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Formation of native insulin from the scrambled molecule by protein disulphide-isomerase

Jian-Guo TANG, Chih-Chen WANG and Chen-Lu TSOU*
Institute of Biophysics, Academia Sinica, Beijing 100080, China

The formation of native insulin either from scrambled insulin or from the separated A chain and B chain *S*-sulphonates by protein disulphide-isomerase was demonstrated with yields of 20–30 % as measured by h.p.l.c. analysis, receptor binding and stimulation of lipogenesis. The h.p.l.c. profile of the reaction products shows that, among all the possible isomers containing both chains, the native hormone is by far the predominating product and consequently the most stable under certain conditions.

INTRODUCTION

Under appropriate conditions the reduced insulin A and B chains can be re-oxidized together to give the native hormone with reasonably good yields, and with A chain in excess yields of 50 % or better can be obtained (Du *et al.*, 1961, 1965). As the total number of possible products when reduced A and B chains of insulin are oxidized together is immense indeed, taking into consideration all the oligomers that can be formed by random joining of the chains (Chance *et al.*, 1981; Wang *et al.*, 1987), it has been suggested that the insulin A and B chains are capable of recognizing each other and that a correct pairing of the chains precedes the eventual formation of the disulphide bridges of the native hormone (Du *et al.*, 1961). However, in spite of the confirmation in different laboratories (Zahn *et al.*, 1966; Katsoyannis *et al.*, 1967) of the resynthesis of insulin from its chains in good yield and its successful application to the production of human insulin from the chains obtained separately by recombinant DNA technique (Chance *et al.*, 1981), the suggestion that the A and B chains of insulin do indeed contain sufficient structural information so as to be able to pair correctly in solution and that under certain conditions the insulin structure is the most stable of all the possible oxidation products containing both chains does not seem to have been generally accepted (Anfinsen & Scheraga, 1975; Brandenburg *et al.*, 1977; Hillson *et al.*, 1984). This is probably partly due to the fact that previous workers (Varandani & Natz, 1970) failed to obtain the native hormone from 'scrambled' insulin with randomly joined disulphide bonds through thiol/disulphide exchange reactions catalysed by protein disulphide-isomerase under the conditions in which proinsulin can be obtained in 25 % yield from the scrambled molecule. It is known that this isomerase is able to promote the reshuffling of the disulphide bridges in a number of scrambled and hence inactive proteins, resulting in the formation of native conformation with full recovery of activity (Freedman & Hillson, 1980; Freedman *et al.*, 1984). It is now shown that in the presence of protein disulphide-isomerase and dithiothreitol at pH 7.5 and 4 °C native insulin can indeed be formed from scrambled insulin or a mixture of the separate *S*-sulphonated chains in 20–30 % yield.

EXPERIMENTAL

Scrambled insulin was prepared by air oxidation of the reduced chains in 0.1 M-glycine/NaOH buffer, pH 10.8, at 4 °C containing 4.2 M-guanidine for 36 h followed by dialysis and freeze-drying. It had a residual activity about 4 % of that of native insulin. Oxidized A and B chains were prepared separately from the respective reduced chains under similar conditions to those used for the mixed chains except that the oxidation was carried out in the absence of guanidine. Sulphitolysis of insulin to give the *S*-sulphonates of the chains was carried out in accordance with Paynovich & Carpenter (1979) with yields usually about 85 %, and the reduced chains were then prepared from the *S*-sulphonated chains by reduction with an excess of dithiothreitol as described previously (Wang & Tsou, 1986). Protein disulphide-isomerase was prepared as described by Hillson *et al.* (1984), and had a specific activity of 750 units/g of protein with scrambled pancreatic ribonuclease A as the substrate as defined by Ibbetson & Freedman (1976).

Reverse-phase h.p.l.c. was carried out with a Waters Associates h.p.l.c. 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 μBondapak C_{18} (10 μm particle size, 30 cm × 3.9 mm internal diam.) from Waters. Elution was with a linear gradient of solvent A [40 % (v/v) methanol in 0.1 % (v/v) trifluoroacetic acid] and 30–100 % of solvent B [90 % (v/v) methanol in 0.1 % (v/v) trifluoroacetic acid] in 20 min. The insulin contents of the reaction products were also determined by competition of receptor binding with ^{125}I-insulin (Mori & Wond, 1984) and an assay for its biological activity as measured by the stimulation of lipogenesis (Moody *et al.*, 1974).

RESULTS

From scrambled insulin

When a preparation of scrambled insulin with an initial activity of 4 % was treated with protein disulphide-isomerase and dithiothreitol at pH 7.5 at 4 °C, h.p.l.c. analysis (Wang & Tsou, 1986) of the products shows the

* To whom correspondence should be addressed.

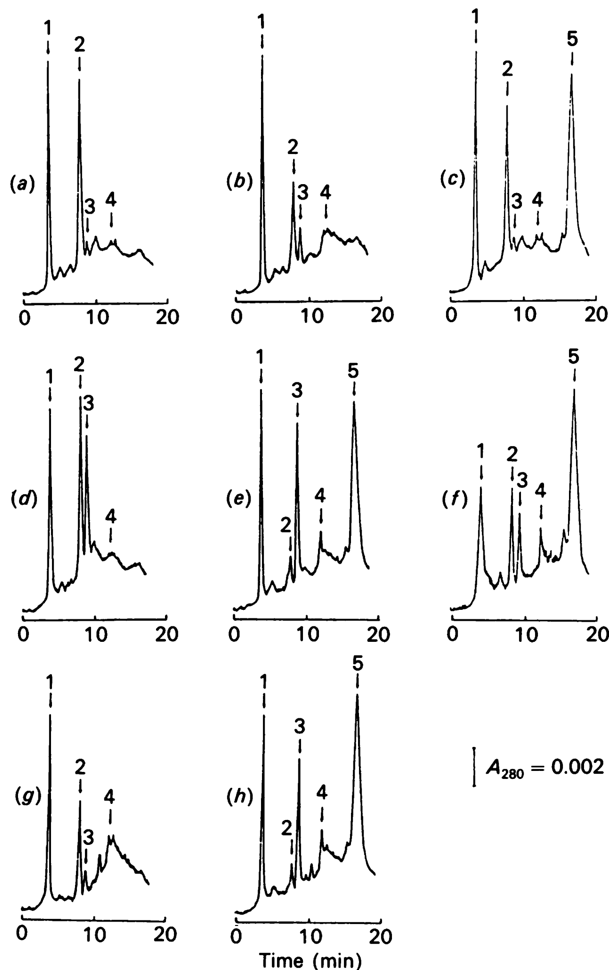

Fig. 1. H.p.l.c. profiles of the regeneration of native insulin from the scrambled molecule and the separate chains

Arrows 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 protein disulphide-isomerase. The position for native insulin was identified by the addition of an authentic sample and co-elution with the reaction product. Other peaks are identified both by the h.p.l.c. profiles of the separately oxidized chains and by amino acid composition analysis. Some of the oxidation products of B chain are eluted at the same position as inactive products containing both chains at peak 2. (*a*) Scrambled insulin obtained by oxidation of the reduced chains in 4.2 M-guanidine; (*b*) reaction product after treatment with dithiothreitol; (*c*) reaction product after treatment with protein disulphide-isomerase; (*d*) as (*a*) with the addition of authentic insulin; (*e*) reaction product after treatment with both protein disulphide-isomerase and dithiothreitol; (*f*) oxidized A and B chains in a molar ratio of 1:1 after treatment with both protein disulphide-isomerase and dithiothreitol; (*g*) *S*-sulphonated A and B chains in a molar ratio of 1:1 after treatment with dithiothreitol; (*h*) as (*g*) after treatment with both protein disulphide-isomerase and dithiothreitol.

reshuffling of the disulphide bonds to form the native hormone as shown in Figs. 1(*a*)–1(*e*). Fig. 1(*a*) shows the h.p.l.c. profile of a scrambled insulin preparation with major peaks consisting of oxidation products of separate A (peak 1) and B (peak 4) chains and inactive products containing both chains joined presumably with incorrect disulphide linkages (peak 2), as identified by comparisons with the h.p.l.c. profiles of the separately oxidized chains and by amino acid analysis (Table 1). In addition, a small peak (peak 3) can be discerned in Figs. 1(*a*)–1(*c*), at the position for the native hormone as shown in Fig. 1(*d*) by co-elution of the reaction mixture with authentic insulin. Although the addition of either dithiothreitol (Fig. 1*b*) or the enzyme alone (Fig. 1*c*) had very little effect on the composition of the products obtained, a comparison of Figs. 1(*a*) and 1(*e*) shows that, by treatment with both, the peak for the incorrectly joined products disappears nearly completely whereas the peak corresponding to the native hormone is greatly intensified. The yield of the native hormone obtained is usually about 20–25% from scrambled insulin preparations (Table 2*a*).

The nature of peak 3 is further identified by the fact that receptor binding assay of a sample collected from the eluate of the h.p.l.c. column shows it has the same activity as an authentic sample of insulin (Fig. 2). Furthermore, peak 3 has the expected amino acid composition of insulin (Table 1). It should be pointed out that, as far as can be resolved by h.p.l.c. analysis, the product with native disulphide linkages is by far the predominant product obtained among all the possible isomeric structures containing both chains.

The insulin contents of the reaction products were also determined by competition of receptor binding with ^{125}I-insulin and the results are shown in Fig. 3. Results of assay for the biological activities of these products as measured by the stimulation of fat-cell lipogenesis are shown in Fig. 4. The yields calculated from Figs. 3 and 4 are listed in Table 2(*a*). The agreement between the yields obtained by h.p.l.c. analysis and by biological assay indicates that the peak that is eluted at the same position as active insulin consists predominantly, if not solely, of a product with full insulin activity.

From the separately oxidized chains

It is possible to obtain the native hormone from a mixture of the separately oxidized A and B chains with protein disulphide-isomerase and dithiothreitol, as shown by h.p.l.c. analysis (Fig. 1*f*), receptor binding and lipogenesis assay. The results are summarized in Table 2(*b*) and the yield, about 10%, is not as good as that obtained either from scrambled insulin or from the *S*-sulphonated chains. It is possible that the oxidation of the separate chains does not necessarily lead to single-chain products but rather to oligomers formed by random joining of more than one molecule of the respective chains. This is especially likely for the B chain, as shown by the complexity of its h.p.l.c. profile (Fig. 1*a*). It is also considerably less soluble than the reduced chain.

From the *S*-sulphonated chains

The joining of the *S*-sulphonated A and B chains to form native insulin as catalysed by the isomerase gives much better yields as compared with the joining of the oxidized chains. The results are shown by the h.p.l.c. profiles of the reaction products in the presence of dithiothreitol alone (Fig. 1*g*) and in the presence of both the enzyme and dithiothreitol (Fig. 1*h*). The insulin contents of the reaction products were also determined by competition of receptor binding with ^{125}I-insulin and

Table 1. Amino acid analysis of the h.p.l.c. peaks of scrambled insulin before and after treatment by protein disulphide-isomerase

Peaks 1, 2 and 4 were from a scrambled insulin preparation as shown in Fig. 1(*a*), and peak 3 was the same preparation after treatment with protein disulphide-isomerase as shown in Fig. 1(*e*).

	Amino acid composition (mol of residue/mol)						
	Found				Expected		
Amino acid	Peak 1	Peak 2	Peak 3	Peak 4	A chain	B chain	Insulin
Asx	1.9	1.7	3.0	2.0	2	1	3
Thr	0	1.3	0.8	0.9	0	1	1
Ser	2.1	1.6	2.7	1.2	2	1	3
Glx	4.0	5.2	7.1	4.8	4	3	7
Pro	0	0.9	0.4	0.7	0	1	1
Gly	1.0	4.5	3.9	3.0	1	3	4
Ala	1.0	3.0	3.0	2.1	1	2	3
Val	1.7	4.6	4.5	2.9	2	3	5
Ile	0.7	0.1	0.8	0	1	0	1
Leu	2.0	5.8	6.1	4.0	2	4	6
Tyr	0.1	0.4	3.7	2.0	2	2	4
Phe	0	3.7	2.7	2.1	0	3	3
Lys	0	1.4	1.2	1.4	0	1	1
His	0	2.3	2.0	1.5	0	2	2
Arg	0	1.3	1.0	0.9	0	1	1

Table 2. Regeneration of native insulin from the scrambled molecule and the separate chains

(*a*) Reaction conditions were: scrambled insulin, 0.6 mg/ml; protein disulphide-isomerase, 0.68 mg/ml; dithiothreitol, 17 μM. The dithiothreitol/disulphide molar ratio in scrambled insulin was 0.055:1. (*b*) Reaction conditions were: oxidized A and B chains in a molar ratio of 1:1, 0.2 mg/ml; protein disulphide-isomerase, 0.23 mg/ml; dithiothreitol, 13 μM. The dithiothreitol/disulphide molar ratio of the chains was 0.13:1. (*c*) Reaction conditions were: *S*-sulphonated A and B chains in a molar ratio of 1:1, 0.6 mg/ml; protein disulphide-isomerase, 0.68 mg/ml; dithiothreitol 375 μM, with a thiol/*S*-sulphonate ratio of 1.25. All reactions were carried out in 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA for 24 h at 4 °C. Abbreviations: PDI, protein disulphide-isomerase; DTT, dithiothreitol.

	Content of native insulin (%)		
	H.p.l.c.	Receptor binding	Lipogenesis
(*a*) From scrambled insulin			
Scrambled insulin	5.3	4.2	2.3
In the presence of PDI	5.0	4.5	4.5
In the presence of DTT	7.9	7.6	5.9
In the presence of PDI and DTT	33.5	25.5	22.8
(*b*) From the oxidized chains			
Oxidized chains (A/B ratio 1:1)	0	< 0.2	< 0.2
In the presence of DTT	0	0.7	1.3
In the presence of PDI and DTT	13.7	10.5	10.0
(*c*) From the *S*-sulphonated chains			
Sulphonated chains (A/B ratio 1:1)	0	0	0
In the presence of DTT	5.7	5.4	3.3
In the presence of PDI and DTT	27.4	21.5	33.0

the lipogenesis assay, as shown in Figs. 3 and 4 respectively. The yields, about 25–30%, determined by h.p.l.c. analysis, receptor binding as well as lipogenesis assay are listed in Table 2(*c*).

DISCUSSION

Many proteins contain disulphide linkages, which are formed, presumably, by joining up of the nascent cysteine thiol groups oxidatively (Freedman, 1984; Kaderbhai & Austen, 1985) either during or after the completion of the synthesis of the peptide chain. The formation of the correct disulphide linkages is crucial for the conformational integrity and consequently the biological activity of the protein molecules concerned, as evidenced by the fact that random joining of the thiol groups under unfolding conditions usually leads to the formation of scrambled molecules with very little, or completely

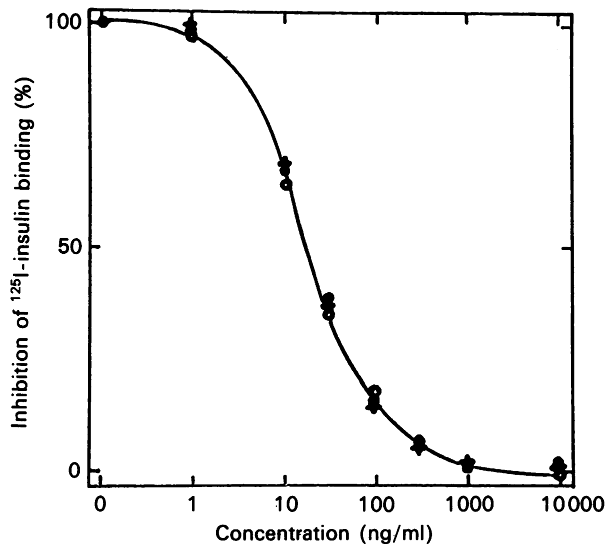

Fig. 2. Receptor binding assay of peak 3 isolated from the eluate from the h.p.l.c. column of a sample of scrambled insulin after treatment with protein disulphide-isomerase

Peak 3 from a similar eluate to that shown in Fig. 1 (*e*) was carefully collected to avoid as far as possible any contamination by peak 2 and then rechromatographed to give a single peak. From 0.6 mg of protein disulphide-isomerase-treated scrambled insulin 0.086 mg of this component, purified as above, was obtained. The receptor-binding activity of this component (+) was compared with that of insulin (○) and insulin dissolved in 70% (v/v) methanol (●).

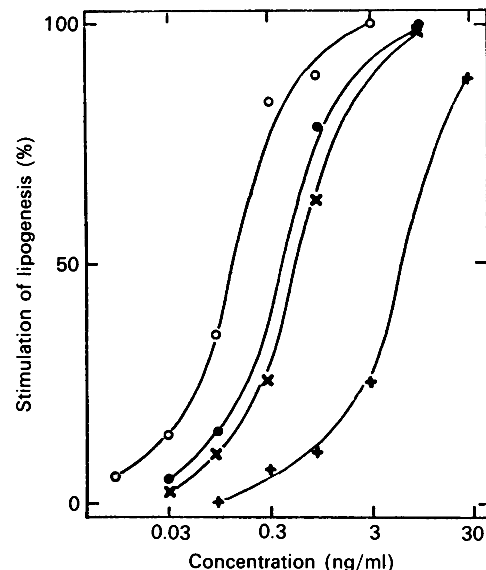

Fig. 4. Effect of insulin and the reaction products on the stimulation of lipogenesis in isolated rat adipocytes

Stimulation, expressed as a percentage of the maximum, is presented as a function of the concentrations of authentic insulin (○), of the products from treatment of scrambled insulin with protein disulphide-isomerase and dithiothreitol (×), of the products from treatment of the mixed *S*-sulphonated chains with protein disulphide-isomerase and dithiothreitol (●) and of scrambled insulin (+).

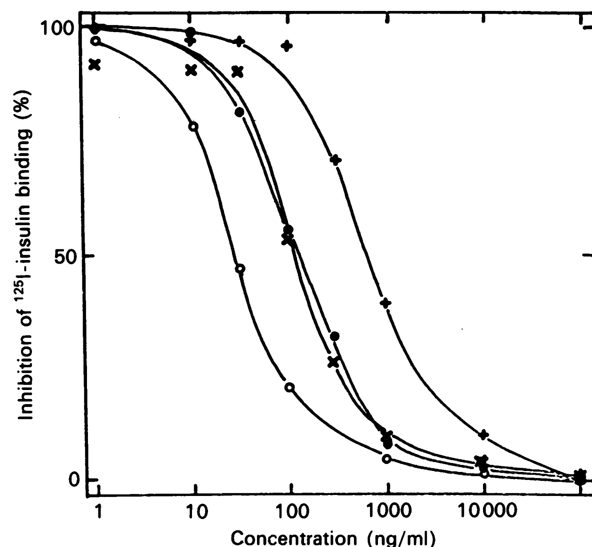

Fig. 3. Competition of insulin and the reaction products to receptor binding with ^{125}I-insulin

The inhibition of ^{125}I-insulin binding to rat liver membrane receptor, expressed as a percentage of the maximum, is presented as a function of the concentrations of authentic insulin (○), of the products from treatment of scrambled insulin with protein disulphide-isomerase and dithiothreitol (×), of the products from treatment of the mixed *S*-sulphonated chains with protein disulphide-isomerase and dithiothreitol (●) and of scrambled insulin (+).

devoid of, biological activity. Although protein disulphide-isomerase has been successfully applied to the regeneration of native disulphide bonds and biological activities from a number of proteins containing scrambled disulphide linkages (Freedman & Hillson, 1980; Freedman *et al.*, 1984), these have been carried out mostly with proteins consisting of a single peptide chain or with multichain proteins that are encoded by separate genes and the chains joined together post-translationally, such as the immunoglobulins (Roth & Koshland, 1981). Varandani & Nafz (1970) reported that it was possible to increase the content of proinsulin to 37% from a preparation of the scrambled molecule containing originally 12% native proinsulin, but very little insulin was obtained from a scrambled insulin preparation containing about 2% insulin under similar conditions. The addition of the connecting C peptide increased the yield to about 12.8%. However, results presented in the present paper show that under appropriate conditions native insulin can indeed be obtained without the addition of C peptide with a yield of 25–30% from a preparation of scrambled insulin containing originally 4% of the native hormone. This yield compares favourably with that given by Varandani & Nafz (1970) for proinsulin. In preliminary work the effect of the amount of added isomerase on the regeneration of insulin from scrambled insulin was studied. It was found that an 8-fold variation of protein disulphide-isomerase (enzyme/substrate ratio 0.02–0.16:1) affected the rate of reaction, but had little effect on the final extent of insulin regeneration. This substantiates the catalytic role of protein disulphide-isomerase in generating native insulin.

Similarly to the successful resynthesis of insulin from

the reduced chains by oxidation (Du *et al.*, 1961, 1965; Zahn *et al.*, 1966; Katsoyannis *et al.*, 1967), of the conditions employed a low temperature has been found to be important for the formation of native insulin from the scrambled molecule. The yield decreases progressively with increasing temperature. That low temperature favours the formation of native insulin has also been reported by Katzen & Tietze (1966) during the oxidation of the reduced chains in the presence of the isomerase, although with lower yields than that obtained in the present study from the *S*-sulphonates of the chains. It is conceivable 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, which 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 of the scrambled molecules to form the native hormone.

The successful generation of insulin from either the scrambled molecule or the separate chains indicates that the chains indeed contain enough structural information so that isomerization of the disulphide bonds leads to the formation of the native hormone in reasonably good yield. The role of the connecting C peptide in proinsulin is probably limited to bringing the two chains together so as to favour the formation of interchain disulphide bonds. This is in accord with the finding that the oxidation of the reduced chains with the *C*-terminal part of the B chain linked to the *N*-terminal of the A chain by a cross-linking reagent (Brandenburg & Wollmer, 1973), which presumably contains no structural information, also gives good yield of the molecule with the native disulphide structure.

The total number of all the possible oxidation products containing one or both of the chains is immense indeed, as has been calculated by Chance *et al.* (1981) as well as by Wang *et al.* (1987). Even if one considers only the products containing one each of the chains, as apparently Pruitt *et al.* (1966) did, there are 12 possible isomers. The h.p.l.c. profile of the products of the unscrambling reaction shows that, among the isomers containing both chains, the native hormone is by far the predominant product. It is therefore evident that, under suitable conditions, of all the possible oxidation products the native insulin structure is the most stable, and not a metastable form as has been previously suggested (Anfinsen & Scheraga, 1975; Brandenburg *et al.*, 1977; Hillson *et al.*, 1984).

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