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Interaction and Reconstitution of Carboxyl-Terminal-Shortened B Chains with the Intact A Chain of Insulin†

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ABSTRACT: With the *S*-(thiomethyl)-A chain and despentapeptide(26–30) and desoctapeptide(23–30) *S*-(thiomethyl)-B chains of insulin at pH 10.8 and a molar ratio of A/B = 1.5, difference spectra of the mixed against the separated chains with negative peaks at 245 and 295 nm and a weak positive peak at 278 nm indicate interaction of the chains leading to Tyr environmental changes as in the case for the intact chains. With the shortened B chains, freshly dissolved from lyophilized powders, it takes some 2 h for the difference spectra to approach completion whereas with the solutions of the shortened B chains left standing overnight at pH 10.8 and 4 °C the difference spectra, similar in shape to that described above, appear almost immediately after mixing. Solvent perturbation with 20% ethylene glycol suggests some ordered structure for the despentapeptide but not for the desoctapeptide B chain. The interactions of the A chain with the shortened B chains appear to be weaker as compared to that with the intact B chain as shown by decreasing reconstitution yields for the intact, despentapeptide, and desoctapeptide B chains respectively with the A chain. The above results indicate that the C-terminal portion of the B chain is important not only for the activity of insulin but also for the correct pairing of the chains.

Although it was suggested some years ago (Du et al., 1961) that the insulin A and B chains pair correctly in solution so that the reoxidation of the reduced chains leads to a fairly good yield of the native hormone, the interaction of the chains has only been recently demonstrated in this laboratory with the *S*-(thiomethyl)-A and -B chains of insulin (Hua et al., 1984, 1985). At pH 10.8 and a molar ratio of the chains of A/B = 1.5, where the resynthesis yield of the hormone by reoxidation of the reduced chains was optimal (Du et al., 1965), both the absorption and the CD[1] spectra of the individual chains show significant changes upon mixing, indicating the interaction of the *S*-(thiomethyl) chains by secondary forces leading to a partial transfer of the Tyr residues from an exposed and hydrophilic to a buried and hydrophobic environment and an increase in ordered secondary structure. Moreover, it is particularly interesting to note that both these changes in absorbance and in ellipticity take about 90 min to approach completion after the mixing of the intact chains. This would seem to suggest that some sort of conformational readjustment of either one or both of the chains is required for their interaction with each other, and this conformational change is a relatively slow process. The presence of a Pro residue at B-28 might be significant in this respect as it is well-known that the cis–trans isomerization of peptide bonds containing the imide nitrogen of prolyl residues is a slow process (Brandts et al., 1975).

A series of insulin derivatives shortened at the C-terminal of the B chain have been prepared, and the role of the C-terminal residues on insulin activity has been studied (Brandenburg, 1981; Lei et al., 1981; Zhang, 1983). It has been found that insulin activity decreases with the increase of the number of residues removed. Despentapeptide(B26–30)-insulin retains about 20% of the activity of native insulin (Gattner, 1975; Zhu et al., 1984) whereas the desoctapeptide(B23–30) derivative is almost completely inactive (Kikuchi et al., 1980). It is therefore considered that the C-terminal sequence of the B chain is important for the binding of the insulin molecule to its receptor and hence to its biological activity.

In this paper, the pairing and the formation of the corresponding insulin derivatives from the C-terminal-shortened despenta- and desoctapeptide B chains with the intact A chain have been studied. The results obtained indicate that the C-terminal sequence of the B chain is important not only for insulin activity but also for the pairing and, hence, the formation of the correct insulin derivatives.

Materials and Methods

Reagents. Porcine insulin was a product of Novo. Pepsin was obtained from E. Merck. DTNB, TPCK-trypsin, collagenase (type II), and albumin (Bovine, fraction V) were Sigma products. Acetyltyrosine ethyl ester was from Dongfong Biochemicals, Shanghai. DTT was from Serva, mercaptoethanol from Fluka, and TFA from Pierce. Urea was a local product twice recrystallized according to Morangos and

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[1] Abbreviations: ATEE, acetyltyrosine ethyl ester; CD, circular dichroism; DP, despentapeptide(B26–30); DO, desoctapeptide(B23–30); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TPCK, L-1-[(*p*-toluenesulfonyl)amino]-2-phenylmethyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Constantinides (1974). Methanol was a local product redistilled before use. ^{125}I-Labeled insulin, prepared by the chloramine T method (Hunter & Greenwood, 1962) was a kind gift from Dr. Z. Liu of the Central Laboratory of the Navy General Hospital. All other reagents were local products of analytical grade used without purification.

Truncated Insulin Derivatives. DP- and DO-insulins were prepared from porcine insulin. Limited proteolysis by pepsin at pH 2.5 and 4 °C for 90 min was used for the preparation of DP-insulin followed by purification through a Sephadex G-50 column as previously described (Insulin Research Group, 1976). DO-insulin was obtained from insulin by specific degradation with TPCK-trypsin and purified by DEAE ion exchange chromatography at pH 8.1 following essentially the procedure of Goldman and Carpenter (1974). The purity of these derivatives was checked by amino acid analysis as well as by HPLC.

Separation and S-Thiomethylation of Truncated B Chains. The truncated insulin derivatives were reduced with mercaptoethanol or DTT essentially as described for the native hormone (Hua et al., 1984). The reduced chains were then separated through a DEAE-Sephadex A-25 column at pH 6.5–6.8 buffered by 0.1 M imidazole. The use of a buffer 1 pH unit lower than that used for the intact chains was found to be essential for the successful separation of the shortened B chains from the intact A chain. N-Terminal analysis of the separated chains showed that they were not contaminated with each other. The complete absence of intact insulin was demonstrated with HPLC (Wang & Qian, 1985). The S-thiomethylation of the chains was then carried out as described before for the intact chains.

S-Sulfonated Chains. The chains of porcine insulin were prepared according to Paynovich and Carpenter (1979).

Reconstitution of Insulin Derivatives. DP-insulin was mixed with the S-sulfonated A chain in 0.1 M Tris-HCl buffer containing 8 M urea and 2 mM EDTA at pH 8.1 to make a molar ratio of the chains of A/B = 1.5 and a total protein concentration about 12 mg/mL. The mixture was reduced at 37 °C under nitrogen for 40 min with DTT, the molar excess of which was calculated in terms of the SH content of DTT to the sulfur atoms of DP-insulin and the added A chain. HPLC analysis of the reduction products showed that there was still a trace of intact insulin left when the molar excess of DTT was 0.5-fold whereas at an excess of 1.2-fold almost no insulin peak could be discerned. A 2.5-fold excess of DTT was routinely used to ensure that the reduction reaction proceeded to completion. Determinations of protein SH groups in the presence of excess DTT by the method of Zahler and Cleland (1968) also showed that the reduction had been complete.

The reduction products were then precipitated with 20 volumes of cold acetone and washed twice with a mixture of 95% ethanol and acetone in a ratio of 1:20 by volume. The precipitate was finally washed with cold acetone and then dried under vacuum. The protein recovery during the precipitation and washing was usually about 90%, but the extent of reduction decreased to about 70–80% of the original value due, no doubt, to oxidation while the precipitate was being washed. The reduced chains were then dissolved in 0.1 M Gly–KOH buffer, pH 10.8, at a protein concentration of about 8 mg/mL in an open glass container at about 4 °C. The pH of the solution was adjusted, and the extent of reduction was checked intermittently during oxidation. It usually took 36–40 h for complete disappearance of the SH groups. The reoxidation product was then desalted by passage through a Sephadex G-25, fine, column at pH 8.5 and lyophilized. It was dissolved in 0.1% TFA to a protein concentration of 5 mg/mL, and 25-μL samples were taken for analysis by HPLC.

Determinations. Determinations of SH groups in the absence and presence of excess DTT were carried out with DTNB according to Ellman (1959) and Zahler and Cleland (1968), respectively. The N-terminal residues of the insulin chains were determined with (dimethylamino)azobenzene 4′-isothiocyanate as described by Chang et al. (1978). Amino acid analysis was carried out on a Beckman 121 MB analyzer after hydrolysis in 6 N HCl at 110 °C for 22 h, and nitrogen determinations were performed on a Perkin-Elmer 240 C machine. Reverse-phase HPLC was carried out with a Waters Associations HPLC system consisting of two M 6000A solvent delivery units, an M 660 solvent programmer, and a U6K universal chromatograph injector coupled to an M 440 UV spectrophotometer and an Omniscribe two-channel chart recorder. The column used was a μBondapak C_{18} (10 μm, 30 cm × 3.9 mm i.d.) from Waters. Elution was carried out with selected gradients (Wang & Qian, 1985) of solution A (40% methanol with 0.1% TFA) and solution B (90% methanol with 0.1% TFA).

Protein concentrations were routinely determined by absorbance at 278 nm. The millimolar absorption coefficients of both DP- and DO-insulin were found to be 4.4 with the protein concentrations initially determined both by the method of Lowry et al. (1951) using insulin as the standard and by amino acid analysis. The millimolar absorption coefficients for the *S*-(thiomethyl)-A chain, the DP *S*-(thiomethyl)-B chain, and the DO *S*-(thiomethyl)-B chain were found to be 3.4, 1.8, and 1.6, respectively. Amino acid analysis was used for the initial protein concentration calculation for the A chain, and both amino acid and nitrogen analysis were employed for the B-chain derivatives. The Receptor binding assay was carried out according to Kahn et al. (1977).

Difference Spectra. Determinations of the difference spectra of the mixed against the separated chains were carried out essentially as described by Hua et al. (1984). For solvent perturbation measurements, the method as described by Herskovits (1967) was employed with ATEE as the model compound. A Cary 219 spectrophotometer was used for these studies.

Results

Difference Spectra. Like the intact chains, the C-terminal-shortened B chains of insulin also interact with the A chain as evidenced from the difference spectra of the mixed against the individual chains. A molar ratio of the chains of A/B = 1.5 and pH 10.8 were employed for interaction studies as under these conditions the best yield of resynthesis of insulin from the chains can be obtained (Du et al., 1965). Also, like the intact chains (Hua et al. 1984), the difference spectra change with time and take some 2 h to reach completion as illustrated in Figure 1 for the DP and DO derivatives, respectively. The shape of the difference spectra also resembles that for the intact chains, showing negative peaks at 245 and 295 nm and a weak position peak at 278 nm. As in the case of the intact chains, the above would seem to indicate a transfer of some of the Tyr residues, partly ionized, from an exposed and hydrophilic to a buried and essentially nonpolar environment.

The above results were obtained with the truncated B chains freshly dissolved from lyophilized powders. Very unexpectedly, when the solutions of the shortened B chains were left to stand at pH 10.8 and 4 °C overnight before the difference spectra measurements were made, although similar difference spectra were observed, these were obtained almost immediately upon

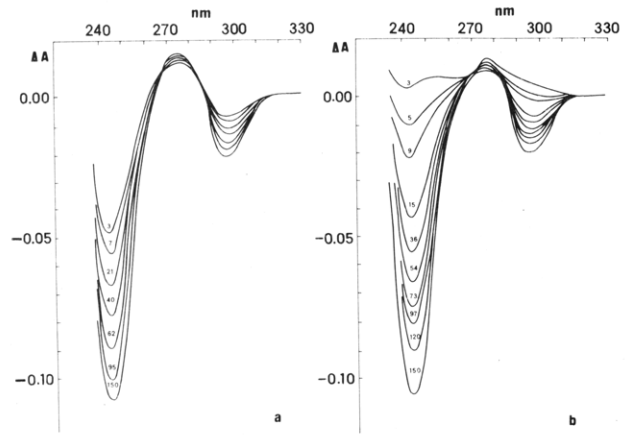

FIGURE 1: Interaction difference spectra of *S*-(thiomethyl)-A chain and fresh prepared shortened *S*-(thiomethyl)-B chains. The concentrations of the A and shortened B chains were 0.25 mM and 0.17 mM, respectively, in Gly-KOH buffer at pH 10.8. Tendem cuvettes were employed with the sample beam cuvette containing the mixed chains in one compartment and the buffer in the other compartment and the reference beam containing A chain in one compartment and B chain in the other compartment. (a) DP derivative; (b) DO derivative. In both (a) and (b), the chains were freshly dissolved and the difference spectra taken immediately after mixing of the chains. The numbers indicate the time in minutes after mixing.

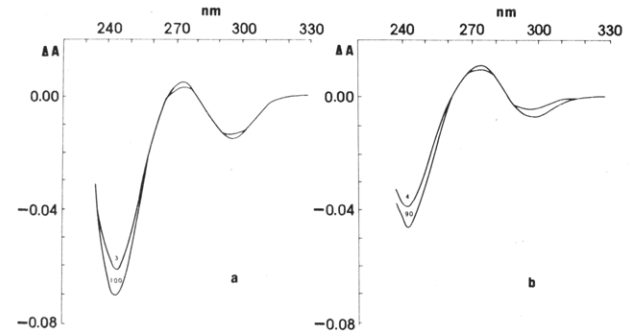

FIGURE 2: Interaction difference spectra of *S*-(thiomethyl)-A chain and aged shortened *S*-(thiomethyl)-B chains. Conditions were as in Figure 1 except that the shortened B chains were dissolved in the buffer employed and left to stand overnight at 4 °C before being mixed with the A chain and the difference spectra being taken. (a and b) DP and DO derivatives, respectively.

mixing and remained unchanged in the course of several hours (Figure 2) for both the DP and DO derivatives. For the intact B chains, similar results were obtained with either freshly dissolved samples or with solutions that have stood overnight at pH 10.8 and 4 °C.

Solvent Perturbation. The results of solvent perturbation studies with 20% ethylene glycol were summarized in Table I. The R_m values [as defined by Herskovits (1967)] observed suggested that as for intact B chain the single Tyr residue at B-16 is half buried for the DP derivative whereas it is completely exposed for the DO derivative. It has been shown previously from CD measurements (Hua et al., 1985) that some ordered secondary structure is present in the intact *S*-(thiomethyl)-B chain. This seems to be also the case for the DP B chain whereas the DO derivative appears to be structureless in the sense that the single Tyr residue is exposed. Results presented in Table I also show that, unlike the intact B chain, the mixing of the shortened B chains with the A chain has no appreciable effect on the extent of exposure of the Tyr residues.

Reconstitution of DP- and DO-insulin. The interaction of the shortened B chains with the intact A chain has also been studied by reconstitution from reoxidation of the reduced

Table I: Solvent Perturbation of Separated and Mixed *S*-(Thiomethyl)-A Chain and Shortened *S*-(Thiomethyl)-B Chains[a]

sample	$\Delta A_{293}/A_{276}$ chains	$\Delta A_{293}/A_{276}$ ATEE[b]	fraction of Tyr exposed R_m	fraction of Tyr exposed calcd[c]
ATEE		0.042		
A	0.024		0.58	
DP(B26–30) B	0.027		0.64	
DO(B23–30) B	0.048		1.14	
A + DP B	0.025		0.60	0.60
A + DO B	0.032		0.76	0.80

[a] The concentrations of the chains were, for the separated chains, [A] = 0.25 mM and [B] = 0.17 mM and for the mixed chains [A] = 0.125 mM and [B] = 0.085 mM. These were in 0.1 M Gly–KOH buffer, pH 10.8. The perturbant was 20% ethylene glycol. [b] The concentrations of ATEE used were in the range of the concentrations of Tyr residues as contained in the chains, and the value given was the average of a series of very similar values obtained at different ATEE concentrations. [c] Calculated from the values of the separated chains assuming no interaction.

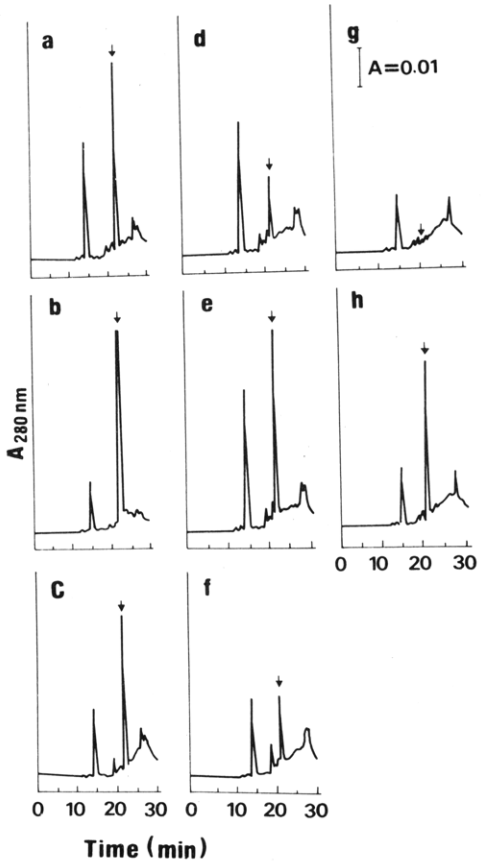

FIGURE 3: HPLC elution profile of resynthesis products of insulin and derivatives from the chains. Conditions for the resynthesis experiments and the reverse-phase HPLC system were described in the text. Elution was with a linear gradient of solvent A (40% methanol, 0.1% TFA) up to 80% of solvent B (90% methanol, 0.1% TFA) in 30 min. Arrows indicate the position of the resynthesized molecules, which was identified in each case by the addition of an authentic sample and coelution with the recombination product. (a) Insulin; (b) insulin with the addition of authentic insulin; (c) as (a), in the presence of 20% ethylene glycol; (d) DP-insulin; (e) DP-insulin with the addition of an authentic sample; (f) as (e), in the presence of 20% ethylene glycol; (f) DO-insulin; (g) DO-insulin with the addition of an authentic sample.

chains to form the truncated insulin derivatives under conditions where optimal reconstitution of insulin from the intact chains can be obtained. The reoxidation products were analyzed by the HPLC technique and by receptor binding assay. These results are summarized respectively in Figure 3 and in

Table II: Resynthesis of Insulin and Derivatives from the Chains

insulin or derivative	ethylene glycol (%)	yield (%) HPLC[a]	yield (%) receptor binding
authentic insulin			100
DP-insulin			17
resynthesized insulin		31.8	23
insulin	20	24.6	20
DP-insulin		9.3	8
DP-insulin	20	11.5	12
DO-insulin		1–3	

[a] The yield was calculated on the basis of the total absorbance at 280 nm of the resynthesis mixture and that of the peak corresponding to the resynthesized insulin or the desired derivative. The values listed were usually averages of several determinations. Conditions for resynthesis were as described in the text.

Table II. Figure 3a shows the chromatographic plot of the reoxidation products of the intact chains. Addition of an authentic sample of insulin to the reoxidation product shows that the middle peak is that for insulin (Figure 3b), and control experiments have shown that the front and the tail peaks are oxidation products of A and B chains, respectively. The presence of 20% ethylene glycol had no appreciable effect on the resynthesis yield of the hormone from its chains (Figure 3c). The results for the DP and DO derivatives are shown in Figure 3d–h, adding authentic samples of the insulin derivatives in each series to identify the DP- and DO-insulins formed. It can be seen that although a significant amount of DP-insulin can be obtained and this is not affected by ethylene glycol, there is only a trace of DO-insulin formed.

Table II lists the resynthesis yields as determined by both the HPLC analysis and the receptor binding assay. By HPLC analysis, the yield for the DP derivative, about 10%, was considerably lower than that for insulin itself, and that for the DO derivative was not more than 1–3%. The resynthesis yield for insulin was lower than that obtained earlier by Du et al. (1965). Receptor binding assay gave similar results with somewhat lower activities for all the samples tested as compared with those obtained by HPLC analysis.

Discussion

Interaction of Shortened B Chains with A Chain. Separated insulin chains have usually been considered to be devoid of ordered secondary structure (Wu & Yang, 1981; Du et al., 1982). However, it has recently been shown in this laboratory that both the reduced and the *S*-(thiomethyl)-protected A and B chains are not entirely structureless in solution as shown both by the partial shielding of Tyr residues from solvent perturbation with ethylene glycol and by direct CD measurements (Hua et al., 1984, 1985). It has now been shown in the present study that the single Tyr residue at B-16 is also partially protected from perturbation by ethylene glycol in the DP *S*-(thiomethyl)-B chain but not in the DO derivative, indicating that the removal of five amino acids from C-terminal of the B chain has not grossly affected its conformation in this respect whereas the further removal of three amino acid residues leads to the complete exposure of this Tyr residue in the DO B chain. It is to be remembered that, in spite of some differences in the side chains, the backbone structure of insulin is largely preserved in DP-insulin (Liang et al., 1983). The detailed structure of DO-insulin is not yet available, but there is evidence to suggest that compared to DP-insulin further change has occurred and its structure could be significantly different from that of the native hormone (Gattner, 1975; Lu et al., 1981).

Very similar difference spectra of the mixed against the separated chains have been obtained for the truncated B chains as compared to the intact chain showing strong negative peaks at 245 and 295 nm and a weak positive peak at 280 nm. The ratio of the peak heights at 245 and 295 nm is about 5, suggesting that these peaks are due to a partial transfer of ionized Tyr residues from an exposed and hydrophilic to a buried and nonpolar environment upon interaction of the truncated B chains with the A chain as observed for the intact chains. Unlike the results obtained for the intact chains, no difference has been observed for the extent of exposure of the Tyr residues upon mixing of the A chain with the C-terminal-shortened B chains by solvent perturbation studies with ethylene glycol. This would seem to suggest that the presence of this solvent has weakened the interaction of the truncated B chains with the A chain to such an extent that no environmental change of the Tyr residues has occurred. It is known that ethylene glycol does affect the conformation of some proteins by weakening the hydrophobic interactions (Herskovits, 1965; Herskovits & Laskowski, 1968), and it seems likely that the shortened B chains are liable to be thus affected. However, the presence of 20% ethylene glycol has not affected the resynthesis yield of DP-insulin from the respective chains; the interaction as indicated by the change in absorption of the Tyr residues probably does not play a decisive role in the correct pairing of the chains.

Resynthesis of DP- and DO-insulins. DP-insulin can be resynthesized with a fairly good yield whereas not more than a trace of DO-insulin can be obtained from the respective chains. This has been shown by HPLC analysis as well as by receptor binding assay, and the results obtained by receptor binding assay, although somewhat lower than those of HPLC analysis, are in general agreement with the latter. The amino acids at the C-terminal part of the B chain appear to affect not only the biological activity of this hormone but also the recognition and correct pairing of the chains. This is in accord with the suggestion by Schwartz and Katsoyannis (1978) and by Gattner et al. (1981) that the yields of resynthesis of insulin analogues from the chains are correlated with the biological activity of these analogues.

It should be pointed out that although similar difference spectra have been observed for the mixed against the individual chains of the DP and DO derivatives as compared to the intact chains, very different resynthesis yields are obtained, especially for the DO derivative. It appears that the partial transfer of Tyr residues from an exposed to a buried environment is not essential for the correct pairing of the chains. As the presence of 20% ethylene glycol does not affect the resynthesis of either insulin or the DP derivative (Table II), the shielding of Tyr residues in the mixed intact chains against solvent perturbation (Hua et al., 1984) no longer occurs in the shortened chains. This would seen to indicate that the correct pairing and, consequently, successful resynthesis of the chains are not much affected by the presence of 20% ethylene glycol and probably involve multiple secondary interactions of the side-chain functional groups, the nature of which remains to be clarified.

Time Course of Interaction. The fact that the interaction of the *S*-(thiomethyl) chains takes some time to reach completion as observed both by difference spectrum and CD measurements has been ascribed to a slow conformational adjustment required for the correct pairing of the chains (Hua et al., 1984, 1985). A similar time course has been observed for the interaction of the truncated B chains with the A chain when the solutions of the shortened *S*-(thiomethyl)-B chains are freshly prepared. Unexpectely, when solutions of these

B-chain derivatives aged overnight, the maximal absorbance values of the difference spectra were obtained within a very short time. On the other hand, aging has no appreciable effect on the course of interaction with the intact chains. It appears that some slow conformational change has taken place after dissolution of the intact and shortened B chains, and this is required for their interaction with the A chain. Moreover, the intact and the truncated B chains are somewhat different in that for the intact B chain, even after an overnight standing, further conformational changes are required for the interaction to take place whereas this is either unnecessary or is a rapid process for the shortened B chains.

Evidently, removal of several amino acids at the C-terminal of B chain has produced a marked effect on the conformation of the entire peptide chain. Moreover, it is all together unexpected that conformational changes of peptides of the length of the insulin chains can be such slow processes (Careri et al., 1975). Whether the cis–trans isomerization of the peptide bond that contains the imide nitrogen of the single Pro residue near the C-terminal end of the chain at B-28 can play any role in this process would require careful consideration.

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Registry No. DP, 102283-04-5; DO, 102283-03-4; S-thiomethylated porcine insulin A chain, 102342-78-9; S-thiomethylated despentapeptide(26–30) porcine insulin B chain, 102342-79-0; S-thiomethylated desoctapeptide(23–30) porcine insulin B chain, 102342-80-3; despentapeptide(26–30) porcine insulin B chain, 41432-74-0; desoctapeptide(23–30) porcine insulin B chain, 77282-70-3.

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