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# New Cyclization Reaction at the Amino Terminus of Peptides and Proteins

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Mild oxidation with periodate of the 1-amino-2-ol moiety of N-terminal seryl or threonyl peptides and proteins leads to a terminal aldehyde function O═CH−CO− which usually may be exploited for bioconjugate formation (e.g., via oximation with an O-alkyl hydroxylamine). We report that, when followed by a prolyl residue, the O═CH−CO− group can undergo a rapid cyclization and dehydration reaction through nucleophilic attack by the amide nitrogen of the third amino acid residue of the chain. We have characterized the resulting heterocycle, which is stable in aqueous acid, by mass spectrometry and NMR. Quantitative oximation can nevertheless be achieved in such cases by performing a one-pot oxidation-oximation without isolation of the intermediate aldehyde, as is demonstrated with cholera toxin B subunit.

## INTRODUCTION

The oxidation by periodate of N-terminal 1,2-amino alcohols Ser (R = $CH_2OH$) or Thr [R = $CH(OH)CH_3$] residues $NH_2$−CHR−CO− of a polypeptide to an aldehyde function O═CH−CO− has been known for many years (*1*). More recently, this mild oxidation reaction has been exploited for the synthesis of bioconjugates through formation of hydrazone, thiazolidine, or oxime (−ON═CH−CO-) bonds (e.g., refs *2*−*8*). Usually, the polypeptide aldehyde is stable in aqueous solution at pH values between 2 and 5 and may be lyophilized and stored as a powder at −20 °C (*4*). In some cases, however, the aldehyde can undergo side reactions. For example, formation of a 1,3,4-oxadiazolino peptide containing two peptide aldehyde units has been observed during attempted hydrazone formation (*9*).

We have observed that some polypeptides and proteins, including Cholera toxin B subunit (CTB), undergo rapid and quantitative oxidation to yield a compound which may be isolated and has the mass calculated for the expected O═CH−CO-polypeptide species, but oximation occurs very slowly indeed (half time estimated in weeks under conditions where normally the half time is of the order of very few minutes). The first protein which gave such difficulties was a $\beta$-lactamase (*5*), and in that case, the problem was solved by careful optimization of the conditions of oxidation of the N-terminus and storage of the aldehyde, but such an approach was not successful with CTB. We were able to reproduce the phenomenon of oxidation followed by very slow oximation using a small synthetic peptide consisting of the first six residues of the $\beta$-lactamase, Thr-Pro-Val-Ser-Glu-Lys-amide. In this article, we show that, when followed by a prolyl residue, the O═CH−CO− group formed by oxidation of N-terminal Ser or Thr can undergo a rapid cyclization and dehydration reaction through nucleophilic attack by the amide nitrogen of the third amino acid residue of the chain. The resulting heterocycle, which is stable in aqueous acid, was characterized by mass spectrometry and NMR. To achieve quantitative oximation in such cases, we developed a one-pot oxidation-oximation without isolation of the intermediate aldehyde and demonstrate here with CTB that it is possible to prepare in good yield a hybrid construct of which 60% of the mass is the B subunit and 40% is totally synthetic. CTB conjugates are potentially useful as vaccines: CTB can supply T helper epitopes and, if the construct assembles as its noncovalent pentamer, such constructs would be expected to elicit a mucosal immune response (e.g., refs *10* and *11*).

## EXPERIMENTAL PROCEDURES

**Materials.** Synthetic peptides were prepared using Boc chemistry on a model 430A synthesizer (Applied Biosystems Inc.) modified according to Kent (*12*). $^{15}N$ (99 atom %) $N^t$-Boc-L-valine was purchased from Isotec. Methylbenzhydrylamine resin (NovaBiochem, Switzerland) was used to obtain peptide amides directly upon cleavage with HF. Synthetic peptides were purified by high-pressure liquid chromatography (HPLC), acetonitrile was removed by rotary evaporation at room temperature, and product was recovered by lyophilization. Purified peptides gave a single peak on analytical HPLC and the expected mass spectrum. The aminooxyacetyl peptide $NH_2OCH_2CO$-$Gly_3$[Lys(Ser)]$_5$Gly-OH was prepared as previously described (*13*). Cholera toxin B subunit (CTB, Inaba strain) was obtained from the Swiss Serum and Vaccine Institute, Berne, Switzerland. Sodium metaperiodate and guanidine hydrochloride (MicroSelect grade) were purchased from Fluka, Buchs, Switzerland. Boc-aminooxyacetic acid was from Calbiochem-NovaBiochem, Läufelfingen, Switzerland.

**Chromatography.** High-pressure liquid chromatography was performed using a column of Nucleosil 300A 5 $\mu$m $C_8$ (Macherey Nagel, Oensingen, Switzerland), 250

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× 4 mm id for analytical work (0.6 mL/min, 2 mL loop) and a radial compression system (210 × 25 mm id, Nova-Pak HR 60A 6 μm $C_{18}$, 20 mL/min, 50 mL loop) for preparative work, monitoring at 214 or 229 nm for analytical or preparative work, respectively. Linear gradients were established between solvent A [1 g of trifluoroacetic acid (TFA) added to 1 L of HPLC grade water and then vacuum filtered and degassed] and solvent B (1 g of TFA added to 100 mL of water, brought to 1 L with gradient grade acetonitrile then vacuum filtered and degassed). Gel filtration was performed on a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) at a flow rate of 0.4 mL/min using 0.25 M phosphate buffer, pH 7.0 (counterion sodium), which was 1 mM in EDTA, monitoring at 280 nm.

**Mass Spectrometry.** Spectra were obtained using electrospray ionization on either a Trio 2000 (infused at 2 μL/min) or Platform II instrument (infused at 7 μL/min); both machines were from Micromass, Altrincham, U.K. Samples of synthetic peptides were dissolved in water/acetonitrile 1:1 by volume, whereas protein samples were dissolved in water/acetonitrile/acetic acid (49.5:49.5:1 by volume).

**NMR spectroscopy** (*14*) was performed on a Bruker AMX-2−600 spectrometer operating at 14.1 T. Peptide samples were dissolved in $D_2O$ at pD 4.7 (DCl). After acquisition of all data, samples were reanalyzed by HPLC to check for possible decomposition. Two-dimensional experiments were performed at 298 K. $^1H/^1H$-DQF−COSY spectra were acquired using 512 × 16K data points and a spectral width of 12 ppm in both dimensions. A shifted sine bell function was applied to the indirectly detected dimension during data processing. The heteronuclear HSQC (*15*−*17*) and HMBC experiments (*18*−*19*) were performed with 128 × 4K data points and spectral widths of 90 × 10.69 ppm in the carbon and proton dimension, respectively. Folded signals could easily be identified as such. For the processing, sine bells were applied in both dimensions.

**Oxidation of TPVSEK-amide.** To a solution of purified hexapeptide amide (17.6 mg, 26.8 μmol of peptide, 28 mL of water, 2.8 mL of 10% ammonium bicarbonate solution) was added 5.6 mL of sodium metaperiodate solution (0.184 g/L in water, 4.8 μmol) with vortex mixing. As soon as possible, the reaction was quenched by mixing in 5.6 mL of ethyleneglycol (10 M in water) and injecting the reaction mixture onto preparative HPLC. After a 5 min isocratic period, a linear gradient of 1% B/min was established. Once the three components had eluted (within 30 min), the column was washed briefly at 50% B then reequilibrated at 100% A. Organic solvent was removed by rotary evaporation at room temperature and product recovered by lyophilization. From 85 mg of peptide, we isolated 39 mg of peak 1, 4.6 mg of peak 2 and 4.6 mg of peak 3.

**Oximation of Oxidized TPVSEK-amide.** For the study at pH 4.6, the buffer was 0.1 M acetate, counterion sodium. In separate experiments, both forms of the oxidized hexapeptide amide (peaks 2 and 3, 0.3 mg in 60 μL buffer) were treated with aminooxyacetic acid (60 μL, 5 mM in buffer). For the study at pH 7.0, a phosphate buffer was used (0.1 M, counterion sodium) in place of acetate. The oximation reactions were monitored by HPLC, injecting 10 μL volumes with a gradient from 0 to 35% B over 30 min.

**Oxidation and Oximation of CTB.** CTB was supplied as a lyophilized powder in the presence of buffer salts, including Tris which might be expected to react with periodate. Vial contents (1 mg of protein) were therefore dissolved in 1 mL of water, and the protein was isolated by gel filtration as the noncovalent pentamer. The protein was concentrated by ultrafiltration (Centricon, Amicon, Beverly, MA, 10000 molecular weight cutoff, precentrifuged twice with 1 mL of phosphate chromatography buffer to remove glycerol which interferes with the oxidation step) to 400 μL. Methionine (50 μL, 0.2 M in the phosphate buffer) was added to protect against thioether oxidation, then oxidation of the N-terminal Thr residue was initiated by addition of sodium periodate (25 μL, 40 mM in the phosphate buffer). After 5 min in the dark, excess periodate was quenched with ethyleneglycol (60 μL, 0.5 M in water), then 4 min later, 27.8 mg of aminooxyacetyl peptide $NH_2OCH_2CO$-$Gly_3[Lys(Ser)]_5$Gly-OH was added, followed by 50 μL of acetic acid. An aliquot (15 μL) was removed for HPLC and mass spectrometric analysis after 30 min, whereupon the sample was placed at −20 °C overnight. The next day, 500 mg of guanidine hydrochloride was added and the protein oxime isolated by gel filtration and ultrafiltration to a volume of about 400 μL. Oxidation of the Ser residues on the Lys side chains of the peptide attached to CTB was achieved by adding methionine (150 μL, 0.2 M in phosphate buffer) followed by periodate (62.5 μL, 80 mM in phosphate buffer). After 5 min in the dark, the reaction was quenched with 1,3-diamino-2-propanol (Fluka, 300 μL, 0.5 M in water adjusted to pH 7.8 with acetic acid). Four minutes later, 500 mg of guanidine hydrochloride was added, and the oxidized protein isolated again by gel filtration and concentrated by ultrafiltration to about 400 μL. A second 400 μL sample (from a separate experiment) was pooled at this stage. To the pool (800 μL), aminooxyacetyl peptide $CH_3CO$-$DC(CH_2CONH_2)$-TLIDALLGDPHK$(COCH_2ONH_2)$-$NH_2$ was added (5.7 mg), and the solution made 4% in acetic acid. For every microliter of this solution, 1 mg of guanidine hydrochloride was added and the oximation left overnight. Final product was isolated by semipreparative HPLC (1 mg after lyophilization) and characterized by mass spectrometry.

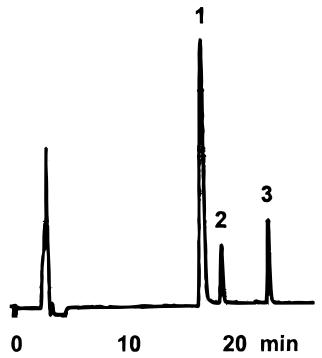



**Figure 1.** Preparative HPLC chromatogram of the oxidation of TPVSEK-amide with a limited quantity of periodate. After a 5 min isocratic period at 100% A, a gradient of 1%/min solvent B was applied, monitoring at 229 nm. Mass spectrometry and NMR identified components as peak 1, unmodified peptide; peak 2, hydrated glyoxylyl peptide $(HO)_2CHCO-$PVSEK-amide; peak 3, cyclic isomer of the expected O=CHCO−PVSEK-amide.

## RESULTS AND DISCUSSION

**Oxidation of TPVSEK-amide.** To study the rapid oxidation of the terminal 1-amino-2-ol moiety of **1**, oxidation with periodate was performed with limiting amounts of periodate and a reaction time of a few seconds (to allow mixing). This allowed isolation of three components by HPLC (Figure 1). Peak 1 was identified by retention time and mass spectrum as unreacted starting

peptide (abundant signal at *m/z* 660.0 corresponding to the protonated molecular ion, most intense peak at *m/z* 330.9, the doubly protonated molecule, data not shown). Peak 2 gave a mass spectrum with signals at *m/z* 614.5, 632.5, and 654.4. The most intense peak *m/z* 632.5 corresponds to the hydrated form of the aldehyde, $(HO)_2CHCO-$PVSEK-amide (**2**) with one proton, and *m/z* 654.4 corresponds to sodium cationization of the same species. The small signal at *m/z* 614.5 in the spectrum of peak 2 became the most intense peak in the spectrum of peak 3, where it was accompanied by *m/z* 636.6. These two signals correspond to O=CHCO−PVSEK-amide (i.e., the aldehyde) with one proton and with one sodium ion, respectively, although we show below that peak 3 is actually a cyclic isomer (**3**) of the aldehyde. Peak 2 material, upon storage in aqueous solution at pH 4.6 (sodium acetate buffer), was found by HPLC and mass spectrometry to transform to peak 3 material over a number of days, but was more stable in 0.1% TFA at pH 2 (not shown). Thus, the signal at *m/z* 614.5 in the spectrum of Peak 2 material could be due to dehydration of the hydrate within the mass spectrometer to give either the aldehyde itself or its isomer (**3**). Peaks 1, 2, and 3 were found to be stable for days in aqueous solution at pH 2 at room temperature, and for months as lyophilized powders at −20 °C (not shown).

**Oximation of oxidized TPVSEK-amide** (peaks 2 and 3) was studied at pH 4.6 and pH 7.0. It has previously been shown that oximation proceeds faster at lower pH, and very slowly indeed at neutral pH (*7*). Oximation of peak 2 (the hydrated aldehyde) with aminooxyacetic acid at pH 4.6 was found to proceed extremely rapidly. Figure 2a shows almost quantitative reaction after 130 min: reaction halftime was approximately 10 min judging by earlier time points (not shown). The expected oxime product was identified by mass spectrometry through its protonated molecular ion at *m/z* 687.7. A trace of peak 3 material is also formed, which elutes 2 min after the oxime (ox). Oximation of peak 3 (which has the mass of the aldehyde) proceeded extremely slowly at pH 4.6, with very little product formed after 130 min (Figure 2b). The oxime was nevertheless being formed (it elutes 2 min earlier than peak 3 and is just visible in Figure 2b), and it represented about 12% after 35 h (product confirmed by mass spectrometry, not shown).

At pH 7.0, almost exactly the same chromatograms were obtained during oximation of peak 2 (Figure 2c) and peak 3 (Figure 2d). After 10 min, both showed presence of peak 2 and peak 3, with virtually no oxime product. At this pH, peaks 2 and 3 clearly interconvert rapidly: equilibrium is almost attained after 10 min, but not quite since we notice that Figure 2c is slightly richer in the starting peak 2 whereas Figure 2d is still slightly richer in the starting peak 3. In both cases, oxime formation proceeded very slowly, reaching about 30% after 10 h (not shown). Thus, although oximation proceeded very slowly at pH 7.0 for both peaks 2 and 3, it nonetheless proceeded more rapidly than for peak 3 at pH 4.6 (but much less rapidly than for peak 2 at pH 4.6).

These results were difficult to understand. For years it has been known that small aliphatic aldehydes (such as heptanal), many peptides and proteins carrying a O=CH−CO−NH−CHR−CO− group (where R is the side chain of a genetically coded amino acid), many polypeptides carrying a $-NHCH_2CH=O$ group, and many peptides carrying a O=CH−CO− group attached to the side chain of Lys undergo rapid and almost quantitative oximation at mildly acid pH. In the case of the oxidized small synthetic peptide TPVSEK-amide, the hydrated

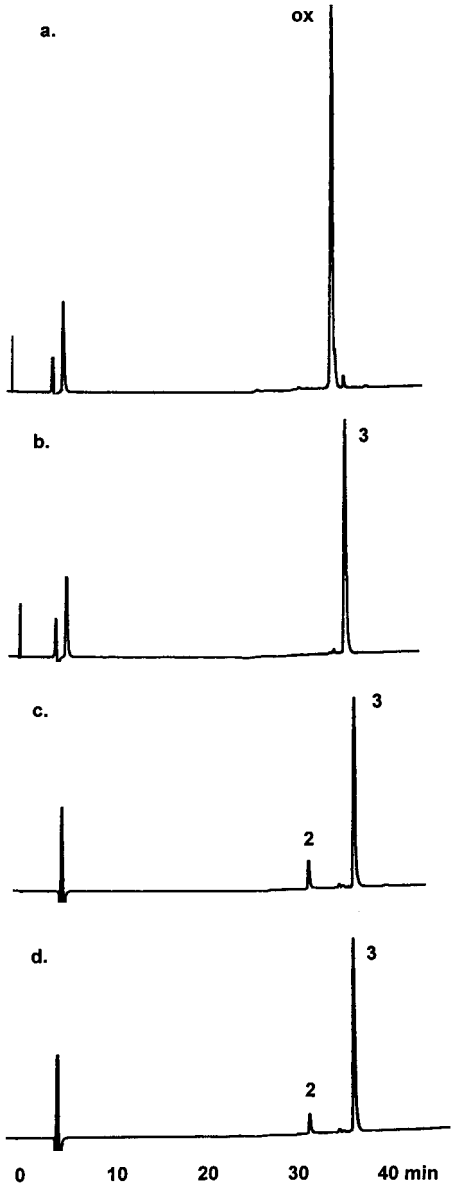



**Figure 2.** Analytical HPLC chromatograms of oximation reactions of peaks 2 and 3 of Figure 1. Remaining material is labeled 2 or 3, accordingly. Oxime product (ox) was identified by mass spectrometry. After a 5 min isocratic period at 100% A, a gradient of 1.17%/min solvent B was applied, monitoring at 214 nm. (a) Peak 2, pH 4.6, 130 min. (b) Peak 3, pH 4.6, 130 min. (c) Peak 2, pH 7, 10 min. (d) Peak 3, pH 7, 10 min.

aldehyde (peak 2) was able to undergo rapid oximation at pH 4.6, but the apparent aldehyde (peak 3) was not. This led us to investigate by NMR spectroscopy the structure of peaks 1, 2, and 3.

**NMR Analysis.** The $^1H/^1H$-DQF−COSY spectra of compounds **1**, **2**, and **3** allowed assignment of all resonances of hydrogen atoms bound to carbon (*14*). Since the amino acid residues differ all in their spin systems, the assignment was straightforward and unambiguous. Data for $^1H$ chemical shifts are compiled in Table 1.

Comparison of the $^1H$-spectra of **1** and **2** immediately confirmed the Thr residue as the site of oxidative cleavage. The three signals of Thr of **1** are missing in compound **2** and are replaced by a singlet at $\delta$ (ppm) 5.58. This is the resonance of the H−C(2) proton of the newly formed dihydroxyacetyl residue [the former H−C(α) of Thr]. That we were dealing with a hydrated aldehyde was corroborated by an HSQC-experiment (*15*−*17*) which correlates this proton to a $^{13}C$ signal at $\delta$ (ppm) 86.0 via

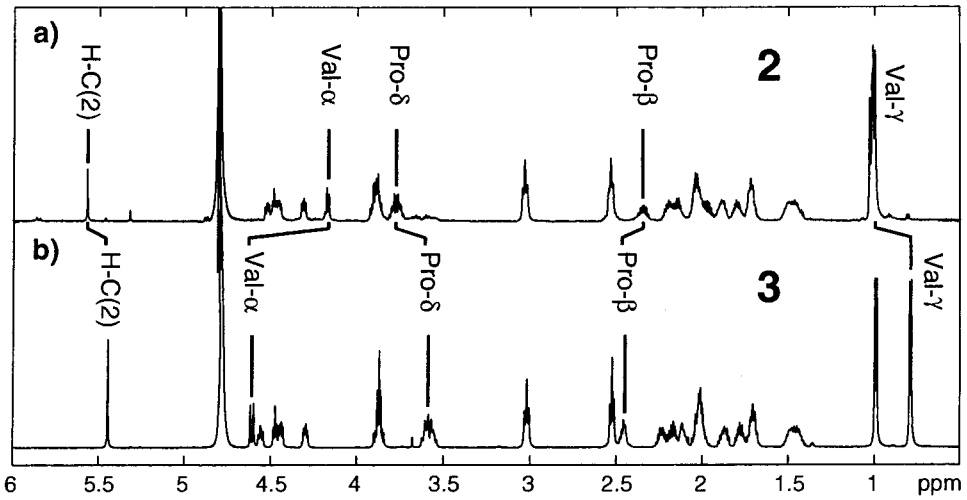



**Figure 3.** $^1$H NMR spectra recorded at 600 MHz in $D_2O$ of (a) compound **2** and (b) compound **3**, with assignment of those resonances which change significantly upon cyclization.

**Table 1. $^1$H Chemical Shifts for the Amino Acid Residues of Compounds 1, 2, and 3 at 600 MHz in $D_2O$ (pD 4.7)**

| residue | position | 1 | 2 | 3 |
|---|---|---|---|---|
| Thr (T) or modified residue[a] | H−C(2) | 4.26 | 5.58 | 5.46 |
| | H−C(β) | 4.20 | | |
| | $H_3C(\gamma)$ | 1.39 | | |
| Pro (P) | H−C(α) | 4.58 | 4.52 | 4.58 |
| | $H_2C(\beta)$ | 1.93/2.36 | 1.97/2.34 | 2.03/2.47 |
| | $H_2C(\gamma)$ | ∼2.10 | ∼2.03 | 2.04/2.13 |
| | $H_2C(\delta)$ | 3.72/3.83 | 3.75/3.77 | 3.57/3.61 |
| Val (V) | H−C(α) | 4.12 | 4.17 | 4.63 |
| | H−C(β) | 2.10 | 2.14 | 2.25 |
| | $H_3C(\gamma)$ | 0.97/1.00 | 0.99/1.01 | 0.81/1.01 |
| Ser (S) | H−C(α) | 4.46 | 4.47 | 4.49 |
| | $H_2C(\beta)$ | 3.86/3.88 | 3.86/3.88 | 3.89 |
| Glu (E) | H−C(α) | 4.45 | 4.44 | 4.45 |
| | $H_2C(\beta)$ | 2.00/2.19 | 1.96/2.18 | 2.04/2.18 |
| | $H_2C(\gamma)$ | 2.50 | 2.51 | 2.55 |
| Lys (K)−(amide) | H−C(α) | 4.30 | 4.30 | 4.31 |
| | $H_2C(\beta)$ | 1.78/1.86 | 1.79/1.88 | 1.80/1.89 |
| | $H_2C(\gamma)$ | 1.42/1.46 | 1.45/1.49 | 1.47/1.50 |
| | $H_2C(\delta)$ | 1.70 | 1.70 | 1.72 |
| | $H_2C(\epsilon)$ | 3.03 | 3.01 | 3.04 |

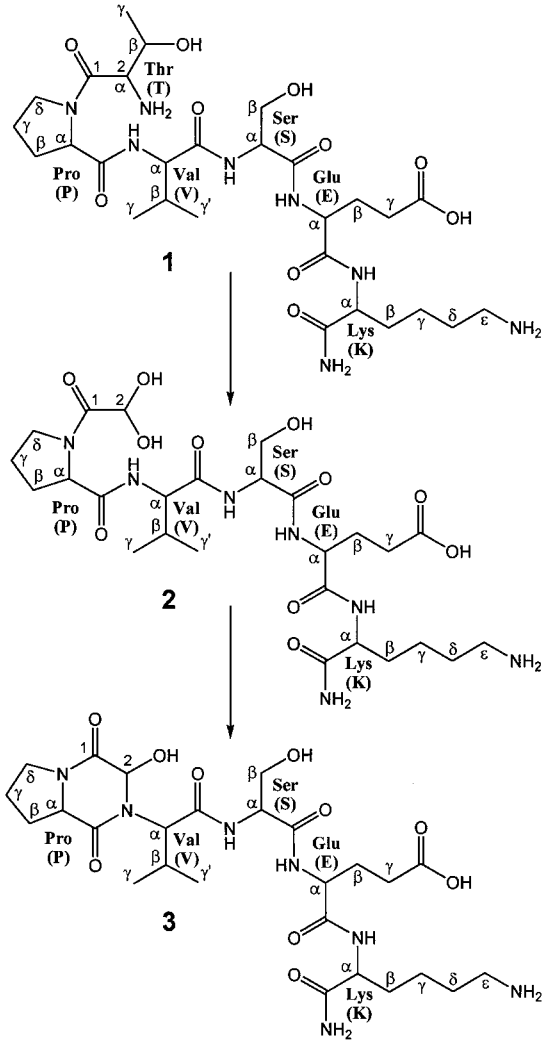
[a] Dihydroxyacetate for **2**, and H−C(2) of the new six-membered heterocycle for **3**.

$^1J$ scalar coupling. The $^1$H chemical shifts of the remaining five residues (Pro, Val, Ser, Glu, and Lys-amide) show a remarkable similarity between compounds **1** and **2**. Clearly, these amino acid residues were not altered.

However, when comparing compounds **2** and **3** (Table 1 and Figure 3), it was evident that the dehydration reaction resulted in significant changes of the Pro and Val resonances. One of the methyl groups of Val had undergone an upfield shift of 0.2 ppm, whereas H−C(α) of Val changed from 4.17 to 4.63. A significant change (5.58 → 5.46) was observed also for the H−C(2) resonance of the oxidized residue. The signals of the three remaining amino acid residues Ser, Glu, and Lys-amide are almost identical for **2** and **3**. These findings clearly show that only Val and Pro sense the new molecular environment in **3**. Bond formation between the nitrogen atom of Val and C(2) of the oxidized residue, i.e. the disguised aldehyde, best accounts for our observations (Scheme 1).

The conclusions found strong support by a HMBC experiment (*18, 19*), which unveiled scalar $^1$H/$^{13}$C coupling between H−C(α) of Val and C(2) of the oxidized residue. The absolute value of this $^3J_{C/H}$ coupling is 4.6 Hz. The existence of the crucial C(2)−$N_{(Val)}$ bond was

**Scheme 1. Structures of Unoxidized Peptide (1), Hydrated Glyoxylyl Peptide (2), and Cyclic Isomer of Glyoxylyl Peptide (3)**




eventually ascertained by specific $^{15}$N-labeling of compound **3** in the $N_{(Val)}$ position. The $^{13}$C NMR spectrum of this labeled material [$^{15}N_{(Val)}$-**3**] shows $^1J_{C/N}$ coupling for three carbon atoms, which are C(2) [at $\delta$ = 79.9, $^1J_{C/N}$ = 7.6 Hz], $C(\alpha)_{(Val)}$ [at $\delta$ = 64.4, $^1J_{C/N}$ = 8.4 Hz], and $CO_{(Pro)}$ [at $\delta$ = 175.9, $^1J_{C/N}$ = 12.8 Hz]. In addition, $^{15}N_{(Val)}$ couples

**Table 2. Ease of Oximation of Various Oxidized Polypeptides**

| N-terminal sequence | source | oximation after isolation of oxidized polypeptide |
|---|---|---|
| SAKELR..... | IL-8 | easy |
| SLAADT..... | MIP-1α | easy |
| SQLHSG..... | GCSF fragment | easy |
| TPVSEK..... | β-lactamase | possible[a] |
| TPVSEK-amide | synthetic | difficult |
| SPYSSD..... | Rantes | difficult |
| TPQNIT..... | Cholera toxin B subunit | difficult |

[a] After much optimization (*5*).

over two bonds with $C(\alpha)_{(Pro)}$ [at $\delta = 61.58$, $^2J_{C/N} = 5.4$ Hz]. These values are close to what is expected for **3** (*20*), and they leave no doubt that $N_{(Val)}$ is bound to three carbon atoms. The ring closure creates, with high stereoselectivity, a new center of chirality. We see a single epimer of **3**. The absolute configuration at H−C(2), however, is unknown.

The cyclization process **2** → **3** is entropically favored by the rigidity of the proline ring, which keeps the two reacting centers in proximity. This propensity to cyclize is probably responsible for the difficulty sometimes encountered to oximate a polypeptide which, by mass spectrometry, has the mass of the aldehyde. All of our difficult oximations have involved X-Pro sequences, where X is Thr or Ser (Table 2, where only a small selection of easily oximated polypeptides is shown).

Studying "glyoxylyl-Pro-Ile-Val-amide" made by treatment of diisopropylthioacetyl peptide with N-bromosuccinimide and isolation under acidic conditions, Qasmi et al. (*21*) found the mass of the aldehyde form (which is the same as that of the cyclic form) by isobutane CI mass spectrometry, but found evidence by NMR (in DMSO) and FAB mass spectrometry (thioglycerol matrix) that the polypeptide can also exist in hydrated form, as we found for our synthetic peptide. By proton NMR, they found no aldehydic proton at all for "glyoxylyl-Pro-Ile-Val-amide", but found a singlet at δ(ppm) 9.2−9.3 integrating 0.5 H for the aldehyde proton of "glyoxylyl-Phe-Pro-Ile-Val-amide" and "glyoxylyl-Asn-Phe-Pro-Ile-Val-amide".

**Oxidation of CTB** and oximation with the carrier molecule $NH_2OCH_2CO\text{-}Gly_3[Lys(Ser)]_5Gly\text{-}OH$ under the "one-pot" conditions described led to quantitative conversion (Figure 4a) to the expected oxime $CTB{=}NOCH_2CO\text{-}Gly_3[Lys(Ser)]_5Gly\text{-}OH$: relative molecular mass found by electrospray ionization mass spectrometry, 12 938 Da (theoretical mass, 12 937 Da). CTB normally exists as a noncovalent pentamer, but treatment with acid and organic solvents leads to the monomeric form exclusively. Oxidation of the side-chain Ser residues of the ligated carrier molecule followed by isolation of the oxidized protein possessing five aldehyde groups and oximation with the peptide $CH_3CO\text{-}DC(CH_2CONH_2)TLIDALLGDPH{-}K(COCH_2ONH_2)\text{-}NH_2$ ($NH_2OCH_2CO$-peptide) yielded 1 mg of product from 2 mg of starting CTB. Characterization by mass spectrometry showed this product (relative molecular mass, 21 102 Da, theoretical mass, 21 101 Da) to be the expected branched hybrid $CTB{=}NOCH_2CO\text{-}Gly_3[Lys(COCH{=}NOCH_2CO\text{-peptide})]_5Gly\text{-}OH$.

A similar product was made earlier (*22*) using the "one-pot" conditions and different form of the peptide, $CH_3CO\text{-}DC(SCH_2CH_2NHCOCH_2ONH_2)TLID\text{-}ALLGDPH{-}NH_2$. At that time, we did not know why it was necessary to use "one-pot" conditions for the first oximation step, while it was possible to isolate the oxidized protein prior to the second oximation step. By performing the "one-pot" oxidation-oximation at pH 6.5 instead of 7.0, and quenching after 2 min, it was possible to isolate four components from the reaction mixture (Figure 4b). Mass spectrometry identified peaks 1−4: peak 1 was the expected oxime (found, 12 938; calcd, 12 937); peak 2 was unoxidized CTB (found, 11 602; calcd, 11 604); peak 3 was hydrated glyoxylyl CTB $(HO)_2CHCO{-}CTB$ (found, 11 580; calcd, 11 577); and peak 4 had the mass expected for "glyoxylyl-CTB" but was most probably the cyclic isomer; found, 11 558; calcd, 11 559. Incubation of peak 3 material with $NH_2OCH_2CO\text{-}Gly_3[Lys(Ser)]_5Gly\text{-}OH$ led to formation of peak 1 material (oxime), while oximation of peak 4 material failed. Use of methanol, which leads to hemiacetal formation, in place of acetonitrile as cosolvent for electrospray ionization shifted the mass of peak 3 to, found, 11 589 and, calcd, 11591, but did not shift the other masses, in particular that of peak 4, providing further evidence of lack of a free aldehyde moiety for peak 4. Taken together with the mass spectrometric and NMR results obtained with the model peptide, it would seem that cyclization of glyoxylyl-Pro polypeptides to a cyclic isomer which oximate with difficulty may also occur with proteins.

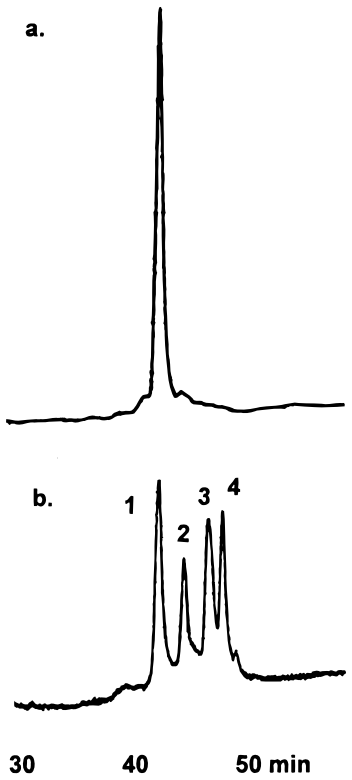



**Figure 4.** Analytical HPLC chromatograms of oximation reactions of oxidized CTB with $NH_2OCH_2CO\text{-}Gly_3[Lys(Ser)]_5\text{-}Gly\text{-}OH$. Compounds were identified by mass spectrometry. After a 5 min isocratic period at 35% B, a gradient of 0.2%/min solvent B was applied, monitoring at 214 nm. (a) "One-pot" oxidation-oximation under optimized conditions, showing quantitative conversion to the oxime. (b) "One-pot" reaction at suboptimal pH and time.

## CONCLUSIONS

It is generally an advantage to isolate an oxidized (glyoxylyl-) polypeptide prior to oximation to avoid the necessity to use an excess of aminooxy component over the aldehydes coproduced during the oxidation reaction (formaldehyde from Ser, acetaldehyde from Thr, and formaldehyde from oxidation of the quenching agent ethyleneglycol). However, particularly when the N-ter-

minal Ser/Thr residue to be oxidized is followed by Pro, we recommend "one-pot" oxidation-oximation to avoid possible formation of the cyclic isomer of the aldehyde described here.

We have not studied the biological properties of the heterocyclic isomer of the aldehyde. It might be interesting as a scaffold for combinatorial chemistry of small (or even protein-sized) molecules, or for other purposes.

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