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# The insulin A and B chains contain structural information for the formation of the native molecule

## Studies with protein disulphide-isomerase

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It has been shown previously [Tang, Wang & Tsou (1988) Biochem. J. 255, 451–455] that, under appropriate conditions, native insulin can be obtained from scrambled insulin or the *S*-sulphonates of the chains with a yield of 25–30 %, together with reaction products containing the separated A and B chains. The native hormone is by far the predominant product among the isomers containing both chains. It is now shown that the presence of added C peptide has no appreciable effect on the yield of native insulin. At higher temperatures the content of the native hormone decreases whereas those of the separated chains increase, and in no case was scrambled insulin containing both chains the predominant product in the absence of denaturants. Both the scrambling and the unscrambling reactions give similar h.p.l.c. profiles for the products. Under similar conditions cross-linked insulin with native disulphide linkages can be obtained from the scrambled molecule or from the *S*-sulphonate derivative with yields of 50 % and 75 % respectively at 4 °C, and with a dilute solution of the hexa-*S*-sulphonate yields better than 90 % can be obtained. The regenerated product is shown to have the native disulphide bridges by treatment with CNBr to give insulin and by the identity of the h.p.l.c. profile of its peptic hydrolysate with that for cross-linked insulin. It appears that the insulin A and B chains contain sufficient information for the formation of the native molecule and that the role of the connecting C peptide is to bring and to keep the two chains together.

## INTRODUCTION

It was suggested some years ago that, among all the possible isomeric structures, the native insulin structure is the most stable, on the basis of a reasonably good yield of the hormone when the reduced A and B chains were reoxidized together (Du *et al.*, 1961, 1965). The successful resynthesis has since not only been confirmed in different laboratories (Zahn *et al.*, 1966; Katsoyannis *et al.*, 1967) but has also been applied to the production of human insulin from the chains obtained separately by a recombinant DNA technique (Chance *et al.*, 1981). However, both the fact that insulin can be obtained from its chains in reasonably good yield and the related suggestion that the insulin A and B chains contain sufficient information for the formation of the native structure have not met with general recognition (Anfinsen & Scheraga, 1975; Brandenburg *et al.*, 1977; Hillson *et al.*, 1984; Lehninger, 1984; Alberts *et al.*, 1989). This is probably partly due to the facts that previous workers failed to obtain native hormone from 'scrambled' insulin with randomly joined disulphide bonds through thiol–disulphide exchange reactions catalysed by protein disulphide-isomerase (PDI) and that treatment of insulin itself by this enzyme led to the destruction of native insulin structure (Givol *et al.*, 1965; Varandani & Nafz, 1970). Varandani & Nafz (1970) also reported that some insulin was obtained from scrambled molecules in the presence of added C peptide, suggesting that structural information was conferred on the whole molecule by this peptide.

In a previous paper we showed that, in the presence of PDI and dithiothreitol (DTT) at pH 7.5 and 4 °C, native insulin can indeed be formed from scrambled insulin in 25–30 % yield (Tang *et al.*, 1988). It is now shown that the yield decreases with increasing temperature and that the addition of C peptide has no appreciable effect. The decrease in insulin yield is due to the formation of products containing only one of the chains, and in no case were products with isomeric structures containing both chains the predominating products for both the scrambling and unscrambling reactions. With $N^{\alpha A1}N^{\epsilon B29}$-cross-linked insulin, molecules containing native disulphide bonds can be obtained in better than 90 % yield from the hexa-*S*-sulphonate derivative. It is suggested that the sequences of the A and B chains provide the necessary information for the formation of the native insulin structure.

## EXPERIMENTAL

Bovine insulin was from Sigma Chemical Co. Scrambled insulin, the *S*-sulphonates of the chains and PDI were prepared as described previously (Tang *et al.*, 1988) except that scrambled insulin was obtained by oxidation of the reduced chains in 6 M-guanidinium chloride and had a residual activity 1–2 % of that of native insulin. C peptide was a synthetic product kindly provided by Professor Niu of the Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, China (Niu *et al.*, 1981). CNBr and pepsin with a specific activity of 100 munits/mg were from Merck. Carbonylbis-(L-methionine *p*-nitrophenyl ester) was a product of Pierce Chemical Co. Other reagents were local products of analytical grade used without further purification.

CBM-insulin, cross-linked at the α- and ε-amino groups of Gly-A1 and Lys-B29 residues respectively, was prepared in accordance with Busse & Carpenter (1976) except the column chromatography on DEAE-cellulose was carried out twice. It had a receptor-binding activity 1.8 % of that of native insulin, and an amino acid analysis showed the presence of the expected amount of methionine. Scrambled CBM-insulin was prepared by dissolving CBM-insulin (15 mg; final concentration 5.2 mM) in

Abbreviations used: CBM-insulin, $[N^{\alpha A1}N^{\epsilon B29}$-carbonylbismethionyl-insulin; DTT, dithiothreitol; PDI, protein disulphide-isomerase.

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0.5 ml of 0.1 M-Tris/HCl buffer, pH 8.1, containing 2 mM-EDTA and 6 M-guanidinium chloride and reduced with 32 mM-DTT for 40 min at 37 °C. The solution was dialysed against 50 mM-$NH_4HCO_3$ to remove excess DTT and then diluted to 30 ml with 0.1 M-glycine/NaOH buffer, pH 10.8, containing 6 M-guanidinium chloride followed by air oxidation at 15 °C for 24 h. The product was then dialysed against 50 mM-acetic acid and freeze-dried. Scrambled CBM-insulin thus prepared contained 5% CBM-insulin, which is also approximately the amount expected from random joining of the thiol groups. The *S*-sulphonate of CBM-insulin was prepared by sulphitolysis in 1 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and 6 M-guanidinium chloride for 1 h at 37 °C. The reaction mixture contained 2.6 mM-CBM-insulin, 178 mM-$Na_2SO_3$ and 56 mM-$Na_2S_4O_6$. It was then dialysed against 50 mM-$NH_4HCO_3$ and freeze-dried. Conditions for the PDI-catalysed reactions were as described previously (Tang *et al.*, 1988). Unless otherwise specified, the PDI-catalysed reactions were carried out with 103 $\mu$M-insulin or its derivatives, 6.4 $\mu$M-PDI and the specified concentration of DTT in 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA at 4 °C for 24 h. The partial inactivation of insulin and destruction of CBM-insulin structure in the presence of PDI is referred to as the scrambling reaction, and the regeneration of native insulin from either the scrambled preparation or the *S*-sulphonates of the chains and the formation of CBM-insulin with the native disulphide linkages from a scrambled preparation or its *S*-sulphonated derivative by PDI are simply described as the unscrambling reaction.

Digestion of insulin and derivatives with pepsin was carried out by dissolving the protein at a concentration of 1 mg/ml in 0.1 M-HCl and hydrolysing with 0.02 mg of pepsin/ml for 24 h at 37 °C, at the end of which the initial material had completely disappeared from the h.p.l.c. profile.

CNBr treatment of CBM-insulin was carried out in accordance with Busse & Carpenter (1976) by treatment of 0.2 mg of protein in 50 $\mu$l of 70% (v/v) formic acid with 10 mg of CNBr at 25 °C for 2 h. The content of regenerated insulin in the product was analysed by a receptor-binding assay.

The reverse-phase h.p.l.c. analysis of insulin and derivatives was carried out as described previously (Wang & Tsou, 1986; Tang *et al.*, 1988) with a Waters Associates h.p.l.c. system. The insulin or CBM-insulin contents of the reaction products were determined by integration of the respective h.p.l.c. peaks. For the h.p.l.c. analysis of the peptic hydrolysate, the same reverse-phase column was used but with a solvent of acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid and a gradient (curve 7 on the Waters instrument) from 0 to 70% (v/v) acetonitrile in 35 min. The eluate was monitored at 220 nm with a Hitachi tunable-wavelength effluent monitor. Insulin contents of the reaction products were also determined by competition of receptor binding with $^{125}$I-insulin (Mori & Wond, 1984) and by the stimulation of lipogenesis (Moody *et al.*, 1974).

## RESULTS

### Amount of DTT required for the unscrambling reaction

It was shown in a previous paper (Tang *et al.*, 1988) that the presence of DTT is necessary for the unscrambling of insulin. Figs. 1 and 2 show the effect of DTT concentration on the yield of native insulin for scrambled insulin and from the *S*-sulphonates of the chains respectively. The insulin contents of the reaction products were determined from the h.p.l.c. profile and by biological assay. It is to be noted that for the unscrambling of scrambled insulin a catalytic amount of DTT is enough for the rearrangement of the disulphide bonds, but for the *S*-sulphonates of the chains close to the stoichiometric amount of DTT for the following reaction (Frank *et al.*, 1981) is required to produce the optimal yield:

$$R(SH)_2 + R'(SSO_3^-)_2 \rightarrow R(S)_2 + R'(S)_2 + 2HSO_3^-$$

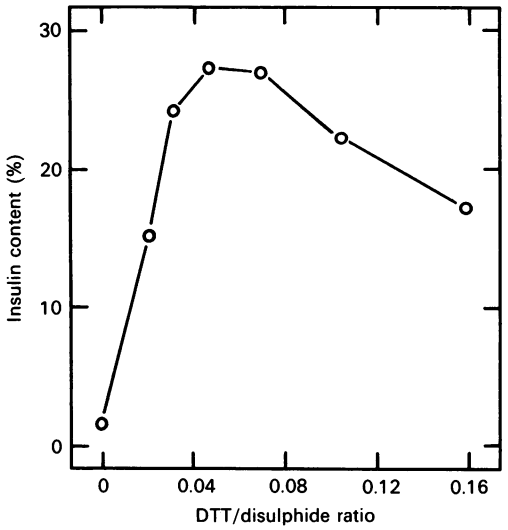


**Fig. 1. Effect of DTT concentration on the yield of native insulin from the scrambled preparation**

For experimental conditions see the text.

### Effect of temperature on the unscrambling reaction

The h.p.l.c. profiles of the products for the unscrambling of scrambled insulin at different temperatures are shown in Fig. 3, showing the decrease in peak height for native insulin with increasing temperature. The unscrambling of an equimolar mixture of the A chain and B chain *S*-sulphonates at a thiol/*S*-sulphonate ratio of 1.25 and a final DTT concentration of 375 $\mu$M produced h.p.l.c. profiles closely similar to those shown in Fig. 3 at the respective temperatures. The identities of the respective peaks have been previously characterized (Tang *et al.*,

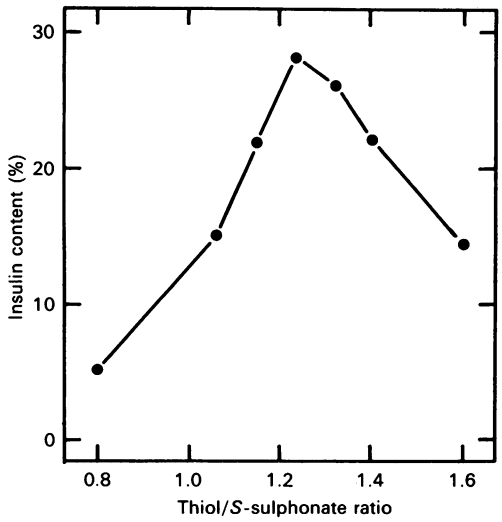


**Fig. 2. Effect of DTT concentration on the yield of native insulin from a mixture of the *S*-sulphonates of A and B chains**

The molar ratio of A and B chains was 1:1 and the final protein concentration was 0.6 mg/ml. For other experimental conditions see the text.

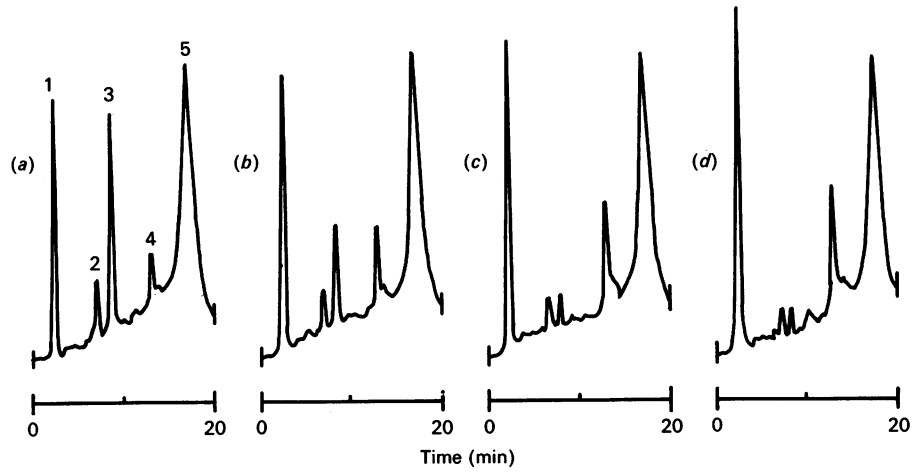


**Fig. 3. H.p.l.c. profiles at different temperatures for the unscrambling of scrambled insulin**

As identified in a previous paper (Tang *et al.*, 1988), peaks 1–5 indicate respectively the positions of oxidized A chain, inactive products containing both chains joined presumably with incorrect disulphide linkages, native insulin, oxidized B chain and PDI. The reaction mixture contained 17 $\mu$M-DTT and scrambled insulin with a DTT/disulphide ratio of 0.055. The h.p.l.c. profiles of unscrambling products obtained at 4, 15, 26 and 37 °C are shown in (*a*), (*b*), (*c*) and (*d*) respectively.

1988). It is to be noted that with the decrease in the peak for native insulin at higher temperatures the peaks for the separate chains increase, but in no case is the peak corresponding to products containing both chains higher than the insulin peak. The decrease in yield of insulin is therefore not due to the increase in the formation of $A_1B_1$ isomers with incorrect disulphide bridges, which should be eluted at peak 2, immediately preceding peak 3. Further, lowering the temperature to −5 °C in 20% (v/v) ethylene glycol did not increase the yield of the unscrambling reaction, nor had altering solvent polarity with 0.5 M-KCl any effect.

### Treatment of insulin with PDI

The h.p.l.c. profiles of the products of the scrambling of insulin at different temperatures are closely similar to those of the unscrambling reaction shown in Fig. 3. The yield of insulin at 37 °C is in agreement to that reported by Givol *et al.* (1965) and by Varandani & Nafz (1970). However, like the unscrambling reaction, there is no significant formation of products containing both chains. With increasing temperature the gradual decrease in the insulin peak was accompanied by increases in the peaks for the separate chains, but not the peak at the position expected for the $A_1B_1$ isomers of insulin (Tang *et al.*, 1988). Fig. 4 compares the contents of native insulin at different temperatures for both the scrambling and the unscrambling reactions. It is also clear from a comparison of the respective h.p.l.c. profiles (not shown) that, no matter whether one started from insulin, scrambled insulin or the *S*-sulphonates of the chains, similar relative amounts of the final products were obtained in the presence of DTT and PDI.

### Effect of C peptide

Varandani & Nafz (1970) reported that no native insulin could be obtained from scrambled insulin unless the C peptide was added. The implication is that the C peptide contained structural information that it somehow conferred on the A and B chains so as to promote the formation of the native hormone. However, in our hands the addition of an equivalent amount of C peptide (0.35 mg/ml, 104 $\mu$M) to scrambled insulin (0.6 mg/ml, 103 $\mu$M), as used by Varandani & Nafz (1970), had no appreciable effect on the yield of native insulin in the presence of PDI and DTT at either 37 °C or 4 °C (results not shown).

### Unscrambling and scrambling reactions of CBM-insulin and its *S*-sulphonate

Under appropriate conditions both scrambled CBM-insulin and its hexa-*S*-sulphonate can be unscrambled with PDI and DTT to give CBM-insulin with the correct disulphide bridges. The h.p.l.c. profiles of the products of the unscrambling reaction from scrambled CBM-insulin and the hexa-*S*-sulphonate are shown in Fig. 5. Addition of DTT alone generates 8.3% and

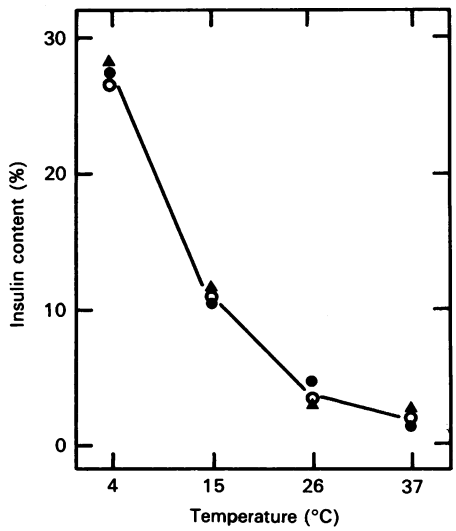


**Fig. 4. Contents of native insulin at different temperatures for the scrambling and unscrambling reactions**

The experimental conditions for scrambled insulin were as given in Fig. 3 legend. Conditions for the other reactions were as described in the text. Curves represented by ▲—▲, ○—○ and ●—● are for reactions starting from native insulin, scrambled insulin and an equimolar mixture of the *S*-sulphonates of the A and B chains respectively.

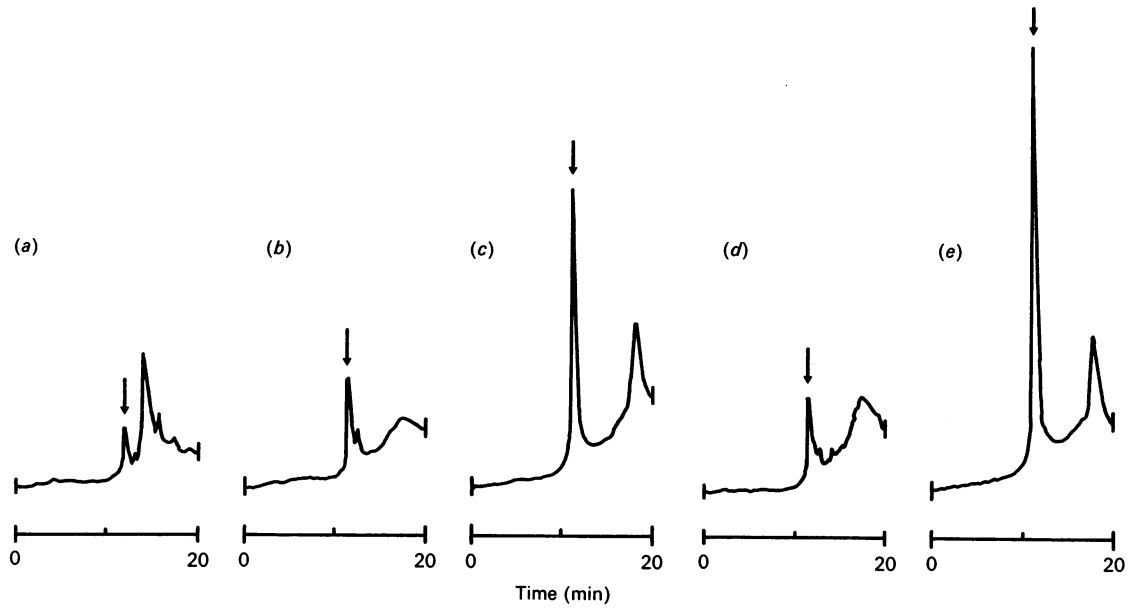


**Fig. 5. H.p.l.c. profiles of the unscrambling products from scrambled CBM-insulin and CBM-insulin hexa-*S*-sulphonate**

The arrow indicates the positions of CBM-insulin with native disulphide bonds. Scrambled products with incorrect disulphide linkages are eluted after CBM-insulin and are presumably more hydrophobic. (*a*) H.p.l.c. profile of scrambled CBM-insulin. (*b*) After reaction of the scrambling products with DTT alone at a DTT/disulphide ratio of 0.16. (*c*) As (*b*), but after reaction with both DTT and PDI. (*d*) Reaction products of the *S*-sulphonate of CBM-insulin with 360 $\mu$M-DTT at a thiol/*S*-sulphonate ratio of 1.2. (*e*) As (*d*), but reaction products obtained in the presence of both DTT and PDI. The second peak in (*c*) and (*e*) is the peak for PDI as a sharp peak at the end of the elution.

11.6% of CBM-insulin from scrambled CBM-insulin and the hexa-*S*-sulphonate respectively, whereas in the presence of both DTT and PDI yields of 50% and 75% were obtained respectively, as shown by the appearance of a peak at the position of authentic CBM-insulin, the identity of which is demonstrated below. At high DTT concentrations the species with native disulphide bonds decreases, probably as a result of the formation of partially reduced products. For the unscrambling from the hexa-*S*-sulphonate derivative, the thiol/*S*-sulphonate ratio is optimal around 1.2, close to the value required by the stoichiometry of the reaction. The unscrambling of CBM-insulin is a faster reaction than that for insulin and approaches completion within about 0.5 h at 4 °C. In contrast with insulin, the unscrambling of CBM-insulin is favoured in dilute solutions, so as to avoid the formation of oligomers. In fact, for the dimeric molecule, the total number of possible isomers will be 5243, as calculated by the equation given by Wang *et al.* (1987). Indeed, with CBM-insulin hexa-*S*-sulphonate, when the protein concentration is lowered to 0.1 mg/ml yields better than 90% can be obtained at 4 °C.

The addition of DTT and PDI to CBM-insulin leads to a partial scrambling of the molecule, resulting in a decrease in the species containing the correct disulphide bridges. The h.p.l.c. profile of the products is similar to that obtained in the unscrambling reaction.

### Effect of temperature on the scrambling and unscrambling reactions of CBM-insulin

The h.p.l.c. profiles of the products for both the unscrambling and the scrambling reactions of CBM-insulin, as well as the unscrambling reaction of CBM-insulin hexa-*S*-sulphonate, at different temperatures were compared. In all cases the yields of the product corresponding to the species containing the native disulphide bridges decrease at higher temperature, as shown in Fig. 6. There was also an increase of products eluted at higher concentrations of methanol, these presumably being more hydrophobic in nature than CBM-insulin (results not shown).

Comparison of the temperature effects on the scrambling and unscrambling reactions of insulin and cross-linked insulin clearly shows (Figs. 4 and 6) that the native structure of CBM-insulin is less sensitive to temperature than is the corresponding structure of insulin. Fig. 6 also shows that CBM-insulin decreases at

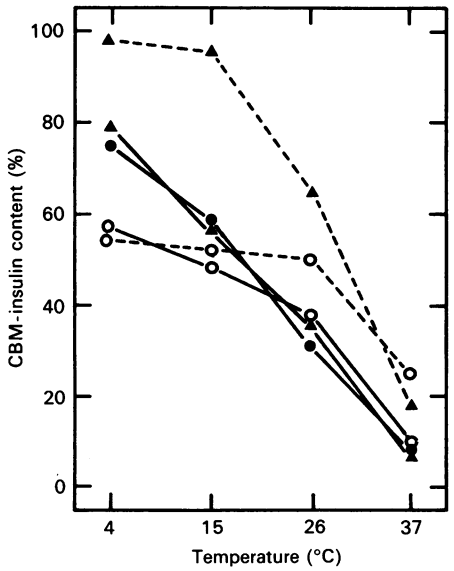


**Fig. 6. Effect of temperature on the scrambling and unscrambling reactions for CBM-insulin**

The scrambling of CBM-insulin at DTT/disulphide ratios of 0.16 and 0.055 are shown in curves ▲—▲ and ▲--▲ respectively. Unscrambling reactions are shown by curves ○—○ and ○--○ for the unscrambling of scrambled CBM-insulin at DTT/disulphide ratios of 0.16 and 0.055 respectively. Curve ●—● shows the unscrambling of CBM-insulin hexa-*S*-sulphonate with a thiol/*S*-sulphonate ratio of 1.2. For other experimental conditions see the text.

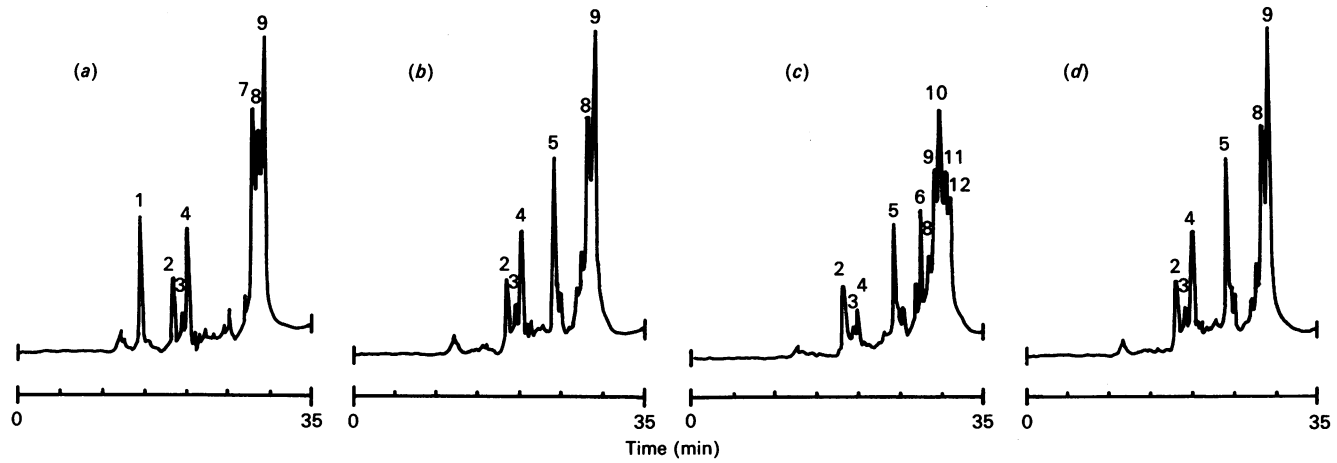


**Fig. 7. H.p.l.c. profiles of the products of peptic digestion**

The h.p.l.c. profiles of the products of peptic digestion of (*a*) insulin, (*b*) CBM-insulin, (*c*) scrambled CBM-insulin and (*d*) regenerated CBM-insulin are shown. Regenerated CBM-insulin was obtained by collecting the h.p.l.c. eluate at the position expected for CBM-insulin from the reaction product of PDI-treated CBM-insulin hexa-*S*-sulphonate. It was freeze-dried after removal of methanol by evaporation, redissolved and then digested by pepsin. For other experimental details and a discussion of the respective peaks in the profile see the text.

higher DTT/disulphide ratio for both the scrambling and the unscrambling reactions, probably as a result of the formation of partially reduced products.

### Identification of the unscrambling product

As CBM-insulin is much less biologically active than insulin (Busse & Carpenter, 1976; Brandenburg *et al.*, 1977), contamination by a small amount of insulin in the CBM-insulin preparation could seriously interfere with determination of the activity of the unscrambling product. A fingerprinting technique and cleavage by CNBr to regenerate insulin were used for the identification of the product obtained from the unscrambling reactions. The peak eluted at the expected position of CBM-insulin during h.p.l.c. separation was collected and hydrolysed with pepsin, and the h.p.l.c. profile of the products was compared with those for the hydrolysates of insulin, CBM-insulin and scrambled CBM-insulin, as shown in Fig. 7. It is evident from a comparison of Figs. 7(*a*) and 7(*b*) that peaks 1 and 7 from the insulin hydrolysate disappeared and were replaced with a new peak, 5, in the profile for the peptic hydrolysate of CBM-insulin. This is as expected, since the *C*- and *N*-terminal peptides of B and A chains respectively would be joined by the cross-linking reagent. From the expected sites of peptic cleavage of insulin chains, peak 1 is very probably the relatively hydrophilic peptide B-(26–30), peak 7 the hydrophobic peptide A-(1–4) and peak 5 the two peptide fragments connected by the cross-linking reagent. The h.p.l.c. profile of the peptide fragments of the scrambled product (Fig. 7*c*) is considerably different from that obtained from CBM-insulin by virtue of the decrease in the heights of peaks 8 and 9, which are no doubt the peptides containing the native disulphide linkages, and the appearance of a number of new peaks, 6, 10, 11 and 12, which are very probably peptides containing disulphide linkages formed during the scrambling reaction. It is possible that the cleavage sites of the scrambled molecule are not completely identical with those of CBM-insulin, because of conformational changes brought about the formation of disulphide linkages not present in CBM-insulin. It is to be noted that peaks 10, 11 and 12 are more hydrophobic than the peptides containing native disulphide linkages, as represented by peaks 8 and 9. That the principal unscrambling product is indeed CBM-insulin is shown by the complete identity of the h.p.l.c. profiles of their peptic hydrolysates (Figs. 7*b* and 7*d*).

The identity of the regenerated CBM-insulin has further been shown by the formation of insulin after treatment with CNBr as described by Busse & Carpenter (1976), with a yield similar to that obtained by these authors.

## DISCUSSION

In spite of reports from different laboratories that good yields of insulin can be obtained when the reduced A and B chains were reoxidized together (Du *et al.*, 1961, 1965; Zahn *et al.*, 1966; Katsoyannis *et al.*, 1967; Chance *et al.*, 1981), it has not been generally recognized that insulin can be obtained from its chains with a yield better than expected by random formation of the disulphide bridges, and consequently the suggestion has been made that the A and B chains contain sufficient information for the formation of the native structure (Anfinsen & Scheraga, 1975; Brandenburg *et al.*, 1977; Hillson *et al.*, 1984; Lehninger, 1984; Alberts *et al.*, 1989). Apparently, the calculation on the yield of random formation of the disulphide linkages made by some authors was based on the consideration of the formation of the $A_1B_1$ isomers only (Pruitt *et al.*, 1966). In fact, had the formation of the disulphide linkages been completely random, monomeric as well as oligomeric products containing one or both of the chains could be formed, and the total possible number of products would be immense indeed, as has been calculated by Kauzmann (1959) and Chance *et al.* (1981) and more recently corrected for the number of $A_2$ isomers by Wang *et al.* (1987). Completely random joining of the thiol groups to form disulphide linkages would result in a negligible yield of the native molecule, as has been shown by the oxidation of the reduced chains in the presence of either 6 M-guanidinium chloride or 0.1% SDS (Qian & Tsou, 1987).

Givol *et al.* (1965) and Varandani & Nafz (1970) reported that when insulin was treated with PDI at 37 °C the native structure was completely destroyed, and that, although it was possible to increase the content of proinsulin in a preparation of the scrambled molecule by treatment with PDI from 12% to 37%, very little insulin was obtained from a scrambled insulin prep-

aration containing about 2% insulin under similar conditions. We have now shown that, under suitable conditions, 25–30% insulin could be obtained for both the scrambling and unscrambling reactions. The main difference between our experimental conditions and those of previous workers is a difference in temperature. As for the resynthesis of insulin from the reduced chains, a temperature of 4 °C was used, whereas both Givol *et al.* (1965) and Varandani & Nafz (1970) used 37 °C. Indeed, we can confirm the results obtained by these authors in that we have shown that on increasing the temperature to 37 °C the final yields of insulin decrease markedly for both the scrambling and the unscrambling reactions. However, h.p.l.c. analysis of the products shows that, with increasing temperature, the decrease in native insulin among the reaction products is accompanied by an increase, not of products containing both chains, but of products containing only one of the chains. In all cases the peak corresponding to products containing both chains also decreases slightly with increasing temperature. In fact Givol *et al.* (1965) did produce evidence suggesting that the precipitate formed during the scrambling reaction of insulin with PDI contained products with only one of the chains.

The addition of the connecting C peptide has been reported (Varandani & Nafz, 1970) to increase the yield of the unscrambling reaction of scrambled insulin by PDI to about 12.8% at 37 °C. However, in the present study the addition of a similar amount of C peptide was found to have no appreciable effect on the yield at either 4 °C or 37 °C. Nevertheless with the two chains cross-linked with carbonylbismethionyl, a yield of 50% could be obtained from the scrambled molecule at 4 °C. Under carefully controlled conditions and with a dilute solution of CBM-insulin hexa-*S*-sulphonate, yields over 90% can be obtained. It is also to be recalled that oxidation of the $N^{\alpha A1}N^{\epsilon B29}$-cross-linked reduced chains gives the native disulphide linkages in good yields (Brandenburg & Wollmer, 1973; Busse & Carpenter, 1976), as obtained for native proinsulin from the oxidation of reduced proinsulin reported by Steiner & Clark (1968) and by Bullesbach *et al.* (1980) or from the *S*-sulphonate derivative as reported by Frank *et al.* (1981). As our results and those reported by different authors were obtained with different chemical cross-linking reagents, it is most unlikely that the cross-linking moiety contains any structural information required to assist the correct pairing of the chains. The above results suggest strongly that the role of the connecting C peptide in proinsulin is limited to bringing and to keeping the A and B chains together during both the oxidation and the unscrambling reactions. The necessary structural information is provided entirely by the A and B chains themselves.

The formation of the native disulphide bridges is favoured by low temperatures, as shown previously during the oxidation of the reduced chains (Du *et al.*, 1961, 1965; Katzen & Tietze, 1966). It was suggested in a previous paper (Tang *et al.*, 1988) that, for the separate chains, low temperatures would decrease the mobility of the chains in solution and consequently increase the opportunity for the chains to be correctly paired. This is likewise essential not only for the separately oxidized chains but also for the scrambled molecules containing both chains, even though the chains could be connected together all the time during the rearrangements of the disulphide bridges. The latter consideration applies especially to the unscrambling of cross-linked insulin and its *S*-sulphonate derivative. The formation of the native disulphide linkages in the cross-linked molecules is less sensitive to an increase in temperature. The fact that they are still sensitive to increasing temperature suggests that some other factors are to be considered. The insulin chains are known to have the tendency to polymerize (Lu & Tsao, 1962; Lu & Cui, 1981), especially the B chain, and this decreases the formation of the $A_1B_1$ isomers. The tendency towards self-association of the individual chains would be decreased but probably not completely prevented by cross-linking the two chains, with the formation of oligomeric CBM-insulin, especially at high concentrations, as evidenced by the increase in yield of the unscrambling reaction in dilute solutions. It is not understood why low temperature would favour the pairing of A and B chains at the expense of oligomerization of the separate chains, unless different forces with different temperature coefficients are responsible for the above two reactions.

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