrease in binding affinity of strain PAO to PK99H (>10,000-fold). This was unexpected when one considers that Pro¹³⁵ or Met¹³⁶ individually had only small effects on binding affinity. In contrast, when the Thr¹³⁸ substitution, which had the largest effect on binding affinity as a single substitution in the PAK sequence (79-fold), was combined with either Pro¹³⁵ or Met¹³⁶ as a double mutant, it was unable to account for the >10,000-fold decrease in binding affinity caused by the triple mutant (PAO sequence) (Fig. 6).

In the case of K122-4, which differed from the PAK sequence in four positions, two of the substitutions, Leu¹³⁸ for Ile and Asn¹³⁵ for Glu, did not cause significant de-

creases in binding affinity for PK99H as single mutants (1.4- and 4-fold decreases). The most significant substitution was Lys¹³⁶ for Gln in the PAK sequence. This single amino acid change accounted for a 950-fold decrease in affinity. In similar fashion to K122-4, KB7 differed from the PAK sequence at four positions, one of which (Ala¹³⁵ for Glu) did not cause a significant decrease in binding affinity (2.9-fold). However, any of the single mutants—Lys¹³⁶ for Gln, Arg¹³⁸ for Ile, and Asn¹⁴⁰ for Lys—resulted in large decreases in binding affinity (950-, 440-, and 2,000-fold, respectively). By analogy with the PAO sequence, a combination of any of the two amino acid changes indicated above could readily account for the 3,100-fold decrease in affinity of KB7 sequence.

The CD4 sequence varied from the PAK sequence in two positions, Glu¹³⁴ for Asp and Met¹³⁶ for Gln. The Met¹³⁶ substitution can account for the decreased affinity of the CD4 sequence for PK99H. The Glu¹³⁴ for Asp substitution is the only naturally occurring substitution that enhanced binding affinity over the native PAK sequence (5-fold).

Discussion

An important issue to understanding antigen–antibody interactions is the underlying principles that determine the binding affinity and specificity of an antibody. There are two kinds of epitopes, continuous and discontinuous epitopes (Atassi & Smith, 1978). The linear or continuous epitopes can be defined as the minimum linear sequence required for maximum antibody binding. The epitope can be mapped and the relative importance of individual side chains can be determined by systematically walking a single alanine substitution through a synthetic peptide known to contain the epitope (an Ala residue in the native sequence is replaced by a Gly residue). For example, the monoclonal antibody PK99H used in this study had been shown to bind to or near the adherence binding domain of *Pseudomonas* PAK pilin (Doig et al., 1990), which was located at the C-terminus of the pilin protein. Our data also showed that PK99H bound to the synthetic 17-residue C-terminal peptide in its native conformationally constrained form (a disulfide bond between cysteine residues 129 and 142) or in its reduced form (lacking the disulfide bond). However, the affinity constant for PAK pili binding to PK99H is not directly comparable with that of the 17-residue peptide due to the polydispersed size distribution of purified pili (Doig et al., 1990). It is assumed that the deletion of a side chain through an alanine substitution will examine the importance of the side chain for antibody binding while having a minimum effect on the conformation of the peptide and its ability to fold into the antibody binding pocket.

In this study, this replacement approach suggested that the epitope was contained in the 7-residue sequence 134–140 (Fig. 2). In fact, the antibody can bind strongly to

PAK(134–140)NH₂ (the deduced epitope of PK99H) conjugated to BSA as observed in direct ELISA (data not shown). This result was further verified by preparing peptides of varying lengths at the N- and C-terminal regions of the suspected epitope (Fig. 3). Using our criteria, this epitope was found to contain six of seven side chains involved in antibody binding; two of which are critical (Phe¹³⁷ and Lys¹⁴⁰), four that are important (Asp¹³⁴, Glu¹³⁵, Ile¹³⁸, and Pro¹³⁹), and one that is nonessential (Gln¹³⁶) (Fig. 5). It is interesting that deletion of the lysine side chain (Lys¹⁴⁰ → Ala) resulted in a 1,100-fold decrease in binding affinity (Fig. 5), whereas deletion of the residue resulted in a >10,000-fold decrease in affinity (Fig. 3). Surprisingly, the minimum sequence required for antibody binding was 136–140, which contains a non-essential side chain. Deletion of Gln¹³⁶ to the sequence 137–140 resulted in a >10,000-fold decrease in binding affinity (Fig. 3). These results show the importance of the peptide backbone of residues Gln¹³⁶ and Lys¹⁴⁰ in antibody binding. Thus, it is important to distinguish between the side chain and backbone requirements for antibody binding when discussing the importance of an amino acid residue. These results agree with those of previous workers; Hodges et al. (1988) showed that the four monoclonal antibodies directed to the cytoplasmic carboxyl-terminus of bovine rhodopsin recognized linear epitopes from 4 to 11 residues, and the minimum sequences required for antibody binding, which were shorter than the epitope, always contained the critical side chains. The number of critical side chains in an epitope varied from three to four.

Geysen et al. (1987a) reported that they have not observed any epitopes in which more than 5 residues were contact residues. Their definition would refer to the residues we have called critical residues; that is, antibody binding is lost or significantly decreased (>1,000-fold) when the original (parent) residue is replaced with residues of dissimilar character. Recently, Xing et al. (1991) have also reported a minimum epitope sequence of 5 residues for three monoclonal anti-mucine antibodies (BC1, BC2, and BC3), and the number of critical residues found was 4 for BC1 and BC2, and 1 for BC3. In addition, the epitope length reported for other monoclonal antibodies falls into the range of 5–10 residues (Anderson et al., 1988; Kövamees et al., 1990; Scott et al., 1990), and the minimum sequence found is between 5 and 7 residues (Anderson et al., 1988).

It has been previously reported that antibody binding of small peptides (2–7 residues) is dominated by nonspecific ionic and hydrophobic interactions, and only peptides of 15–20 residues in length could undergo meaningful specific binding (Shi et al., 1984; Berzofsky, 1985). In this study, there are three observations which support that the binding of the 7-residue epitope sequence to PK99H is specific. First, the ability of the 7-residue peptide to inhibit PK99H binding to PAK pili is very similar to that

of the 17-residue peptide in either its oxidized or reduced form. In addition, the inhibition profiles of the single alanine-substituted 7-residue peptide analogs (Fig. 5) are similar to those of the 17-residue peptide analogs (Fig. 2). This observation showed that the binding of a conformationally constrained peptide such as the disulfide-bridged 17-residue peptide could be mimicked by a small 7-residue peptide. Second, PK99H could react with PAK and CD4 peptides only, but had extremely low affinity for the PAO, K122-4, or KB7 peptides (Fig. 6). Third, the small peptide FIPK, which constitutes the four most important side chains for antibody binding, did not compete with PAK pili binding to PK99H. These data strongly support the conclusion that the 7-residue peptide binds specifically to its monoclonal antibody. Worobec et al. (1985) showed that the binding of synthetic peptides to polyclonal anti-EDP208 pilus antibodies was enhanced as the peptide was shortened from 12 residues to 5 residues, which defined the epitope. Though these small peptides probably lack conformational properties (free in solution), they can readily fold into a preformed antibody binding pocket and also maintain the sequence-specific and conformational properties when bound. This is important, because large synthetic peptides containing a small linear epitope may not readily fold into the antibody binding pocket. Kodama et al. (1991) have reported a 5-residue epitope sequence responsible for the strain-specificity of the monoclonal antibody SF8/5E11 directed against the transmembrane protein of simian immunodeficiency virus of macaque monkey by site-specific mutagenesis. However, they failed to demonstrate specific reactivity of this monoclonal antibody to 25-residue peptides containing the epitope.

Strain-specificity

An antibody produced against an immunogen can be either cross-reactive or strain-specific to various heterologous strains depending on the nature of the mutations in the epitope sequence and the highly specific binding nature of antibody. In our studies, we not only mapped the strain-specific epitope recognized by PK99H (Ac-PAK(134–140)NH₂), but also determined the importance of individual residues from other strains (Table 1) that varied in sequence from the PAK epitope. The strain-specificity of PK99H for strains PAO, K122-4, and KB-7 can be explained by the accumulated sequence changes in these strains. It is our finding that at least two sequence changes in the epitope were required to decrease binding affinity and achieve strain-specificity with PK99H. Cross-reactivity of PK99H to CD4 can be explained by the fact that there was only one side chain responsible for decreasing binding affinity compared to the PAK sequence. On the other hand, a single amino acid change in an epitope sequence could affect antibody binding to result in strain-specificity (substitution of the critical residues, Phe¹³⁷

and Lys¹⁴⁰). Pathogens escape immune surveillance of the host (McKeating et al., 1989) and survive under the pressure of natural selection through mutations of immunogenic regions. However, the pathogen must compromise this desire with the ability to maintain its adherence properties as in the case of this epitope, which is in the adherence binding domain.

Materials and methods

Bacterial pili

The bacterial pili employed in this study were obtained from the *P. aeruginosa* strain PAK/2pfs. Purification of pili was as previously described (Paranchych et al., 1979).

Peptide synthesis

The sequences of peptides synthesized are listed in Table 1 and Figures 2, 3, 5, and 6. The peptide analogs were named according to the position and amino acid used in substitution, *P. aeruginosa* strain, and the sequence of peptide corresponding to the native protein. For instance, (Ala¹³⁴) AcPAK(128–144)OH denotes a PAK pilin synthetic peptide comprising the sequence from residues 128 to 144 with position 134 substituted with an alanine. All peptides were *N*^α-acetylated at the N-terminus and were denoted with an Ac. An OH denotes a peptide with a free α -carboxyl group at the C-terminus, whereas an NH₂ denotes a peptide with an amide at the α -carboxyl group of the C-terminus.

Peptide synthesis was performed following the general procedure for solid-phase synthesis described by Erickson and Merrifield (1976) with modifications made by Parker and Hodges (1985) and Hodges et al. (1981) on either a Beckman model 990 (Fullerton, California) or an Applied Biosystems model 430A (Foster City, California) peptide synthesizer. Syntheses of peptides with a C-terminal lysine and a free α -carboxyl group were started with tBoc lysine-OCH₂-Pam resin (Applied Biosystems; 1% cross-linked, 0.67 mmol Lys/g). The synthesis of (Ala¹⁴⁴)AcPAK(128–144)OH was initiated by esterification of the cesium salt of tBoc-alanine to copoly (styrene, 1% divinylbenzene) chloromethyl resin (Pierce Chemical Co.; 0.9 mmol amino groups/g). In addition, syntheses of peptide amides were carried out using benzhydrylamine resin (Bachem Inc., Torrance, California; 1% cross-linked, 0.92 mmol amino groups/g).

After synthesis, peptides were cleaved from the resin support by anhydrous hydrogen fluoride (20 mL/g resin) containing 10% (v/v) anisole and 2% (v/v) 1,2-ethanedithiol as scavenging reagents for 45 min at –5 °C. The solvent mixture was then removed under reduced pressure. The resin was washed with anhydrous diethyl ether (three times, 30 mL total), and peptide was extracted with 30% acetic acid (three times, 20 mL total). The crude

peptide solution was diluted with distilled water and lyophilized.

Peptide purification

Purification of crude peptides was accomplished by reversed-phase (RP) HPLC on either a semipreparative, C₁₈, RP-P Synchropak column (250 × 10 mm internal diameter [I.D.], SynChrom Inc., Linden, Indiana) or an analytical Synchropak RP-P column (250 × 4.6 mm I.D.). A linear AB gradient of 0.2–0.5% B/min (depending on the peptides) at a flow rate of 2 mL/min for the semipreparative column and 1 mL/min for the analytical column was used. Solvent A was 0.05% TFA in water, and solvent B was 0.05% TFA in acetonitrile. The absorbance was recorded at 210 nm.

The purity and authenticity of the peptides were examined by means of analytical HPLC, amino acid analysis, and mass spectrometry. Analytical HPLC was done on an Aquapore RP-300, C₈ reversed-phase column (220 × 4.6 mm I.D.; Brownlee Labs, Santa Clara, California). Purified peptides were hydrolyzed with 6 N HCl with 0.1% phenol in sealed, evacuated tubes at 110 °C for 22 h, and amino acid analyses were performed on either a Durrum Model D-500 high-pressure automatic analyzer (Durrum Instrument Corp., Palo Alto, California) or a Beckman System 6300 high performance automatic analyzer (Beckman, Palo Alto, California). The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolysate was used to calculate the concentration of the peptide. The molecular weight and purity of the peptide was checked with a BIOION 20 Plasma desorption mass spectrometer (Bio-Ion Nordic AB, Uppsala, Sweden) to further ensure the authenticity of the purified peptide.

Peptide oxidation

For those 17-residue peptides containing two cysteines in their sequence (Fig. 2), air oxidation was performed by stirring a solution of the peptide (0.1 mg/mL) in 100 mM NH₄HCO₃, pH 8.2, overnight at room temperature. Completion of oxidation was examined by treating 100 μ L of peptide solution with 10 μ L of a 1-mg/mL aqueous solution of *N*-ethylmaleimide (NEM) (Lee et al., 1990). The HPLC chromatograms of NEM-treated and untreated peptides were compared. Only reduced peptide with free cysteine residues could react with NEM, and the modified peptide eluted off the column with a retention time much later than that of the oxidized or reduced peptide (Lee et al., 1990). The HPLC chromatograms and mass spectrometry showed that the oxidized peptides were all in monomeric form. NH₄HCO₃ in the peptide solution was removed by first acidifying the peptide solution with acetic acid and then subsequent lyophilizations.

Monoclonal antibody

Monoclonal antibody, PK99H, was prepared as described by Doig and coworkers (1990). Ascites tumors were produced by injecting 10^6 hybridoma cells into pristine primed BALB/c male mice. Ascites fluid was collected daily and the hybridoma cells were removed by centrifugation. Partial purification was accomplished with ammonium sulfate fractionation and subsequently dialyzed against PBS, pH 7.4. Antibody was then purified by HPLC on a protein G affinity column (Chromato-Chem, Missoula, Montana).

Competitive ELISA

Competitive ELISA was carried out according to the principles of Voller et al. (1974) with modifications described previously (Doig et al., 1990). The wells on the ELISA plate were coated with 100 μ L of 2 μ g/mL PAK pili in 0.01 M carbonate buffer, pH 9.5, and incubated for 6 h at room temperature. The plate was then washed three times with 0.05% (w/v) BSA in PBS, pH 7.4 (buffer A). Two hundred microliters of 5% (w/v) BSA in PBS was added in each well to block nonspecific adsorption of antibodies, and the plate was stored at 4 °C overnight. Peptide solutions at various concentrations were mixed with monoclonal antibody PK99H (295 μ g protein/mL in stock solution, and final dilution in each well was 5×10^{-6} of the stock solution) in buffer A and incubated for 1 h at room temperature. For ELISA using the reduced 17-residue peptide, reducing conditions were maintained by including 0.5 mM DTT in the solution. The plate was then washed two times with buffer A. One hundred microliters of antibody mixture was added into corresponding wells and incubated for 2 h at 37 °C. The plate was washed five times with buffer A, and 100 μ L of goat anti-mouse IgG immunoglobulin conjugated to horseradish peroxidase (Jackson Laboratories, California) in buffer A was added. The mixture was incubated at 37 °C for another 2 h. The plate was then washed five times with buffer A, and 125 μ L of substrate solution containing 1 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) and 0.03% (v/v) hydrogen peroxide in 10 mM sodium citrate buffer, pH 4.2, was added. The reaction was stopped by the addition of 125 μ L of 4 mM sodium azide, and the absorbance at 405 nm was determined by using a Titertek Multiskan Plus MK II microplate reader (Flow Lab Inc., McLean, Virginia). The apparent association constant (K_a) of the monoclonal antibody for each peptide analog can be calculated by the formula $K_a = 1/I_{50}$ as described by Nieto et al. (1984). By utilizing the K_a values of the peptide analogs (K_S) and the native sequence (K_N), one can determine the loss of binding free energy ($\Delta(\Delta G)$) of each peptide analog as compared with the native peptide through the following procedure: $\Delta(\Delta G) = RT \ln(K_N/K_S)$ (Bhattacharyya & Brewer, 1988).

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