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New Protein Cross-Linking Reagents That Are Cleaved by Mild Acid

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ABSTRACT: New homo- and heterobifunctional cross-linking reagents have been synthesized. These reagents are based on ortho ester, acetal, and ketal functionalities that undergo acid-catalyzed dissociation but are base stable. The protein-reactive group in all the homobifunctional reagents is a maleimide group; the heterobifunctional acetal cross-linker has a maleimide group at one end and an *N*-hydroxysuccinimide ester at the other. These reagents have been used to cross-link diphtheria toxin (DT) to itself to give covalently cross-linked DT dimer or to conjugate DT monomer to the anti-CD5 antibody, T101. The hydrolysis of these cross-linked proteins was studied as a function of pH. Cleavage rates vary from minutes to hours at the pH of acidified cellular vesicles (~pH 5.4), ortho esters being the fastest, acetals the slowest, and ketals intermediate, but the cross-linked products are approximately 100 times more stable at the vascular pH of 7.4 and 1000 times more stable at a storage pH of 8.4 in all cases. The utility of these reagents in the reversible blockade of a toxic protein functional domain was demonstrated by using cross-linked DT dimer where the blocking and unblocking of toxin binding sites correlates with cellular toxicity. Of the different cross-linkers described, the acetone ketal, bis(maleimidoethoxy)propane (BMEP), appears to be the most promising in the construction of highly efficacious immunotoxins.

Bifunctional cross-linking reagents have found widespread application in protein chemistry in recent years. Many aspects of protein structure and function and relationship to surrounding proteins have been investigated by using these reagents (Davies & Stark, 1970; Hucho et al., 1975; Hadju et al., 1976; Peters & Richards, 1977). A variety of cross-linkers are commercially available—homobifunctional, heterobifunctional, noncleavable, and cleavable. Most commonly used are heterobifunctional reagents that have maleimide and *N*-hydroxysuccinimidyl ester functionalites separated by a spacer group (Kitagawa & Aikawa, 1976; Kitagawa et al., 1981; Yoshitake et al., 1982; Gitman et al., 1985). Specific

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coupling of two proteins via a noncleavable thioether bond is achieved by introducing a sulfhydryl group into one of the proteins (Traut et al., 1973; Jue et al., 1978). Much of the earlier work on linking toxins to immunoglobulins relied extensively on these reagents.

More recently, it has been recognized that cleavable cross-linkers have some advantages over their noncleavable counterparts in certain applications. One specific example is in the construction of immunotoxins by coupling the A chain of a toxin to an antibody. The first A chain immunotoxins were constructed with disulfide (-s-s-) cross-links because they were meant to be analogues of the parent toxin A-s-s-B, and for DT[1] it was known that A and B must dissociate to expose ADP-ribosyltransferase activity (Chang & Neville, 1977). These conjugates are usually prepared by using the heterobifunctional reagent SPDP (Carlsson et al., 1978) and are apparently more efficacious than those having a noncleavable thioether linkage (Jansen et al., 1982; Masuho et al., 1982). Most of the currently available reagents with cleavable linkages have drawbacks. The aforementioned disulfide linkage introduced with SPDP has to be employed under strickly nonreducing conditions in the absence of free sulfhydryl groups in order to avoid disulfide exchange. Such an environment is incompatible with many enzymes. Other cleavable cross-linkers contain a *vic*-glycol unit as the labile bond (Coggins et al., 1976); these, however, are cleaved under rather harsh conditions (periodate oxidation), which excludes their use in cross-linking easily oxidizable proteins like glycoproteins. Yet another commercially available cleavable cross-linker is EGS (Abdella et al., 1979; Baskin & Yang, 1980) where an ester linkage serves as the labile moiety. Here again, the conditions for cleavage (hydroxylamine) are incompatible with proteins having an Asn-Gly residue, which is known to be attacked by hydroxylamine (Bornstein & Balian, 1977).

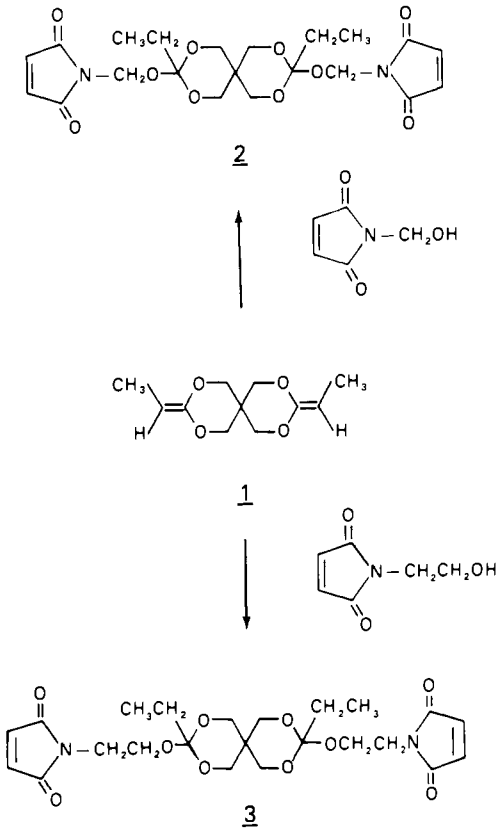
The need for a reagent that permits cleavage of the cross-linked product under mild conditions has been largely unmet. A notable exception is the heterobifunctional reagent reported by Blättler et al. (1985) which forms an acid-labile link. Although this reagent possesses some of the properties desirable in a cleavable cross-linker (e.g., release of one of the two cross-linked proteins in an unmodified form under mildly acidic conditions), it also suffers from significant drawbacks. Some of these deficiencies are the following: the relationship between hydrolytic rate and H^+ ion concentration is nonlinear (30-fold change in hydrolysis for a 600-fold change in H^+); the rate of hydrolysis of the cross-linked product is very slow at the pH of intracellular vesicles (<25% hydrolysis at pH 5.5 in 10 h); finally, the reagent is available only through a fairly lengthy sequence of reactions.

Our interest in developing new cleavable cross-linking reagents stemmed mainly from our research in the area of immunotoxins where a major objective was to develop highly efficacious toxin–antibody conjugates with potential for in vivo therapy. Immunotoxins constructed by conjugation of the native toxins, containing both A and B chains, to antibodies are more toxic than those having only an A chain attached to an antibody (Youle & Neville, 1982; Neville, 1986). The B chain of a toxin is apparently involved in more than mere binding to cell surface receptors. There is good evidence that in the case of ricin a functional galactose binding site on the B chain is required inside the cell for maximum efficacy (Youle et al., 1981; Leonard et al., 1985; Youle & Colombatti, 1987). Although external addition of B chains to A chain immunotoxins enhances their toxicity somewhat, these conjugates are still far less toxic than the parent toxins (Esworthy & Neville, 1984). For diphtheria toxin based immunotoxins inclusion of increasing portions of the B chain is associated with increased efficacy (Colombatti et al., 1986; Greenfield et al., 1987). However, nontarget cell toxicity is concomittantly increased (Pappenheimer et al., 1982; Greenfield et al., 1987). The obvious conclusion is that, for maximum efficacy, immunotoxins should incorporate substantial portions of the B chain or intact toxins. Even an intact toxin coupled irreversibly to a monoclonal antibody, however, has diminished toxin potency toward toxin-sensitive cells and presumably also toward cells targeted by the antibody (Youle & Neville, 1980). In view of this, we deemed the design and application of suitable cleavable cross-linking agents to conjugate toxins, mutant toxins, or large toxin fragments to antibodies a fruitful area of research to explore. It is generally accepted that antibodies bound to cell surface antigens enter the cell by receptor-mediated endocytosis and are internalized into acidified vesicles known as endosomes (de Duve, 1983). The pH drop experienced by the conjugate in passing from the cell surface to these acidified vesicles is approximately 2 pH units (from pH 7.4 to pH 5.4; Heiple & Taylor, 1982; Mellman et al., 1986; Tycko & Maxfield, 1982). Consequently, immunotoxins constructed with an acid-labile link between the toxin and antibody could be expected to cleave upon passage through these endosomes and release toxin free from the steric constraints of the antibody. Such a scheme should provide highly potent conjugates since it is known that free toxins translocate across membranes and cause cell death by inhibition of protein synthesis extremely efficiently (Yamaizumi et al., 1978; Hudson & Neville, 1985). Successful implementation of this strategy, however, places some severe constraints on the nature of the cross-linking reagent. It has to be cleaved fairly rapidly under very mildly acidic conditions (pH 5.5) yet be quite stable at the intravascular pH of 7.4; it should be easy to synthesize and stable toward long-term strorage; it should release the monomeric proteins either in native form or modified in a manner that does not significantly affect their activity.

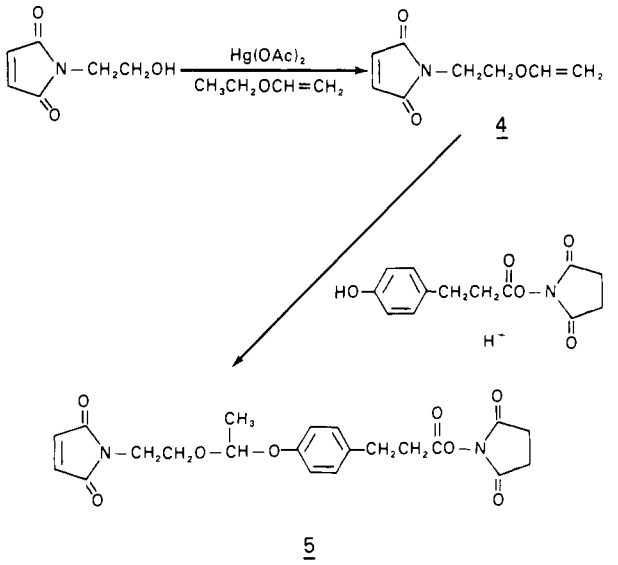
We have developed a number of cross-linking reagents that span a wide range of acid-catalyzed hydrolytic rates. These are based on acetals, ketals, and ortho esters—functionalities well established to be hydrolytically acid labile and base stable (Cordes, 1967; Cordes & Bull, 1974). We deliberately chose to synthesize a variety of cross-linking reagents with diverse hydrolytic rates because one of the major unknowns in the mechanism of action of immunotoxins is the length of time a conjugate spends in an acidified intracellular compartment. Our initial synthetic efforts have been directed toward homobifunctional reagents since these are easier to prepare. We have subsequently made progress in extending our methodology to heterobifunctional cross-linkers. Most of the cross-linkers described in this report are maleimide derivatives of ketals, acetals, and ortho esters in which the maleimide group functions as a highly efficient Michael acceptor toward sulfhydryl groups which can be selectively generated on proteins. Conjugates of DT with the anti-CD5 antibody T101 have been prepared by using these cross-linkers and shown to

[1] Abbreviations: DT, diphtheria toxin; SPDP, *N*-succimindyl 3-(2-pyridyldithio)propionate; EGS, ethylene glycol bis(succinimidyl succinate); MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; CD5, human T-cell surface membrane epitope characteristic of pan T cells; DMF, *N,N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin of the γ subclass; NaP_i, a mixture of mono- and dibasic sodium phosphate; DMSO, dimethyl sulfoxide; 90/10/1 buffer, 90 mM Na_2SO_4, 10 mM sodium phosphate, pH 8.5, and 1 mM EDTA; BMEP, bis(maleimidoethoxy)propane; SDS, sodium dodecyl sulfate; RT, room temperature.

Scheme I: Synthetic Route for Ortho Ester Cross-Linkers **2** and **3**



Scheme II: Synthetic Route for Heterobifunctional Acetal **5**



cleave upon acidification, releasing monomeric DT.

We have also covalently cross-linked DT dimer with these reagents and measured hydrolytic rates as a function of pH. This is a convenient system to monitor hydrolysis especially with homobifunctional cross-linkers. We have also used DT dimer cross-linked with our reagents as a model to demonstrate the reversible blockade of a toxin functional domain. This latter concept is of great significance for the in vivo application of immunotoxins because an undesirable property of a toxin (e.g., its binding to nontarget cells) can be blocked outside the cell but unraveled intracellularly to allow the toxin to exhibit its full potency.

Experimental Procedures

Materials, General Methods, and Syntheses

Melting points were measured on a Thomas Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian XL 200 spectrometer using tetramethylsilane as an internal standard. Mass spectra were recorded on an Extrel-Finnigan 1015 instrument. Reverse-phase HPLC was carried out on a Hewlett-Packard 1090 instrument with a diode array detector. Diketene acetal **1** was provided by SRI International, Menlo Park, CA 94025. Other materials like toxins, cells, etc. were purchased and handled as previously described (Hudson & Neville, 1987). Assays for the inhibition of protein synthesis were carried out according to the protocol outlined previously (Hudson & Neville, 1985).

Synthesis of New Reagents. The synthetic route for the ortho ester cross-linkers **2** and **3** is shown in Scheme I.

(*a*) *Ortho Ester Cross-Linker* **2**. *N*-(Hydroxymethyl)-maleimide (250 mg, 1.97 mmol), prepared from maleimide according to the procedure of Tawney et al. (1961), was dissolved in anhydrous ether (50 mL) and the solution treated with the spiro diketene acetal **1** (220 mg, 1.04 mmol; Ng et al., 1986). The homogeneous reaction mixture was stirred under an argon atmosphere at RT for 1–2 h. At the end of this time a slight turbidity was observed. The reaction mixture was filtered and concentrated to remove most but not all of the solvent. Hexane was added dropwise to initiate crystallization. The mixture was cooled in a refrigerator to complete crystallization. Filtration gave white crystals of **2** in 75% (350 mg) yield, mp 143–146 °C dec. ^{1}H NMR (CD_3CN) δ 0.86 (t, J = 7 Hz, 6 H), 1.78 (q, J = 7 Hz, 4 H), 3.27–4.12 (m, 8 H), 4.94 (s, 4 H), and 6.90 (s, 4 H) ppm.

(*b*) *Ortho Ester Cross-Linker* **3**. **3** was prepared in a similar manner to **2** starting with *N*-(hydroxyethyl)maleimide (Miyadera et al., 1971) and the ketene acetal **1**. The final product in this case was a colorless oil. ^{1}H NMR (CD_3CN) δ 0.81 (t, J = 7 Hz, 6 H), 1.63 (q, J = 7 Hz, 4 H), 3.10–4.00 (m, 16 H), and 6.80 (s, 4 H) ppm.

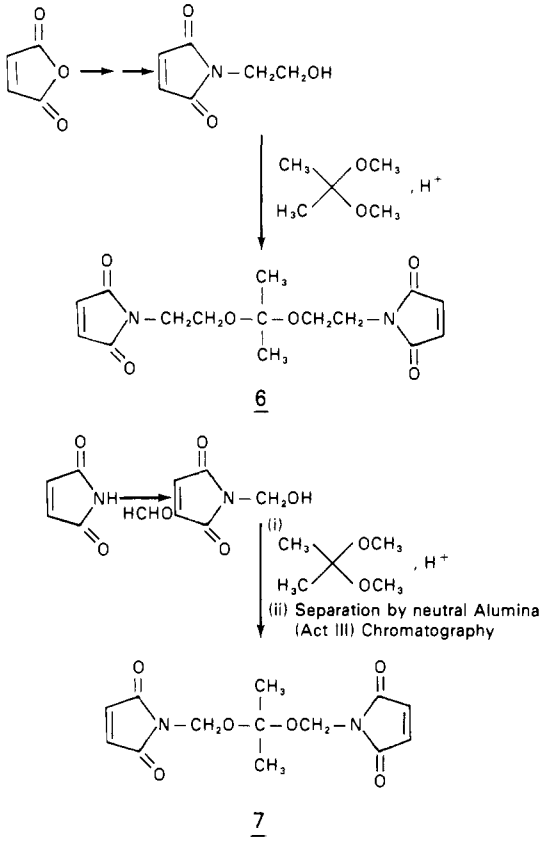
Synthesis of Acetal and Ketal Cross-Linkers. The synthetic route for the heterobifunctional acetal cross-linker **5** is shown in Scheme II.

(*a*) *Maleimido Vinyl Ether* **4**. *N*-(2-Hydroxyethyl)maleimide (1.18 g, 8.4 mmol), prepared by the method of Miyadera et al. (1971), was suspended in ethyl vinyl ether (25 mL) and mercuric acetate (70 mg) from a freshly opened bottle added. The mixture was heated at reflux with stirring under an argon atmosphere for 24 h. After the reaction mixture was allowed to cool to RT, anhydrous K_2CO_3 (1.5 g) was added and stirring continued of a few more minutes. The mixture was filtered and the precipitate washed thoroughly with ethyl acetate. The washings were combined with the filtrate and evaporated to dryness in vacuo to yield an oily residue. This oily residue was chromatographed over a neutral alumina (50 g, Merck, activity 3) column using CH_2Cl_2–hexane (1:1) for elution. The initial fractions were collected and combined to yield 2-maleimidoethyl vinyl ether as a colorless liquid in 14% yield (200 mg).[2] ^{1}H NMR (CD_3CN) δ 3.72 (t, J = 5 Hz, 2 H), 3.84 (t, J = 5 Hz, 2 H), 4.00 (d of d, J = 7 and 2 Hz, 1 H), 4.20 (d of d, J = 14 and 2 Hz, 1 H), 6.45 (m, 1 H), and 6.80 (s, 2 H) ppm.

(*b*) *Cross-Linking Reagent* **5**. 3-(4-Hydroxyphenyl)-propionic acid *N*-hydroxysuccinimide ester (120 mg, 0.46

[2] The relatively low yield is based on the total amount of *N*-(2-hydroxyethyl)maleimide used and does not take into account the significant amount of this starting material that is left unconsumed at the end of the reaction.

Scheme III: Synthetic Route for Ketal Cross-Linking Reagents **6** (BMEP) and **7**



mmol) was dissolved in anhydrous ethyl acetate (10 mL) and **4** (200 gm, 1.2 mmol) added. The reaction mixture was stirred at RT under argon and a solution of *p*-toluenesulfonic acid (0.5 mg) in anhydrous ethyl acetate (u mL) added as catalyst. After a reaction time of 6 h, a few drops of pyridine were introduced to neutralize the acid and the reaction mixture was evaporated to dryness to yield a pale yellow viscous oil. Hexane was added to this residue, and the mixture was refrigerated for several hours. The white solid that precipitated was collected, washed with hexane, and dried to give a quantitative yield of crude **5**. Crystallization from CH_2Cl_2–hexane yielded white crystals, mp 134–137 °C. ^1H NMR (CD_3CN) δ 1.40 (d, J = 6 Hz, 3 H), 2.78 (s, 4 H), 2.95 (br s, 4 H), 3.54–3.80 (m, 4 H), 5.45 (q, J = 6 Hz, 1 H), 6.60 (s, 2 H), 6.86 (d, J = 9 Hz, 2 H), and 7.19 (d, J = 9 Hz, 2 H) ppm. MS (CI, NH_3) m/z 448 $(M + NH_4)^+$.

Synthesis of Homobifunctional Ketal Cross-Linking Reagents **6** *and* **7**. The synthetic route for ketals **6** and **7** is illustrated in Scheme III.

(*a*) *Maleimido Ketal* **6**. *N*-(2-Hydroxyethyl)maleimide (141 mg, 1 mmol) was suspended in dry benzene (10 mL) and 2,2-dimethoxypropane (75 mg, 0.72 mmol) added. The mixture was stirred at RT under argon and *p*-toluenesulfonic acid (0.25–0.5 mg) added as catalyst. After the reaction mixture became homogeneous, the solvent was evaporated off under vacuum and another portion of 2,2-dimethoxypropane (50 mg, 0.48 mmol) in dry benzene (10 mL) added. Stirring was continued for a further 2 h at RT. A few drops of pyridine were added to neutralize the acid. The solvents were removed by evaporation under vacuum, and benzene (10 mL) was added to the residue. After stirring thoroughly, the mixture was filtered and the benzene-soluble fraction evaporated to dryness. The residue was crystallized from acetone–hexane to yield 80 mg (50%)[3] of white fluffy crystals, mp 124–126 °C. ^1H NMR (CD_3CN) δ 1.20 (s, 6 H), 3.44 (t, J = 6 Hz, 4 H), 3.55 (t, J = 6 Hz, 4 H), and 6.75 (s, 4 H) ppm. MS (CI, NH_3) m/z 340 $(M + NH_4)^+$; (EI) m/z 307 $(M - CH_3)^+$.

(*b*) *Maleimido Ketal* **7**. **7** was prepared in a similar manner to **6** with toluene as solvent. The reaction product in this case was contaminated with *N*-(hydroxymethyl)maleimide and the mixed ketal. Separation could be effected on a short column of neutral alumina, activity 3, using CH_2Cl_2 for elution. The initial fractions that eluted were collected, combined, and evaporated to yield a colorless oil that crystallized on standing. This was further purified by recrystallization from CH_2Cl_2–hexane to give white crystals of **7**, mp 106–107 °C. ^1H NMR (CD_3CN) δ 1.39 (s, 6 H), 4.95 (s, 4 H), and 6.83 (s, 4 H) ppm.

Methods

Hydrolysis of Cross-Linker **2**. The hydrolysis of cross-linker **2** was followed by isocratic reverse-phase HPLC analysis on a 2-cm, 5-μm, Supelcosil LC 18 column using 40:60 CH_3CN/10 mM potassium phosphate buffer, pH 7.2, for elution. The cross-linker eluted with a retention time of ~7.5 min at a flow rate of 0.2 mL/min and did not suffer significant hydrolysis within this time period. A stock solution of **2** in CH_3CN (5 mM) was prepared as well as 10 mM potassium phosphate buffer solutions adjusted to the pH values 5.24, 6.10, and 7.15. Hydrolysis was initiated by diluting 1 mL of the stock solution of **2** in CH_3CN 10-fold with the buffer solution at the appropriate pH. Samples were withdrawn at periodic intervals and analyzed for the disappearance of **2**.

Cross-Linking of Proteins with Acid-Cleavable Reagents. (*a*) *Cross-Linking with Cleavable Heterobifunctional Reagents*. In general, reaction conditions are similar to those for the noncleavable linker MBS with the exception that the coupling appears more efficient with **5**. For the synthesis of anti-CD5–DT and anti-CD5–ricin, the toxins, between 1 and 5 mg/mL, were derivatized with an equimolar amount of **5** dissolved in DMF and added at pH 8–8.5. Unreacted reagent was removed by passage over a small G25-F (Pharmacia) column. The maleimide-derivatized toxin was reacted with thiolated antibody, 2 mol of SH groups/mol (2:1 toxin:antibody), achieved by treatment of 15 mg/mL T101 (gift from Hybritech Corp., San Diego, CA) with 0.6 mM iminothiolane, pH 8.0, for 60 min and subsequent passage over G25-F. Both proteins were allowed to react for 20 min. Reactants and products were separated by HPLC over Zorbax GF-250 column (Du Pont) run in 90 mM Na_2SO_4, 10 mm NaP_i, pH 8.5, and 1 mM EDTA (90/10/1 buffer). Yields varied between 20 and 50% of input antibody. All thiolations were performed under argon in the presence of 1 mM EDTA to inhibit disulfide bond formation. Any IgG monoclonal can be conjugated by this procedure.

(*b*) *Cross-Linking with Acid-Cleavable Homobifunctional Reagents*. (*1*) *Proteins That Form Natural Aggregates*. For example unnicked diphtheria toxin (DT) dimer was isolated by fractionation of previously frozen DT in 10 mM NaP_i, pH 7.1, using a 60 × 2.5 cm TSK column (LKB). Dimer was thiolated by using 1 mM iminothiolane (IT) (Pierce) at pH 8.0 in 0.4 M sodium borate for 20 min. Dimer was freed from excess IT and any monomer by GF-250 chromatography and reacted with a 7-fold molar excess of **2** or **3** over the free SH concentration (~1.5 mol of SH/mol of DT monomer) for 5 min at pH 8.5. The solution was diluted 10 times with H_2O and made 40% in DMSO to dissociate un-cross-linked species,

[3] Not optimized.

and the cross-linked dimer was isolated by chromatography on a GF-250 column.

(2) *Proteins That Do Not Form Noncovalent Aggregates.* For example, for isolation of anti-CD5–DT conjugate cross-linked with **6**, nicked DT monomer, 10–15 mg/mL in 0.4 M sodium borate, pH 8.0, was derivatized with 1 mM iminothiolane for 60 min and freed of residual IT by G25-F chromatography in pH 8.5 buffer. The derivatized DT containing 1 mol of SH/mol was reacted with a 15-fold mol/mol excess of **6** dissolved in dry DMF and after 5 min rechromatographed on G25-F. The derivatized DT was now a mixture of internally cross-linked DT and DT containing 0.7 free maleimide group/mol. A 20-fold excess of this DT mixture was reacted with thiolated T101 as described above at pH 8.5 for 30 min. Reactants and products were separated on GF-250 chromatography. Yield of cross-linked conjugate was 70–90% based on input antibody. Previous work has shown that antibody toxin conjugates synthesized as described with a 10-fold or greater excess of toxin over antibody are a mixture of mono- and biotoxin conjugates (Marsh & Neville, 1986). In order to maximize the bitoxin component for hydrolytic studies, we isolated the leading edge of the conjugate peak which preceded the antibody peak by 0.82 min (GF-250, 15 × 0.7 cm column run at mL/min). A more detailed characterization of these conjugates necessitated the use of an irreversible cross-linker, 1,6-dimaleimidohexane. This homobifunctional reagent, which has approximately the same degree of flexibility as the acid cleavable **6**, yields toxin antibody conjugates with identical retention profiles to those formed with **6** (GF-250 chromatography) but greater stability. To explore the range of conditions required to achieve more than one cross-linked antibody to a single toxin, we increased the thiolation of toxin to 4 mol of SH groups/mol and reduced the molar ratio of toxin to antibody to 1:1. Fractionation was performed as before, and fractions with elution times preceding antibody by 1.0, 1.2, and 1.35 min were subjected to SDS gel electrophoresis using miniature (Phast System, Pharmacia) 7.5% polyacrylamide gels. The fraction with the longest retention time consisted primarily of material corresponding to 200 000–210 000 daltons, consistent with a monotoxin conjugate, and some material at 320 000 daltons consistent with antibody dimer. The two faster eluting fractions contained higher molecular weight species that remained trapped in the upper gel.

Hydrolysis of Acid-Cleavable Conjugates Resulting in the Release of the Individual Proteins. Cross-linked proteins were stored at pH 8.5 in 90/10/1 buffer at 4 °C. Acid pulsing was achieved by adding an amount of buffer of higher buffering capacity to attain the desired lower pH at 25 °C. The reaction was stopped by injection into a Zorbax GF-450 size exclusion HPLC column operated at pH 8.5 in 0.1% SDS. Monomer and dimeric DT and antibody–DT are all separable by size and quantitated by utilizing a UV detector fed to an integrator. The loss of the cross-linked species was quantitated by the appearance of the un-cross-linked species. Below pH 5.5, acid pulsing of DT was performed in the presence of 1% SDS to prevent precipitation of DT.[4]

Blocking and Unblocking of a Protein Toxin Functional Domain with the Aid of an Acid-Cleavable Cross-Linker (Figure 1). DT dimer (unnicked) was isolated and cross-linked with **3**, forming a covalently stable dimer which could not be dissociated with 40% DMSO. Over 90% of the cross-linked dimer toxicity was lost as assayed by inhibition of protein synthesis on Vero cells following a 30-min incubation, wash, and reincubation for 2.5 h. The dimer was acid pulsed from pH 8.5 to pH 6.5 for 2 h and chromatographed in 90/10/1 buffer on GF-250. Approximately $^1/_2$ of the dimer was converted to monomer. Both peaks were isolated.

[4] Hydrolytic rates in the presence of SDS may be significantly accelerated due to catalysis by this surfactant (Cordes & Bull, 1974).

Table I: Half-Lives and Observed First-Order Rate Constants of Hydrolysis of Cross-Linker **2** at Various pH Values[a]

pH	$t_{1/2}$ (min)	k_{obsd} (min^{-1})	pH	$t_{1/2}$ (min)	k_{obsd} (min^{-1})
5.24	11	5.76×10^{-2}	7.15	145	4.86×10^{-3}
6.10	75	9.38×10^{-3}			

[a] Hydrolysis carried out in acetonitrile/phosphate buffer (1:10).

Table II: Comparison of First-Order Hydrolytic Rates of DT Dimers Cross-Linked with **2** and **3** following Acidification to pH 6.4[a]

cross-linked product	$t_{1/2}$ (min)	k_{obsd} (min^{-1})
DT~**2**~DT	60	1.17×10^{-2}
DT~**3**~DT	165.5	4.14×10^{-3}

[a] The loss of dimers with time correlates with the appearance of monomers.

Results

The ortho ester cross-linkers **2** and **3** were our initial targets because of the established sensitivity of this functionality to acid-catalyzed hydrolysis. Ortho ester polymers prepared by the addition of the appropriate diols to the ketene acetal **1** have been used in the slow release of subcutaneously implanted steroid contraceptives (Heller et al., 1983, 1980). The addition of alcohols to ketene acetals takes place under very mild conditions and usually requires no catalysts (De Wolfe, 1974). Addition of *N*-(hydroxymethyl)- or *N*-(hydroxyethyl)maleimide to the ketene acetal **1** proceeded smoothly in good yield to afford the cross-linkers **2** and **3**. The hydrolysis of the cross-linker **2** was studied at the pH values 5.24, 6.10, and 7.15, and the results are shown in Table I. In this pH range the hydrolytic rate should reflect cleavage of the ortho ester bonds only and not be influenced by the maleimide function. The linear relationship between the rate constant and $[H^+]$ holds only between pH 5.24 and 6.10 but breaks down at higher pH ($t_{1/2}$ should be 841 min at pH 7.15, assuming linearity, but is actually only 145 min). General-acid catalysis could well be responsible for this as documented in other examples of ortho ester hydrolysis (Cordes & Bull, 1974).

2 and **3** were used to covalently cross-link DT dimers as described under Methods, and the hydrolytic stability of the cross-linked product was determined at various pHs. DT has a natural tendency to aggregate to the dimeric form (Carroll et al., 1986), which facilitates cross-linking with homobifunctional reagents like **2** and **3**. The dissociation of cross-linked DT dimers into monomers with time following acidification to pH 6.4 is a first-order process. Interestingly, cross-linker **3** is more than 2.5 times as stable as **2** at this pH (Table II). The faster rate of hydrolysis of DT dimer cross-linked with **2** as compared to **2** itself ($t_{1/2}$ of 60 min at pH 6.4 vs 75 min at pH 6.10) is probably because the cross-linked protein hydrolysis is carried out in purely aqueous solution whereas hydrolysis of **2** is done in acetonitrile diluted 10-fold with aqueous buffer. Hydrolytic rates are influenced by the ratio of organic to aqueous solvent employed—the higher the organic solvent content, the slower the hydrolysis (Cordes, 1967).

We have demonstrated the effectiveness of these ortho ester reagents in the blocking and unblocking of a protein toxin functional domain. Dimerization of DT (cold induced) has

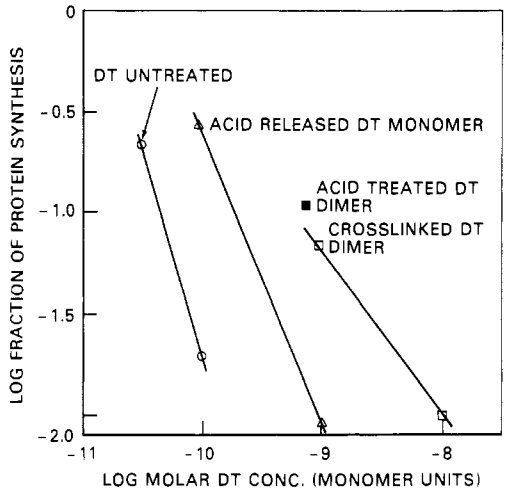


FIGURE 1: Increasing concentration of DT progressively inhibits protein synthesis of vero cells. Dimers cross-linked by **3** are less toxic, requiring 30-fold higher concentrations for equal inhibition. pH 6.5 treatment of cross-linked dimer for 2 h converts about half the dimer into monomer with partial restoration of the original toxicity.

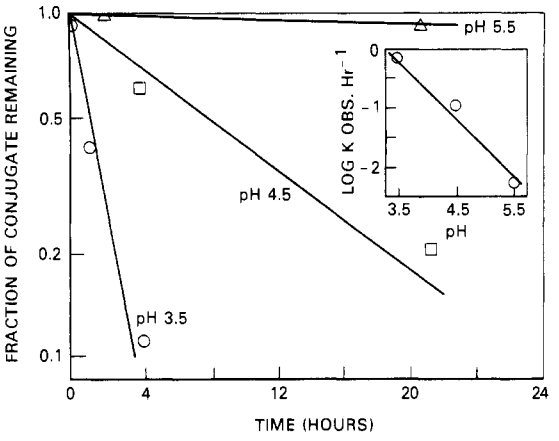


FIGURE 2: The fractional loss of the immunotoxin T101–DT constructed with cross-linker **5** plotted on a log scale is followed by the appearance of free T101 antibody at pH 3.5, 4.5, and 5.5. The slope at each pH defines the observed first-order rate constant of dissociation, k, and in the insert log k is plotted vs pH, revealing a linear dependence, a characteristic of specific-acid catalysis.

been shown to result in a functional loss of the DT binding domain and a corresponding loss of toxicity. Treatment of dimers with DMSO converts them to monomers (unnicked) and restores toxicity and binding (Carroll et al., 1986). DT dimer (unnicked) was cross-linked with **3** and approximately $^1/_2$ of the dimer was converted to monomer upon acidification at pH 6.5 over 2 h. Dimer toxicity was unchanged, but the monomer toxicity was partially restored (Figure 1). Although DT dimers are stable for days at 37 °C, cross-linked DT dimers that have been treated with 40% DMSO dissociate rapidly after cross-linker hydrolysis. The 0.5 log loss of toxicity of the acid-released unnicked DT monomer compared to untreated DT is a consequence of the slight modification of the protein after cleavage of the ortho ester bonds.

A drawback of these reagents is their sensitivity to moisture, making long-term storage impractical. Freshly prepared samples in typical NMR solvents ($CDCl_3$, CD_3CN, etc.) rapidly deteriorate on storage at ambient temperature as is apparent from periodic monitoring of their NMR spectra. Chemical manipulations needed to convert these reagents into the more desirable heterobifunctional cross-linkers are also precluded because of this instability. Furthermore, cross-linked products utilizing **2** and **3** hydrolyze at a measurable rate between pH 7 and 8. Consequently, the utility of these ortho esters in immunotoxin construction is rather limited.

Our experience with ortho esters led us to consider other candidates like acetal- and ketal-based cross-linkers which are somewhat more stable toward acid-catalyzed hydrolysis. We rationalized that these would be very stable at pH >7.4 yet would be cleaved fairly rapidly in the pH range 5–6. Their greater stability would also permit chemical manipulations to achieve variations in hydrolytic rates. We were encouraged by a report on the acid-reversible protection of acetaminophen with an acetal function (Hussain et al., 1978). Cross-linker **5** was designed after this "prodrug" and was synthesized in a straightforward manner in spite of the low yield of the vinyl ether intermediate **4**.

The commercially available Bolton–Hunter reagent was used to introduce the *N*-hydroxysuccinimidyl ester moiety. Immunotoxins were constructed by coupling DT to the anti-CD5 T101 antibody using **5** as described. The acid-induced dissociation of this conjugate is shown in Figure 2 over a 3 pH unit range. Within experimental error the observed first-order rate constant is linearly related to the H^+ ion concentration. The $t_{1/2}$ is estimated to be 139 h at pH 5.5—too slow a hydrolytic rate for our specific purposes.

Table III: Comparison of Cross-Linked Protein Dissociation Half-Lives at pH 5.5

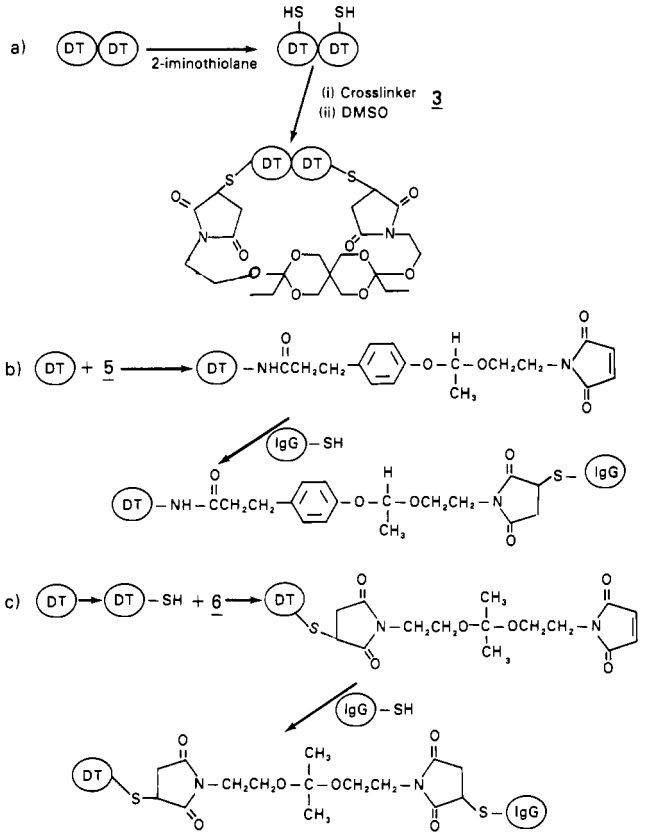
cross-linker	type	acid-cleavable group	reactive groups	$t_{1/2}$ at pH 5.5 (h)
2[a]	homobifunctional	ortho ester	dimaleimide	0.1
3[a]	homobifunctional	ortho ester	dimaleimide	0.3
6	homobifunctional	ketal	dimaleimide	0.7
5	heterobifunctional	acetal	maleimide, NHS ester	139

[a] Half-lives determined at pH 6.5 ± 0.1 for **2** and **3** and extrapolated to pH 5.5, assuming a linear dependence of k_{diss} on $[H^+]$. All measurements at 25 °C.

It is of interest to note that the kinetics of hydrolysis of cross-linked toxins fails to exhibit a lag period (Figure 2). A lag period should be seen if there is more than one cross-link per conjugate. This is because the kinetics changes from a single-hit survival curve to a multitarget model and approaches first order only after a delay (Hutchinson & Pollard, 1961). Since our coupling procedures were chosen to minimize the density of cross-linkages (see Methods), the absence of a lag period suggests that on the average we have introduced only one cross-link per conjugate.

Ketals are known to have faster rates of acid-catalyzed hydrolysis than acetals (Cordes, 1969) because of enhanced stabilization of the intermediate carbonium ion by an alkyl group compared to H (i.e., R_2C^+ is more stable than RHC^+). Among ketals, acyclic ketals of acetone are about the most acid labile. A simple ketal exchange reaction between 2,2-dimethoxypropane and *N*-(2-hydroxyethyl)maleimide afforded the highly symmetrical ketal **6** [2,2-bis(maleimidoethoxy)-propane (BMEP)] in good yield. **6** is a crystalline compound that shows no significant decomposition on storage. After considerable experimentation, conditions were worked out for the preparation of conjugates of toxin with antibody using the homobifunctional **6**. Good yields were generally obtained, and unwanted side products have been kept to a minimum. A typical procedure for the construction of DT monomer–T101 using **6** is described under Methods. The hydrolytic stability of these conjugates have been determined, and Table III shows a comparison of the half-times of cross-linked protein dissociation at 25 °C for the different types of acid-cleavable

Scheme IV: Construction of Toxin Conjugates with Acid-Cleavable Cross-Linkers[a]



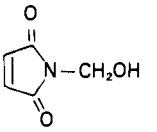
[a] (a) Intramolecular cross-linking of DT dimer with ortho ester **3**. (b) Formation of an immunotoxin between DT and the antibody T101 using heterobifunctional acetal **5**. (c) Coupling of DT to an IgG by use of ketal **6** (BMEP).

reagents described in this report. Between 25 and 37 °C hydrolytic rates change by less than 2-fold. As is apparent, these cross-linkers cover a wide range of hydrolytic rates and exhibit pH stability in the order acetal > ketal > ortho ester, in general agreement with the literature. Scheme IV illustrates the use of these cross-linkers in the construction of toxin conjugates.

Discussion

Ortho ester, ketal, and acetal groups have been used for a long time as acid-labile and base-stable protecting groups in organic chemistry but to date have not found any application in protein chemistry. We decided to design cleavable cross-linking agents that incorporate these functionalities for three reasons: (1) The chemistry of these groups is well established, (2) they are stable toward base and can be stored and manipulated at pH 8–9, and (3) the mechanism of their acid-catalyzed hydrolysis including correlation of rates with structure has been studied in detail. Most compounds containing these groups hydrolyze by specific-acid catalysis with observed rate constants of dissociation linearly related to hydrogen ion concentration. This linear dependence gives the widest possible change in dissociation rates at any two different pH values and is a desirable feature in a cross-linker that utilizes a 2 pH unit change (from vascular to acidified vesicle compartment) to achieve a transformation from a relatively stable state to a labile state.

It was recognized at an early stage that it would be much easier to synthesize homobifunctional reagents that incorporated these functionalities rather than heterobifunctional ones even though the latter are more desirable for the purpose of coupling two different proteins. The maleimide group was chosen as the functional group for attachment of the proteins in all the homobifunctional reagents described. This group is very reactive and fairly specific for sulfhydryl groups, which can be conveniently generated in a controlled fashion on proteins by using 2-iminothiolane. In order to implement our strategy, we needed alcohols derivatized with the maleimido group. Two such derivatives which we have employed extensively are the primary alcohols **8** and **9**.



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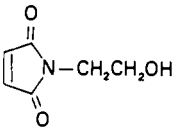
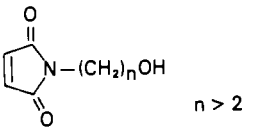


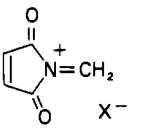
9

(Hydroxymethyl)maleimide (**8**) is easily prepared in one step from maleimide. The synthesis of *N*-(2-hydroxyethyl)-maleimide (**9**), through somewhat longer, is also straightforward. These two building blocks have served as versatile starting materials for all the cross-linkers reported in this article. Our scheme permits us to extend the lengths of these cross-linkers merely by starting with longer chain alcohols **10**:



10

In general, we have found that cross-linkers utilizing **9** give higher yields of cross-linked products than those constructed with **8**. The hydrolytic rates also differ significantly in the two cases. In the synthesis of ketals by exchange reactions involving **8**, care must be exercized to keep the amount of acid catalyst at a minimum and reaction conditions as mild as possible. Under more drastic conditions, this alcohol readily eliminates H_2O to give the iminium ion **11** which can be trapped by another molecule of alcohol to yield the ether **12**. Under the conditions described for the preparation of **7** no contamination by **12** was observed.



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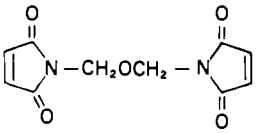
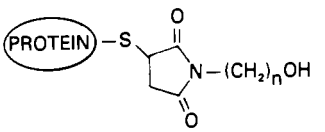


12

When designing these reagents, we realized that the monomeric proteins released from the cross-linked product would be modified with the residue **13**:



13

Such a modification could conceivably alter the activity of the protein (e.g., attenuate the cytotoxicity of a toxic protein) and defeat the purpose of these cross-linkers. However, we have established that the modified toxins released after acid-induced cleavage are not significantly lower in toxicity from the parent toxin (~0.5 log in the case of unnicked DT at the level of thiolation described under Methods). Thus even with this slight drawback these cross-linking reagents should prove useful in a number of applications.

Of all the cross-linkers described here, the acetone ketal BMEP (**6**) seems to be the most promising in terms of ease of preparation, thermal stability, hydrolytic stability at pH above neutrality, and rate of dissociation under mildly acidic conditions. Anti-CD5–ricin immunotoxins prepared with BMEP show enhanced toxicity over those utilizing conventional noncleavable cross-linking reagents (U.S. Patent Application No. 7-204, 163; pending June 1, 1988). Details of these studies will be published elsewhere. We are presently investigating various routes to a heterobifunctional reagent that retains the acetone ketal function as the acid-labile unit. The ortho ester cross-linkers **2** and **3**, though somewhat limited in utility for reasons previously discussed, should nevertheless find application in those instances where rigidity of structure (imposed by the spiro ring system) and very rapid rates of hydrolysis in acid milieu are desirable. Work is currently in progress to modify the structure of the heterobifunctional reagent **5** in order to increase its sensitivity to acid-catalyzed dissociation. One way to accomplish this is to replace the Bolton–Hunter reagent in the reaction with vinyl ether **4** with a more acidic phenol. The acetal product would then have a better leaving group, thereby enhancing its rate of acid-catalyzed hydrolysis.

A major utility of these cleavable cross-linkers is in the construction of therapeutic protein prodrugs. A specific property of an administered therapeutic protein or an agent attached to a protein is blocked outside of the target cell but is unblocked within the target cell. This achieves a higher therapeutic ratio by reversibly altering functionalities that direct proteins to certain cells or organs. Lifetime within the vascular compartment, clearance by preexisting antibodies, and antigenic stimulation can also be modified. This concept of reversible blockade of a protein functionality has been demonstrated in the cross-linked DT dimer system, where we have shown that blocking and unblocking of the protein toxin binding sites correlates with cellular toxicity.

Finally, it should be pointed out that these reagents are not limited to cross-linking proteins but can be used in any circumstance where it is necessary to link two species and release them under mildly acidic conditions. The only constraint is that the components to be linked have either sulfhydryl or amino groups. Drug–antibody conjugates and reversible coupling of proteins to matrices for synthetic and chromatographic purposes are among numerous potential applications that can be envisaged.

ACKNOWLEDGMENTS

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Registry No. **1**, 65967-52-4; **2**, 118377-59-6; **3**, 118377-60-9; **4**, 77932-11-7; **5**, 118377-61-0; **6**, 118377-62-1; **7**, 118377-63-2; *N*-(hydroxymethyl)maleimide, 5063-96-7; *N*-(hydroxyethyl)maleimide, 1585-90-6; ethyl vinyl ether, 109-92-2; 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester, 34071-95-9; 2,2-dimethoxypropane, 77-76-9.

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Interactions of Colicin A Domains with Phospholipid Monolayers and Liposomes: Relevance to the Mechanism of Action†

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ABSTRACT: The colicin A polypeptide chain (592 amino acid residues) contains three domains which are linearly organized and participate in the sequential steps involved in colicin action. We have compared the penetrating ability in phospholipid monolayers and the ability to promote vesicle fusion at acidic pH of colicin A and of protein derivatives containing various combinations of its domains. The NH_2-terminal domain (171 amino acid residues), required for translocation across the outer membrane, has little affinity for dilauroylphosphatidylglycerol (DLPG) monolayers at all pHs tested. The central domain has a pH-dependent affinity, although lower than that of the entire colicin A. The COOH-terminal domain contains a high-affinity lipid binding site, but in addition an electrostatic interaction is required as a first step in the process of penetration into negatively charged DLPG films. In contrast to the constructs containing the ionophoric domain, the NH_2-terminal domain alone has no fusogenic activity for liposomes. These results are discussed with regard to the mechanism of entry and action of colicin A in sensitive cells. Our results suggest the existence of a pH-dependent interaction between the receptor binding domain (amino acid residues 172–388) and the pore-forming domain of colicin A (amino acid residues 389–592).

Colicin A is a 592-residue bactericidal protein (Morlon et al., 1983), known to exert its lethal effect through an ability to depolarize the cytoplasmic membrane of sensitive *Escherichia coli* cells (Schein et al., 1978). Studies of the properties of this colicin in artificial membranes have led to the conclusion that depolarization occurs as a result of formation by the colicin of a nonspecific ion channel in the inner membrane (Schein et al., 1978; Konisky, 1982; Pattus et al., 1983a). Colicin A first interacts with its specific receptor at the cell surface (Cavard & Lazdunski, 1981), is then translocated across the outer membrane, and finally reaches the cytoplasmic membrane to insert its pore-forming domain (Lazdunski et al., 1988). In this process, the colicin A polypeptide chain must switch from a water-soluble conformation to one that is more stable in the membrane. The domains associated with the three steps defined above are organized in three distinct regions of the polypeptide chain (Baty et al., 1988). The COOH-terminal domain which carries the ionophoric activity (Martinez et al., 1983) has been crystallized (Tucker et al., 1986), its three-dimensional structure at 6.0 Å is resolved (Tucker, unpublished result), and the 2.7-Å resolution should become available in the near future.

The mechanics of colicin uptake are poorly understood. This is a difficult problem since the number of colicin molecules entering a cell is below the level of detection and a single colicin molecule is theoretically sufficient to kill a sensitive cell. Since translocation through membranes occurs during uptake, membrane lipids may play a role in one or more of the steps described above.

Earlier studies on the interaction of colicin A with lipid model membranes showed that its affinity for lipid increases drastically at acidic pH (Pattus et al., 1983b). Moreover, colicin A was found to promote efficient fusion of lipid vesicles at acidic pH. Fusion was observed not only with pore-forming colicins (A,E1) but also with colicins that contain nuclease activities (E2,E3) (Pattus et al., 1985a). The appearance of a lipid binding site in colicin E3 at acidic pH has been demonstrated (Escyuer et al., 1986) as well as an acidic pH requirement for insertion of colicin E1 into artificial membrane vesicles (Davidson et al., 1985).

Such a requirement resembles the pH dependence in vitro for the actions of diphteria toxin (Sandvig & Olsnes, 1980),

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