Protein−Protein Interactions in Colicin E9 DNase−Immunity Protein Complexes. 1. Diffusion-Controlled Association and Femtomolar Binding for the Cognate Complex†

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Received May 18, 1995; Revised Manuscript Received August 7, 1995®

ABSTRACT: The cytotoxic activity of the secreted bacterial toxin colicin E9 is due to a nonspecific DNase housed in the C-terminus of the protein. A kinetic and thermodynamic analysis of complex formation for both the holotoxin and the isolated DNase domain with the cytoplasmic inhibitor of this enzyme, the immunity protein Im9, is presented. The dissociation constant for each complex was calculated from the ratio of the association and dissociation rate constants. Association was monitored by stopped-flow fluorescence and comprises at least two steps for both complexes, an initial fluorescence enhancement followed by a fluorescence quench. The data are consistent with a two-step binding mechanism in which the rate of formation of an encounter complex (k_1) is rate determining and essentially diffusion controlled (4.0×10^9 M^{-1} s^{-1} for colicin E9) in buffer of low ionic strength. This encounter complex then rearranges to the final stable complex. Sequential stopped-flow experiments using 5-hydroxy-L-tryptophan labeled DNase domain support the two-step mechanism and further show that the rate of encounter complex rearrangement is significantly faster than its dissociation. The overall rate of dissociation of the colicin E9−Im9 complex (k_{off}) was determined by radioactive subunit exchange to be 3.7×10^{-7} s^{-1}. Thus, the K_d for the complex (k_{off}/k_1) is 9.3×10^{-17} M, which corresponds to a change in free energy on binding of −21.9 kcal mol^{-1} at 25 °C. The affinity of the complex between the isolated DNase domain of colicin E9 and Im9 was very similar to that of the full size protein ($K_d = 7.2 \times 10^{-17}$ M, $\Delta G = -22$ kcal mol^{-1}). The effects of pH, temperature, and salt on complex formation were also analyzed. Altering the pH of the medium (between pH 5 and 9) had little effect on the association and dissociation rate constants. The temperature dependence of the equilibrium constant yielded values for ΔH of −6.4 kcal mol^{-1} and for ΔS of 52.3 cal mol^{-1} K^{-1} at 25 °C. The association rate constant decreases by over two orders of magnitude in the presence of 250 mM NaCl, suggesting that electrostatic steering plays an important role in the rapid formation of the encounter complex.

The basis of the affinity and specificity of protein−protein interactions is fundamental to the study of biological recognition, protein engineering, and drug design. In recent years our understanding of how proteins interact with other proteins has increased dramatically as structural and thermodynamic studies of several protein complexes have emerged. For example, the study of proteinase−inhibitor complexes (Vincent & Lazdunski, 1972; Empie & Laskowski, 1982; Bode & Huber, 1992), antibodies and protein antigens (Davies & Padlan, 1990; Kelley & O'Connell, 1993), hormone−receptor complexes (Cunningham & Wells, 1989; De Vos et al., 1992), and ribonuclease−inhibitor complexes (Lee et al., 1989; Schreiber & Fersht, 1993; Guillet et al., 1993) have all contributed to this expanding information base on protein recognition. In the present study, we have investigated the binding of a small DNase inhibitor protein known as Im9[1] to its target endonuclease.

Colicin E9 (61kDa) is a member of the E colicin family of plasmid-encoded bacterial toxins that are inducibly expressed, through the stress response of the producing organism, following DNA damage and then secreted into the extracellular medium (Jakes, 1982; Luria & Suit, 1987). This class of bacterial toxin recognizes and binds the BtuB receptor of *Escherichia coli* cells through a central protein domain, whereas translocation across the bacterial cell membranes is dependent upon an N-terminal domain and cytotoxicity mediated by a C-terminal domain (Ohno-Iwashita & Imahori, 1980, 1982; Benedetti et al., 1991). E group colicins can be divided into three cytotoxic classes: colicin E1 is a pore-forming ionophore (Cramer et al., 1983; Pattus et al., 1990), colicins E3 and E6 are RNases (Bowman et al., 1971; Senior & Holland, 1971; Akutsu et al., 1989), and colicins E2, E7, E8, and E9 are nonspecific DNases (Schaller & Nomura, 1976; Toba et al., 1988; Chak et al., 1991; Eaton & James, 1989).

Associated with the production of each nuclease-type colicin is the synthesis of a specific, highly acidic immunity (Im) protein (9.5 kDa) which binds to the basic endonuclease

† This work was supported by the Wellcome Trust and by the U.K. Science and Engineering Research Council.

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® Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: Im9, immunity protein of colicin E9; ColE9, colicin E9; E9 DNase, the isolated 15 kDa endonuclease domain of colicin E9; IPTG, isopropyl β-D-thiogalactoside; 5OH-Trp, 5-hydroxy-L-tryptophan; [^{3}H]Im9, Im9 protein biosynthetically radiolabeled with tritiated leucine.

0006-2960/95/0434-13743$09.00/0

domain of the toxin, forming a stoichiometric complex that neutralizes the action of the toxin in the producing cell (Jakes & Zinder, 1974; Sidikaro & Nomura, 1974; Wallis et al., 1992a). The resulting 71 kDa heterodimeric complex is released into the extracellular medium by the action of a third inducible protein, the lysis protein (Pugsley & Schwartz, 1983), and it is as this complex that the cytotoxic process is initiated.

The four DNase E colicins have nearly identical amino acid sequences in the regions specifying receptor binding and translocation, whereas their DNase domains are ~80% identical and the corresponding immunity proteins (Im2, Im7, Im8, and Im9) are ~60% identical in sequence [reviewed in Lau et al., (1992)]. Despite this level of sequence identity, the toxins have different immunity specificities such that, in general, each immunity protein protects cells only against its cognate colicin (Cooper & James, 1984), the implication being that each immunity protein will only recognize its specific colicin DNase. The region of the immunity proteins which defines nuclease specificity has been narrowed down by homologous recombination experiments to lie between residues 16−43 (Wallis et al., 1992b). The issue of colicin DNase−immunity protein specificity is dealt with further in the accompanying paper (Wallis et al., 1995).

Thermodynamic studies of protein−protein interactions center on the determination of the dissociation constant (K_d) for a protein complex (Janin & Chothia, 1990). Little is known about the affinity of an immunity protein for an E colicin DNase except that it is presumed to be very high since the two proteins copurify from bacterial growth media, following mitomycin induction, and can only be separated under denaturing conditions (Jakes & Zinder, 1974; Wallis et al., 1992a). We report the use of rapid reaction and subunit exchange experiments to determine the association and dissociation rate constants, and hence K_d, for the interaction between colicin E9 and its cognate immunity protein, Im9. By studying the interaction between the isolated 15 kDa E9 DNase domain with Im9, we have also addressed the question of whether the stability of the complex is due solely to interactions between the C-terminal DNase domain and Im9 or whether other parts of the toxin are also involved. This represents the first detailed binding study of any colicin−immunity protein complex, and the results are discussed with respect to other well characterized protein complexes.

EXPERIMENTAL PROCEDURES

Protein Purifications and Protein Determinations. Purification of colicin E9, from which the endogenous immunity protein has been removed, the E9 DNase domain of the toxin and the Im9 protein have all been described previously (Wallis et al., 1992a, 1994). Tritiated Im9 protein ([^{3}H]Im9) was prepared from a 1 L culture of *E. coli* NCIMB 11219 (*trp his arg thr leu*) containing plasmid pRJ345, which overexpresses the *imm9* gene from the trc promoter in the vector pTrc99A (Pharmacia), in M9 media supplemented with glucose (0.2%), L-threonine, L-tryptophan, L-arginine, L-histidine (50 μg mL^{-1}), thiamine (0.01%), ampicillin (20 μg mL^{-1}), and L-leucine containing L-[4,5-^{3}H]leucine (75 Ci/mmol, Amersham). The subsequent overproduction and purification was carried out as described previously (Wallis et al., 1992a). The specific radioactivity of the purified [^{3}H]Im9 was determined to be 0.3 μCi/μmol.

5-Hydroxy-L-tryptophan (5OH-Trp) was incorporated into the E9 DNase domain on its overproduction in minimal media. A 2 L culture of *E. coli* NCIMB 11219 containing the plasmid pRJ352, which overexpresses the E9 DNase−Im9 complex (Wallis et al., 1994), was incubated in M9 media supplemented with glucose (0.2%), L-threonine, L-tryptophan, L-arginine, L-histidine, L-leucine (50 μg mL^{-1}), thiamine (0.01%), and ampicillin (20 μg mL^{-1}). The culture was grown at 37 °C with shaking to an OD_{550} of 1.0. The cells were then harvested by centrifugation and resuspended in an equal volume of the same medium except that 5-hydroxy-L-tryptophan was substituted for L-tryptophan. The culture was incubated for a further hour, and then expression of the complex was induced by the addition of IPTG (1 mM). After 6 h incubation at 37 °C the culture was harvested by centrifugation and the labeled E9 DNase was isolated as described by Wallis et al. (1994).

Protein concentrations were determined by amino acid analysis and from the absorbance at 280 nm. The molar absorption coefficients determined for the proteins are as follows, colicin E9, 89 670 M^{-1}cm^{-1}; E9 DNase, 17 550 M^{-1} cm^{-1}; and Im9, 11 400 M^{-1} cm^{-1}.

Stopped-Flow Measurements. Stopped-flow kinetic measurements were made using an Applied Photophysics biosequential stopped-flow spectrofluorimeter (slit widths = 5 nm) using an excitation wavelength of 280 nm, or 315 nm when using 5OH-Trp-labeled proteins, and monitoring the fluorescence emission above 320 nm. Unless otherwise stated, all reactions were performed in 50 mM Mops buffer, pH 7.0, at 25 °C. Four thousand data points were recorded over the course of each reaction, and at least ten runs were averaged for each measurement. Kinetic traces were analyzed using the Workstation software supplied by Applied Photophysics Ltd. Association between colicin E9 and Im9 or between the E9 DNase domain and Im9 was monitored under both second-order reaction conditions, using equimolar concentrations of each protein (final concentration = 0.35 μM), and pseudo-first-order conditions, using at least a 4-fold excess of Im9. In the former case, the initial fluorescence enhancement k_1 was calculated by fitting the data to

$$F = F_0 + \Delta F[N]_0^2 k_1 t/(1 + [N]_0 k_1 t) \quad (1)$$

where $[N]_0$ is the initial concentration of the nuclease and Im9, F is the fluorescence at time t, F_0 is the initial fluorescence, and ΔF is the total change in fluorescence divided by the protein concentration (Schreiber & Fersht, 1993). Eq 1 applies to the specific case where the initial concentrations of nuclease and Im9 are equal. The fluorescence quench that was also observed was fitted to a first-order rate equation. Under pseudo-first-order conditions the two rates were fitted to a double-exponential equation.

Dissociation Kinetics. Dissociation rate constants were determined by subunit exchange. An excess of unlabeled Im9 protein (54 μM) was incubated with preformed, stoichiometric complex between either colicin E9 or the E9 DNase domain and [^{3}H]Im9 protein (6 μM). The dissociation rate was followed by monitoring the disappearance of [^{3}H]Im9 from the complex with time. Complex was separated from unbound Im9 by gel filtration using a Superdex-75 column (Pharmacia) equilibrated in 50 mM potassium phosphate, pH 7.0. The levels of radioactivity across the protein peaks were determined using a Rack-

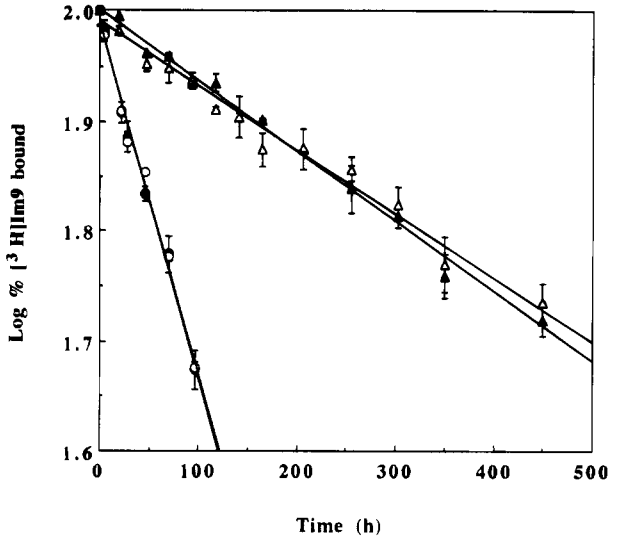

FIGURE 1: Dissociation kinetics of ColE9−Im9 (open symbols) and E9 DNase−Im9 (closed symbols) complexes. An excess of unlabeled Im9 protein (54 μM) was incubated with preformed complex between either colicin E9 or the E9 DNase domain and [^{3}H]Im9 (6 μM) in 50 mM Mops, pH 7.0 (△, ▲), and buffer containing 200 mM NaCl at 25 °C (○, ●). At different time points protein samples were removed, the components separated by FPLC gel filtration, and the levels of radioactivity determined in a scintillation counter. The graph shows the average of duplicate observations and the standard errors for each determination. The dissociation rate constants (k_{off}) were calculated from the slopes of these lines which were fitted by linear regression.

BetaExcel liquid scintillation counter. Unless otherwise stated, all reactions were carried out in 50 mM Mops buffer, pH 7.0, at 25 °C. The data were fitted using first-order kinetics.

RESULTS

Colicin E9 and its cognate immunity protein Im9 are known to form a very tight, stoichiometric complex that can only be separated under denaturing conditions, and this interaction inactivates the nuclease activity of the toxin (Wallis et al., 1992a). For this reason, rather than a direct estimation of the equilibrium dissociation constant, the K_d value was determined from the ratio of the dissociation (k_{off}) and association (k_{on}) rate constants, as has been described for a number of other tight protein complexes (Vincent & Lazdunski, 1972; Empie & Laskowski, 1982; Lee et al., 1989; Longstaff et al., 1990; Schreiber & Fersht, 1993). In the following study, k_{on} and k_{off} were determined for Im9 in complex with the DNase domain of the 61 kDa ColE9 holotoxin and the isolated 15 kDa E9 DNase domain.

Dissociation Kinetics (k_{off}). The dissociation rate constant k_{off} for both the ColE9−Im9 and E9 DNase−Im9 complexes was estimated by subunit exchange using [^{3}H]Im9 and fitting the data using first-order kinetics. The exchange process was generally only followed to 50% because of the very slow dissociation rates that were observed. Similar off-rates were determined, however, using freshly mixed and preformed complexes (several weeks) suggesting that there are no additional slow rates that significantly increase the affinity of binding. Figure 1 shows the dissociation rates of the ColE9−Im9 and E9 DNase−Im9 complexes measured in the presence and absence of 200 mM NaCl. Very similar rates were obtained for each complex. Rate constants of 3.7×10^{-7} s^{-1} ($t_{1/2}$ = 520 h) and 4.1×10^{-7} s^{-1} ($t_{1/2}$ = 470 h) in the absence of salt and 2.1×10^{-6} s^{-1} ($t_{1/2}$ = 92 h) and 2.2×10^{-6} s^{-1} ($t_{1/2}$ = 86 h) in 200 mM NaCl were observed for the full size and truncated complexes, respectively.

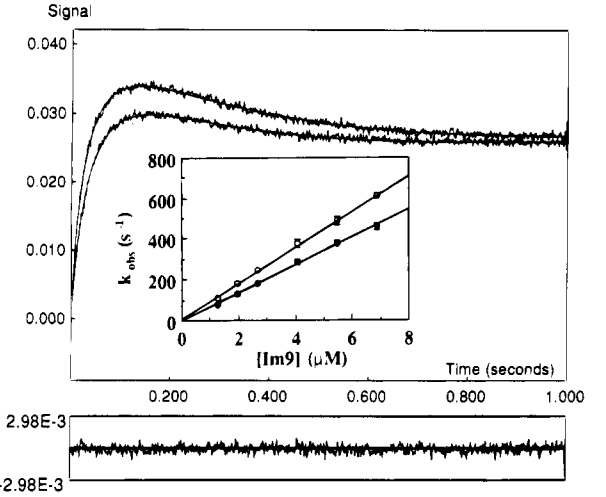

FIGURE 2: Association kinetics of the ColE9−Im9 complex (lower) and the E9 DNase−Im9 complex (upper trace) monitored by stopped flow fluorescence under second-order conditions. Equimolar concentrations of the two proteins (0.70 μM) in 50 mM MOPS buffer, pH 7.0, containing 200 mM NaCl were mixed at 25 °C, and the change in emission fluorescence (>320 nm) was monitored with time. The initial fluorescence enhancement and the fluorescence quench were fitted to second-order (eq 1 in Experimental Procedures) and first-order rate equations, respectively. The residuals for the DNase−Im9 fit are shown below the figure. (Inset) Complex formation monitored under pseudo-first-order reaction conditions using a minimum of a 4-fold excess of immunity protein over colicin E9 (●) or the E9 DNase domain (○; 0.35 μM). The association and dissociation rate constants k_1 and k_{-1} were determined from the gradient and intersect of lines of best fit, respectively. The duplicate data were fitted by linear regression.

Association Kinetics (k_{on}). We have reported previously that on complex formation between Im9 and either ColE9 or the E9 DNase domain, there is an enhancement (~15−20%) of the native tryptophan emission fluorescence (Wallis et al., 1992a, 1994). This property was used to follow the association of the proteins following rapid mixing by stopped-flow. For both complexes, association appeared to proceed by a two-step process (over the concentration ranges examined) in which a second-order fluorescence enhancement was followed by a first-order fluorescence quench (Figure 2). On the basis of these data, we propose a two-step binding profile in which colicin E9 or the E9 DNase domain (N) and the Im9 protein (I) associate to form an encounter complex (NI*) which then undergoes a conformational change to the final stable complex (NI) (eq 2). We

$$N + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NI^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} NI \qquad (2)$$

have interpreted all our data in terms of this model.

Under second-order reaction conditions, in which the concentrations of both proteins are equal (0.35 μM), the association rate constant k_1 was estimated by fitting the initial fluorescence enhancement to a second-order rate equation (eq 1 in Experimental Procedures) and the quench phase ($k_2 + k_{-2}$) to a first-order rate equation. In 50 mM Mops, pH 7.0, and at 25 °C values for k_1 of 4.0×10^9 M^{-1} s^{-1} and 5.7×10^9 M^{-1} s^{-1} were determined for the ColE9−Im9 and E9 DNase−Im9 complexes, respectively. Under these conditions the rate constants are close to the upper limit of

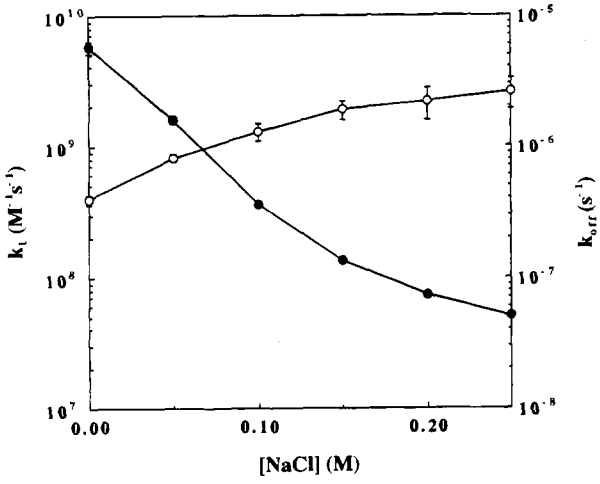

FIGURE 3: Dependence of salt concentration on the association and dissociation rates of the E9 DNase−Im9 complex. Values for k_{off} (○) and k_1 (●) were determined in 50 mM Mops, pH 7.0, at 25 °C as described in the legends to Figures 1 and 2, respectively.

detection for the stopped flow apparatus (dead time ~ 0.5ms), since the reaction time course is essentially complete within 10 ms. The quench phase was also very similar between the two complexes with rate constants of 6.8 s^{-1} and 4.4 s^{-1}, respectively.

Salt Dependence of the E9 DNase−Im9 Interaction. The values of k_1 for the two complexes are very close to the theoretical calculated diffusion limit for uncharged proteins of 7×10^9 M^{-1} s^{-1} at 25 °C (Fersht, 1985). Rate constants for most macromolecular associations are generally well below the encounter limit, one reason being that the upper limit is calculated assuming that the interacting molecules are uniformly reactive [reviewed in Pontius (1993)]. A comparison of protein complexes for which structural data are available, however, reveals that the binding sites generally occupy between 5 and 20% of the total surface area (Janin & Chothia, 1990). One important factor in complexes where association approaches the diffusion-controlled rate appears to be the contribution of electrostatic interactions to binding (Lee et al., 1989; Karshikov et al., 1992; Schreiber & Fersht, 1993; Pontius, 1993). Therefore, to assess the importance of charge interactions in the colicin system, the salt dependence of binding was determined for the E9 DNase−Im9 complex. Figure 1 illustrates the effect of 200 mM NaCl on the dissociation rate constant, whereas Figure 3 summarizes the effect of varying the concentration of NaCl on both the association and dissociation rate constants in the range 0−250 mM. The bimolecular association rate is particularly sensitive to the salt concentration with a decrease of over two orders of magnitude over this range, while the dissociation rate was found to increase by approximately 7-fold (Figure 3). In contrast, the second association step (the fluorescence quench) was found to be relatively insensitive to the salt concentration (data not shown). The diffusion-controlled association process together with the strong salt dependence of k_1 suggest that electrostatic interactions play an important role in Im9 binding to the E9 DNase domain.

In the absence of salt the association rate constant is too fast to follow under pseudo-first-order conditions. However, since k_1 is particularly sensitive to the salt concentration, this made it possible to investigate both the ColE9−Im9 and the E9 DNase−Im9 association under pseudo-first-order conditions (inset to Figure 2). This was done in the presence of 200 mM NaCl and using at least a 4-fold excess of immunity protein over nuclease (0.35 μM). Rate constants of 7.0×10^7 M^{-1} s^{-1} and 9.0×10^7 M^{-1} s^{-1} were determined for the ColE9−Im9 and E9 DNase−Im9 complexes, respectively, in 50 mM Mops, pH 7.0, containing 200 mM NaCl at 25 °C. These values were very similar to those determined under second-order conditions in the same buffer conditions (6.3×10^7 M^{-1} s^{-1} and 8.6×10^7 M^{-1} s^{-1}, respectively).

Estimation of k_{-1} by Sequential Stopped Flow and Calculation of Dissociation Constants. The equilibrium dissociation constant (K_d) for a two-step binding mechanism, such as the one shown in eq 2, is described by

$$K_d = (k_{-1}/k_1)/[1 + (k_2/k_{-2})] \quad (3)$$

In order to determine the K_d for such a scheme, it was first necessary to establish the rate of rearrangement of the encounter complex relative to its dissociation. This required that the rate constant k_{-1} in the proposed two-step binding model shown in eq 2 be analyzed further. This value is obtained from the intercept to the pseudo-first-order plots shown in the inset to Figure 2. In each case, the value for k_{-1} is so low (<10 s^{-1}) that reliable estimates for both the ColE9−Im9 and E9 DNase−Im9 complexes could not be obtained from these plots.

As an alternative strategy to estimate k_{-1}, a sequential stopped-flow experiment was designed that required 5-hydroxy-L-tryptophan (5OH-Trp) be incorporated into one of the proteins of the complex. 5OH-Trp has an excitation spectrum that is significantly red-shifted compared to that of L-tryptophan enabling selective excitation in a mixture of labeled and unlabeled proteins ($\lambda_{ex} \geq 310$ nm; Ross et al., 1992). Furthermore, we found that 5OH-Trp was a relatively noninvasive probe in this system since the dissociation rate constant of a complex between labeled E9 DNase and unlabeled Im9 was approximately only 2-fold greater than the value for native complex [9.0×10^{-7} s^{-1} ($t_{1/2}$ = 214 h) in 50 mM Mops buffer, pH 7.0].

Since it was unclear which of the interacting proteins was the most appropriate for labeling, the E9 DNase domain and Im9 were separately labeled with 5OH-Trp and used in pilot experiments. Each showed similar association kinetics with their respective unlabeled partner (as detected by fluorescence enhancement following excitation at 280 nm). However, using an excitation wavelength of 315 nm (selective for 5OH-Trp), a significant complexation-induced change in the fluorescence emission spectrum was observed only when the E9 DNase carried the label. Moreover, the profile of the fluorescence change monitored by stopped flow was very similar to that between the native proteins (Figure 4), suggesting that the fluorescence changes which accompany complex formation arise from one or both of the tryptophan residues in the E9 DNase domain.

In order to estimate k_{-1}, and to further test the proposed two-step binding model, 5OH-Trp labeled E9 DNase domain (11 μM final concentration) was mixed with unlabeled Im9 (5.5 μM) by stopped-flow (λ_{ex} = 315 nm). The protein concentrations were chosen such that the first binding step (k_1) was rapid (<50 ms). We then attempted to chase the labeled DNase from the encounter complex by sequential mixing using an equal volume of a 10-fold excess of unlabeled domain. If the dissociation rate $k_{-1} > k_2$, the

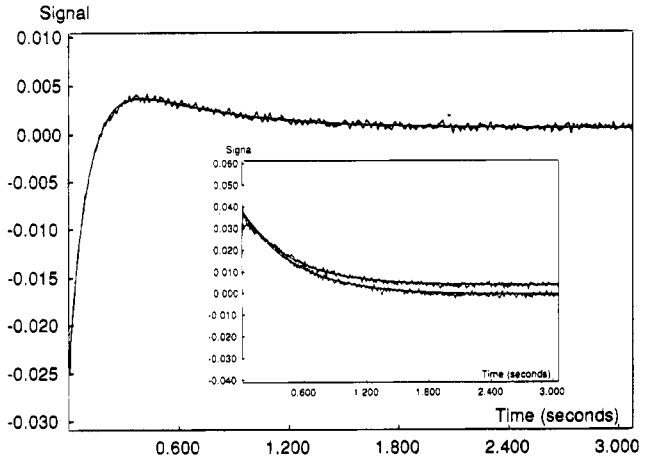

FIGURE 4: Estimation of the rate constant k_{-1} of the E9 DNase−Im9 complex by sequential stopped flow. Association between 5OH-Trp labeled E9 DNase domain (0.7 μM final concentration) and unlabeled Im9 (0.35 μM) in 50 mM Mops containing 50 mM NaCl. The excitation wavelength was 315 nm, and the fluorescence emission was monitored above 320 nm. (Inset) A 2-fold excess of 5OH-Trp labeled nuclease domain (11 μM final concentration) and unlabeled Im9 were mixed by stopped flow and chased after 250 ms with an equal volume of a 10-fold excess of unlabeled E9 DNase domain (lower trace) or buffer (50 mM Mops, pH 7.0; upper trace). The data were fitted in each case to a first-order rate equation.

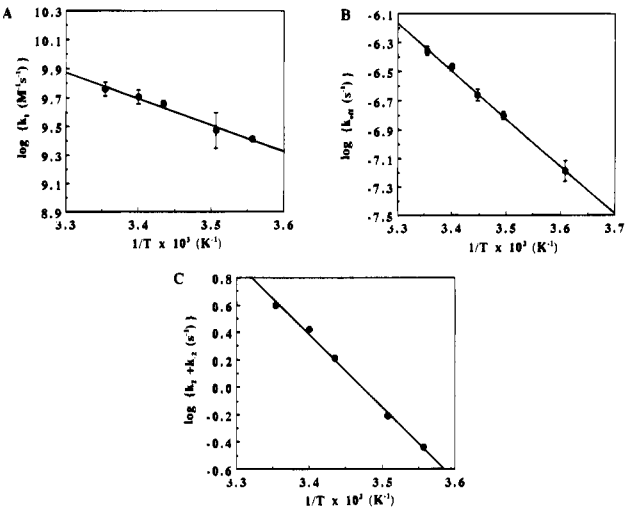

FIGURE 5: Temperature dependence of the association and dissociation rate constants of the E9 DNase−Im9 complex. Arrhenius plots for the bimolecular association rate constant k_1 (A), the dissociation rate constant k_{off} (B), and the fluorescence quench step, $k_2 + k_{-2}$ (C). The data were fitted by linear regression (the plots were linear over the temperature range tested) with R values of 0.979, 0.997, and 0.997, respectively.

fluorescence should decrease as the labeled DNase in the encounter complex is replaced by unlabeled DNase. Chasing with either buffer or unlabeled E9 DNase domain after time intervals between 50 and 250 ms revealed very little change in either the rate constant or the amplitude of the second binding step (the fluorescence quench) under all conditions tested (inset to Figure 4), indicating that k_{-1} is at least an order of magnitude less than k_2.

The absolute value of k_{-1} could not be obtained from the sequential stopped-flow experiments; nevertheless, the results show that the encounter complex rearranges faster than it dissociates. This, in turn, indicates that k_1 must be rate limiting for the forward direction. On the basis of this observation, two alternatives can be considered for the reaction scheme described by eq 3. Where $k_2 \gg k_{-2}$, the dissociation constant simplifies to $K_d = k_{-1}k_{-2}/k_1k_2$, where $k_{on} = k_1$ and k_{off}, measured by the exchange experiment, is $k_{-1}k_{-2}/k_2$, hence $K_d = k_{off}/k_1$. Alternatively, if $k_2 < k_{-2}$ the binding kinetics approach those for a one step reaction where again $K_d = k_{off}/k_1$, but now k_{off} approximates to k_{-1}. While the latter extreme case seems unlikely, since a two step association profile is observed for each complex, the relative values of k_{-1} and k_{-2} cannot be determined from these experiments.

The K_d value for the ColE9−Im9 complex was calculated from the ratio of k_{off}/k_1 to be 9.3×10^{-17} M in 50 mM Mops, pH 7.0, at 25 °C. The presence of 200 mM salt increased the K_d by three orders of magnitude to 3.0×10^{-14} M. These values are similar to those of other nuclease−inhibitor complexes such as angiogenin in complex with placental ribonuclease inhibitor ($K_d = 10^{-16}$ M; Lee et al., 1989) and barnase in complex with its inhibitor barstar ($K_d = 10^{-14}$ M; Schreiber & Fersht, 1993). Under the same conditions and in the absence of salt, the K_d of the E9 DNase−Im9 complex is 7.2×10^{-17} M, whereas in the presence of 200 mM salt this also increases by three orders of magnitude to 2.4×10^{-14} M. The calculated binding energy of the truncated E9 DNase complex (−22.0 kcal mol^{-1}, in the absence of salt) is very similar to that of the full size complex (−21.9 kcal mol^{-1}, in the absence of salt), indicating that it is the interaction of the DNase domain of the ColE9 molecule with the Im9 protein that determines the stability of the complex.

Table 1: Thermodynamic Parameters for the Interaction between the E9 DNase Domain and the Im9 Protein at 25 °C[a]

(A) Activation Parameters

activation parameters	k_1	k_{off}	$k_2 + k_{-2}$
$\Delta G^{\ddagger}$ (kcal mol^{-1})	4.1 (±0.1)	26.0 (±0.1)	16.6 (±0.1)
$\Delta H^{\ddagger}$ (kcal mol^{-1})	7.9 (±1.7)	14.3 (±2.2)	23.6 (±2.7)
$\Delta S^{\ddagger}$ (cal mol^{-1} K^{-1})	12.7 (±6.0)	−39.2 (±7.7)	23.5 (±9.4)

(B) Equilibrium Binding Parameters

ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
−22.0 (± 0.1)	−6.4 (± 3.8)	52.3 (±13.1)

[a] All experiments were conducted in 50mM Mops pH 7.0.

Temperature Dependence of the Interaction of the E9 DNase Domain with Im9. In order to further characterise the E9 DNase−Im9 complex, the temperature dependence of binding was determined in the range 4−25 °C. Figure 5 shows Arrhenius plots for k_1, k_{off} and the fluorescence quench step ($k_2 + k_{-2}$) and the values for the corresponding activation parameters, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$, are listed in Table 1. The data indicate that the activation energies of both the bimolecular association and the rearrangement steps are enthalpically determined with positive entropic terms in each case, whereas the activation energy of the dissociation process is both enthalpically and entropically determined with similar contributions from each term. The bimolecular rate constant k_1 shows a relatively small dependence on temperature over the range tested (Figure 5A). The calculated activation energy for this process is similar to that for other diffusion limited reactions (Gutfreund, 1972) and supports our contention that association of the colicin complex is diffusion controlled.

On the basis of the temperature dependence of k_1 and k_{off}, the effect of temperature on the dissociation constant ($K_d = k_{off}/k_1$) could be determined and the thermodynamic param-

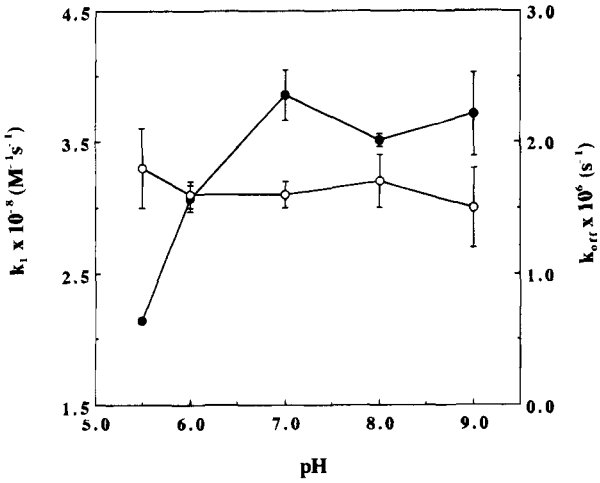

FIGURE 6: pH dependence of the association (●) and dissociation (○) rate constants of the E9 DNase−Im9 complex. Rate constants were determined in 50 mM buffer (Mes, pH 5.5−6; Mops, pH 7; and Tris, pH 8−9) containing 100 mM NaCl at 25 °C.

eters ΔH and ΔS were estimated using the van't Hoff equation (Table 1). The data indicate that both the entropic and enthalpic terms favor complex formation for the E9 DNase−Im9 complex.

Effect of pH on the E9 DNase−Im9 Complex. The ionization of amino acid residues in interacting proteins can have dramatic effects on the stability of a protein complex. For example, the dissociation constant of the barnase−barstar complex decreases by approximately two orders of magnitude over the pH range 5−9, largely as a result of deprotonation of His102 in barnase (Schreiber & Fersht, 1993). Figure 6 shows the pH dependence of the association and dissociation rates of the E9 DNase−Im9 complex in the pH range 5.5−9.0 containing 100 mM NaCl. Salt was included in the reaction buffer to minimize ionic strength effects. Measurements could not be made below pH 5.5 due to precipitation of Im9. The association and dissociation rates were found to vary only slightly over the entire pH range tested. The fluorescence quench step was also found to be relatively insensitive to changes in pH between 5.5 and 9 (data not shown).

DISCUSSION

Protein inhibitors of enzymes have been a major source of information concerning protein−protein interactions. Proteinase inhibitors (Vincent & Lazdunski, 1972; Empie & Laskowski; 1982; Longstaff et al., 1990) and, more recently, RNase inhibitors (Lee et al., 1989; Schreiber & Fersht, 1993; Hartley, 1993; Guillet et al., 1993; Buckle et al., 1994) have provided a number of important insights into how proteins interact with other proteins.

The only reported inhibition of a DNase enzyme where both thermodynamic and structural data are available is that of DNase I by actin. The dissociation constant for the inhibition of DNase I by actin is $\sim 10^{-9}$ M (Mannherz et al., 1980), and inhibition of enzymic activity occurs by steric hindrance of important active site groups; hydrogen bonds, electrostatic and hydrophobic interactions are all involved in the interaction (Kabsch et al., 1990). The mechanism by which Im9 inhibits its target endonuclease is unknown since there are as yet no structures for the E9 DNase−Im9 complex, although the secondary structure for Im9 has been determined by NMR (Osborne et al., 1994). While there may well be similarities between the two systems, a major difference between them is the strength of the protein−protein interaction since the ColE9−Im9 complex has a dissociation constant of 9.3×10^{-17} M at 25 °C, which is around eight orders of magnitude tighter than the DNase−actin complex. This difference in stability is partly explained by a slower association rate constant for actin with DNase I (10^6 M^{-1} s^{-1}) and partly by a faster dissociation rate constant, 10^{-3} s^{-1} (Mannherz et al., 1980) compared to 10^{-7} s^{-1} for the ColE9−Im9 complex.

Electrostatic Steering and Colicin−Immunity Association. The majority of kinetic studies on enzyme−inhibitor complexes have been with proteinase−inhibitor complexes, and these indicate that bimolecular association rate constants typically fall within the range 10^5−10^7 M^{-1} s^{-1} [reviewed in Janin and Chothia (1990)]. In contrast, k_1 for the interaction of colicin E9 with Im9 is 4×10^9 M^{-1} s^{-1} in buffer of low ionic strength, which is close to the diffusion limit. Furthermore, the low activation energy for this process (4 kcal mol^{-1}, Table 1) is consistent with that of a diffusion-controlled reaction (Gutfreund, 1972; Geeves & Gutfreund, 1982).

There are a number of examples of macromolecular reactions, including enzyme-substrate, protein−protein, and protein−DNA interactions, that display association rate constants that approach or in some cases appear to excede the calculated diffusion limit [for reviews, see Berg and von Hippel (1985) von Hipple and Berg, (1989) and Honig and Nicholls (1995)]. In these systems, the interacting components are charged, and it is proposed that electrostatic fields set up around the molecules orient the binding surfaces prior to collision. On binding, reorientation is thought to occur during a series of minicollisions resulting in the final docking of the two proteins (Berg & von Hippel, 1985; von Hipple & Berg, 1989). The properties of the ColE9−Im9 interaction are compatible with such association schemes. The association rate constant k_1 is diffusion controlled and shows a strong dependence on salt concentration, consistent with the charge states of the two proteins; Im9 is highly acidic (pI 4.5), whereas the E9 DNase domain to which it binds is highly basic (pI > 10.5). It is likely therefore that the two proteins are electrostatically steered so that they are appropriately oriented to form the encounter complex, NI*.

Multistep Binding Mechanisms for Protein Complexes. The fluorescence changes that we observe on complex formation between Im9 and either colicin E9 or the E9 DNase domain suggest that association occurs in at least two steps. Two-step reaction schemes have been proposed for a number of protein complexes, where the protein components form an encounter complex which then undergoes a conformational change to the final stable complex (Luthy et al., 1973; Lee et al., 1989; Schreiber & Fersht, 1993; Pontius, 1993). However, it is unusual to actually observe the second step spectroscopically, as in the present case. Instead, rearrangement of encounter complexes are often inferred, as in the case of placental ribonuclease inhibitor binding to angiogenin where the presence of an encounter complex is concluded from the nonlinearity of pseudo-first-order plots of the association rate constant, k_{obs} (Lee et al., 1989). While it is unclear what the rearrangement step represents, we can say that the spectral changes emanate from the E9 DNase since 5OH-Trp labeled Im9 does not show any fluorescence

enhancement on complex formation (when excited at 315 nm) in contrast to the DNase domain.

k_1 in eq 2 is rate determining for association, but which step is rate determining for dissociation? At present, we cannot pinpoint which of the two off-rates, k_{-1} or k_{-2}, is the slower step since the exchange experiment (Figure 1) measures the overall off-rate. An upper limit can, however, be put on k_{-1} from the sequential stopped flow experiment as $\leq 0.5\ s^{-1}$ (an order of magnitude less than the fluorescence quench step, $k_2 + k_{-2}$).

The biphasic association profiles we observe for colicin E9 and Im9 are certainly in keeping with other protein associations; however, the data could also be explained by alternative reaction schemes, such as isomerization of the nuclease as opposed to the protein complex (Bagshaw et al., 1974). Eq 4 could be a plausable mechanism, in which N,

$$N \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} N^* + I \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} N^*I \qquad (4)$$

the nuclease, is in equilibrium with N* a conformationally different species. In this scheme, the slow fluorescence quench phase would represent the isomerization of the remaining, unbound nuclease domain. Reaction schemes of this type have been proposed for many enzyme reactions, where the substrate or enzyme exist in one or more different forms in equilibrium with each other and only one isomer is reactive (Gutfreund, 1972; Halford, 1971). In this case, the rate constants of the rapid fluorescence enhancement would correpond to k'_2, the fluorescence quench to k'_1 and the dissociation rate constant measured by the exchange experiment to k'_{-2}. Although the kinetic data do not discount it as a possibility, this model seems less likely for protein−protein interactions which invariably involve much larger surface areas (typically 5−20%) of both proteins becoming buried in the complex (Janin & Chothia, 1990). In addition, we are unaware of any interprotein complexes that follow such a binding scheme.

In a second alternative reaction scheme, the second phase of the association process (the fluorescence quench) could be due to self-association of either the protein components or the complex. Previous studies have, however, indicated that both colicin E9 and the nuclease domain form a 1:1 complex with Im9, with no evidence of dimerization of the isolated species or the complex (Wallis et al., 1992a, 1994).

Eq 5 represents a third alternative reaction mechanism.

$$\begin{array}{ccc} N + I & \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} & NI \\ {\scriptstyle k'_1}\downarrow\uparrow{\scriptstyle k'_{-1}} & & \\ NI^* & & \end{array} \qquad (5)$$

In this scheme, the initial phase leads to the partitioning of products between a stable complex NI and an intermediate complex form NI*, off the reaction pathway, by a diffusion-controlled process. The slow second phase represents transformation of NI* to NI in a flux through the free components, N and I. In this case, the dissociation rate constant measured by the subunit exchange experiment would correspond to k''_{-2} and the rate constant of the slow fluorescence quench step to $k''_{-1}k''_2/(k''_2 + k''_1)$. Again, while this scheme cannot be discounted from the kinetic data, the presence of two (or more) distinct binding modes for the E9 DNase and Im9 seems less likely than the mechanism depicted in eq 2, especially in view of the currently understood mechanisms by which diffusion-controlled, electrostatically steered associations can occur (Berg & von Hippel, 1985; von Hippel & Berg, 1989).

Since the colicin E9−Im9 interaction has not been studied before, we investigated the temperature dependence of all the measured kinetic constants (Table 1) to gain some insight into the driving forces for complex formation. The thermodynamic parameters obtained from the temperature dependence of K_d indicate that both entropic and enthalpic terms favor binding. Since protein association will result in a decrease in the translational and rotational entropy, a positive entropic term would be consistent with the exclusion of water molecules from the protein surfaces on complex formation; either ordered water molecules surrounding nonpolar groups (the hydrophobic effect) or associated with charged groups.

In conclusion, the Im9 protein forms an extremely tight complex with its cognate toxin colicin E9, which neutralizes the nuclease activity of the toxin, and the stability of this complex is due solely to the interaction with the E9 DNase domain. In order that the toxin can exhibit its nuclease activity, the immunity protein must, at some point, dissociate from the toxin. Data for the RNase toxin cloacin DF13 (specific for *Enterobacter cloacae* and *E. coli* cells) suggest that its immunity protein is left behind in the medium as the toxin binds to the extracellular receptor and begins its passage through the outer cell membrane (Krone et al., 1986). This poses an intriguing question for the nuclease class of toxins; with a dissociation constant of 10^{-17} M, how does the toxin free itself of the inhibitor so that it can enter a bacterial cell? One way this might occur is through the unfolding of the toxin as it passes through the outer membrane. Although no evidence for this exists for the nuclease class of colicins, this seems to be the mechanism of import for pore-forming bacteriocins such as colicin A (Benedetti et al., 1992).

ACKNOWLEDGMENT

We gratefully acknowledge Alan Dawson and Andrew Leech (Norwich), Clive Bagshaw (Leicester), and Alan Cooper (Glasgow) for their help and advice with the kinetic and thermodynamic analysis and Ann Reilly for expert technical assistance. In addition, we would like to thank all those in C.K.'s, R.J.'s and G.R.M.'s labs for helpful discussions during the course of the work.

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BI9511200