C500 Rotation Report

Two Step Disulfide Bond formation in Insulin Synthesis

Indiana University

Department of Chemistry

C500

Spring 2006

By:

Jonathan M. Meyers

Introduction:

Insulin is a small globular protein comprised of two short peptide chains linked via two disulfide bonds. In human insulin the A-chain is 21 amino acids long with cysteine residues at positions 6, 7, 11, and 20. The B-chain is 30 amino acids long and contains two cysteines at positions 7 and 19. There is an additional intra-chain disulfide bond between cysteines 6 and 11 on the A-chain (Figure 1). These three disulfide bonds are vital to determining the three dimensional structure and therefore the function of insulin.

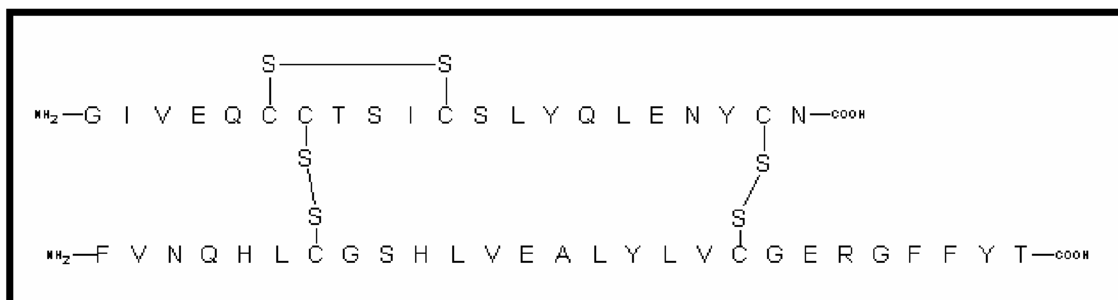

Figure 1: Native Human Insulin, showing two inter-chain and one intra-chain disulfide bonds.

In the search for useful insulin analogs, synthetic production is a favorable tool for small scale synthesis. Any synthetic method must include the oxidation of A and B chain cysteines to form disulfide bonds. Currently two distinct methods are used to form these bonds. The first method involves creating all three disulfide bonds simultaneously, either by air oxidation, or by the addition of an oxidizing agent such as iodine. This method relies on the random combination of the two chains and the formation of conformers in a thermodynamically driven equilibrium. The process has been optimized for the combination of natural A and B chains, however when analogs are used yields can decrease significantly. This means that optimization of each combination of analogs must be carried out to give maximum yield. The second, and newer, method is the step by step, regioselective formation of the three bonds using orthogonal cysteine protecting groups[1].

While this method reduces the formation of undesired conformers it has two major drawbacks: time and low yield. The independent de-protection, disulfide bond formation, and subsequent purification for each step is time consuming. Additionally the yield in this type of chain combination is very low. Akaji et. Al. reported 0.6 mg of fully oxidized insulin from 37 mg of protected B-chain and 24 mg of protected A-chain starting materials[1].

Due to the large number of analogs that must be synthesized and characterized it would be beneficial if the synthesis time could be shortened while increasing the yield of desired product. One possible method for achieving this is a two step formation of the three disulfide bonds. A two step synthesis would be beneficial if the yield is high enough, because it would decrease the number of purifications therefore decreasing the amount of product lost and decreasing the amount of time needed to achieve complete disulfide bond formation. For each individual mechanism of two step disulfide bond formation there are six ways to achieve total disulfide oxidation. Table 1 list the six possible combinations for a two step approach.

Path	Step 1	Step 2
1	A7-B7 and A20-B19	A6-A11
2	A7-B7 and A6-A11	A20-B19
3	A20-B19 and A6-A11	A7-B7
4	A7-B7	A20-B19 and A6-A11
5	A20-B19	A7-B7 and A6-A11
6	A6-A11	A7-B7 and A20-B19

Table 1: Any mechanism of two step disulfide bond formation in Insulin can proceed through six different paths.

Here we report our attempt to form the two inter-chain bonds simultaneously and follow that by the formation of the intra-chain disulfide bond (Path 1). This is

accomplished by synthesizing the A-chain with protected cysteines at 6 and 11, and activating the A-chain cysteines with good leaving groups for nucleophilic attack by a deprotonated sulfhydryl. No additional reagents are needed to form the disulfide bonds in the first step, and the third bond is formed after de-protection by iodine oxidation.

Experimental:

<u>Peptide Synthesis</u>

B-Chain (SH)$^{7, 19}$

B-chain (SH)$^{7, 19}$ was obtained by reducing biosynthetic human insulin B-chain with S-sulfonate protecting groups at Cys^{7} and Cys^{19}. The biosynthetic B-chain was obtained from Lilly. Reduction was carried by reacting 100 mg of B-chain (SSO_3)$^{7, 19}$ with 150 mg of dithiothretiol in 10 ml of 7 M Tris/Urea buffer. The reduced B-chain was purified using reverse phase FPLC. The reduction produced an 84% yield. Figure 2 shows the HPLC and mass spec. of the final product.

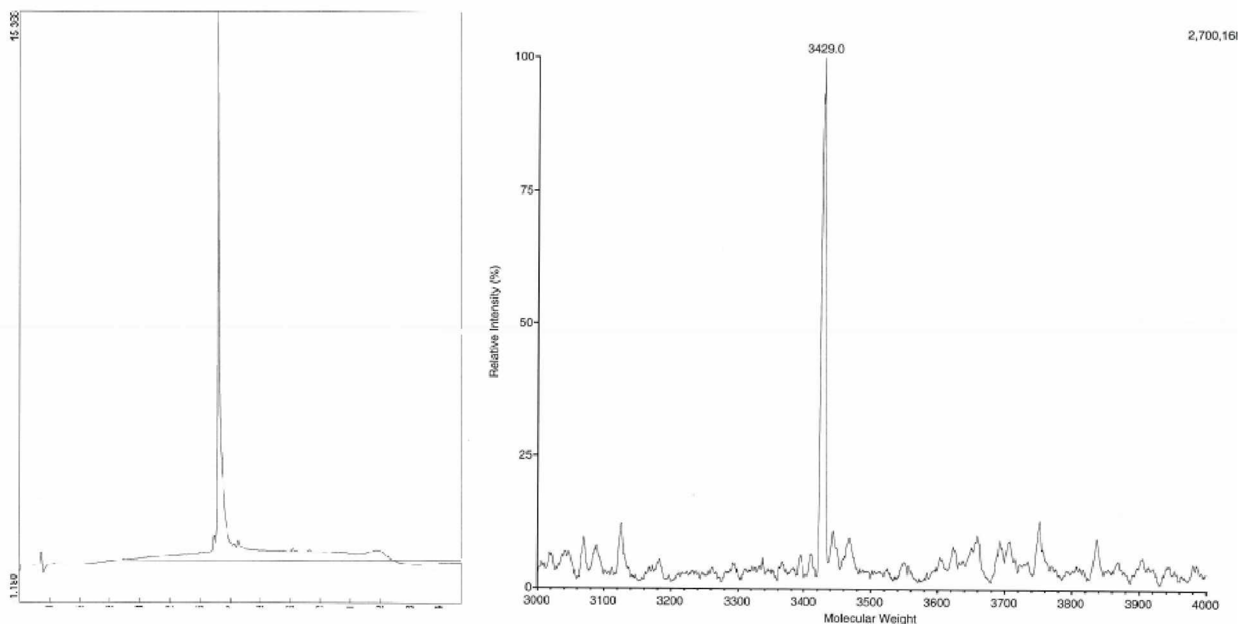

Figure 2: The figure on the left is an analytical HPLC, and the figure of the right is the mass spec. of the final product of reduction of Biosynthetic B chain (SSO3) with DTT.

A Chain $(ACM)^{6, 11}$ $(SSO_3)^{7, 20}$

A-chain was synthesized and purified by Dr. Jie Han using solid state matrix and the t-boc synthesis system. Purity was verified by HPLC and mass spec.

<u>*Chain Combination Reactions*</u>

pH 4

A-Chain $(ACM)^{6, 11}$ $(SSO_3)^{7, 20}$ was solubilized in 7 M Urea (pH 4, 4^{0C}) at a concentration of 2.5 mg/ml. This was added to the molar equivalent of B-chain $(SH)^{7, 19}$ to produce a total peptide concentration of 5 mg/ml. The solution was incubated in an ice bath for three hours. An analytical HPLC taken at time 1hr and 3 hr, and a MALDI mass spec at time 3 hr.

Chain Combination at pH 8.5 and 10

A Chain (ACM)[6, 11] (SSO_3)[7, 20] was solubilized in 30 to 50 mM glycine buffer (pH 8.5 or 10, 4^{0C}). This was added to the molar equivalent of native B-chain to produce a total peptide concentration of either 2.5 or 5 mg/ml depending on the reaction. The solution was incubated in an ice bath for three hours. An analytical HPLC taken at time 1hr and 3 hr, and a MALDI mass spec at time 3 hr.

Results and Discussion:

We chose to protect the cysteines involved in the formation of the intra-chain disulfide bond with acetamidomethyl (ACM, figure 3). The A-chain cysteines at 7 and 20 were activated by sulfitolysis with S-sulfonate as the leaving group (figure 4). B-chain was synthesized with free sulfhydryls at position 7 and 19 (figure 4). The B-chain and activated A-chain were solubilized and allowed to react for three hours. After three hours reaction was stopped by dilution with aqueous solution of 50% Acetonitrile in 0.1 TFA. An analytical HPLC was then taken and an aliquot was submitted for MALDI mass spec.

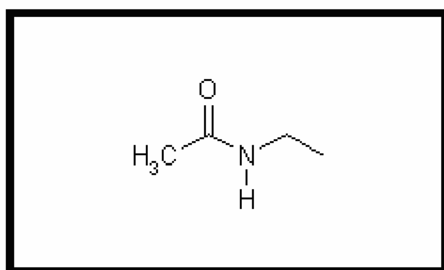

Figure 3: An Acetomidomethyl (ACM) Group

	pH	Temperature	Total Peptide Conc.
1	4	0°C	5.0 mg/ml
2	8.5	Room	2.5 mg/ml
3	8.5	0°C	2.5 mg/ml
4	8.5	0°C	5.0 mg/ml
5	10	0°C	2.5 mg/ml
6	10	0°C	5.0 mg/ml

Table 2: Reaction Conditions for Chain Combinations

Six sets of reaction conditions were used to see the effect of temperature, pH, and concentration on the chain combination (Table 2). The molar ratio of A-chain to B-chain

was 1:1 in all attempted combinations. In all experiments the A-chain was solubilized in buffer then added to powdered B-chain. The major product in all five experiments in basic buffer was cyclic B-chain or B-chain dimer. The reaction in acidic buffer showed no change in reactants after three hours. In all six attempts no appreciable product was seen. In the acidic reaction the low pH may have prevented sufficient deprotonation of the free sulfhydryls on the B-chain. The fact that no product was seen in the reactions in basic buffer may be a result of the high reactivity of the B-chain cysteines. The local environment of the B-chain may make its cysteines too reactive for this type of chain combination. The current explanation is that the deprotonated B-chain sulfhydryl attacks the activated A-chain cysteine side chain and displaces the S-sulfonate to form the disulfide bond. This disulfide bond is short lived however, as the A-chain cysteine is quickly replaced by a more reactive B chain cysteine to form either the cyclic B chain or the B-chain dimer.

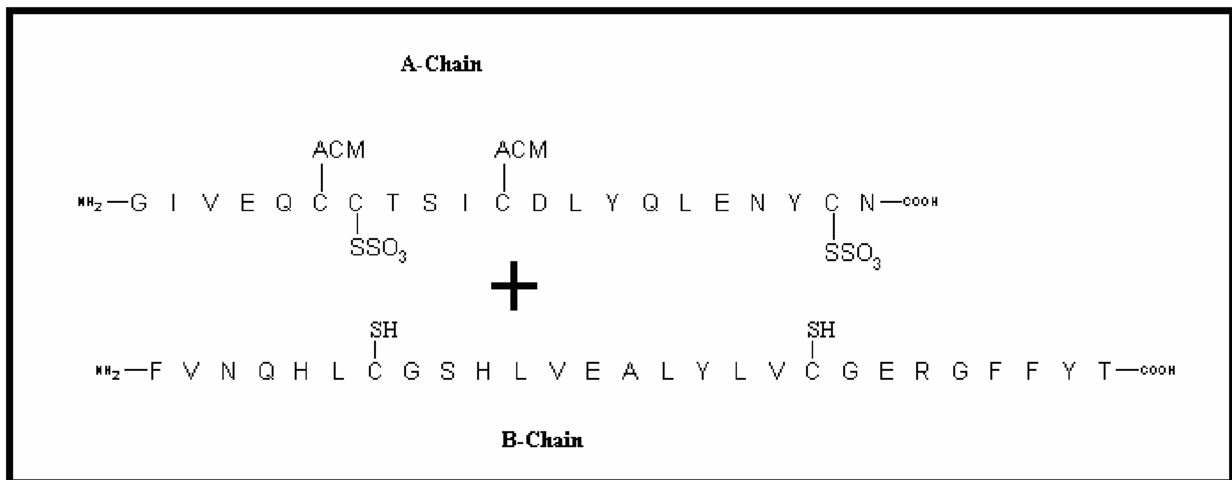

Figure 4: Shows the activated Asp 12 A-Chain and the native B chain.

This problem has led to the investigation of the alternative reaction where the B-chain cysteines are activated with the leaving group and the A-chain is synthesized with

free sulfhydryls at 7 and 20. Preliminary results have shown significant product formation. Future work will concentrate on quantifying the yield of this reaction, as well as exploring synthesis where one bond is formed in the first step and two in the subsequent step.

Conclusion:

From these experiments it is evident that the B-chain free sulfhydryl is too labile for stable formation of disulfide bonds between A and B chains. Instead it drives the formation of either B-chain dimer or cyclic B. However new data suggest that the two step synthesis may still be a vialble alternative to simultaneous three bond formation or three step regioselective bond formation. Future efforts should focus attempting the other paths of insulin bond formation as well as quantifying the yield of any successful reaction schemes.

References:

1. Akaji, K., Fujino, K., Tatsumi, T., and Kiso, Y., *J. Am. Chem. Soc.* **1993**, 115, 11384-11392.