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 cysteines. 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-
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 et al., 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>
<thead>
<tr>
<th>Peptide</th>
<th>Length(s)</th>
<th>Cys</th>
<th>Bridge pattern</th>
<th>Sequencea</th>
<th>Referencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conotoxin GI, N</td>
<td>13</td>
<td>4</td>
<td>1-3, 2-4</td>
<td>ECCNPACGRHYSC-NH₂</td>
<td>1, 2</td>
</tr>
<tr>
<td>Conotoxin GI, A</td>
<td>13</td>
<td>4</td>
<td>1-4, 2-3</td>
<td>ECCNPACGRHYSC-NH₂</td>
<td>1</td>
</tr>
<tr>
<td>Conotoxin GI, B</td>
<td>13</td>
<td>4</td>
<td>1-2, 3-4</td>
<td>ECCNPACGRHYSC-NH₂</td>
<td>1</td>
</tr>
<tr>
<td>Insulin</td>
<td>(30, 21)</td>
<td>6</td>
<td>1-4, 2-6, 3-5</td>
<td>(InsB) FVNOQHLGCISHLEVEYLVCGERFFYTPKA-OH</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(InsA) GIVEQCCAVCSLYQLENYCN-OH</td>
<td>3</td>
</tr>
<tr>
<td>Endothelin</td>
<td>21</td>
<td>4</td>
<td>1-4, 2-3</td>
<td>CCCCSSLMDKEVVYFCCHLDIIM-OH</td>
<td>4</td>
</tr>
<tr>
<td>Apatin</td>
<td>18</td>
<td>4</td>
<td>1-3, 2-4</td>
<td>CINCKAPELALCARRCQOH-NH₂</td>
<td>5</td>
</tr>
<tr>
<td>Enterotoxin STp(5-18)</td>
<td>14</td>
<td>6</td>
<td>1-4, 2-5, 3-6</td>
<td>CCCECNAPACAGCY-OH</td>
<td>6</td>
</tr>
<tr>
<td>Conotoxin GV1A</td>
<td>27</td>
<td>6</td>
<td>1-4, 2-5, 3-6</td>
<td>CKSOGSSCSSOTSYNNCRSCNOYTKRCY-NH₂</td>
<td>7</td>
</tr>
</tbody>
</table>

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 |

\[\text{References:} \] Nishiuchi and Sakakibara (1982); Gray et al. (1984); Ryle et al. (1955); Kumagaye et al. (1988); Callewaert et al. (1968); Shimonishi et al. (1987); Nishiuchi et al. (1986); Olivera et al. (in prep.); McIntosh et al. (in prep.); Shon et al. (in prep.); Janatova and Gray (in prep.); 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 (10⁻⁷–10⁻⁹ 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).

Alkylation 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% deamination 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%/40%, 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 ace tone, 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-
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, 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 Na₂EDTA 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 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 iodoacetamide 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 I₂).

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

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.

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

<table>
<thead>
<tr>
<th>pH</th>
<th>Min</th>
<th>N</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R</th>
<th>%/min</th>
<th>Rel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.5</td>
<td>69.5</td>
<td>13.3</td>
<td>2.8</td>
<td>5.7</td>
<td>0.9</td>
<td>6.8</td>
<td>36.3</td>
<td>1.00</td>
</tr>
<tr>
<td>4.0</td>
<td>0.5</td>
<td>71.8</td>
<td>10.7</td>
<td>6.7</td>
<td>3.9</td>
<td>0.8</td>
<td>5.4</td>
<td>32.9</td>
<td>0.91</td>
</tr>
<tr>
<td>3.0</td>
<td>0.5</td>
<td>79.8</td>
<td>6.4</td>
<td>6.7</td>
<td>1.3</td>
<td>nd</td>
<td>4.9</td>
<td>24.2</td>
<td>0.67</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>82.5</td>
<td>8.4</td>
<td>6.0</td>
<td>0.6</td>
<td>nd</td>
<td>2.0</td>
<td>3.2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*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 A220 that was measured under each component. Percentage of disulfides reduced per minute. Rate, relative to that at pH 5.0.
Disulfide analysis of complex peptides

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 ([l, 1, 13; 3-15] and [l-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].

Conotoxin GI and isomers

Conotoxin GI
[2-7; 3-13]

The natural bridging pattern in this 13-residue bicyclic peptide has been established as [2-7; 3-13] (Nishiuichi & Sakakibara, 1982; Gray et al., 1984); the other isomers [2-3; 7-13] and [2-13; 3-7] have also been described (Nishiuichi & 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*), 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 I₂. 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-
**Insulin**

**Insulin B**


**Insulin A**

C10.N–OH

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-
Disulfide analysis of complex peptides

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.

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(Cam2)) was purified and subjected to further partial reduction, producing Ins3(Cam2, SH2). This was alkylated with 4-VP to give Ins3(Cam2, Pe2). 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 alklylation 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
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 several experiments. a: Alkylation of Ins3 with iodoacetamide gives small reoxidation peak 1, minor reagent peaks 2, and Ins3* (= Ins3(Cam1)), c: Reduction of Ins3* with TCEP (10 mM, pH 3, 20 °C, 8.5 min) gives residual Ins3*, mainly Ins6* (= Ins6(Cam2)), and small amounts of alkylated A and B chains (InsA*, InsB*) from overreduction of Ins3*. d: Alkylation with 4-VP gives mainly Ins6** (= Ins6(Cam2, Pe2)), plus a little reoxidation to Ins3*. e: Reduction with TCEP (10 mM, pH 3, 65 °C, 10 min) gives InsA** (= InsA(Cam1, Pe2)) and InsB*, which were finally alkylated with iodoacetic acid (not shown).

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**Table 3. Peptides from partial reduction of insulin**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Time (min)</th>
<th>Yield (%)</th>
<th>Reduction products</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.8</td>
<td>0.15</td>
<td>A only</td>
<td>R2</td>
</tr>
<tr>
<td>N</td>
<td>15.0</td>
<td>73.45</td>
<td>2, 3, 4</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>16.8</td>
<td>5.46</td>
<td>5, 6</td>
<td>R1</td>
</tr>
<tr>
<td>3*</td>
<td>17.9</td>
<td>4.59</td>
<td>(1), 6, 7</td>
<td>R2</td>
</tr>
<tr>
<td>4</td>
<td>19.2</td>
<td>0.61</td>
<td>1, 5, 6</td>
<td>R1</td>
</tr>
<tr>
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<td>2.59</td>
<td>B only</td>
<td>B</td>
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<tr>
<td>A</td>
<td>20.6</td>
<td>0.96</td>
<td>A only</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>21.3</td>
<td>0.38</td>
<td>A; B; fast</td>
<td>R13</td>
</tr>
<tr>
<td>6</td>
<td>21.7</td>
<td>11.81</td>
<td>A; B; slow</td>
<td>R12</td>
</tr>
</tbody>
</table>

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

**Bacterial enterotoxin**

*Enterotoxin ST<sub>p</sub>(5-18)*

C<sup>1</sup>, C<sup>2</sup>, E.L.C<sup>3</sup>, C<sup>6</sup>, N.P.A.C<sup>10</sup>.A.G.

C<sup>13</sup>, Y–OH [1-6; 2-10; 5-13]

Synthetic enterotoxin ST<sub>p</sub>(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 (Enter0N) 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 (Enter01, 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 Enter0N with TCEP gave fully reduced peptide (Enter0R), which co-eluted with Enter01, 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 Enter01 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 Enter01, was also straightforward, and the major products (Enter02 or Enter02(Cam2)) 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...
Disulfide analysis of complex peptides

Fig. 8. Pathways of reduction for insulin. The native molecule (N) can be attacked at each of the three disulfides, giving products R1, R2, and R3. These in turn can be reduced to R12, R13, and R23 (which is a mixture of A' and B). The third stage of reduction (R123) 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.

two stages of reduction and then alkylated or was reduced and alkylated in two discrete steps. Sequencer analysis of Entero2(Cam4) 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 J021

Conotoxin GVIA

C\textsuperscript{1}.K.S.O.G.S.S.C\textsuperscript{8}.S.O.T.S.Y.N.C\textsuperscript{15}.C\textsuperscript{16}.R.S.C\textsuperscript{19}.N.O.Y.T.K.R.C\textsuperscript{26}.Y-NH\textsubscript{2}\space[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 NaBH₄ 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):

\[
R_1-S-S-R_2 + X_3P + H_2O \rightarrow R_1SH + R_2SH + X_3PO.
\]

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., ConoN₁, 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 (X₃PH⁺) rather than the phosphate (X₃P), because its pKᵢ is 7.66 (Podlahra & Podlahova, 1973). The results suggest that a group with pKᵢ of approx. 3 is influential. A likely explanation is that reduction by X₃PH⁺ 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⁺(CH₂·CH₂·COOH)₃·CH₂·CH₂·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.,...
**Disulfide analysis of complex peptides**

1972). In another context an attempt was made to use TCEP for removing a tert-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**

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

^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.

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 non-answer 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. Secondly, at pH 7 there is likely to be highly preferential labeling of Cys 1, due to its pKₐ 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-
Disulfide analysis of complex peptides

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 $10^5\cdot10^7$, 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 breakdown (10–20%) of Cys(Cam) to a series of products. In early cycles of a sequence analysis, there is also some ambiguity about 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 vinylpyr-
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 GV1A 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 spectrometry (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 conotoxin 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


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


