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Disulfide structures of highly bridged peptides: A new strategy for analysis

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Abstract

A new approach is described for analyzing disulfide linkage patterns in peptides containing tightly clustered cystines. Such peptides are very difficult to analyze with traditional strategies, which require that the peptide chain be split between close or adjacent Cys residues. The water-soluble tris-(2-carboxyethyl)-phosphine (TCEP) reduced disulfides at pH 3, and partially reduced peptides were purified by high performance liquid chromatography with minimal thiol–disulfide exchange. Alkylation of free thiols, followed by sequencer analysis, provided explicit assignment of disulfides that had been reduced. Thiol–disulfide exchange occurred during alkylation of some peptides, but correct deductions were still possible. Alkylation competed best with exchange when peptide solution was added with rapid mixing to 2.2 M iodoacetamide. Variants were developed in which up to three alkylating agents were used to label different pairs of thiols, allowing a full assignment in one sequencer analysis. Model peptides used included insulin (three bridges, intra- and interchain disulfides; -Cys·Cys- pair), endothelin and apamin (two disulfides; -Cys·x·Cys- pair), conotoxin GI and isomers (two disulfides; -Cys·Cys- pair), and bacterial enterotoxin (three bridges within 13 residues; two -Cys·Cys- pairs). With insulin, all intermediates in the reduction pathway were identified; with conotoxin GI, analysis was carried out successfully for all three disulfide isomers. In addition to these known structures, the method has been applied successfully to the analysis of several previously unsolved structures of similar complexity. Rates of reduction of disulfide bonds varied widely, but most peptides did not show a strongly preferred route for reduction.

Keywords: analysis; conotoxin; disulfide; endothelin; enterotoxin; insulin; peptide; TCEP

Disulfide bridges between cysteine residues are a key structural element of many secreted proteins and peptides, being especially abundant in some hormones, enzymes, plasma proteins, inhibitors, and venom proteins. Motifs containing repeated clusters of cysteine residues also appear in extracellular domains of membrane-bound receptors. With the smaller molecules, biological activity depends strictly on correct pairing of the cysteines; the same is presumed to be true generally.

Analyzing the connectivities is thus an important facet of structure determination, but it can take a prohibitively large investment of time and material. The traditional strategy has been to break the peptide chain with proteases and isolate bridged fragments (Ryle et al., 1955; Spackman et al., 1960). When cysteines are well dispersed, as with ribonuclease (Spackman et al., 1960), one may obtain each disulfide as part of a unique enzymatic fragment and thus learn which residues are paired. When they are tightly clustered, however, enzymes rarely yield a full set of diagnostic fragments. For insulin, Sanger also had to use acid hydrolysis for chain cleavage and obtained only low yields of definitive peptides (Ryle et al., 1955). The "diagonal method" (Brown & Hartley, 1966) simplifies the purification of cystine peptides but does not solve the problem of generating a complete set. One can sometimes supplement an incomplete digest with a single step of Ed-

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Abbreviations: TCEP, tris-(2-carboxyethyl)-phosphine. Amino acids are indicated by the standard three- or one-letter abbreviations, with the addition of Hyp or O for hydroxyproline; Cys(Cam), *S*-carboxamidomethylcysteine; Cys(Cm), *S*-carboxymethylcysteine; Cys(Pe), *S*-pyridylethylcysteine; PTH-, phenylthiohydantoin derivative of amino acid; TFA, trifluoroacetic acid; 4-VP, 4-vinylpyridine; BPTI, bovine pancreatic trypsin inhibitor; HPLC, high performance liquid chromatography. *Oxidation status of Cys residues:* Square brackets enclose pairs of numbers separated by semicolons; numbers within a pair are separated by a hyphen when the residues are connected by a disulfide bridge or by a comma when the residues are present as thiols. Thus, [2, 7; 3-13]-conotoxin GI indicates a peptide with thiols on residues Cys 2 and Cys 7, and a disulfide linking residues Cys 3 and Cys 13. *Informal nomenclature of peptides:* To assist discussion of peptides before structures are assigned, peptides are also given a trivial name related to their elution from HPLC, and/or reduction state. Native peptides are designated as N, and fully reduced peptides as R. Thus, native insulin is referred to as InsN, and fully reduced endothelin is referred to as EndoR. Partially reduced intermediates are labeled with letters and/or numbers as indicated in figures (e.g., ConoA$_1$).

man degradation to split between Cys residues (Callewaert et al., 1968) and thus obtain additional information. To handle especially difficult problems, several groups have recently used peptide synthesis (Nishiuchi & Sakakibara, 1982; Hoeprich & Doolittle, 1983; Nishiuchi et al., 1986; Shimonishi et al., 1987), building molecules with one or more of their bridges specified and comparing them with natural material. With two bridges, completely defined syntheses can be done for all three isomers (Nishiuchi & Sakakibara, 1982), but 15 isomers result from three bridges, and the approach has to be more piecemeal (Nishiuchi et al., 1986; Shimonishi et al., 1987).

An attractive option would be to unpick the disulfides while leaving the peptide chain intact (Gray et al., 1984). This has rarely been attempted because of the "scrambling problem": thiols and disulfides rapidly exchange partners, destroying the original linkage pattern. I report here a solution to this problem and describe a method that has been applied successfully to several difficult test cases.

The method hinges upon the ability to produce and separate partially reduced peptides, while limiting the exchange reactions. Scrambling is largely suppressed at pH 2–3, because its usual mechanism is attack on the disulfide by thiolate anion (RS^-) rather than by un-ionized thiol (RSH) (Ryle & Sanger, 1955). Reduction by agents such as mercaptoethanol or dithiothreitol actually proceeds via the exchange reaction and thus will not occur at low pH. Trialkylphosphines, on the other hand, are highly reactive toward disulfides and can be employed at low pH (Ruegg & Rudinger, 1977). The water-soluble TCEP (Levison et al., 1969; Burns et al., 1991) has proved to be excellent for this purpose. Unlike many other phosphines, it is a stable crystalline compound applied readily in aqueous medium.

Analysis of a bridged peptide involves four steps: (1) partial reduction at pH 3 using TCEP; (2) separation of products at pH 2 by reversed-phase HPLC; (3) alkylation of free thiols; (4) sequencer analysis to determine location of labeled cysteines. The greatest risk of exchange is during the alkylation step: therefore, kinetically forcing conditions must be used to enhance the intermolecular alkylation relative to the intramolecular exchange.

A primary advantage of this approach is its underlying simplicity, because only a few peptides are produced and every one is relevant. Typically, only one intermediate is needed to define a two-bridge system, two for a three-bridge system, etc. Redundant information is almost always obtainable for confirming assignments. This redundancy can be used for detecting exchange and for identifying the correct partners.

The strategy was designed for peptides that can be sequenced directly (10–50 residues) but is not limited to these. It has been applied to both intra- and interchain bridges of fragments from larger proteins. Table 1 gives a summary of model peptides used to develop the method and several molecules for which disulfide structures were analyzed for the first time. They cover a range of difficulties, with one or more pairs of adjacent Cys residues, -Cys·x·Cys- sequences, and multichain peptides. To provide a realistic test, analyses were usually carried through

Table 1. *Peptides used in method development*[a]

Peptide	Length(s)	Cys[b]	Bridge pattern[c]	Sequence[d]	Reference[e]
Conotoxin GI, N	13	4	1-3, 2-4	ECCNPACGRHYSC-NH_2	1, 2
Conotoxin GI, A	13	4	1-4, 2-3	ECCNPACGRHYSC-NH_2	1
Conotoxin GI, B	13	4	1-2, 3-4	ECCNPACGRHYSC-NH_2	1
Insulin	(30, 21)	6	1-4, 2-6, 3-5	(InsB) FVNQHLCGSHLVEALYLVCGERGFFYTPKA-OH	3
				(InsA) GIVEQCCASVCSLYQLENYCN-OH	3
Endothelin	21	4	1-4, 2-3	CSCSSLMDKECVYFCHLDIIW-OH	4
Apamin	18	4	1-3, 2-4	CNCKAPETALCARRCQQH-NH_2	5
Enterotoxin STp(5-18)	14	6	1-4, 2-5, 3-6	CCELCCNPACAGCY-OH	6
Conotoxin GVIA	27	6	1-4, 2-5, 3-6	CKSOGSSCSOTSYNCCRSCNOYTKRCY-NH_2	7
Conus peptides					
"Scratcher"	12	6	Two -Cys·Cys- pairs; shortest 3-bridge peptide		8
"Kappa"	22	6	Two -Cys·Cys- pairs; blocked N-terminus; broad HPLC peaks; rapid exchange		9
"J021"	25	6	One -Cys·Cys- pair; very thermostable		10
C4a fragment	(19, 5, 21)	6	Three chains; two -Cys·Cys- pairs; broad HPLC peaks		11
Echistatin	49	8	Four bridges; -Cys·Cys- and -Cys·x·Cys- pairs; thermostable; rapid exchange		12

[a] The upper part of the table gives details of model peptides used. The lower part indicates peptides for which structures will be presented in separate publications, so that only a general description is included here.
[b] Number of Cys residues.
[c] Linkage pattern, numbers referring to order of Cys residues, not absolute position.
[d] Standard one-letter code, with addition of O = hydroxyproline.
[e] References: [1]Nishiuchi and Sakakibara (1982); [2]Gray et al. (1984); [3]Ryle et al. (1955); [4]Kumagaye et al. (1988); [5]Callewaert et al. (1968); [6]Shimonishi et al. (1987); [7]Nishiuchi et al. (1986); [8]Olivera et al. (in prep.); [9]MacIntosh et al. (in prep.); [10]Shon et al. (in prep.); [11]Janatova and Gray (in prep.); [12]Gray (1993).

with starting amounts of peptide in the range of 1–10 nmol. This is an important factor when dealing with scarce products in very dilute solution (10^{-7}–10^{-5} M).

Experimental procedures

Materials and methods

Peptides were obtained from various sources and were purified by reversed-phase HPLC before use: endothelin (gift of Dr. M. Verlander, Bachem, Inc., Torrance, California); bacterial enterotoxin STp(5-18) (gift of Dr. Y. Shimonishi, Institute for Protein Research, Osaka); insulin, apamin (Sigma, St. Louis, Missouri); conotoxins GI, [Hyp 5]-GI, and GVIA were synthesized as described previously (Gray et al., 1984; Rivier et al., 1987).

TCEP was synthesized by the method of Burns et al. (1991).

Alkylating agents. 4-VP was purchased from Aldrich (Milwaukee, Wisconsin), distilled under vacuum, and stored frozen as 200-μL aliquots under nitrogen. A working batch, stored at 4 °C, was used only until there was visible discoloration and then was discarded. Iodoacetamide from Sigma was stored at 4 °C and used without further purification. Iodoacetic acid from Sigma was recrystallized from petroleum ether until totally free of color and stored at 4 °C.

Other reagents. Tris, citric acid, sodium citrate, acetic acid, ascorbic acid, phosphoric acid, acetonitrile, and TFA were of the highest reagent grade or HPLC grade available.

Amino acid sequencing was carried out by Dr. Robert Schackmann of the Utah Regional Cancer Center, using an ABI Model 477A, with automatic sample injection and analysis. Values given in the figures are not corrected for losses or carryover from previous cycles. Cys(Cam) and Cys(Cm) are corrected for approximately 20–25% deamidation of the former.

HPLC. All experiments were done using a Beckman Altex pumping system with column effluent monitored at 220 or 280 nm (Waters Lambda Max detector coupled to Shimadzu integrating recorder C-R1B). The column used throughout the work was a Vydac C18 reverse-phase (#218TP54, 5-μm particle size, 300-Å pore, 4.6 × 250 mm). Mobile phases were: A, 0.1% TFA in water (v/v); B, 0.092% TFA in acetonitrile/water (60%, v/v). Linear gradients were used, typically at a flow rate of 1 mL/min, with steepness and endpoints appropriate to the peptide under analysis (see Results for individual peptides, and Discussion). Peak fractions were collected manually into 1.5-mL polypropylene tubes. Some brands of tubes are treated with oils or plasticizers during manufacture; when used with the acetonitrile-containing buffers, the additives may give spurious peaks on HPLC and may also form a surface film that severely retards drying of samples. Life Science Products (Denver, Colorado) provides suitable tubes (#8510-GMT). Peptides were either used immediately or stored in solution at −76 or −10 °C. Partially reduced peptides were never concentrated to dryness.

Reduction of peptides by TCEP

TCEP stock solutions (20 mM) were prepared in acetate, citrate, or phosphate buffers of various pH values in the range 2–7. Solutions above pH 5 were made immediately prior to use, whereas others could be kept for several days.

Partial reductions were carried out by mixing appropriate volumes of TCEP solution and peptide solution (typically 10^{-6}–10^{-5} M in HPLC eluent, 0.1% TFA, with varying amounts of acetonitrile), followed by incubation at room temperature. Reactions were terminated by injection of the mixture onto the HPLC column after appropriate dilution with 0.1% TFA to ensure adsorption of the peptide and its reduction products. TCEP and buffer salts were not retained significantly. Acetonitrile was sometimes helpful in preventing loss of peptide as reduction proceeded. In such cases, it was added to peptide stock solutions before addition of TCEP.

When sufficient experience had been gained with respect to reduction rates and thiol–disulfide rearrangements (see Results, below), partial reductions were routinely carried out using a 20-mM solution of TCEP in 0.17 M citrate, pH 3. This stock solution is referred to as TCEP3 and can be stored at room temperature for weeks with little deterioration.

Complete reduction of peptides was accomplished by incubation with 10 mM TCEP for 10 min at 65 °C. Longer reaction times and/or higher temperatures may be necessary with unusually stable peptides.

Rapid alkylation procedure

Some partially reduced peptides exchange rapidly when the pH is raised to a level at which alkylation can proceed (see Results). Favorably positioned pairs of thiols may also undergo reoxidation to disulfides, a reaction that is further catalyzed by heavy metal ions. The following rapid alkylation with iodoacetamide is recommended for routine use in order to minimize these problems. It is unnecessary to use such forcing conditions with fully reduced peptides.

Iodoacetamide solution. Iodoacetamide (100 mg) is weighed directly into a 1.5-mL polypropylene tube. To this is added 200 μL of 0.5 M Tris acetate, pH 8, con-

taining 2 mM Na_2EDTA. The mixture is heated at 65 °C until a clear solution is obtained, with no residual crystals. This supersaturated solution, approx. 2.2 M iodoacetamide, can be cooled to room temperature and used within 5–10 min without precipitation occurring. During reagent preparation, the solution should be protected from light to minimize photolytic production of iodine, which is a very potent oxidizing agent for thiols.

Alkylation of peptide. Peptide solution (250 μL), as collected in HPLC effluent (pH 2), is drawn into a glass syringe fitted with a fine-tipped needle. It is then forcibly squirted into the iodoacetamide solution while the latter is stirred vigorously on a vortex mixer. Mixing is discontinued as soon as the peptide solution is added. Reaction is quenched after 20–30 s by acidification with 400 μL of 0.5 M citric acid, and the mixture is applied immediately to the HPLC column. The column is washed with a low percentage of buffer B until the effluent reaches an absorbance below 0.05, when the gradient may be started. Larger volumes of peptide solution can be alkylated in batches, with as many as three being applied successively for a single HPLC run. Beyond this there is a tendency for the baseline to remain elevated and noisy.

Note that it is essential to dilute the peptide solution into the iodoacetamide solution and not vice versa. Otherwise, the pH of the peptide solution is transiently raised before an adequate concentration of alkylating agent is present; interchange and/or reoxidation may then be favored.

Iodoacetic acid. A similar procedure can be applied, replacing the iodoacetamide solution with one in which 0.5 M iodoacetic acid is buffered directly with Tris base or NaOH to pH 8, and 5 mM Na_2EDTA is included. It appears to be less successful than iodoacetamide for very labile peptides.

Alkylation with 4-VP

Direct addition of 4-VP. Until significant disulfide exchange was encountered with endothelin (see below), peptide intermediates were alkylated by direct addition of 4-VP to the HPLC effluent. At the level of 3 μL per 0.5 mL of effluent (final concentration 60 mM), the vinylpyridine acts as both alkylating agent and base, raising the pH to approx. 7. This procedure was discontinued for most partially reduced peptides but was used when there was no danger of interchange.

Alkylation with 4-VP was also used to provide positive identification of Cys residues that remained bridged after partial reduction. Peptides that had been alkylated with iodoacetamide or iodoacetic acid were purified by HPLC as described above. They were then fully reduced and alkylated with 4-VP. Two procedures were employed for this step, applied to peptide fractions directly as eluted from HPLC:

1. Reduction at pH 3: The peptide fraction was mixed with an equal volume of TCEP3 and heated at 65 °C for 10 min. It was then reapplied to the column to obtain fully reduced peptide, typically in 0.5 mL of eluent. To this was added 4-VP (3 μL) with mixing, followed by 3 μL of pyridine. The tube was then incubated in the dark for 15–20 min at room temperature, an equal volume of buffer A was added, and the sample was injected into the HPLC. Because of the strong citrate buffer present, 4-VP is ineffective when added directly to the reduction mixture.
2. Reduction at pH 8: The peptide fraction (0.5 mL) was mixed with an equal volume of 0.25 M Tris acetate, pH 8, containing either 20 mM TCEP or 10 mM dithiothreitol. After incubation of the solution at 65 °C for 10 min, 5 μL of 4-VP was added, and incubation was continued in the dark for another 15–20 min at room temperature. The pH was lowered by the addition of 0.5 M citric acid, before injection of the sample onto HPLC.

As with the other alkylation procedures, the column was eluted at a low percent B until the effluent absorbance decreased below 0.05. Fully alkylated peptides were eluted by an appropriate gradient and dried under vacuum for sequencer analysis.

Reagent notes. (1) Cleanliness of the vinylpyridine is important, as polymeric products are generated on storage or upon exposure to light. These may elute as an extended series of peaks, obscuring the peptide derivative. (2) Because TCEP has a poor shelf life in solution at pH 8, a fresh preparation should be used. (3) Sodium iodoacetate is available in high purity and is used widely for alkylation. However, it proved less satisfactory than the recrystallized iodoacetic acid buffered with Tris or NaOH, in that reoxidation of labile thiol pairs was more of a problem. This may result from higher contamination by heavy metals and/or traces of iodine. (4) Solutions of iodoacetamide and iodoacetic acid must be fresh preparations because they react slowly with Tris to produce H^+ (which lowers pH) and I^- (which can generate I_2).

Results

The methods described above evolved from experience gained with a wide variety of peptides containing disulfide. Results presented are chosen to highlight different aspects of the chemistry.

Endothelin and apamin

Endothelin

$C^1.S.C^3.S.S.L.M.D.K.E.C^{11}.V.Y.F.C^{15}.$
H.L.D.I.I.W-OH [1-15; 3-11]

Apamin

$C^1.N.C^3.K.A.P.E.T.A.L.C^{11}.A.R.R.C^{15}.$
$Q.Q.H\text{-}NH_2$ [1-11; 3-15]

Although several small conotoxins were analyzed before these peptides, endothelin was the first for which disulfide exchange was a serious problem. They are presented first to bring this issue forward.

Endothelin's bridges are linked [1-15; 3-11] (Kumagaye et al., 1988; Yanagisawa et al., 1988). Partial reduction at pH 5 gave a well-resolved pattern (Fig. 1a), but more complex than expected. Four products (EndoA-EndoD), two of them minor, were obtained with elution times between those of native (N) and fully reduced (R) forms. EndoA was repurified to minimize contamination by EndoB and alkylated by the direct addition of 4-VP to a final concentration of 50 mM (pH ~7). Analysis by HPLC suggested that approx. 30% of the material had been reoxidized to N; this was verified by sequencing, which showed a lack of any Cys labeling. The remainder eluted as a single major peak with a small shoulder. Sequencer analysis of this product showed extensive labeling of Cys 1 and Cys 15, minor labeling (10%) of Cys 11, but none of Cys 3. This indicated that EndoA resulted from reduction of a Cys 1-Cys 15 disulfide and was enough to define the linkage pattern.

Attempts to label EndoB and EndoC by this method were unsuccessful. Again, there was about 30% reoxidation to N, plus a very sharp doublet of peaks in both cases. Sequencing of the components of this doublet from EndoB showed that they were the pyridylethyl derivatives of [1, 11; 3-15] and [1, 15; 3-11]. There was a small amount of cross-contamination but essentially no labeling of Cys 3. These results, along with the presence of more than two intermediate peaks in the original chromatogram, suggested that disulfide exchange was faster than alkylation. More rigorous conditions were thus sought for the reduction and alkylation steps.

Reduction as a function of pH. Reduction of endothelin was investigated in the pH range 2-5, on the expectation that both exchange (mediated by $R\text{-}S^-$ rather than R-SH) and reduction (mediated by R_3P rather than R_3PH^+) would be slowed by increasing acidity. Extent of reduction was estimated by integration of HPLC peaks, assuming equal extinction coefficients at 220 nm for the various peptides. This is not strictly accurate, but the errors are unlikely to exceed 20%. As shown in Table 2, there was surprisingly little change in reduction rate between pH 5 and 3, but a sharp drop was observed between pH 3 and 2. In contrast, the HPLC profile after reduction at pH 3 (Fig. 1b) showed a dramatic change from that obtained at pH 5 (Fig. 1a). EndoA was still the major intermediate, but EndoB had increased greatly at the expense of C and D. A qualitatively similar shift was observed at pH 5 by using higher concentrations of TCEP and shorter reaction times. These results suggested strongly that EndoA and B were direct reduction products of endothelin, whereas EndoC and D were derived from them by rearrangement.

Direct observation of rearrangement. EndoB was rechromatographed to remove residual EndoA. It was then allowed to stand at 20 °C in the HPLC effluent (pH 2), and samples were analyzed after various times. EndoC and D were generated within short times, with EndoA being formed only after significant accumulation of the other products. This lag in the production of EndoA would be expected because the complementary reduction products of a bicyclic peptide cannot interconvert directly by a single exchange reaction (see Discussion). Conversion of EndoB to C and D proceeded with a half-life of about 2 h under these conditions of pH and temperature. No attempt was made to obtain a final equilibrium distribution of the various products. Analogous experiments with EndoA and C showed that they can interconvert directly by exchange and also that EndoC can generate small amounts of EndoB and D.

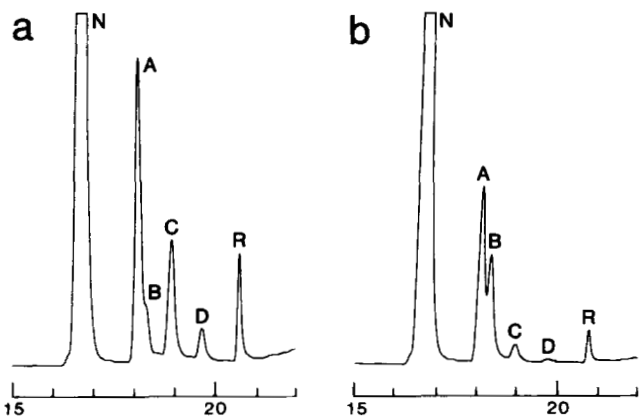

Fig. 1. Reduction of endothelin by TCEP. **a:** 10 mM, pH 5, 20 °C, 2 min. **b:** 10 mM, pH 3, 20 °C, 2 min. Gradient 35-75% B in 20 min.

Table 2. *Reduction of endothelin by TCEP as a function of pH*[a]

pH	Min	N	A	B	C	D	R	%/min[b]	Rel.[c]
5.0	0.5	69.5	13.3	2.8	5.7	0.9	6.8	36.3	1.00
4.0	0.5	71.8	10.7	6.7	3.9	0.8	5.4	32.9	0.91
3.0	0.5	79.8	6.4	6.7	1.3	nd	4.9	24.2	0.67
2.0	3.0	82.5	8.4	6.0	0.6	nd	2.0	3.2	0.09

[a] Samples of endothelin, approx. 1 nmol in 50 μL of 0.1% TFA, were mixed with an equal volume of TCEP (20 mM) in 0.5 M citrate or acetate buffer at pH 2-5. After the indicated times, the samples were injected into the HPLC at a flow rate of 1.0 mL/min, and a gradient of acetonitrile in 0.1% TFA was applied (35-75% B in 20 min). Values in the body of the table represent percentage of the integrated A_{220} that was measured under each component.

[b] Percentage of disulfides reduced per minute.

[c] Rate, relative to that at pH 5.0.

Alkylation by iodoacetamide. The most successful attempts to alkylate the very labile EndoB and C were with the rapid alkylation procedure described above. Alkylation was fast enough to compete with thiol-disulfide exchange but not to prevent it entirely. Figure 2 shows chromatograms of the major alkylation products of EndoA, B, and C, and it is clear that all were heterogeneous. The chromatogram from EndoB (Fig. 2b) contained three more or less equal components: two of these corresponded in position to the main peaks from EndoA and C (Fig. 2a,c), while the earliest one (B*) seemed likely to be the bona fide EndoB derivative. The main peaks (A* and C*) from EndoA and C both contained small amounts of the putative EndoB derivative plus main peaks with small trailing or leading shoulders, respectively. Sequencer analysis (Fig. 2d,e) confirmed that EndoA* and B* were the natural isomers ([1, 15; 3-11], and [1-15; 3, 11], respectively) derived directly from endothelin. EndoC contained label at all four Cys positions (Fig. 2f), so was presumably a mixture derived from disulfide exchange (see Discussion).

Apamin is shorter and more hydrophilic than endothelin. Its cysteines are located in positions identical to those of endothelin, but their linkage pattern is different [1-11; 3-15] (Callewaert et al., 1968). Reduction of apamin was rapid; however, some exchange occurred even at pH 3, as evidenced by the presence of at least three intermediates. One of these was dominant and could be identified as a primary reduction product by the same approach used with endothelin. Rapid alkylation of this partially reduced apamin with iodoacetamide gave a major peak, which by sequence analysis appeared to be a 2:1 mixture of the natural isomer [1, 11; 3-15] and the rearrangement product [1-15; 3, 11].

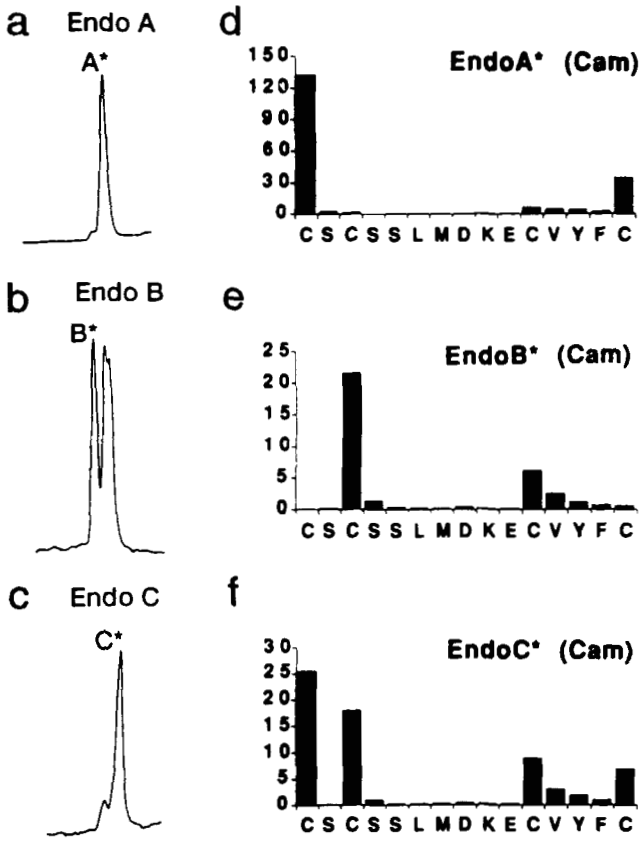

Fig. 2. a–c: HPLC profiles of endothelin products (Fig. 1) labeled with iodoacetamide by the rapid alkylation procedure, rechromatography of major peaks; gradient 45–60% B in 30 min. **d–f:** Sequencer analysis of labeled products as indicated in a–c. Ordinates indicate yield (pmol) of PTH-Cys(Cam).

Conotoxin GI and isomers

Conotoxin GI

$E.C^2.C^3.N.P.A.C^7.G.R.H.Y.S.C^{13}\text{-}NH_2$
[2-7; 3-13]

The natural bridging pattern in this 13-residue bicyclic peptide has been established as [2-7; 3-13] (Nishiuchi & Sakakibara, 1982; Gray et al., 1984); the other isomers [2-3; 7-13] and [2-13; 3-7] have also been described (Nishiuchi & Sakakibara, 1982; Zhang & Snyder, 1991). Native peptide (ConoN) was analyzed successfully by reduction at pH 5, conditions that were later found to be insufficiently rigorous for general use. Reduction at this pH gave one main intermediate ($ConoN_1$), which was identified as [2, 7; 3-13] after direct alkylation with 4-VP. Several sequence analogs ([Hyp 5]-conotoxin GI, desamido-conotoxin GI, [Ala 9]-conotoxin GI, conotoxin GIA) were also analyzed readily by this approach (data not shown). A broader investigation of conotoxin GI was undertaken when the more rigorous conditions had been established and was extended to include all three bridging isomers.

Preparation of isomers. Synthetic native toxin (ConoN) was fully reduced with TCEP, and open-chain peptide (ConoR) was isolated by HPLC. A solution of iodine (20 mM in methanol) was added directly to 0.5 mL of column effluent, pH 2, to give a final concentration of 0.4 mM I_2. After 20 s at room temperature, excess iodine was destroyed by addition of 0.1 mL of 0.3 M ascorbic acid, and the mixture was reapplied to HPLC after dilution with 0.4 mL of 0.1% TFA (Fig. 3a). This step resulted in essentially quantitative conversion to a mixture of three products, ConoA, B, and N, with ConoB eluting close to the position of ConoR. There was no detectable iodination of Tyr or His under these conditions, the elution times of such products being known from previous work (Gray et al., 1984).

Analysis of isomers. The three putative bridging isomers were each subjected to a partial reduction and alkylation. Material from each of peaks ConoA, B, or N was mixed with an equal volume of TCEP (20 mM, pH 3), incubated for 3 min at room temperature, and injected onto HPLC. In each case, two new peaks were generated in ad-

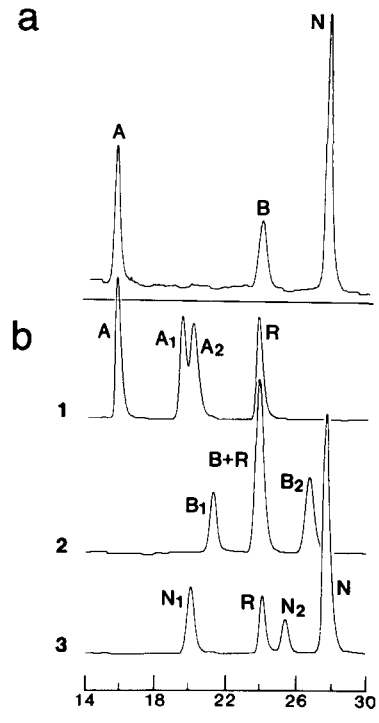

Fig. 3. HPLC separation of conotoxin GI isomers A, B, and N, and their partial reduction products. Gradient 10–30% B in 40 min. **a:** Oxidation of reduced conotoxin GI (R) by 0.4 mM I_2, 20 °C, 20 s. **b:** Reduction of individual isomers by TCEP, 10 mM, pH 3, 20 °C, 3 min.

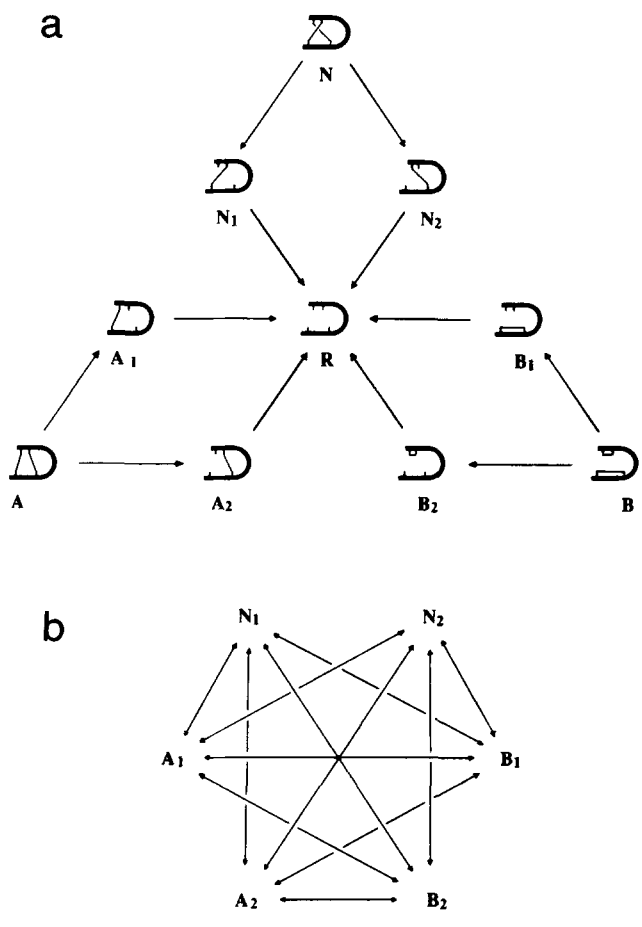

Fig. 4. a: Reduction pathways of disulfide-bridged isomers N, A, and B of conotoxin GI, and their partial reduction products as identified in Figure 3. **b:** Network of permissible thiol–disulfide exchange reactions among the monocyclic isomers of conotoxin GI; note that members of a complementary pair (A_1, A_2, etc.) cannot interconvert directly.

dition to unchanged peptide and ConoR (Fig. 3b). All of these intermediates (ConoA_1, A_2, B_1, B_2, N_1, and N_2) alkylated cleanly with iodoacetamide, utilizing the rapid procedure. Two of the alkylated peptides, ConoA_2(Cam) and ConoB_1(Cam), were then fully reduced with TCEP and secondarily labeled with iodoacetic acid and 4-VP, respectively. The doubly labeled peptides ConoA_2(Cam, Cm) and ConoB_1(Cam, Pe) were mixed and analyzed together on the sequencer. Presence of Cys(Cm) at cycles 3 and 7 established clearly that ConoA_2 is [2, 13; 3-7]; likewise, Cys(Pe) at cycles 7 and 13 showed that ConoB_1 is [2, 3; 7-13]. Because ConoN_1 was known to be [2, 7; 3-13] from the experiment described previously, the complete set of singly bridged and doubly bridged isomers is thus assigned (Fig. 4a).

Insulin

Insulin B

F.V.N.Q.H.L.C^{7}.G.S.H.L.V.E.A.L.Y.L.V.C^{19}.
G.E.R.G.F.F.Y.T.P.K.A-OH

Insulin A

G.I.V.E.Q.C^{6}.C^{7}.A.S.V.C^{11}.S.L.Y.Q.L.E.N.Y.
C^{20}.N-OH [B7- A7; B19-A20; A6-A11]

Analysis of insulin's disulfides by Ryle et al. (1955) was a landmark in protein chemistry. The protein contains two interchain bridges and one intrachain bridge and includes a pair of adjacent Cys residues. Its behavior toward TCEP was therefore of great interest. Two analyses were carried out by different approaches, each starting with 6.4 nmol of peptide. The correct bridging pattern was deduced readily, and the full set of intermediates in the reduction process was identified.

Reduction of insulin by TCEP. This proceeded more slowly than with conotoxin GI or endothelin. Figure 5 shows the HPLC profile after reduction of insulin at pH 3 (9 mM TCEP, 12 min, room temperature). Positions of native insulin (InsN) and the fully reduced InsA and InsB chains are indicated on the figure. Six additional peaks (designated Ins1–Ins6) were also present, plus a slightly elevated baseline after InsA. When reduction was carried out at pH 5 instead of pH 3, Ins3 and Ins6 were the dominant products.

Identification of bridges using a single label with 4-VP. Peptides Ins3 and Ins6 were alkylated by direct ad-

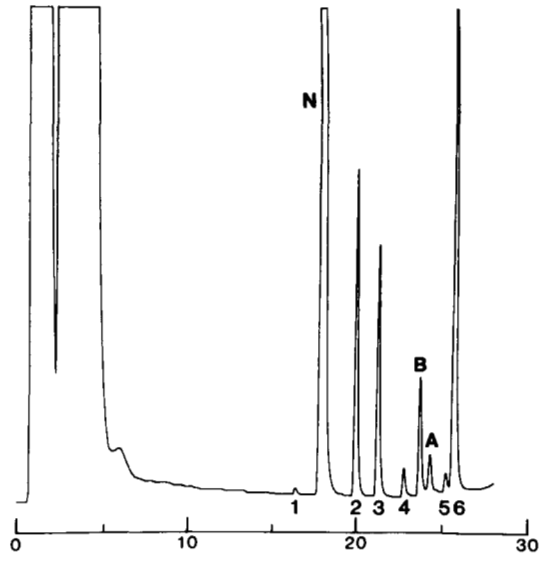

Fig. 5. HPLC separation of insulin and products of partial reduction (9 mM TCEP, pH 3, 20 °C, 12 min). Gradient 35–85% B in 25 min. All peaks were collected and reduced further to produce the assignments made in Table 3 and Figure 8.

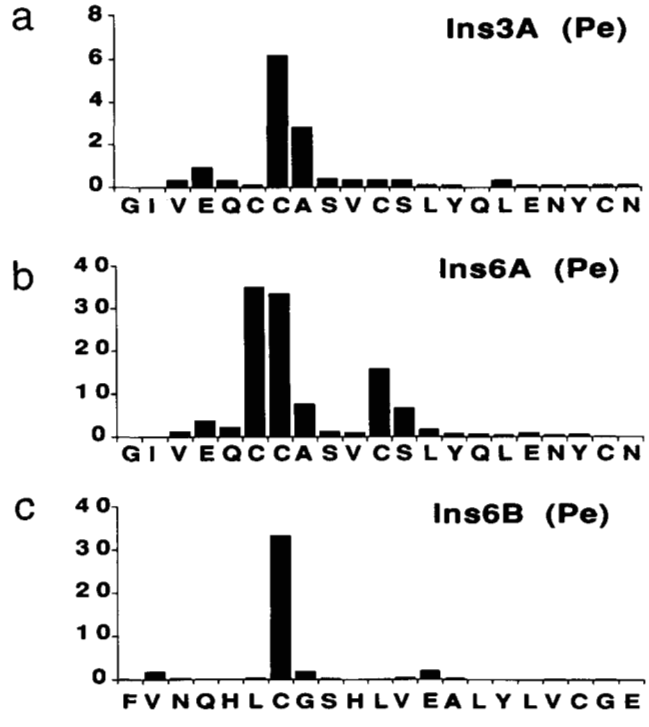

Fig. 6. Sequencer analysis of insulin peptides 3 and 6 (Fig. 5), which had been labeled by direct addition of 4-VP. Ordinates indicate yield (pmol) of Cys(Pe) at each sequencer cycle. Small peaks at cycle 4 (A) and cycle 13 (B) arise from interference by a side-product of Glu. **a:** A chain from peptide 3. **b, c:** A and B chains from peptide 6.

dition of 4-VP. Despite the nonforcing conditions, this resulted in very effective labeling. With Ins3 a single major peak (89%) was obtained, while 4–8% was reoxidized to native insulin. A similar yield (88%) of the major alkylation product was obtained with Ins6, plus 6–8% of a byproduct. The minor products were not investigated; they could have arisen by alkylation of other reactive groups, by oxidation, or by disulfide exchange. After complete reduction of the major alkylation products from Ins3 and Ins6 (10 mM TCEP, pH 3, 65 °C, 10 min), the variously modified InsA and InsB chains were isolated by HPLC. The two InsA products were quite distinctive, whereas the two InsB products appeared to be identical. On sequencer analysis of the A chain from Ins3, Cys(Pe) was found only at cycle A7 (Fig. 6a); cycles A6 and A11 were additionally labeled in the A chain from Ins6 (Fig. 6b). Only cycle B7 was labeled in the B chain from Ins6 (Fig. 6c). Although it was not separately sequenced, the apparently identical B-chain product from Ins3 is expected to be labeled at position B7. These results establish that one interchain bridge links A7 and B7, while the intrachain bridge is between A6 and A11. By difference, therefore, the second interchain bridge is between A20 and B19, and the analysis is complete.

Identification of bridges using a triple label. Given the availability of several alkylating agents, experiments were carried out to test the usefulness of consecutive labeling of the bridges (Fig. 7). Approximately 600 pmol of Ins3 were isolated and labeled with iodoacetamide. The labeled product (Ins3(Cam_2)) was purified and subjected to further partial reduction, producing Ins3(Cam_2, SH_2). This was alkylated with 4-VP to give Ins3(Cam_2, Pe_2). Complete reduction of this product gave the modified InsA and InsB chains, which were separated by HPLC and finally alkylated with iodoacetic acid. This last step gave poor yields of the triply derivatized InsA; several more rapidly eluting peaks were obtained, probably arising from alkylation of the pyridylethyl groups on Cys 6 and Cys 11. Sequencer analysis of the main products established bridges A6–A11 and A7–B7, but insufficient signal was present to identify A20–B19 positively. In another experiment where the alkylation sequence was (Cam, Cm, Pe), complete reoxidation of the A6–A11 bridge occurred at the second step, no Cys(Cm) was obtained, and A6, A11, and A20 were all labeled with Cys(Pe).

Behavior of Ins2. Brief reduction of insulin gave only Ins2, Ins3, and Ins4, suggesting that these each arise by the cleavage of one of the three bridges. As mentioned above, although Ins2 is a major product at pH 3, it is much less evident after reduction at pH 5. This is probably due to its lability, rather than lack of production. Alkylation by direct addition of 4-VP was unsuccessful with this peptide: it readily reoxidized to native insulin and also gave strong evidence of disulfide exchange. Much cleaner alkylation was achieved with iodoacetamide, but the analysis was not pursued. The identities of this and

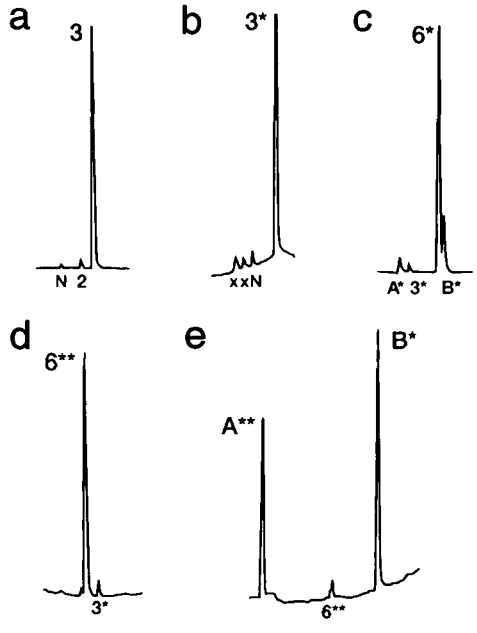

Fig. 7. Stepwise reduction and alkylation of insulin. Gradient 35–85% B in 25 min. Each chromatogram shows the main peak from the previous chromatogram, processed as follows. **a:** Repurification of Ins3 (Fig. 5) pooled from several experiments. **b:** Alkylation of Ins3 with iodoacetamide gives small reoxidation peak N, minor reagent peaks X, and Ins3* (= Ins3(Cam_2)). **c:** Reduction of Ins3* with TCEP (10 mM, pH 3, 20 °C, 8.5 min) gives residual Ins3*, mainly Ins6* (= Ins6(Cam_2)), and small amounts of alkylated A and B chains (InsA*, InsB*) from overreduction of Ins3*. **d:** Alkylation with 4-VP gives mainly Ins6** (= Ins6(Cam_2, Pe_2)), plus a little reoxidation to Ins3*. **e:** Reduction with TCEP (10 mM, pH 3, 65 °C, 10 min) gives InsA** (= InsA(Cam_1, Pe_2)) and InsB*, which were finally alkylated with iodoacetic acid (not shown).

other intermediates were established by a different approach, described below.

Identification of intermediates by "chase experiments." While the identities of Ins3 and Ins6 were analyzed directly, the others were established by creating a network of precursor-product relationships. The various products from the reduction of insulin (Fig. 5) were collected from HPLC and stored at −10 °C while residual InsN was put through several cycles of partial reduction and HPLC. This was necessary because steady-state levels of some products were very low. Each intermediate was then repurified as a single pool to concentrate it and to remove contamination derived either from the initial chromatography or from any rearrangement products that might have arisen during storage. Treatment of each peptide with TCEP for varying times gave one or more products from the set [InsA, InsB, Ins1–Ins6]. The results, summarized in Table 3, show that all possible intermediates in the reduction pathway (Fig. 8) were identified, and that no rearrangement products were present in significant amounts. Only the slightly elevated baseline in the region of InsA and B is not explained.

Table 3. *Peptides from partial reduction of insulin*[a]

Peptide[b]	Time[b] (min)	Yield[b] (%)	Reduction products[c]	Identification[d]
1	13.8	0.15	A only	R_{23}
N	15.0	73.45	2, 3, 4	N
2	16.8	5.46	5, 6	R_1
3[e]	17.9	4.59	(1), B, 6	R_2
4	19.2	0.61	1, B, 5	R_3
B	20.0	2.59	B only	B
A	20.6	0.96	A only	A
5	21.3	0.38	A, B; fast	R_{13}
6	21.7	11.81	A, B; slow	R_{12}

[a] Peptides were produced by partial reduction of insulin by TCEP and separated by HPLC as described in Figure 5.

[b] Nomenclature, elution times, and yields are for the chromatogram shown in Figure 5. Yields are based on integrated absorbance, which is only approximately to be equated with mole %.

[c] Peptide peaks were collected from several HPLC runs, and further reductions with TCEP were carried out. Products listed are those found at short reaction times.

[d] Deduced structures, according to nomenclature of Figure 8.

[e] Insufficient amounts of peptide 3 were analyzed to see the expected minor peak of peptide 1.

Bacterial enterotoxin

Enterotoxin ST_p(5-18)

$C^1.C^2.E.L.C^5.C^6.N.P.A.C^{10}.A.G.$
$C^{13}.Y\text{-}OH$ [1-6; 2-10; 5-13]

Synthetic enterotoxin STp(5-18) was readily reduced at pH 3, but the products had a small range of elution times on HPLC, using a gradient of 25–50% B/25 min. This made it advantageous to use the stepwise approach to reduction. In this gradient, native tricyclic peptide (EnteroN) eluted as a very sharp peak at 17.3 min. After 5 min reduction with 2 mM TCEP at pH 3, approx. 19% of the original was converted to a bicyclic species (Entero1, broad peak at 19.7 min), and another 4% gave a mixture of monocyclic forms (sharp peaks centered on 20.9 min). Prolonged treatment of EnteroN with TCEP gave fully reduced peptide (EnteroR), which co-eluted with Entero1, albeit with a sharper profile. However, the reduction conditions were easily controlled to provide intermediates.

The enterotoxin does seem to have a preferred route of reduction: it unpeeled from the amino end. The first reduction was quite selective, and alkylation of Entero1 proceeded cleanly with either iodoacetamide or iodoacetic acid: Cys 1 and Cys 6 were labeled (Fig. 9a). The second partial reduction step, of either alkylated or nonalkylated Entero1, was also straightforward, and the major products (Entero2 or Entero2(Cam_2)) were easily isolated. However, rapid exchange and reoxidation made the second alkylation difficult. Only the most vigorous conditions with iodoacetamide were useful. The same main product was obtained whether toxin was taken through

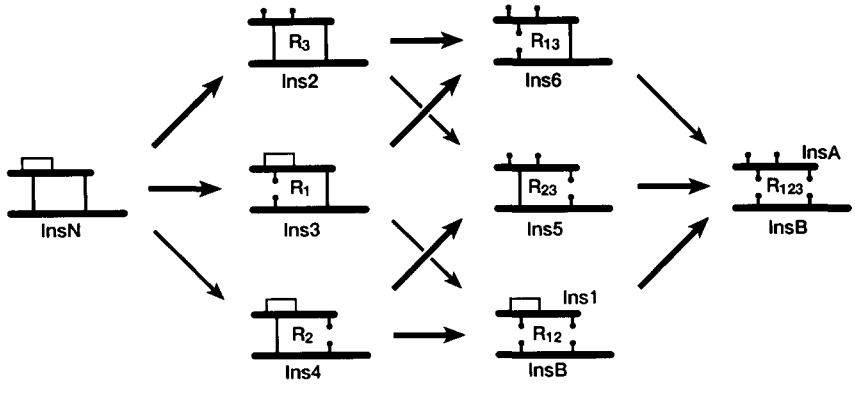

Fig. 8. Pathways of reduction for insulin. The native molecule (N) can be attacked at each of the three disulfides, giving products R_1, R_2, and R_3. These in turn can be reduced to R_{12}, R_{13}, and R_{23} (which is a mixture of A′ and B). The third stage of reduction (R_{123}) produces fully reduced A and B chains. All of these are present in the chromatogram (Fig. 5) and identified in Table 3. Arrow thickness denotes relative rates as fast (thick) and slow (thin). Intermediates 1, 4, and 5 achieve only a low steady-state level because their production rates are relatively low, while their destruction rates are relatively high.

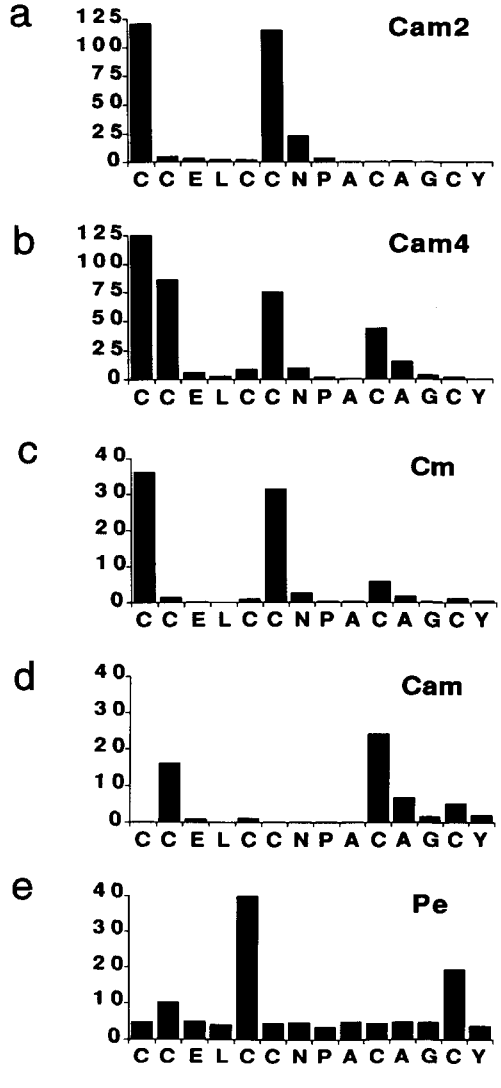

Fig. 9. Sequencer analysis of enterotoxin STp(5-18), variously labeled with Cam, Cm, and Pe. Ordinates indicate yield (pmol) at each sequencer cycle. **a, b:** Alkylation of singly reduced and doubly reduced peptide, reacted with iodoacetamide. **c–e:** Analysis of peptide reduced and alkylated in a stepwise manner, alkylating first with iodoacetic acid, second with iodoacetamide, and third with VP.

two stages of reduction and then alkylated or was reduced and alkylated in two discrete steps. Sequencer analysis of Entero2(Cam_4) showed the presence of Cys(Cam) at cycles 1, 2, 6, and 10 (Fig. 9b), defining a second bridge between Cys 2 and Cys 10. By difference, the third bridge must be Cys 5–Cys 13.

Sequencer analysis of the product from a triple-label experiment is also shown in Figure 9c–e. This sample was labeled first with iodoacetic acid, second with iodoacetamide, and third with 4-VP. The main peak obtained by HPLC was predominantly the "correctly" labeled peptide, with 25% of a component that had rearranged at the second alkylation step: thiol of Cys 2 attacked Cys 5 of the [5-13] bridge to create a new bridge [2-5] and thiol on Cys 13. Subsequent analysis of a purified sample of the minor component (data not shown) confirmed this interpretation. The disulfide assignments made in this work are in complete agreement with those of Shimonishi et al. (1987).

Omega conotoxins GVIA and JO21

Conotoxin GVIA

C^1.K.S.O.G.S.S.C^8.S.O.T.S.Y.N.C^{15}.C^{16}.R.S.C^{19}.
N.O.Y.T.K.R.C^{26}.Y-NH_2 [1-16; 8-19; 15-26]

Conotoxin GVIA is a tricyclic peptide from *Conus geographus* (Rivier et al., 1987) whose bridge pattern was established by the multiple synthesis approach (Nishiuchi et al., 1986). Attempts to reduce it with TCEP were initially unsuccessful. At room temperature the peptide resisted reduction, even after prolonged reaction in the presence of various denaturants such as 6 M urea, 6 M guanidine hydrochloride, 50% acetic acid, 50% acetonitrile. Reduction at 65 °C appeared to be an all-or-none melting and reduction process, with essentially no intermediate products. Small but usable amounts (2–5%) of intermediates were produced at temperatures between 41 and 48 °C. Analysis of GVIA was not pursued further. A more ex-

treme example of the same behavior was encountered with conotoxin J021, a previously undescribed peptide from *C. purpurascens* (Olivera et al., in prep.). In this case, a more determined effort was made to solve the problem, and a temperature (62 °C) was found at which partial reduction occurred with reasonable yields of intermediates. These were successfully alkylated by iodoacetamide and sequenced to determine the disulfide pattern, which proved to be the same as other omega conotoxins (Shon et al., in prep.).

Discussion

Peptides containing tightly clustered disulfides have often proved extraordinarily difficult to analyze, for the reasons mentioned in the introduction: there is no reliable method for splitting the peptide chain between adjacent or closely spaced Cys residues, and partial reduction of the bridges invites disaster from disulfide scrambling. Partial reduction has therefore been limited mainly to cases where some bridges are effectively buried within a stable protein domain (for example, inter- versus intrachain bridges in immunoglobulins [Pink & Milstein, 1967]). With the relatively stable conotoxin GI, partial reduction by $NaBH_4$ in the presence of iodoacetic acid gave unambiguous results (Gray et al., 1984), although some exchange was detected. On the other hand, alkylation of partially oxidized enterotoxin led to incorrect assignments (Houghten et al., 1984). The present work describes a way to circumvent the exchange problem, opening a new route to disulfide assignment.

Reduction of disulfides by trialkylphosphines

In contrast to exchange-reduction by thiols, trialkylphosphines reduce disulfides irreversibly and stoichiometrically (Ruegg & Rudinger, 1977):

$$R_1\text{-S-S-}R_2 + X_3P + H_2O \rightarrow R_1SH + R_2SH + X_3PO.$$

On the whole, however, they have found little acceptance in protein and peptide chemistry because the readily available compounds, such as tributylphosphine, have been very hydrophobic, toxic, foul-smelling, and subject to rapid oxidation by air.

Ruegg and Rudinger (1977) briefly discussed TCEP, comparing it unfavorably to tributylphosphine in terms of reactivity with proteins. Report of a convenient synthesis of TCEP, and its selective behavior toward simple organic disulfides (Burns et al., 1991), was the stimulus for the present work. Buckwalter et al. (1992) independently described the use of TCEP for selective reduction of a disulfide in somatotropin.

With tributylphosphine, Ruegg and Rudinger (1977) pointed out that although reduction can be carried out at acid pH, it is much slower than at the slightly alkaline pH they recommend. It is precisely the ability of trialkylphosphines to reduce disulfides at acidic pH that is of interest in the present context, because it is in this regime that thiol-disulfide exchange is minimized (Ryle & Sanger, 1955). Burns et al. (1991) used pH 4.5–5 in their work and found rapid reduction of organic disulfides. Anticipating that disulfide exchange would be slow enough, my initial experiments with peptides were also carried out at pH 5. It soon became clear that although these conditions appeared to be safe for some partially reduced peptides (e.g., $ConoN_1$, EndoA, Ins3), significant exchange was occurring within a few minutes with others (e.g., EndoB).

Reduction of endothelin at lower pH produced the rather surprising result (Table 2) that there was only a slow decline in the rate of reduction between pH 5 and 3, but a sharp decrease between pH 3 and 2. Throughout this range TCEP should exist predominantly as the trialkylphosphonium (X_3PH^+) rather than the phosphine (X_3P), because its pK_a is 7.66 (Podlaha & Podlahova, 1973). The results suggest that a group with pK_a of approx. 3 is influential. A likely explanation is that reduction by X_3PH^+ is facilitated by an ionized carboxyl, and that it is the titration of the last of the carboxyethyl groups of TCEP that is responsible for the sharp drop in reactivity. Under these conditions the active reducing agent must be $HP^+(CH_2 \cdot CH_2 \cdot COOH)_2 \cdot CH_2 \cdot CH_2 \cdot COO^-$. The low pH range proved to be essential for success of the method, and pH 3 was adopted as a standard condition for carrying out the reduction. Lower pH will provide more suppression of exchange, but this is offset by the need for longer reduction times.

Selectivity and kinetics of reduction

In its action on organic disulfides, TCEP was found to be kinetically selective in that rate of reduction was dependent on the degree of strain in the disulfide rather than on its thermodynamic stability (Burns et al., 1991). An obvious possibility for peptide analysis, therefore, would be to carry out the reduction in a stepwise fashion: stoichiometrically reduce one bridge, alkylate it, and then reduce another, etc. This proved quite infeasible for two main reasons.

First, reduction of peptides is idiosyncratic, depending more on accessibility to the very hydrophilic reagent than on local disulfide conformation. Although experiments were not designed to address kinetics and selectivity per se, but to find conditions suitable for structural analysis, some pertinent observations could be made. In many cases (e.g., the isomers of conotoxin GI, endothelin) there was almost no selectivity, with both disulfides breaking at comparable rates. A significant difference was observed with the three bonds of insulin: two (A6–A11 and A7–B7) reduced quite readily, while the third (A20–B19) was severalfold slower (see Fig. 5). The slow reaction with the second interchain bridge may perhaps be attributed to its being more highly buried and in a more hydrophobic environment than either of the other bonds (Blundell et al.,

1972). In another context an attempt was made to use TCEP for removing a *t*-butylthio protecting group from synthetic peptides. Little reduction occurred, even at 65 °C, again suggesting difficulty with hydrophobic environments. Bacterial enterotoxin STp did appear to have a preferred sequence of opening of its three bridges. With some other peptides (e.g., conotoxins GVIA and J021) reduction was dependent on the global stability of the molecule: all bonds were equally resistant up to some point, when all became sensitive (Shon et al., in prep.).

Second, given that concentrations of peptide are usually low (10^{-6}–10^{-5} M), reduction with stoichiometric amounts of TCEP would be impractical because the second-order reaction takes so long to complete that disulfide exchange may be serious, even at pH 3. Rather than use TCEP in the micromolar range, it proved better to use it in large excess, varying the concentration and reaction time to get an appropriate degree of reduction. It is important to do exploratory experiments to find appropriate conditions because peptides vary enormously in their reduction rates: for endothelin, 1 min reaction with 10 mM TCEP3 at room temperature gave a good distribution of products, whereas conotoxin J021 required 10 min at 62 °C. Test experiments can be done with small amounts of peptide, starting with mild conditions such as 10 mM TCEP, pH 3, for 2 min at room temperature. Reaction is then extended or cut back as appropriate. Little is lost by this approach because unreduced peptide is recovered in good yield (typically 90%) and can be used for further experiments. As a comparative guide, Table 4 gives approximate rates of reduction for several disulfides, expressed as fraction reduced per min under standard conditions using 10 mM TCEP, pH 3.

Table 4. *Relative rates of reduction by TCEP at pH 3*

Substrate	Bridges	Disulfide reduced	%/min[a]
Conotoxin GI-N	[2-7; 3-13]	2-7	6
		3-13	8
Conotoxin GI-A	[2-13; 3-7]	3-7	10
		2-13	12
[Hyp 5]-GI	[2-7; 3-13]	2-7	8
Endothelin	[1-15; 3-11]	1-15	16
		3-11	14
Insulin	[A6-A11; A7-B7; A20-B19]	A6-A11	2
		A7-B7	3
		A20-B19	0.2
Enterotoxin STp-N	[1-6; 2-10; 5-13]	1-6	16
Enterotoxin STp-R1	[1, 6; 2-10; 5-13][b]	2-10	30
Enterotoxin STp-R2	[1, 6; 2, 10; 5-13][b]	5-13	9
Conotoxin GVIA	[1-16; 8-19; 19-26]	All	<0.1

[a] Values given are expressed as % reduction per minute in 10 mM TCEP, pH 3, at 22 °C. They should be regarded as approximate only (±50%), because experiments were not designed for this purpose.

[b] Note that in these cases, the substrate was a peptide that already had been partially reduced as indicated.

Because the desired products are those in which reduction has opened some but not all bridges, conditions must be chosen to optimize yields of these. Steady-state levels are sometimes very low, and this may mean collecting unreduced peptide for recycling, rather than prolonging the reaction. The various partially reduced peptides are frozen until enough has been collected. When ample starting material is available, as with many synthetic peptides, this frugal but tedious approach can be bypassed. In this case, a large amount (e.g., 25–50 nmol) can be reduced in a single batch, yielding enough intermediates for alkylation and sequencing. Individual analyses described in the body of the text were usually made with 1–10 nmol, to gain experience under realistic conditions.

Peptide separation by HPLC

Reverse-phase HPLC with the widely used 0.1% TFA/acetonitrile system (Rivier et al., 1984) was chosen because of its exquisite sensitivity to nuances of hydrophobic character. It was expected that isomers differing only in terms of which of two or three disulfides was opened would thereby expose different parts of their interiors and hence be separable. This was generally realized, as can be seen in typical chromatograms (e.g., Figs. 1, 3, 5), and obviated any need for selectivity of reduction. A dramatic example is the insulin A chain, which elutes earlier by about 17% B when its internal disulfide A6–A11 is intact (Fig. 5, peak 1).

On occasion this simple picture is complicated by peptides that show anomalously broad or skewed peaks. This was encountered several times with multibridged peptides, most commonly when a single bridge of a tricyclic peptide had been opened, creating a destabilized molecule with limited flexibility. During chromatography, different conformers of the peptide may separate faster than they can reequilibrate, resulting in massive peak broadening. It was first seen with conotoxin MI (Gray et al., 1983). In the present context it occurred to some extent with enterotoxin STp, for which the singly reduced peptide peak was twice as broad as the others. This was aggravated by the fact that fully reduced peptide ran at the same position, but the final analysis was unambiguous (Fig. 9). It was a serious problem with other peptides not discussed in detail here (e.g., fragment of human complement C4a [Janatova & Gray, in prep.], conotoxin kappa [McIntosh et al., in prep.] and bovine pancreatic trypsin inhibitor [Gray, unpubl.]). In two of these cases, peak width increased by a factor of 10 or more. Fortunately, further reduction loosened the molecules, and sharp peaks were again obtained.

As a procedural matter, peptides were taken to dryness only when the fully alkylated form was ready for sequencing. Most often, peptides were processed immediately and were ready for injection as soon as the HPLC column was reequilibrated. In this way, a complex set of reactions

could be carried out in quick succession: in a single day, three consecutive reductions and alkylations with different reagents may be carried out on one peptide, or three different intermediates can be alkylated and repurified. Because peptides were always in the presence of sufficient acetonitrile to elute them from a C18 column, losses due to adsorption were small. Peptide recovery, monitored by peak integration, was routinely in the range 85–100%.

Before reinjecting material onto the HPLC column, it is usually necessary to dilute the acetonitrile present in the sample with at least an equal volume of aqueous buffer, or peptides may not be retained. This dilution is intrinsic to several procedures described above, such as alkylation with iodoacetamide or further reduction with TCEP, but not to direct alkylation with vinylpyridine. Most frequently, reduced peptides eluted later than their oxidized counterparts. Alkylation, on the other hand, especially with vinylpyridine, produced more hydrophilic products, whose elution times could be greatly decreased. Additional dilution and a low starting concentration of buffer B are thus recommended to avoid premature elution of peptides with multiple pyridylethyl groups.

Thiolate–disulfide exchange and reoxidation

Both of these side reactions were encountered during the alkylation step. For all practical purposes, alkylating agents attack the thiolate ion and are thereby in competition with disulfide exchange. Likewise, the thiolate is oxidized readily by molecular oxygen. Reoxidation is less of a problem than exchange because it gives a straight nonanswer rather than a misleading one. It is the *labeled* peptide whose structure is analyzed directly in the sequencer, but the *unlabeled* peptide whose structure we need to deduce. Exchange must be minimized by using the most vigorous approach for alkylation of peptides whose behavior is not known in advance. Fortunately, even rapid exchange does not preclude a successful interpretation.

Thus, consider endothelin, a two-bridge peptide [1-15; 3-11]. Partial reduction should give two isomers [1, 15; 3-11] and [1-15; 3, 11], which can now be confidently assigned as EndoA and EndoB (Fig. 1b). They were produced at comparable rates but were quite different with respect to exchange. EndoA was relatively stable and easily alkylated. EndoB was very unstable, rapidly giving rise to EndoC, which in turn gave rise more slowly to EndoA. For purposes of discussion, let us assume that EndoC contains one or both of the exchange isomers C1 [1, 11; 3-15] and C2 [1-11; 3, 15]. The other monocyclic isomers, [1-3; 11, 15] and [1, 3; 11-15], contain two independent chain segments with unfavorable ring sizes (Zhang & Snyder, 1989) and are likely to play only a minor role in rapid exchanges.

Figure 10 diagrams the exchanges available to the four main isomers. For instance, EndoB gives rise to C1 by attack of Cys 3 thiolate on Cys 15 of the disulfide. Note that members of a complementary pair (A, B) or (C1, C2) cannot interconvert directly but only in a two-step process via a member of the other pair. In each of the four isomers shown, a disulfide keeps the chain compact, holding the Cys residues close. Such proximity probably underlies the rapid exchange reactions observed, most notably during attempts to alkylate EndoB and C. When the reactive thiolate is located within a bridged loop (Cys 3 or Cys 11), we may perhaps expect greater reactivity than when it is free to rotate away as with those on terminal segments (Cys 1 or Cys 15). Arrows in Figure 10 are stressed to indicate this idea. This simple picture is consonant with what is known for the relative stabilities of EndoA, B, and C.

Depending on how fast it can be done, alkylation may accurately reflect the original composition of any mixture of isomers or may be totally misleading. When the starting mixture is already at equilibrium, as in folding studies on BPTI (Creighton, 1975; Weissman & Kim, 1991; Goldenberg, 1992), the correspondence should usually be good. Exceptions would be if there were preferential labeling of thiols, or if the equilibrium distribution for partially alkylated peptides were not similar to the original. Starting with a single isomer, especially a less stable one, the product distribution will always be distorted, sometimes massively so.

Nonforcing treatment of EndoB with vinylpyridine at pH 7 led almost exclusively to the pyridylethyl derivatives of EndoA and C1, and even the most forcing conditions gave only about 30% of correctly labeled product (Fig. 2b). Vigorous labeling of EndoC alkylated all four Cys residues (Fig. 2f). A simple interpretation is that EndoC was a mixture of C1 and C2, though others are not ruled out. In the reaction with vinylpyridine, it is now clear that exchange was much faster than alkylation. Secondarily, at pH 7 there is likely to be highly preferential labeling of Cys 1, due to its pK_a being lowered by the adjacent α-amino group, further distorting the pattern. With vasopressin, uncomplicated by exchange, highly preferential alkylation of the α-amino group by acrylamide was observed (data not shown). Note that the "fast" arrows in Figure 10 diverge strongly away from EndoB and converge toward labeled EndoA and C1.

Apamin, which has analogous Cys residues to those in endothelin, but linked in the [1-11; 3-15] arrangement (Callewaert et al., 1968), likewise underwent very rapid exchange. In this case the "native" isomers are analogous to C1 and C2. Other peptides that showed rapid exchange included insulin reduced at the intrachain bond and enterotoxin reduced at the Cys 2–Cys 10 bond.

Given that some exchange is unavoidable and may be severe, how can one avoid being misled? First, a problem should be suspected if more "intermediate" peaks are observed upon HPLC than should arise from the molecule under study: no more than two for a two-bridged peptide, no more than six for a three-bridged one. Thus, endothe-

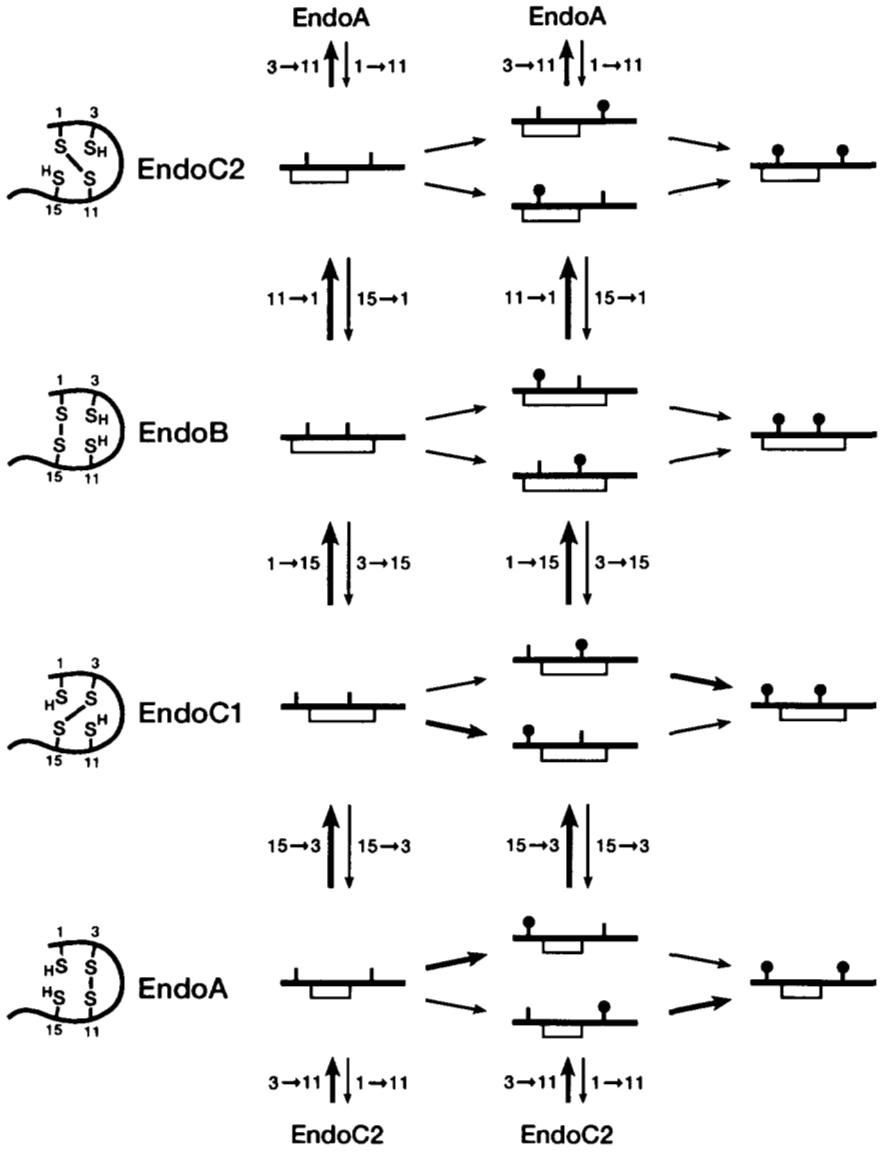

Fig. 10. Disulfide exchange and alkylation reactions of four isomers of partially reduced endothelin. Exchange reactions are shown on the vertical coordinate, with "wrap around" between top and bottom; numbers next to the arrows identify the attacking thiolate and the residue attacked. Alkylation reactions are shown on the horizontal coordinate. Heavy arrows indicate reactions that may be favored (see text). ⊥, Free -SH; T, blocked -SH; ▭, disulfide bridge.

lin (Fig. 1a) raised suspicion. Carrying out the reduction at pH 3 has eliminated most cases of exchange at this stage. Comparing reductions made at two different pHs (Fig. 1) or for different lengths of time will also distinguish primary reduction products from artefacts. Another useful diagnostic test with singly reduced species is to observe the effect of oxidation. Iodine, as used for making conotoxin GI isomers (Fig. 3), cleanly converts each of the various intermediates back to the appropriate starting peptide. Samples of 20 pmol are quite adequate for this test, which was used with conotoxin J021 to confirm that reduction at 62 °C had not led to massive rearrangement of the products (Shon et al., in prep.).

Exchange has more frequently been detected by the finding of multiple products during alkylation (Fig. 2a–c). When only one dominant peak has been present upon HPLC, it has always proved by sequencing to correctly represent the original in cases where the linkages were known in model peptides or where additional information was available from other reduction products. Where there is more than one significant alkylation product, the correct one may be identified by comparing profiles from peptides treated with greater or less stringency (endothelin B, J021; see also Weissman & Kim [1991]).

Alkylation and sequencing

The method was designed for peptides having multiple bridges in a relatively short chain, which could be sequenced directly. Labeling of individual bridges with different alkylating reagents could then reveal the complete disulfide pattern in a single sequencer run. Such reagents must satisfy several criteria: (1) highly reactive toward cysteine, but minimal side reactions with other amino acids;

(2) very soluble so that high concentrations can be used; (3) wash out cleanly at low %B on the HPLC system; (4) available in very high purity to avoid unexpected reactions of minor components, or tailing on HPLC; and (5) give well-behaved PTH derivatives that separate well in the standard HPLC systems.

Experience with rapid thiol–disulfide exchange forced a compromise on this issue. Only concentrated iodoacetamide proved suitable for several labile peptides and must be considered the reagent of choice. It is intrinsically about 10 times more reactive than iodoacetic acid (Creighton, 1975) and also caused much less reoxidation, perhaps related to its being less contaminated by heavy metals and, possibly, iodine. Thus it can be used as purchased and is relatively inexpensive (300 mg may be used to alkylate a single intermediate). The rapid alkylation procedure uses a molar excess of about 10^6–10^7, so adequate purity is crucial. As emphasized earlier, an important detail is the order of mixing: peptide must be added to reagent and not vice versa. In this way, peptide is never at neutral pH except in the presence of a high concentration of iodoacetamide. Limiting the reaction to 15–30 s and then quenching with acid have been adequate to avoid side reactions: all 20 amino acids including Trp, His, Met, and Tyr have been encountered without problem. One exception is peptides containing Cys(Pe), which appeared to be alkylated by iodoacetic acid under less vigorous conditions than those advocated here for iodoacetamide.

A drawback of iodoacetamide is that the resulting Cys(Cam) is not ideal for sequencer analysis. The primary derivative, PTH-Cys(Cam) elutes almost exactly with PTH-Glu, and there is also about 20–25% deamidation to PTH-Cys(Cm), which elutes almost exactly with PTH-Ser. This is not as serious as might be thought, because one is studying peptides of known sequence and asking about Cys labeling, rather than trying to distinguish Cys from Glu or Ser. It does, however, add a slight complication to double-label experiments with iodoacetamide and iodoacetic acid, in that allowance must be made for the contribution of Cys(Cm) arising from deamidation. In early cycles of a sequence analysis, there is also some breakdown (10–20%) of Cys(Cam) to a series of products that elute later. These are rarely significant beyond the third or fourth cycle.

In the most straightforward analysis, peptide intermediates are simply labeled with iodoacetamide and applied to the sequencer separately. An excellent example of this is the enterotoxin (Fig. 9a,b). Alternatively, peptides that have been alkylated with iodoacetamide can be further reduced and labeled with other reagents. With a single peptide, all bridges may be identified uniquely in the same sequencer run, as was also done with enterotoxin (Fig. 9c–e). Although this is elegant, it involves much more manipulation of peptides and the risk of losing material. One useful step, however, has been to completely reduce Cam-labeled peptides and alkylate with vinylpyridine or iodoacetic acid. Yields drop during a sequencer run, and toward the end it can be difficult to decide whether lack of Cys(Cam) is due to lack of labeling or loss of signal. Positive identification of Cys(Pe) or Cys(Cm) resolves this, allowing one to be more confident that disulfide exchange has not occurred. By using different secondary labels on related peptides, a single analysis can also be used to obtain information about both, as was shown with the isomers of conotoxin GI.

Alkylating agents such as vinylpyridine, but not iodoacetamide, are directly compatible with TCEP. It is thus possible to reduce peptides at neutral or basic pH, and concomitantly alkylate the nascent thiols. Such an approach was used previously with other reagents, combining $NaBH_4$ and iodoacetic acid in the analysis of conotoxin GI (Gray et al., 1984). It was not attempted here because it seemed unlikely that an appropriate balance could be struck among factors such as high reagent concentration versus HPLC loading, low reagent concentration versus reaction rate (and hence competition with exchange), etc. Doing the alkylation step without first separating peptides by HPLC has another drawback, in that one loses information about disulfide exchange that may be critical for interpreting results.

Scope and limitations of the method

This work has clearly shown a great potential for TCEP as a disulfide reagent for peptides. Several disulfide structures were solved by the partial reduction approach, which had not otherwise been analyzed directly by chemical methods. In addition to the model peptides described here, a number of previously unknown structures have been solved (Table 1), which will be described in detail in separate publications. Analysis of two-bridge structures is now routinely conducted in a single day and has been applied to intermediates in the synthesis of novel ω-conotoxins (Monje et al., 1993).

The various studies have also defined some limits of the approach, particularly with regard to thiol–disulfide exchange and alkylation. It is likely that the very high rates of exchange found for certain isomers of endothelin and apamin may be close to the upper limit at which identification of the correct products for analysis can occur, but they may also be among the highest rates that will be encountered. No doubt part of the high reactivity derives from close spatial proximity of two disulfides, so that thiols created when one of them is reduced are in prime position to attack the other. One approach to handling such problems would be to disrupt this favored status in some way, and an obvious method is to introduce nicks into the peptide chain. Enzymatic digestion with trypsin or chemical cleavage with cyanogen bromide are both candidates for endothelin, and trypsin should work also for apamin. These nicks would not produce separate peptides corresponding to the individual bridges, but two- or three-

chain molecules in which the partial reduction products should be distinguished quite readily. A similar approach has been used successfully with a trypsin-produced peptide from human complement fragment C4a, which had three disulfides linking three chains (Janatova & Gray, in prep.), and is quite analogous to the analysis of insulin described above. Enzymatic nicking may also be a way to improve chromatography of some, though not all, of the peptides that eluted as broad peaks.

A related problem is that of peptides that are resistant to TCEP, such as conotoxins GVIA and J021 (Shon et al., in prep.). Reduction at high temperature has been applied successfully in such cases, but with increased risk of disulfide exchange. Loosening the structure by chain nicking prior to reduction is another possibility to be explored as the need arises, but it is quite possible that resistance to TCEP may go hand in hand with resistance to proteases. Any such manipulation of the peptides must include safeguards against exchange during digestion.

One limit on application of the method as described concerns peptide size. The largest on which it has been applied directly is echistatin (four bridges in 49 residues, Table 1; Gray, 1993). At some point direct sequencing of the intact peptide chain will become hopelessly inefficient, and analysis of fragments will be necessary. This could be done after digestion of peptides carrying multiple labels or of peptides containing some labeled and some bridged cysteines. If the original molecule has tightly clustered cystines, it is expected that the pieces will contain two or more Cys residues, so that some form of sequencing will still be needed to determine the exact positions of particular labels. Mass spectrometry is a very attractive possibility for this: one would be asking questions about a known peptide, rather than establishing complete sequence de novo. It has already been used for disulfide analysis in a number of cases (Raschdorf et al., 1988; Hidaka et al., 1990; Zhou & Smith, 1990) and seems especially appropriate for handling complex digests of larger proteins. Combined with the method described here for differentially labeling the bridges, it may be feasible to analyze undigested peptides of moderate size. The ability of TCEP to reduce disulfides at acid pH has already been used to advantage with secondary-ion and laser-desorption mass spectroscopy (Craig et al., 1993; Fischer et al., 1993).

The emphasis in this paper has been to demonstrate how TCEP can be used for assignment of disulfides in particularly difficult cases, while kinetic aspects of reduction and exchange were purely incidental. It should be clear from the results with insulin and contoxin GI that the emphasis could easily be turned around. All products on the complex reduction path of insulin were identified (Fig. 8). With conotoxin GI it was also a simple matter to prepare all possible isomers and intermediates (Figs. 3, 4). Intramolecular disulfide exchange reactions of each of these (Fig. 4) can now be studied individually, allowing a complete set of 24 first-order kinetic constants to be measured for the six peptides having both thiols and disulfide.

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References

Blundell, T., Dodson, G., Hodgkin, D.C., & Mercola, D. (1972). Insulin: The structure in the crystal and its reflections in chemistry and biology. *Adv. Protein Chem. 26*, 279–402.

Brown, J.R. & Hartley, B.S. (1966). Location of disulfide bridges by diagonal paper electrophoresis. *Biochem. J. 101*, 214–228.

Buckwalter, B.L., Cady, S.M., Shieh, H.-M., Chaudhuri, A.K., & Johnson, D.F. (1992). Improvement in the solution stability of porcine somatotropin by chemical modification of cysteine residues. *J. Agric. Food Chem. 40*, 356–362.

Burns, J.A., Butler, J.C., Moran, J., & Whitesides, G.M. (1991). Selective reduction of disulfides by tris-(2-carboxyethyl)-phosphine. *J. Org. Chem. 56*, 2648–2650.

Callewaert, G.L., Shipolini, R., & Vernon, C.A. (1968). The disulfide bridges of apamin. *FEBS Lett. 1*, 111–113.

Craig, A.G., Mahe, E., Fischer, W.H., Gray, W.R., & Rivier, J.E. (1993). Strategy to determine disulfide bridge arrangements by selective reduction of cystines using TCEP. Abstract, 41st Annual Conference on Mass Spectrometry, San Francisco.

Creighton, T.E. (1975). Interactions between cysteine residues as probes of protein conformation. *J. Mol. Biol. 96*, 767–776, 777–782.

Fischer, W.H., Rivier, J.E., & Craig, A.G. (1993). *In situ* reduction suitable for MALDI and LSIMS ionization, using tris-(2-carboxyethyl)-phosphine. *Rapid Commun. Mass Spectrom. 7*, 225.

Goldenberg, D.P. (1992). Native and non-native intermediates in the BPTI folding pathway. *Trends Biochem. Sci. 17*, 257–261.

Gray, W.R. (1993). Echistatin disulfide bridges: Selective reduction and linkage assignment. *Protein Sci. 2*, 1749–1755.

Gray, W.R., Luque, F.A., Galyean, R., Atherton, E., Sheppard, R.C., Stone, B.L., Reyes, A., Alford, J., McIntosh, J.M., Olivera, B.M., Cruz, L.J., & Rivier, J.E. (1984). Purification and sequence of a presynaptic peptide toxin from *Conus geographus* venom. *Biochemistry 23*, 2796–2802.

Gray, W.R., Rivier, J.E., Galyean, R., Cruz, L.J., & Olivera, B.M. (1983). Conotoxin M1. Disulfide bonding and conformational states. *J. Biol. Chem. 258*, 12247–12251.

Hidaka, Y., Sato, K., Nakamura, H., Kobayashi, J., Ohizumi, Y., & Shimonishi, Y. (1990). Disulfide pairing in geographutoxin I, a peptide neurotoxin from *Conus geographus*. *FEBS Lett. 4*, 29–32.

Hoeprich, P.D. & Doolittle, R.F. (1983). Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel fashion. *Biochemistry 22*, 2049–2055.

Houghten, R.A., Ostresh, J.M., & Klipstein, F.A. (1984). Chemical synthesis of an octadecapeptide with the biological and immunological properties of human heat-stable *Escherichia coli* enterotoxin. *Eur. J. Biochem. 145*, 157–162.

Kumagaye, S.-I., Kuroda, H., Nakajima, K., Watanabe, T.X., Kimura, T., Masaki, T., & Sakakibara, S. (1988). Synthesis and disulfide structure determination of porcine endothelin: An endothelium-derived vasoconstricting peptide. *Int. J. Pept. Protein Res. 32*, 519–526.

Levison, M.E., Josephson, A.S., & Kirschenbaum, D.M. (1969). Reduction of biological substances by water-soluble phosphines: Gammaglobulin. *Experientia 25*, 126–127.

Monje, V.D., Haack, J., Naisbitt, S., Miljanich, G., Ramachandran, J., Nadasdi, L., Olivera, B.M., Hillyard, D.R., & Gray, W.R. (1993).

A new *Conus* peptide ligand for Ca channel subtypes. *Neuropharmacology 32*(10).

Nishiuchi, Y., Kumagaye, K., Noda, Y., Watanabe, T.X., & Sakakibara, S. (1986). Synthesis & secondary structure determination of ω-conotoxin GVIA: a 27-peptide with three intramolecular disulfide bonds. *Biopolymers 25*(Suppl.), S61–S68.

Nishiuchi, Y. & Sakakibara, S. (1982). Primary & secondary structure of conotoxin GI, a neurotoxic tridecapeptide from a marine snail. *FEBS Lett. 148*, 260–262.

Pink, J.R.L., & Milstein, C. (1967). Disulphide bridges of a human immunoglobulin G. *Nature 214*, 92–94.

Podlaha, J., & Podlahova, J. (1973). Compounds structurally related to complexone I. Tris(carboxyethyl)phosphine. *Collect. Czech. Chem. Commun. 38*, 1730–1736.

Raschdorf, F., Dahinden, R., Maerki, W., Richter, W.J., & Merryweather, J.P. (1988). Location of disulfide bonds in human insulin-like growth factors (IGFs) synthesized by recombinant DNA technology. *Biomed. Environ. Mass Spectrom. 16*, 3–8.

Rivier, J.E., Galyean, R., Gray, W.R., Azimi-Zonooz, A., McIntosh, J.M., Cruz, L.J., & Olivera, B.M. (1987). Neuronal calcium channel inhibitors: Synthesis of ω-conotoxin GVIA and effects on ^{45}Ca-uptake by synaptosomes *J. Biol. Chem. 262*, 1194–1198.

Rivier, J.E., McClintock, R., Galyean, R., & Anderson, H. (1984). Reversed-phase HPLC: Preparative purification of synthetic peptides. *J. Chromatogr. 288*, 303–328.

Ruegg, U.T. & Rudinger, J. (1977). Reductive cleavage of cystine disulfides with tributylphosphine. *Methods Enzymol. 47*, 111–126.

Ryle, A.P. & Sanger, F. (1955). Disulphide interchange reactions. *Biochem. J. 60*, 535–540.

Ryle, A.P., Sanger, F., Smith, L.F., & Kitai, R. (1955). The disulphide bonds of insulin. *Biochem. J. 60*, 541–556.

Shimonishi, Y., Hidaka, Y., Koizumi, M., Hane, M., Aimoto, S., Takeda, T., Miwatani, T., & Takeda, Y. (1987). Mode of disulfide bond formation of a heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *Escherichia coli*. *FEBS Lett. 215*, 165–170.

Spackman, D.H., Stein, W.H., & Moore, S. (1960). The disulfide bonds of ribonuclease. *J. Biol. Chem. 235*, 648–659.

Weissman, J.S. & Kim, P.S. (1991). Only native intermediates are populated in a revised protein folding pathway for BPTI. *Science 253*, 1386–1393.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., & Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature 332*, 411–415.

Zhang, R. & Snyder, G.H. (1989). Dependence of formation of small disulfide loops in two-cysteine peptides on the number and types of intervening amino acids. *J. Biol. Chem. 264*, 18472–18479.

Zhang, R. & Snyder, G.H. (1991). Factors governing selective formation of specific disulfides in synthetic variants of α-conotoxin. *Biochemistry 30*, 11343–11348.

Zhou, Z. & Smith, D.L. (1990). Location of disulfide bonds in antithrombin III. *Biomed. Environ. Mass Spectrom. 19*, 782–786.