INSULIN SYNTHESIS FROM NATURAL CHAINS BY MEANS OF REVERSIBLE BRIDGING COMPOUNDS

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SUMMARY. Insulin and $N^{\alpha B1}$-trifluoroacetylinsulin were crosslinked by diaminodicarboxylic acids, preferably α,α'-diamino-suberic acid (Dsa), using bis-(tert-butyloxycarbonyl)-2.7-diaminosuberoyl-bis-(2.4.5-trichlorophenylate) [$(BOC)_2$-Dsa-$(OTcp)_2$] as coupling reagent. Elimination of the BOC groups by trifluoroacetic acid treatment and Edman degradation resulted in biologically fully active des-Phe^{B1}-insulin and $N^{\alpha B1}$-Tfa-insulin, respectively. The N^{α}group of the natural A-chain tetrasulfonate and the $N^{\varepsilon B29}$ group of the natural B-chain disulfonate, specifically trifluoroacetylated in $N^{\alpha B1}$, were linked together by successive reaction with the above mentioned reagent. After formation of the correct disulfide bridges in high yields (60-75 %) the elimination of the connecting Dsa by Edman degradation yielded $N^{\alpha B1}$-Tfa-insulin, which was biologically fully active.

Low yields of active insulin are recovered, when natural insulin chain sulfonates are reduced and combined by reoxidation[1]. However, when proinsulin, in which the A- and B-chain is connected by a C-fragment to form a single chain protein, was subjected to an analogous reduction and oxidation cycle, as much as 70 % of the initial proinsulin ensued[2]. Since the distance between the ε-amino group of $lysine^{B29}$ and the α-amino group of $glycine^{A1}$ was established by

X-ray analysis[3] of crystalline insulin to be 10 Å, Lindsay[4] has suggested the consideration of an insulin, $N^{\alpha A1}$, $N^{\varepsilon B29}$-crosslinked by dicarboxylic acids as a proinsulin model. These crosslinked insulin derivatives[4,5] indeed produced upon reduction and reoxidation a considerably high yield[6] of the initial crosslinked hormone derivative. However the bridging members cannot be removed from these insulin compounds.

To prepare insulin itself, we had based crosslinking experiments on α,α'-diaminodicarboxylic acids like lanthionine, diaminoheptanedioic acid and preferably α,α'-diaminosuberic acid (Dsa)[7,8] which can be removed by Edman degradation[9] (figure I). First we reacted insulin with $(BOC)_2$-Dsa-$(OTcp)_2$, to explore whether or not a relatively high yield of A_1, B_{29}-crosslinked insulin would result, induced by the close fit of Dsa into the 10 Å gap. Additionally, crosslinked insulin was necessary to work out conditions for the Edman degradation of the connecting diaminodicarboxylic acids. In the case of non-$N^{\alpha B1}$-trifluoroacetylated insulin, biologically fully active des-Phe^{B1}-insulin results, due to the concurrent degradation of the free N-terminal phenylalanine of the B-chain.

Dsa, as a mixture of all the possible diastereomers, was prepared according to the literature[10]. N,N'-Bis-(tert-butyloxycarbonyl)-2.7-diaminosuberic acid ($(BOC)_2$-Dsa, mp. 175 - 177°C) was synthesized following the general procedure[11] for N-tert-butyloxycarbonyl-amino acids. $(BOC)_2$-Dsa-$(OTcp)_2$ (mp.198-201°C; recrystallized from isopropanol) was produced by the method of Pless and Boissonnas[12].

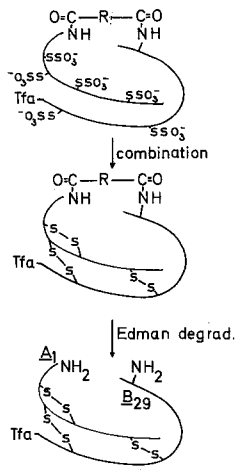

Figure I. Simplified reaction scheme, starting with the A- and $N^{\alpha B1}$-Tfa-B-chain sulfonates, crosslinked with N,N'-diaminodicarboxylic acids. R = -CH·(-NH-BOC)·CH_2·CH_2·CH_2·CH_2·(BOC-NH-)·CH- in the case of N,N'-diaminosuberic acid; R = -CH·(-NH-BOC)·CH_2·S·CH_2·(BOC-NH-)·CH- for lanthionine

All compounds met the required theoretical values of the elemental analyses.

Crystalline beef insulin (3 gr; HOECHST) was reacted with $(BOC)_2$-Dsa-$(OTcp)_2$ (380 mg) and 1.4 ml of N-ethylmorpholine in 250 ml of dimethylsulfoxide for 16 hours. After precipitation of the reaction product by addition of methanol/ether (1:10; v/v) the crude material was purified by partition chromatography on Sephadex LH 20 (upper phase of the system n-butanol-acetic acid-water = 2:1:10, v/v, was used as stationary phase, the aqueous layer as elution medium). The desired compound (26 %) was isolated by lyophilization of the main fraction. Proof of the correct crosslinkage was furnished by Edman degradation and dansylation. After performic acid oxidation neither free A- nor B-chain could be detected electrophoretically. The BOC residues were

split off by treatment with trifluoroacetic acid. Edman degradation of the unprotected A_1, B_{29}-diaminosuberoyl-insulin and purification on Sephadex G 50 (10 % acetic acid as eluent) yielded des-Phe^{B1}-insulin. Amino acid analysis met the required values (2 Phe), and only traces of diaminosuberic acid could be discovered in the hydrochloric acid hydrolysate. The performic acid oxidation mixture gave a pattern in the electrophoresis identical to an original sample of des-Phe^{B1}-insulin treated in the same way. In addition the degradation product was biologically fully active.

Essentially the analogous experiment was carried out with the specifically protected, biologically fully active N$^{\alpha B1}$-Tfa-insulin[13]. After Edman degradation and purification, N$^{\alpha B1}$-Tfa-insulin was isolated.

Since these reactions showed the expected results, namely specific crosslinking in relatively high yields and elimination of the diaminosuberoyl moiety by Edman degradation, the connection of the natural insulin chain sulfonates was attempted. The A-chain tetrasulfonate was reacted with a 1.5 molar excess of $(BOC)_2$-Dsa-$(OTcp)_2$ and 9 molar excess of N-methylmorpholine in dimethylsulfoxide. After 15 hours at room temperature electrophoresis showed only traces of unreacted A-chain. The excess of the bifunctional reagent was separated by precipitating the reaction product with methanol/ether. The ensuing solid material was coupled with the theoretical amount of N$^{\alpha B1}$-Tfa-B-chain disulfonate which was prepared by standard sulfitolysis of N$^{\alpha B1}$-Tfa-insulin. The Tfa group was chosen as the protecting group because it is stable against trifluoroacetic acid and can be removed by treatment with alkali, for instance 0.1 m piperidine[14].

The reaction was kept for 60 hours at room temperature, after which time only traces of the starting materials could be seen by means of electrophoresis. Precipitation by addition of methanol/ether (1:10; v/v) and purification by column chromatography on Sephadex G 50 (0.05 m $NH_4 HCO_3$ solution as eluent) yielded pure insulin A- and $N^{\alpha B1}$-Tfa-B-chain crosslinked between $N^{\alpha A1}$ and $N^{\varepsilon B29}$ with the N,N'-bis-BOC-diaminosuberoyl residue.

This compound was subjected to a reduction/oxidation cycle according to the reaction conditions of Du et al.[15] using a 500 molar excess of mercaptoethanol as reducing agent and an oxidation time of 24-72 hours at 5°C (0.01-2 mg protein ml). The pH of the solution during air oxidation was varied in different batches from 9.0 to 10.6. The crude combination product isolated by lyophilization after acidification to pH 4 with acetic acid was purified by partition chromatography on Sephadex LH 20 in the above mentioned system. The main fraction contained the desired material in yields of 60-75 %. The electrophoretical mobility was the same as exhibited by directly crosslinked $N^{\alpha B1}$-Tfa-insulin[a]. The material was treated with trifluoroacetic acid to remove the BOC residues. It was then subjected to an Edman degradation cycle. After purification on a Sephadex G 50 column, $N^{\alpha B1}$-Tfa-insulin could be isolated in 20-40 % yield based on the amount of linked chain sulfonates from which the combination procedure had been started. This material showed the

a) In the electrophoresis of this material, traces of crosslinked insulin without Tfa group, which has been split off under our conditions of reoxidation could be detected. Further investigation showed that the Tfa group is almost quantitatively saponified under the reoxidation conditions mentioned by Brandenburg[6].

correct amino acid analysis without diaminosuberic acid and exhibited full insulin activity. A sample could be crystallized from phenol-containing citrate buffer at pH 6.2. Upon treatment with 0.1 m piperidine, $N^{\alpha B_1}$-Tfa-insulin yields insulin.

We hope that the above described method will be able to increase the chances of a reasonable approach to a feasible synthesis of insulin.

References:

(1) K. Lübke and H. Klostermeyer, Advan. Enzymol. 33, 445 (1970)

(2) D.F.Steiner and J.L.Clark, Proc.Nat.Acad.Sci.U.S.A. 60, 622 (1968)
D.F. Steiner, J.L. Clark, C. Nolan, A.H. Rubenstein, E.Margiolash, B.Aten and P.E.Oyer, Recent Progr.Hormone Res. 25, 207 (1969)
W.W. Bromer and J.M. Patterson, Fed. Proc. 28, 343 (1969)

(3) T.L. Blundell, G.G.Dodson, D.C. Hodgkin and D.A. Mercola, Advan. Protein Chem. 26, 279 (1972)

(4) D.G. Lindsay, FEBS Lett. 21, 105 (1972)

(5) D. Brandenburg, Hoppe Seyler's Z. Physiol. Chem. 353, 869 (1972)

(6) S.M.L. Robinson, I.Beetz, O.Loge, D.G. Lindsay and K. Lübke, Tetrahedron Letters 12, 985 (1973)
D.Brandenburg and A.Wollmer, Hoppe Seyler's Z.Physiol.Chem. 354, 613 (1973)

(7) R.Obermeier and R.Geiger, Symposium: Chemie des Insulins und Proinsulins, Aachen, Germany, 1973

(8) D. Brandenburg, independently on the above mentioned symposium

(9) Germ. Pat. Appl. filed in October 1972

(10) C.Neuberg, Hoppe Seyler's Z.Physiol.Chem. 45, 92 (1905)

(11) E.Schnabel, Liebigs Ann. Chem. 702, 188 (1967)

(12) J. Pless and R.A. Boissonnas, Helv.Chim. Acta 46, 1609 (1963)

(13) R. Geiger and D. Langner, in preparation

(14) R.F. Goldberger and C.B. Anfinsen, Biochemistry 1, 401 (1962)

(15) Y.-c. Du, R.-q. Siang and C.-e. Tsou, Scientia Sinica 14, 229 (1965)