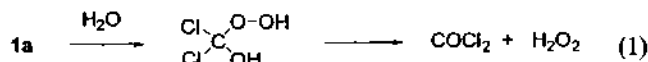
13. (a) For NaOCl, see Zhang, W.; Jacobsen, E. N. *J. Org. Chem.* **1991**, *56*, 2296. (b) For ArI=O, see reference 12b. (c) For H_2O_2, see Pietikainen, P. *Tetrahedron Lett.* **1994**, *35*, 941. (d) For $NaIO_4$, see Pietikainen, P. *Tetrahedron Lett.* **1995**, *36*, 319. (e) For $O_2/Me_3CH=O$, see Yamada, T.; Imagawa, K.; Nagata, T.; Mukaiyama, T. *Chem. Lett.* **1994**, *35*, 941. (f) For Dimethyldioxirane, see Adam, W.; Jeko, J.; Levai, A.; Majer, Z.; Nemes, C.; Patony, T.; Parkany, L.; Sebok, P. *Tetrahedron Asymmetry* **1996**, *7*, 2437.
14. (a) Fu, H.; Look, G.; Zhang, W.; Jacobsen, E. N.; Wong, C-H. *J. Org. Chem.* **1991**, *56*, 6497. (b) Norrby, P-O.; Linde, C.; Akermark, B. *J. Am. Chem. Soc.* **1995**, *117*, 11035. (c) Pospisil, P. J.; Carsten, D. H.; Jacobsen, E. N. *Chem. Eur. J.* **1996**, *2*, 974.
15. Makosza, M.; Wawrzyniewicz, M. *Tetrahedron Lett.* **1969**, 4659.
16. Battioni, P.; Renaud, J. P.; Bartoli, J. F.; Reina-Artikes, M.; Forte, M.; Mansuy, D. *J. Am. Chem. Soc.* **1988**, *110*, 8462.
17. The enantiomeric excess of the compound **4** was determined to be zero, which implies that the catalyst **2** was not involved in cyclopropanation process. For the reference of the related metalloporphyrin-mediated cyclopropanation reaction, see Ziegler, C. J.; Suslick, K. S. *J. Am. Chem. Soc.* **1996**, *118*, 5306.
18. We could not exclude the possibility of dichlorodioxirane, the cyclic isomer of **1a**, involving as the active oxidant. However, the isomerization of carbonyl oxide to the corresponding dioxirane was reporeted to have relatively high activation energy barrier. For the reference, see Hull, L. A. *J. Org. Chem.* **1978**, *43*, 2780.
19. Typical experimental procedure is as follows; To the solution of the olefin (1 mmol), chloroform (20 mL), *n*-Bu_4NBr (0.04 mmol), imidazole (1 mmol) and sodium hydroxide (6 M, 10 mL) in the presence of the catalyst (*S,S*)-**2** (0.1 mmol) was bubbled the oxygen gas for 12 h at room temperature. After the phases were separated, the organic layer was washed with brine solution and dried with anhydrous sodium sulfate. After being concentrated, the mixture was analyzed by GC and GC-MSD spectroscopy or purified by flash column chromatography.
20. There is a possibility of generation of hydrogen peroxide during the reaction as depicted in equation 1.
We have examined the possible involvement of H_2O_2 as



the active oxidant in this process. Pretreatment of dichlorocarbene and oxygen for 1 hr followed by addition of cat **2** and olefin provided no epoxide at all, which indicates no participation of free H_2O_2 as the oxidant.
21. Jernia, D. M.; Daly, J. W. *Science (Washinton D. C.)* **1974**, *185*, 573.

The Role of Highly Conserved Tetrapeptide Sequence of C-Peptide in the Folding of Proinsulin

Ki-Doo Choi, Seung-Gu Chang, and Hang-Cheol Shin*

Hanhyo Institute of Technology, 461-6 Chonmin-dong, Yusong-gu, Taejon 305-390, Korea

Received May 22, 1997

Proinsulin, a single chain insulin precursor, comprises the B-chain, a Arg-Arg sequence, the connecting C-peptide of 31 amino acids, a Lys-Arg sequence, and the A-chain. After folding and the formation of disulfide bridges, insulin is released by a proteolytic cleavage of proinsulin at the two dibasic sites. The presence of a pair of basic residues is considered to be a minimum requirement for conversion.[1]

The role of C-peptide in the folding of proinsulin was speculated to bring the two distant parts of the polypeptide, A and B chains, into proximity for efficient formation of disulfide bridges between the two chains,[2] but the exact role has not been elucidated.

C-peptide is the most variable portion of the proinsulin molecule except the highly acidic region which occurs in the first four positions of the C-peptide. This acidic region is highly conserved in various animal species with the sequence Glu/Asp-X-Glu/Asp, where X is alanine, valine or leucine.[3] The presence of highly conserved acidic residues immediately following dibasic residues may suggest some functional role of this region in the formation of proinsulin structure.

In the present work, we have examined the role of the highly conserved tetrapeptide sequence of C-peptide in the folding of proinsulin. For this purpose, we have produced two analogue forms of proinsulin, in which either the tetrapeptide sequence, Glu-Ala-Glu-Asp, was deleted (D4PI) or replaced with Gly-Gly-Gly-Gly sequence (G4PI) (see Figure 1 for the sequence).

The proinsulin and analogues were produced as fusion proteins which form inclusion bodies in *E. coli* cells. The gene for the fusion protein was placed under the control of the T7 promoter in the expression plasmid pET-3a[4] and the fusion protein was expressed in *E. coli* BL21(DE3) by IPTG induction. N-terminal portion of human tumor necrosis factor-alpha (hTNF-α) was used as a fusion partner, a fusion system described by our group previously.[5] After

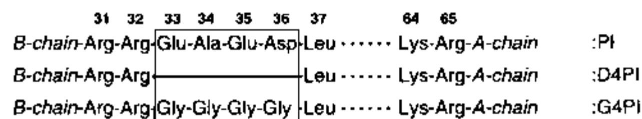


Figure 1. Amino acid sequence at the beginning of native and mutant human proinsulin C-peptides. The first four residues of the C-peptide are boxed and constitute the conserved acidic region. The numbers above each residue refer to the amino acid positions in the full proinsulin sequence.

the sulfonation and isolation process of the proinsulin and analogues, the subsequent refolding experiments were carried out.

Refolding reactions of the sulfonated proinsulin and analogues were performed in 50 mM glycine buffer at various pH and temperature conditions. The reaction was stopped by adding H_3PO_4 to pH 2.5. Typically, 16 μg of protein solution was filtered and loaded onto a Zorbax C8 reverse-phase column. The protein was eluted with solvent A of 0.125 M ammonium sulfate (pH 2.5) and solvent B of 90% acetonitrile using linear gradient of 20-60% solvent B. The refolding yields of the reaction products were determined by comparing the peak size of the correctly folded species in the HPLC profile with the standard. The correct disulfide formation was verified by subsequent finger print analysis using *Staphylococcus aureus* strain V8 protease (Glu-C endoproteinase) that specifically cleaves the peptide bond on the carboxyl side of the glutamic acid residue.[6]

Figure 2 shows the refolding yields of proinsulin (PI), D 4PI and G4PI at various pH conditions. Compared to the analogues, PI exhibits higher folding yields at the pH range of 7.5-9 and maintains lower fluctuation with respect to the pH variations. The two analogues show similar trends each other, the folding yields being proportional to the pH variation up to pH 11, with the highest yield at pH 11.0. This indicates that the repulsive force between Arg-Arg and Lys-Arg is significant to give an influence for the folding of

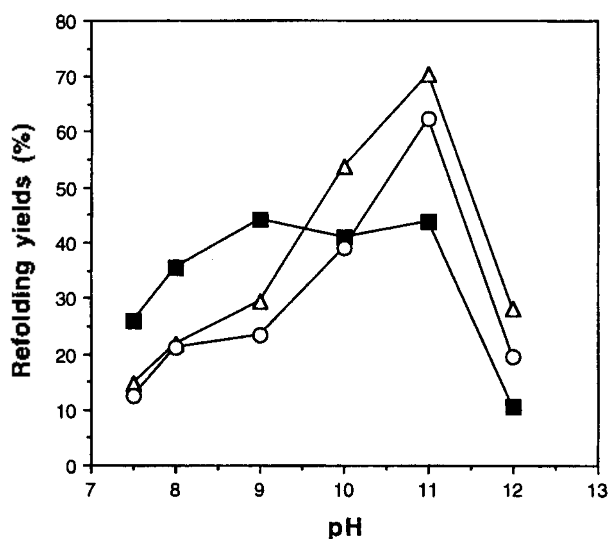


Figure 2. Effect of pH on the refolding of proinsulin (■), D4PI (△) and G4PI (○). Protein concentration=50 μg/mL, Temperature=4 °C.

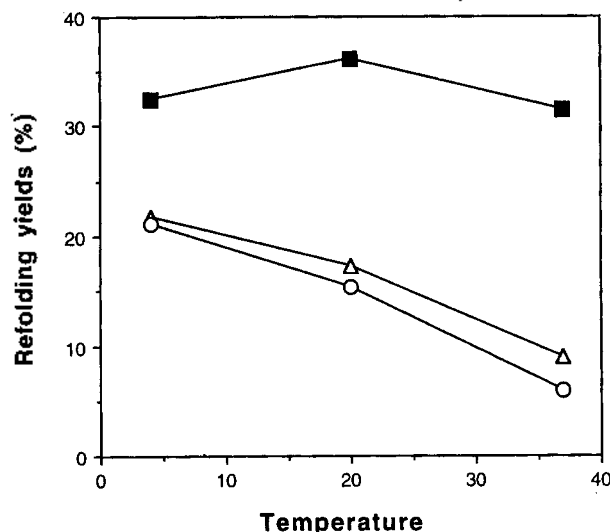


Figure 3. Effect of temperature on the refolding of proinsulin (■), D4PI (△) and G4PI (○). Protein concentration=50 μg/mL, pH=8.0.

proinsulin. At pH 12, the folding yields of the three species drop significantly, probably due to the partial disruption of proinsulin structure at this extreme pH condition.

More dramatic effect was found in the folding of PI and analogues at various temperature conditions (Figure 3). It is generally known that the yield of any refolded protein decreases with increasing temperature. Interestingly, the folding yield of proinsulin is relatively constant, irrespective of the temperature variation. On the other hand, the folding yield of analogues decreases as the temperature increases. At 37 °C, which is the physiological temperature, the folding yield of the two analogues was less than 10%, in comparison with more than 30% found in proinsulin. These results indicate that the highly acidic tetrapeptide sequence confers enhancement in thermal stability during proinsulin folding.

It was reported that reoxidation of cross-linked insulins gives high yields of a product with the correct formation of disulfide bonds.[7] Along with the fact that the native insulin could be generated from the air oxidation of a mixture of the reduced A and B chains,[8] doubts on the structural role of C-peptide were raised.[9] However, since either the cross-linked insulins or insulin itself lack the double dibasic sites in their sequence, the folding of these proteins is not relevant to the folding of proinsulin, in which there may be a close interaction between the two dibasic sites.[10]

The C-peptide has been studied for its possible involvement in the targeting of proinsulin to secretary granules or its conversion into insulin. Reduced yield of conversion was observed for the proinsulin analogue with the deletion of four amino acids immediately following the B-chain/C-peptide junction.[3,11] C-peptide does not appear to play a significant role in targeting proinsulin to granules.[3,12]

Our data, even though studied under *in vitro* conditions, suggest that in addition to the possible role of C-peptide in the conversion, the C-peptide has a role in the folding of proinsulin. Especially, the maintenance of high folding yield in the proinsulin at elevated temperature (37 °C) clearly

shows the important role of this acidic region in the folding of proinsulin.

In our future studies, other regions of C-peptide sequence will be investigated for their involvement in the proinsulin folding. The combined results will provide a clear understanding on the folding behavior of this biologically important prohormone.

Acknowledgment. This work was supported by Hanil Synthetic Fiber, Co.

References

1. Halban, P. A. *J. Biol. Chem.* **1982**, *257*, 13177.
2. (a) Steiner, D. F. *Diabetes* **1978**, *27*, Suppl. 1, 145. (b) Steiner, D. F. *Harvey Lect.* **1984**, *78*, 191.
3. Gross, D. J.; Villa-Komaroff, L.; Kahn, C. R.; Weir, G. C.; Halban, P. A. *J. Biol. Chem.* **1989**, *264*, 21486.
4. Studier, W. F.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. *Methods Enzymol.* **1990**, *185*, 60
5. Kim, D.-Y.; Shin, N.-K.; Chang, S.-G.; Shin, H.-C. *Biotechnol. Tech.* **1996**, *10*, 669.
6. Grau, U. *Diabetes* **1985**, *34*, 1174.
7. Wollmer, A.; Brandenburg, D.; Vogt, H.-P.; Schermutski, W. *Hoppe-Seyler's Z. Physiol. Chem.* **1974**, *355*, 1471.
8. (a) Katsoyannis, P. G. *Biochemistry* **1967**, *6*, 2642. (b) Chance, R. E. In *Peptides, Synthesis, Structure and Function*; Rich, D. H.; Gross, E., Eds.; Proc. 7th American Peptide Symposium, Pierce Chemical Co.: 1981; pp 712-728.
9. Wang, C.-C.; Tsou, C.-L. *TIBS* **1991**, *16*, 279.
10. (a) Taylor, N. A.; Doherty, K. *Biochem. J.* **1992**, *286*, 619. (b) Rhodes, C. J.; Lincoln, B.; Shoelson, S. E. *J. Biol. Chem.* **1992**, *267*, 22719.
11. Kaufmann, J. E.; Irminger, J.-C.; Halban, P. A. *Biochem. J.* **1995**, *310*, 869.
12. Powell, S. K.; Orci, L.; Criak, C. S.; Moore, H. P. H. *J. Cell. Biol.* **1988**, *105*, 1843.

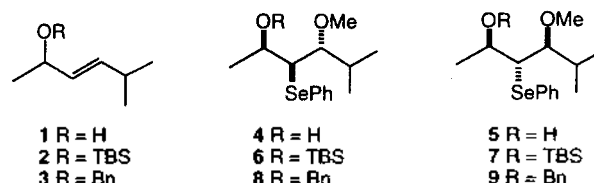
Regio- and Stereochemistry of Methoxyselenenylation of Acyclic Allylic Alcohol Derivatives and Allylic Phenylselenides

Kwan Soo Kim*, Kwang Ook Ahn, and Jong Il Park

Department of Chemistry, Yonsei University, Seoul 120-749, Korea
Received May 26, 1997

Oxyselenenylation of olefin and the subsequent transformation have been appreciated as a useful tool in the synthesis of various important natural products.[1] Regio- and stereochemistry of the electrophilic selenenylation of olefin can be understood with the same rules as those of the usual electrophilic addition to olefin. Outcome of the regio- and stereochemistry in the oxyselenenylation of allylic alcohol derivatives, on the other hand, appears to be influenced by the directing effect of the allylic oxygen.[2,3] We have also reported recently the regio- and stereoselective methoxyselenenylation of acyclic allylic alcohol derivatives.[4] Nevertheless, the results on the regio- and stereochemistry of oxyselenenylation of allylic alcohol derivatives are sometimes contradictory and the origin of the selectivity is yet unclear.[2-5] Herein we report further results on the regio- and stereochemistry in methoxyselenenylation of acyclic allylic alcohol derivatives and allylic phenylselenides as well.

It was difficult to determine whether the outcome of regiochemistry was electronic or steric origin with the allylic alcohol derivatives used in earlier works including our own previous work.[2-5] Therefore, we first prepared (E)-5-methyl-3-hexen-2-ol (**1**) which has a bulky isopropyl group in the opposite side of the allylic hydroxyl group with a double bond between. Methoxyselenenylation of **1** and its derivatives **2** and **3** was completely regioselective and highly diastereoselective to afford mainly 1,3-*anti*-diol derivatives (Table 1, entries 1, 2, and 3). For example, to a solution of



allylic alcohol **1** (1.14 g, 10 mmol) and 2,6-di-*t*-butylpyridine (DTBP, 1.0 equiv.) in methanol (45 mL) was slowly added methanol solution (20 mL) of phenylselenenyl bromide (1.3 equiv.). The reaction was conducted under nitrogen atmosphere, at room temperature, and in the flask covered with aluminum foil. After completion of reaction, the reaction mixture was concentrated and partitioned between ether and water. The organic phase was washed with aq. $NaHCO_3$ followed by chromatography afforded 1,3-*anti*- and 1,3-*syn*-hydroxymethoxide, **4** and **5**, in 98 : 2 ratio in 65% yield.[6] We also prepared (E)-3-methyl-3-penten-2-ol (**10**) and its benzyl ether **11**. Methoxyselenenylation of **10** and **11**, on the other hand, afforded not only 1,3-isomers but also 1,2-isomers **14** and **17**, respectively (Table 1, entries 4 and 5). The relative stereochemistry of the products **4-9** and **12-17** was determined by the same procedure as described in our previous work.[4] The result from the reaction of compounds **1**, **2**, and **3** indicates that the steric hindrance around episelenonium ion is not the determining factor for the regioselectivity in the methoxyselenenylation of