Protein-Protein Interaction Specificity of Im9 for the Endonuclease Toxin Colicin E9 Defined by Homologue-scanning Mutagenesis*

(Received for publication, April 24, 1997, and in revised form, June 25, 1997)

Wei Li‡‡, Caitriona A. Dennis‡‡, Geoffrey R. Moore§, Richard James‡, and Colin Kleanthous‡‡**

From the ‡School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom, §Protein Structure Group, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG, United Kingdom, and ¶School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

The colicin DNase-specific immunity proteins interact with the endonuclease domain of the bacterial toxin colicin E9 with dissociation constants that span the millimolar to femtomolar affinity range. Among the non-cognate interactions Im2 shows the strongest binding toward the E9 DNase domain with a $K_d$ of $10^{-8}$ M, 6 orders of magnitude weaker than that of the cognate immunity protein Im9. Based on a NMR structure of Im9 that shows it to be a 4-helix protein, we have conducted a mutagenic scan in which elements of Im9 secondary structure were substituted into Im2 to precisely delineate regions that define specificity. Eleven chimeras were constructed, and their biological cross-reactivity toward colicins E2 and E9 was evaluated. From this set of mutants seven proteins were purified, and the $K_d$s for their interaction with the E9 DNase domain was measured by a combination of stopped-flow fluorescence and subunit exchange kinetics. Our results show that immunity specificity is dominated by residues on helix II, accounting for 5 orders of magnitude binding specificity relative to Im2, and that packing interactions of helix II with its neighbor helix I and the loop connecting helix III with helix IV play minor roles. The conformational stability of these chimeric proteins was also determined. Proteins displaying an Im9 phenotype were all more stable than the parent Im2 protein, and surprisingly some chimeras were significantly more stable than either Im2 or Im9.

Understanding specificity in protein-protein recognition is fundamental to our knowledge of complex biological processes such as transcription, signal transduction, and immune recognition, yet little is known about how specificity is determined in protein-protein interactions. In this paper we describe the use of homologue-scanning mutagenesis to determine the main structural elements that define the specificity of the immunity protein Im9 in its interaction with the DNase derived from the bacterial toxin colicin E9 (ColE9). 1

Colicins are plasmid-borne toxins produced by the Enterobacteriaceae that have lethal action against other related strains and are classified into groups on the basis of the cell surface receptor to which they bind (reviewed by James et al. (1)). The focus of our work has been the E group colicins, which bind to the product of the chromosomal btuB gene, an essential component of the high-affinity transport system for vitamin B$_12$ in Escherichia coli (2). Following receptor binding, the toxin translocates into the cell and initiates cell death. Each E colicin plasmid codes for the production of a $61$-kDa colicin toxin, a $9.5$-kDa inhibitor (immunity) protein that protects the producing cell against the cytoxic activity of the toxin, and a small lipoprotein (the lysis protein) that releases the resulting $71$-kDa heterodimeric colicin complex from the bacterium.

Colicin-producing cells are naturally resistant to the action of their own toxin but sensitive to the action of other bacteriocins from the same family. This forms the basis for the so-called immunity test by which cells producing different colicins can be identified. Using this biological test the E group has been subdivided into 9 types (ColE1–ColE9), and these fall into three cytotoxic classes (3, 4); the periplasmic membrane-depolarizing (or pore-forming) toxin ColE1 (5, 6), the RNases ColE3, ColE4, ColE5, and ColE6 (7–9), and the DNases ColE2, ColE7, ColE8, and ColE9 (9–13).

We have been studying the DNase colicins and their interaction with immunity proteins as a model system for investigating specificity in protein-protein recognition. The four colicins are almost identical in sequence in the N-terminal regions of the protein that are involved in translocation and receptor binding, but share only $\sim 80\%$ sequence identity in the C-terminal DNase domains. Hence, the specific immunity proteins Im2, Im7, Im8, and Im9 have evolved to counteract the toxicity of each DNase colicin, and these share $\sim 50\%$ sequence identity (9). Im9 binds to either ColE9 or the isolated $15$-kDa E9 DNase domain with a dissociation constant of $10^{-16}$ M in the absence of salt (pH 7.0 and 25°C) rising to $10^{-14}$ M in 200 mM salt (14). Association between the two proteins is diffusion controlled, wherein the proteins are electrostatically steered toward one another to produce an encounter complex, which then undergoes a conformational change to produce the final stable complex (14). The other immunity proteins of this family can also bind the E9 DNase, but with much weaker affinities; the equilibrium $K_d$ values for these complexes are $10^{-4}$ M for Im7, $10^{-6}$ M for Im8, and $10^{-8}$ M for Im2. In each case, binding results in inhibition of enzymatic activity, and the binding affinity is governed by the rate of complex dissociation (15).

ColE9, Im2–($x$–$y$)Im9, mutant construct in which Im9 sequences from $x$ to $y$ have been inserted into Im2; $k$, association rate constant; $k_{off}$, the overall dissociation rate constant; $K_d$, equilibrium dissociation constant; [GdnHCl]$_{50\%}$, concentration of guanidine hydrochloride required to induce 50% protein denaturation.
Our knowledge of the regions of an immunity protein that determine its specificity is limited to the results of homologous recombination experiments between Im9 and Im8 (16). This early work focused on the biological activity of chimeric immunity proteins and showed that the primary determinants for specificity were located in the N-terminal half of the protein. Moreover, substituting Val-34 in Im9 with aspartic acid, the amino acid at this position in Im8 provided the resulting mutant Im9 with some ColE8 cross-reactivity in in vivo assays. Subsequent structural work has shown that Im9 is a 4-helix protein in which position 34 is part of helix II (17, 18) (Figs. 1 and 2). Chak et al. (19) have recently proposed, based on the crystal structure of the Im7 protein, that helices I and II are both involved in determining specificity as well as loop 2 and possibly loop 1 (Fig. 1). In large part their model is based on the observation that these are the most variable sequences in the immunity protein family (Fig. 2). However, this is at variance with recent isotope-edited NMR experiments in which the am-

immunity protein family (Fig. 2). However, this is at variance with recent isotope-edited NMR experiments in which the am-
immunity protein family (20).

DNase are helices II and III, the latter being conserved in the both involved in determining specificity as well as loop 2 and

immunity protein family.

Im2 and ColE9 were tested using a modified procedure of a plate assay described previously (15). 22 × 22-cm LB agar plates containing 100 μg/ml ampicillin were divided into lanes and each lane overlaid with a lawn of exponentially growing E. coli JM83 cells producing a different immunity protein. Two parallel plates were used, one induced with IPTG (1 mM) and the other without. Aliquots (2 μl) of 5-fold serially diluted ColE2 and ColE9 from 3 mg/ml to 1.5 mg/ml were spotted on top of the cells. The plates were incubated overnight at 37 °C and scored the following day for biological protection against the toxin.

Biological Plate Assay—The biological activity of each chimeric immunity protein toward ColE2 and ColE9 was tested using a modified procedure of a plate assay described previously (15). 22 × 22-cm LB agar plates containing 100 μg/ml ampicillin were divided into lanes and each lane overlaid with a lawn of exponentially growing E. coli JM83 cells producing a different immunity protein. Two parallel plates were used, one induced with IPTG (1 mM) and the other without. Aliquots (2 μl) of 5-fold serially diluted ColE2 and ColE9 from 3 mg/ml to 1.5 mg/ml were spotted on top of the cells. The plates were incubated overnight at 37 °C and scored the following day for biological protection against the toxin.

Protein Purification and Protein Determinations—Im9 cells that had been transformed with wild type and mutant immunity genes cloned into pTrc99A were grown in LB broth containing ampicillin (100 μg/ml) and were induced by the addition of 1 mM isopropyl β-D-thiogalacto-

1 cm

amide gel electrophoresis after IPTG induction and double-stranded DNA sequencing using an ALF sequencer (Pharmacia).

Solution structure of Im9 highlighting the major ele-

Protein-Protein Interaction Specificity of Im9

with recent isotope-edited NMR experiments in which the am-

immunity protein family (20).

Materials and Methods

Bacterial Strain and Plasmids—E. coli strain JM83 Hsd R, a coli-

sequence of the other immunity gene. Thus, in the first polymerase chain reaction using imm2 as template, the resulting product will have an imm9 sequence overhang. This product is then used as a “mega” primer for a second polymerase chain reaction, although the sequence that actually primes this reaction is only around 20–30 nucleotides in length. The second polymerase chain reaction product was cloned into pTrc99A and transformed into E. coli JM83. Transformants were screened by streaking out on Luria-Bertani (LB) plates with 100 μg/ml ColE2 or ColE9. The surviving clones were checked by SDS-polyacryl-

amide gel electrophoresis after IPTG induction and double-stranded DNA sequencing using an ALF sequencer (Pharmacia).

Biological Plate Assay—The biological activity of each chimeric immunity protein toward ColE2 and ColE9 was tested using a modified procedure of a plate assay described previously (15). 22 × 22-cm LB agar plates containing 100 μg/ml ampicillin were divided into lanes and each lane overlaid with a lawn of exponentially growing E. coli JM83 cells producing a different immunity protein. Two parallel plates were used, one induced with IPTG (1 mM) and the other without. Aliquots (2 μl) of 5-fold serially diluted ColE2 and ColE9 from 3 mg/ml to 1.5 mg/ml were spotted on top of the cells. The plates were incubated overnight at 37 °C and scored the following day for biological protection against the toxin.

Protein Purification and Protein Determinations—Im9 cells that had been transformed with wild type and mutant immunity genes cloned into pTrc99A were grown in LB broth containing ampicillin (100 μg/ml) and were induced by the addition of 1 mM isopropyl β-D-thiogalacto-

acrose at an optical density at 650 nm between 0.6–0.8. The immu-

nity protein was purified as described previously for Im9 (22). The E9 DNase was purified according to the protocol described by Garinot-

Schneider et al. (23). Protein concentrations were determined using the molar absorption coefficients of 17,550 M⁻¹ cm⁻¹ for the E9 DNase and 11,400 M⁻¹ cm⁻¹ for Im9, Im2, and the chimeric immunity proteins (14).

Electrospray Mass Spectrometry—The masses of all of the purified proteins were verified by mass spectrometry using a VG Platform electrospray mass spectrometer. The tryptic proteins were dissolved in high pressure liquid chromatography grade water, and the concentra-

tions were adjusted to about 0.1 mg/ml. Formic acid was added to a final concentration of 1% just before the injection into a mobile phase of water:acetonitrile:formic acid (1:1:0.001) at a flow rate of 5 μl/min. The scan range was from 700 to 1700 m/z, and 10 transients were collected and averaged for each sample with the raw data processed using the accompanying MassLynx software. Horse heart myoglobin (Sigma) was used as a calibrant. Each protein was analyzed at least twice.

E9 DNase Immunity Protein Dissociation Constants—Dissociation constants were obtained from the ratio of the individual dissociation (k_d) and association (k_a) rate constants at 25 °C in 50 m M Mops buffer, pH 7.0, containing 200 m M NaCl and 1 m M diethylthioctetraline, as described previously by Wallas et al. (14, 15) and Osborne et al. (20). Association of the proteins was monitored by stopped-flow fluorescence under pseudo-first-order conditions using a 4–20-fold excess of immunity protein over E9 DNase (0.35 μ M). The resulting biphasic fluorescence traces were fitted to a double exponential equation, and the bimolecular rate constant was obtained from linear replots of the rate of initial fluores-

cence enhancement versus the immunity protein concentration. Dissociation kinetics for E9 DNase-Im2 complexes were obtained either from radioactive subunit exchange (in which complexes were chased with 


dH2O for 9 for slow dissociation rate constants (~10⁻³ s⁻¹) or from fluo-

rescence chase stopped-flow (in which complexes were chased with a 6-fold excess of Im9) for fast dissociation rate constants (~10⁻¹ s⁻¹).

Differences in binding energy between Im2 mutants and wild type Im2 binding to the E9 DNase were determined according to Equation 1,

\[ \Delta G_{\text{binding}} = RT \ln (k_{\text{mutant}}/k_{\text{wild type}}) \]  

where \( R \) is the gas constant, and \( T \) is the absolute temperature.

Immunotype Protein Stability—Immunotype protein stabilities were determined by guanidine hydrochloride (GdnHCl) denaturation where protein denaturation was monitored by the change in tryptophan fluores-

cence. All of the experiments were carried out on a Shimadzu RF-5000 fluorescence spectrophotometer using an excitation wavelength of 295 nm. The excitation and emission bandwidths were both 5 nm. Each immunity protein (2 μ M) was equilibrated at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, containing GdnHCl (0–3 M) for a minimum of 2 h, and the fluorescence emission was measured at 354 nm. The relative stabilities of the immunity proteins were compared by the concentrations of GdnHCl required to cause 50% protein denaturation (GdnHCl_50%).
RESULTS

Biological Specificity—Eleven chimeric immunity proteins were constructed and cloned into the expression vector pTrc99a, transformed into *E. coli* JM83, and their biological specificity toward colicins scored using an agar plate assay (see “Materials and Methods”). We have previously shown (15) that in the absence of IPTG, JM83 cells containing this expression vector and a non-cognate immunity gene cloned into it are completely sensitive toward the action of ColE9, whereas the same construct containing the *imm9* gene is resistant toward the action of the toxin. Although SDS-polyacrylamide gel electrophoresis indicates the absence of any significant amounts of expressed protein under these conditions, the complete protection of cells containing the pTrc99a+*imm9* construct is most likely due to “leaky” expression (15). On addition of IPTG, >25% cell protein is immunity protein, and now both the Im8 and Im2 containing cells begin to show non-cognate biological cross-reactivity with the latter being the strongest.

The sensitivity of JM83 cells (with and without IPTG induction) containing each of the eleven chimeric immunity proteins generated in this study were tested against serial dilutions of both ColE9 and ColE2. The results for five of these constructs are shown in Fig. 3 along with the data for cells expressing wild type Im2 and Im9. As in our previously published work, both Im2 and Im9 are completely resistant to the action of their cognate colicin with or without induction by IPTG (i.e., no zones of clearance). However, whereas IPTG-induced Im2 provides *E. coli* cells with partial protection toward ColE9, IPTG-induced Im9 shows no such cross-reactivity toward ColE2 (Fig. 3).

When helix II of Im9 is inserted into Im2 (Im2-(30–44)*Im9* mut) the resulting chimera behaves very much like Im9 in that it provides complete protection toward ColE9 (9) and is sensitive toward ColE2 in the absence of induction. It differs from Im9, however, since on induction with IPTG it still retains some residual ColE2 cross-reactivity. By contrast, when the N terminus of Im9 was inserted into Im2 (Im2-(1–29)*Im9*) the resulting chimera behaved exactly like Im2 toward both colicins with or without induction suggesting that helix I is not directly involved in specificity. A further chimeric protein in which the N-terminal half of Im9 was fused to the C-terminal half of Im2 (Im2-(1–44)*Im9*) showed exactly the same biological phenotype as Im2-(30–44)*Im9*. This latter result is in agreement with our previous homologous recombination experiments between Im9 and Im8, which showed that residues in the N terminus of the protein were the most likely determinants of biological specificity (16). The present data show for the first time that of the two helices in the N-terminal half of the protein, helix II is responsible for these differences in specificity.

An intriguing property of both the Im2-(30–44)*Im9* and Im2-(1–44)*Im9* chimeras is that they retain some residual cross-reactivity toward ColE2 even though Im9 itself does not show this behavior (Fig. 3). The region of the protein responsible for this cross-reactivity was identified by introducing further structural elements of Im9 into Im2-(1–44)*Im9*. It was not until the loop connecting helix III and helix IV was introduced (Im2-(1–64)*Im9*) that this residual cross-reactivity was finally lost, and the protein behaved like Im9 ± IPTG (Fig. 3). Interestingly, the same phenotype could be obtained by combining the helix II and loop 2 substitutions (Im2-(30–44, 56–64)*Im9*) but not helix I and loop 2 (Im2-(1–29, 56–64)*Im9*) or loop 2 on its own (Im2-(56–64)*Im9*) both of which behave essentially like Im2. A summary of the biological phenotypes of the bacterial cells expressing the chimeric constructs is given in Table I.

E9 DNase Binding—Although a total of eleven chimeric immunity proteins were constructed (Table I) and induced by IPTG to approximately similar levels as deduced from SDS-polyacrylamide gel electrophoresis (data not shown), only those which showed representative changes in biological specificity were characterized further. Seven chimeras were purified and their masses confirmed by electrospray mass spectrometry. In each case the observed mass of the protein corresponded to the predicted mass ± 1 Da (data not shown).

The *Kd* for each purified chimeric immunity protein binding the E9 DNase was obtained from the ratio of the individual dissociation and association rate constants, *koff* and *k1*, respectively, as summarized under “Materials and Methods” (14, 15). Association kinetics were monitored by stopped-flow fluorescence, and dissociation kinetics were monitored by subunit exchange methods. The association of Im2 and Im9 with the E9 DNase is biphasic in stopped-flow fluorescence experiments in which a fluorescence enhancement representing the bimolecular collision is followed by a fluorescence quench that is thought to emanate from a conformational change in the complex (14). The rate constants for both of these processes (in Mops buffer at pH 7.0, 25 °C and containing 200 mM NaCl) are very similar for both Im2 and Im9 where *k1* ~ 6–9 × 107 M–1 s–1, and the conformational change is ~4–5 s–1 (15). Very similar values were obtained for each of the seven Im2/Im9 chimeric immunity proteins (data not shown) indicating that the association kinetics of these proteins do not play a significant role in determining the stability of the resulting complexes with the E9 DNase. However, the complexes differed quite significantly in the rate of complex dissociation; some behaved like Im2 (for example, Im2-(1–29)*Im9*), which has a *koff* of ~1 s–1, whereas others displayed off rates similar to that of wild type Im9 (for example, Im2-(19–44)*Im9*), which has a *koff* of ~10–8 s–1. The equilibrium dissociation constants for each of the seven purified chimeric immunity proteins are shown in Table I along with data for wild type Im2 and Im9. Also shown in Table I are values for ΔG, which have been calculated relative to Im2 binding the E9 DNase. Hence the ΔG for Im9 is ~8 kcal/mol, reflecting the difference in specificity between these two proteins for the E9 DNase.

The thermodynamic data in Table I show clearly that helix I makes no contribution toward E9 DNase binding specificity (ΔG = 0), and this matches the biological phenotype of the cells expressing the Im2-(1–29)*Im9* mutant. In contrast, helix II contributes ~6 kcal/mol toward E9 DNase binding specificity, and this corresponds to a complete switch in the biological specificity. The sequences among the four immunity proteins are shown at the bottom with **bold letters**.
phenotype of the protein. As further sequences toward the C terminus of Im9 are included in Im2 (Im2-(30–64)Im9) binding is reduced a little, and at the same time the residual cross-reactivity toward ColE2 is lost. Conversely, as helix II is combined with sequences toward the N terminus, including loop 1 and part of helix I, binding affinity increases and even seems to surpass that of the cognate immunity protein Im9. Nevertheless, the residual biological cross-reactivity toward ColE2 is retained in these chimeras.

Stabilities of the Mutant Proteins—Little is known about the

![Fig. 3. Biological phenotypes of chimeric immunity proteins shown by a toxin overlay agar plate assay. 22 × 22-cm agar plates were divided equally into 8 lanes and each lane was overlaid with E. coli JM83 cells harbouring a different immunity protein construct or the vector. A serial dilution, going from left to right, of 3 mg/ml to 1.6 ng/ml of either ColE2 (top of each lane) or ColE9 (bottom of each lane) was dropped onto each lawn of cells. The uppermost lane is the vector control and shows the cells to be sensitive to the action of both toxins. The absence of zones of killing indicates biological cross-reactivity toward the toxin by the expressed immunity protein.](https://example.com/fig3)

**Table I**

Biological phenotypes, binding affinities, and thermodynamic stabilities of chimeric immunity proteins

| Protein | [GdnHCl] \( \times 10^4 \) (M) | \( K_d \) (M) | ΔΔG (kcal/mol) | ColE9\(^9\) (-IPTG) | ColE9\(^9\) (+IPTG) | ColE9\(^9\) (-IPTG) | ColE9\(^9\) (+IPTG) | ColE9\(^9\) (-IPTG) | ColE9\(^9\) (+IPTG) |
|---------|--------------------------------|---------------|----------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Im2     | 1.52                           | 1.47 x 10^{-4} |                | ++                | +++++             | +                | +                | +                 | +                 |
| Im9     |                                |               |                |                   |                   |                   |                   |                   |                   |
| Im2(1-29)Im9 | 29                            | 1.71           | 1.82 x 10^{-4} | -8.1(±0.1)        | +++++             | +                | -                | -                 | -                 |
| Im2(50-64)Im9 | 64                            | 1.27           | 1.31 x 10^{-8} | 0 (±0.2)          | -                 | +                | +                | +++               | +++               |
| Im2(50-44)Im9 | 64                            | 1.58           | 2.26 x 10^{-12}| -5.2 (±0.1)       | +++               | -                | -                | -                 | -                 |
| Im2(25-44)Im9 | 25                            | 1.75           | 3.23 x 10^{-13}| -6.4 (±0.1)       | +++               | +++              | -                | +                 | +                 |
| Im2(19-44)Im9 | 19                            | 1.85           | 2.01 x 10^{-13}| -6.7 (±0.1)       | +++               | +++              | -                | +                 | +                 |
| Im2(1-44)Im9 | 44                            | 1.85           | 0.76 x 10^{-14}| -8.6 (±0.1)       | +++               | +++              | -                | +                 | +                 |
| Im2(1-55)Im9 | 55                            | ND             | ND              | ND                | +++               | +++              | -                | +                 | +                 |
| Im2(1-64)Im9 | 64                            | 2.17           | 2.08 x 10^{-14}| -8.0 (±0.2)       | +++               | +++              | -                | -                 | -                 |
| Im2(56-64)Im9 | 64                            | ND             | ND              | ND                | +++               | +++              | -                | +                 | +++               |
| Im2(30-44)Im9 | 44                            | ND             | ND              | ND                | +++               | +++              | -                | -                 | -                 |
| Im2(1-20-56-64)Im9 | 29                            | ND             | ND              | ND                | -                 | -                | -                | ++                | ++                |

\(^{a}\) Data shown are the means from two independent experiments for which the standard errors were ±3%. ND, not determined.

\(^{b}\) \( K_d \) is the dissociation constant for binding E9 DNase at 25 °C in Mops buffer, pH 7.0, containing 200 mm salt and 1 mm dithiothreitol and was obtained from the ratio \( h_{off}/h_{on} \) as described under "Materials and Methods." Values of \( h_{off} \) and \( h_{on} \) were determined twice, and the standard errors were always below ±15%.

\(^{c}\) Changes in binding energy relative to Im2 calculated according to the relation, \( RT \ln (K_d \text{ mutant}/K_d \text{ wild type}) \).

\(^{d}\) Results from the agar plate assay. ColE9 and ColE2 refer to the protection against ColE9 and ColE2, respectively. The levels of protection are represented by the concentrations of colicins to which cells are immune: +++, >10^{-3} g/ml; ++, 10^{-4}-10^{-3} g/ml; +, 10^{-5}-10^{-4} g/ml; +, 10^{-6}-10^{-5} g/ml; and −, <10^{-6} g/ml.
thermodynamic stabilities of immunity proteins, and since the homolog scan involved moving whole elements of secondary structure between immunity proteins, it was of interest to compare the thermodynamic stabilities of Im2 and Im9 with the chimeras. Guanidine hydrochloride was used as a denaturant, and denaturation was monitored by tryptophan fluorescence spectroscopy. The endonuclease-specific immunity proteins possess a single conserved tryptophan at position 74, and these two immunity proteins. The only chimeric protein that did not show an increase in E9 DNase binding affinity was Im2-(25–44)Im9 with Im2-(19–44)Im9. Interestingly, these sequences have to be added for full E9 DNase binding (Table I). The most important of these appears to be the C-terminal end of helix I (compare the binding energy data for Im2-(25–44)Im9 with Im2-(19–44)Im9). Interestingly, these sequences in isolation (in the form of the Im2-(1–29)Im9 construct) do not affect E9 DNase binding. In other words, the effect of helix I is not additive to that of helix II but is felt only in the presence of helix II. We conclude from these observations that it is the buried residues of helix I that elicit this effect, probably by changing the orientation of helix II. Since helix I and II pack against each other in the Im9 structure, and since both helices are largely variable in sequence between immunity proteins, it seems reasonable that packing interactions between them may play a role in specificity. Tight binding protein complexes are characterized by highly complementary and well packed binding interfaces (27), and so it is quite conceivable that such intramolecular packing interactions could affect intramolecular specificity. It is also noteworthy that packing interactions between these two helices affect the stability of the protein; adding residues 19–30 from Im9 to the Im2-(30–44)Im9 mutant increases $\langle\text{GdnHCl}\rangle_{50\%}$ by $\approx0.2$ M (Table I).

Although neither of the variable loops play important roles in specificity, the results for chimeras containing loop 2 do show some relatively minor effects that require comment. Co-incident with the loss of residual ColE2 cross-reactivity of Im9-like chimeras, the inclusion of loop 2 from Im9 also seems to reduce E9 DNase binding in these proteins (Table I). This chosen to use homologue-scanning mutagenesis combined with biological and in vitro binding assays to determine the regions of the protein that govern its specificity. Homologue scanning was first used by Wells and co-workers (24) to identify the regions of human growth hormone that are required for specific binding to its receptor. Since then it has been used in a number of systems to localize functionally important regions in families of homologous proteins (25, 26). The basic premise of the technique is the substitution of sections of sequence from one protein with analogous sections from a related protein and then testing the resulting chimeras by a functional assay that discriminates between the two wild type starting proteins. Clearly for the strategy to succeed the two proteins must be sufficiently similar in three-dimensional structure to allow accommodation of the novel sequences and so yield chimeras that are stable and can be purified and characterized.

In the present work, we have used this technique to localize the elements of secondary structures of the immunity proteins Im2 and Im9, which define their colicin specificity. The solution structure for Im9 has been determined by NMR (18) and this structure was used as the basis for the scan (Fig. 1). Im2 and Im9 are identical in length (86 amino acids) and share 68.6% sequence identity (Fig. 2) and so it is likely that the structure of Im2 will be similar to that of Im9. Indeed, the structures for the other endonuclease specific immunity proteins Im7 and Im8, which show even less sequence identity to Im9 than Im2, have very similar 4-helix structures (19).2 A further point, which suggests that the Im2 and Im9 structures will be comparable, stems from the results of this work since a total of eleven chimeras were generated, and in each case a stable protein was made suggesting that there was no gross distortion of the protein fold.

Helix II, Loop 2, and Packing Interactions—Our data show that of the four helices in an immunity protein, helix II is the major determinant of colicin specificity that in the context of the binding of Im2 relative to Im9 contributes over 6 kcal/mol E9 DNase binding energy from a total of 8 kcal/mol. Other sequences have to be added for full E9 DNase binding (Table I). The most important of these appears to be the C-terminal end of helix I (compare the binding energy data for Im2-(25–44)Im9 with Im2-(19–44)Im9). Interestingly, these sequences in isolation (in the form of the Im2-(1–29)Im9 construct) do not affect E9 DNase binding. In other words, the effect of helix I is not additive to that of helix II but is felt only in the presence of helix II. We conclude from these observations that it is the buried residues of helix I that elicit this effect, probably by changing the orientation of helix II. Since helix I and II pack against each other in the Im9 structure, and since both helices are largely variable in sequence between immunity proteins, it seems reasonable that packing interactions between them may play a role in specificity. Tight binding protein complexes are characterized by highly complementary and well packed binding interfaces (27), and so it is quite conceivable that such intramolecular packing interactions could affect intramolecular specificity. It is also noteworthy that packing interactions between these two helices affect the stability of the protein; adding residues 19–30 from Im9 to the Im2-(30–44)Im9 mutant increases $\langle\text{GdnHCl}\rangle_{50\%}$ by $\approx0.2$ M (Table I).

Although neither of the variable loops play important roles in specificity, the results for chimeras containing loop 2 do show some relatively minor effects that require comment. Co-incident with the loss of residual ColE2 cross-reactivity of Im9-like chimeras, the inclusion of loop 2 from Im9 also seems to reduce E9 DNase binding in these proteins (Table I). This
affect can be seen in two cases. First, it is seen in chimeras that seem to bind slightly more tightly to the E9 DNase than Im9 itself (Im2-(19–44)Im9 and Im2-(1–44)Im9). This affinity is reduced to wild type Im9 levels when loop 2 from Im9 is added (Im2-(1–64)Im9). Second, this region also reduces the binding of the mutant protein that contains helix II alone (compare Im2-(30–44)Im9 with Im2-(30–64)Im9). Taken together these results suggest a rather paradoxical situation in which loop 2 in Im2 imparts some specificity for its cognate E2 DNase (hence the residual biological cross-reactivity) as well as providing some binding energy for the non-cognate E9 DNase. Substituting this loop in Im2 with that from Im9 removes both effects. Loop 2 also seems to affect the thermodynamic stability of the immunity proteins since the [GdnHCl]50% of chimeras containing this loop is increased by 0.2–0.3 m (see “Results” and Table I).

The observations on specificity concerning loop 2 could be the result of direct effects where loop 2 is involved in DNase binding or indirect effects where loop 2 alters the conformation of binding residues. Two pieces of evidence suggest that the latter is the most likely. First, few amide resonances from this loop in Im9 are perturbed on binding the E9 DNase in 15N edited NMR experiments (20). Second, the solution structure of Im8 shows that the conformation of Tyr-55, a conserved residue in helix III, is markedly different from that of Im9, and the reason for this seems to be the nature of the adjoining residues in loop 2. The importance of this observation stems from the fact that recent mutagenesis data have shown this residue to be critical for DNase binding.

In conclusion, the specificity of an immunity protein for an E.coli DNase domain is complex. Although dominated by residues from a single helix, interactions with other parts of the immunity protein scaffold also play an indirect role. Protein–protein interactions are characterized by relatively large surface areas of each binding partner (typically >600 Å2) becoming buried in the complex (27, 28), and given the very tight binding for the E9 DNase-Im9 complex this is likely to be true for a colicin complex. Since it is clear that other parts of Im9 are also involved in binding the DNase (20), it is interesting that specificity can be controlled almost exclusively by residues from a single helix, a situation reminiscent of some DNA-protein interactions (29).

Acknowledgments—We thank Ann Reilly and Christine Moore for expert technical assistance, and Andrew Leech, Russell Wallis, Kit-Yi Leung, Ansgar Pommer, Theonie Georgiou, and Catriona Giffard for help and advice during the course of this work. We also thank Ruth Boetzel for Fig. 1.

REFERENCES

1. James, R., Kleanthous, C., and Moore, G. R. (1996) Microbiology 142, 1569–1580
2. Di Mastri, D. R., White, D. C., Schnaitman, C. A., and Bradbeer, C. (1973) J. Bacteriol. 115, 506–513
3. Cooper, P. C., and James, R. (1994) J. Gen. Microbiol. 140, 209–215
4. Watson, R. J., Vernet, T., and Visentin, L. P. (1985) Plasmid 13, 205–210
5. Cramer, W. A., Cohen, S., Merrill, A. R., and Song, H. Y. (1996) Mol. Microbiol. 4, 519–526
6. Wallis, K.-Y., Leung, K.-Y., and Kleanthous, C. (1997) Structure 5, 445–458
7. Bowman, C. M., Dahlerg, J. E., Ikemura, T., Konisky, J., and Nomura, M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 964–968
8. Senior, B., and Holland, I. B. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 959–963
9. Lau, C. K., Parsons, M., and Uchimura, T. (1992) in Bacteriocins, Microcins and Lantibiotics (James, R., Lazdunski, C., and Pattus, F., eds) pp. 353–378, NATO ASI Series H, Springer-Verlag, Heidelberg, Germany
10. Schaller, K., and Nomura, M. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2989–2993
11. Toba, M., Masaki, H., and Ohta, T. (1988) J. Bacteriol. 170, 3237–3242
12. Eaton, T., and James, R. (1989) Nucleic Acids Res. 17, 1761
13. Chak, K.-P., Kuo, W.-S., Lu, F.-M., and James, R. (1991) J. Gen. Microbiol. 137, 91–100
14. Wallis, R., Moore, G. R., James, R., and Kleanthous, C. (1995) Biochemistry 34, 13743–13750
15. Wallis, R., Leung, K.-Y., Pommer, A. J., Volber, H., Moore, G. R., James, R., and Kleanthous, C. (1995) Biochemistry 34, 13751–13759
16. Wallis, R., Moore, G. R., Kleanthous, C., and James, R. (1992) Eur. J. Biochem. 210, 923–930
17. Osborne, M. J., Lian, L.-Y., Wallis, R., Reilly, A., James, R., Kleanthous, C., and Moore, G. R. (1994) Biochemistry 33, 12347–12355
18. Osborne, M. J., Breeze, A. L., Lian, L.-Y., Reilly, A., James, R., Kleanthous, C., and Moore, G. R. (1996) Biochemistry 35, 9505–9512
19. Chak, K.-P., Safo, M. K., Ko, W.-Y, Hsieh, S.-Y., and Yuan, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6437–6442
20. Osborne, M. J., Wallis, R., Leung, K.-Y., William, G., Lian, L.-Y., James, R., Kleanthous, C., and Moore, G. R. (1997) Biochem. J. 325, 825–831
21. Sarkar, G., and Sommers, S. S. (1990) BioTechniques 8, 404–407
22. Wallis, R., Reilly, A., Rowe, A. Moore, G. R., James, R., and Kleanthous, C. (1992) Eur. J. Biochem. 207, 687–695
23. Garinot-Schneider, C., Pommer, A. J., Moore, G. R., Kleanthous, C., and James, R. (1996) J. Mol. Biol. 260, 731–742
24. Cunningham, B. C., Druhany, P., Ng, P., and Wells, J. A. (1989) Science 243, 1330–1336
25. Bettler, B., Tezido, G., Raggi, S., Ruegg, D., and Hofstetter, H. (1992) J. Biol. Chem. 267, 185–191
26. Lee, C. W., Luck, M. D., Juppner, H., Potts, J. T., Kronenberg, H. M., and Gardeia, T. J. (1995) Mol. Endocrinol. 9, 1269–1275
27. Janin, J., and Chothia, C. (1990) J. Mol. Biol. 210, 687–695
28. Osawa, M., Wallis, R., Leung, K.-Y., William, G., Lian, L.-Y., James, R., Kleanthous, C., and Moore, G. R. (1997) Biochem. J. 325, 825–831
29. Luisi, B. (1995) in DNA-Protein: Structural Interactions (Lilley, D. M. J., ed), pp. 1–48, IRL Press at Oxford University Press, UK

3 R. Wallis and K.-Y. Leung, unpublished observations.