Intra-A chain disulphide bond forms first during insulin precursor folding

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In this study, we investigated the folding pathway of insulin precursor and compared it with that of insulin-like growth factor I (IGF-I). The intra-A chain disulphide bond was found to form early in insulin precursor folding, whereas the corresponding disulphide bond in IGF-I formed late. Intra-A chain disulphide-bond deleted [A6, A11-Ser] proteins, including proinsulin, insulin, and A chain, were employed for this investigation. Under the same conditions the recombination yield of insulin from S-sulphonates of native A and B chains was 22%, while the yield of [A6, A11-Ser] insulin from S-sulphonates of [A6, A11-Ser] A chain and native B chains was only approx. 7%. This indicated that the intra-A chain disulphide bond may serve to stabilize the A chain folding intermediate so as to facilitate the correct recognition and pairing with the B chain. Time courses of oxidation of reduced insulin A chains, reduced A and B chains,

and reduced proinsulins showed that the intra-A chain disulphide bond formed first during insulin precursor folding. The formation of intra-A chain disulphide bond further accelerated the formation of the other two inter-chain disulphide bonds. The time course of helix structure formation of insulin A chains also indicated that the intra-A chain disulphide bond formed first, and could stabilize partially folded A chain helix structure. The rate of intra-A chain disulphide bond formation was almost the same as that for both helix structure formation and insulin molecule formation, indicating that the formation of the intra-A chain disulphide bond was the rate limiting step for the folding of insulin precursor.

Keywords: CD analysis, folding pathway, insulin-like growth factor I, secondary structure.

INTRODUCTION

The three dimensional structure of a protein is determined by its primary structure and a disulphide bond may serve as a major stabilizing element in protein structure [1–4]. Disulphide bond formation also has great influence on the protein folding pathway, as the stabilization effect of a disulphide bond on folding intermediates may favour the formation of a native molecule. The disulphide-coupled folding pathways of bovine pancreatic trypsin inhibitor [5–7] and bovine pancreatic ribonuclease A [8–11] have been extensively studied. It was found that with the formation of a trypsin inhibitor Cys30–Cys51 disulphide bond, the intermediate was more stable than other one disulphide ones [5]. The formation of a Cys65–Cys72 disulphide loop during the regeneration of ribonuclease A appears to facilitate the subsequent folding events [8]. Insulin and insulin-related proteins, including insulin-like growth factor I (IGF-I), relaxin, and bombyxin-II, contain three motif-specific disulphide bonds. Although the structure of insulin is well known, the role of those three disulphide bonds in the folding pathway of insulin precursor has yet to be elucidated. The role of disulphide bonds in the folding of IGF-I has been well established [12–16]. An IGF-I mutant lacking all three disulphide bonds is inactive and unfolded. Introduction of a Cys18–Cys61 disulphide bond (corresponding to B19–A20 inter-chain disulphide bond in insulin) results in a compact, partially folded state, with low but significant biological activity. Additional but incomplete structural organization and biological activity are observed following the introduction of either Cys6–Cys48 or Cys47–Cys52 disulphide bonds (corresponding to B7–A7 inter-chain and A6–A11 intra-A chain disulphide bonds in insulin respectively) [12]. The refolding of the fully reduced molecule generates various ratios of correctly and incorrectly folded forms, via several inter-

mediates. All of the intermediates contain the disulphide bond between Cys18 and Cys61, indicating that formation of this disulphide is the first step in refolding [13]. The disulphide between Cys47 and Cys52 in IGF-I has been shown to be an unfavourable high-energy bond, which may exist in the native molecule in a strained configuration, and forms late during the folding process [14]. In the native state this disulphide bridge anchors an adjoining amphipathic α -helix (helix 2, residues 42 to 49, corresponding to insulin A chain N-terminal helix) against the hydrophobic core. Since there is no putative folding pathway of insulin precursor, it is then proposed that folding of the insulin motif is directed by a subset of native structural elements, and that these elements form at an early step in the pathway. Formation of helix 2, despite its prominence in the native state, is likely to represent a late step [15]. So far no detailed hypotheses for the folding pathways of relaxin and bombyxin-II have been proposed [17–20]. In the past 40 years much work has been carried out on the recombination of reduced or S-sulphonated insulin A and B chains, as well as proinsulin and its analogues, to their correctly disulphide-bonded molecule [21–29]. Under appropriate conditions, the reduced insulin A and B chains have been re-oxidized to give a reasonably good yield of insulin [21,22], which has been applied to the production of human insulin from the separate chains obtained by recombinant DNA techniques [23]. Good yields of proinsulin or proinsulin analogues could also be obtained from their reduced or S-sulphonated forms [24–26]. In the presence of protein disulphide-isomerase (PDI), insulin, proinsulin or proinsulin analogues with native disulphide linkages could be obtained as well from their scrambled forms, or from S-sulphonated derivatives with good yields [27–29]. Among the three disulphide bonds of insulin, two inter-chain disulphide bonds have been demonstrated to be more easily reduced than that of the intra-A chain [30]. This infers that

Abbreviations used: IGF-I, insulin-like growth factor I; PDI, protein disulphide-isomerase.

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the inter-chain disulphide bonds may play a critical role in the maintenance of the three dimensional structure of insulin. The intra-A chain disulphide bond is buried in a pocket between the chains [31]. It may have some role in the folding of insulin chains to form the native molecule. Is there any difference between this intra-A chain disulphide bond in folding and the corresponding one in IGF-I? To gain some insight into this complicated process, intra-A chain disulphide bond deleted [A6, A11-Ser] proteins, including proinsulin, insulin and A chain, were prepared. The recombination yields from S-sulphonated chains were compared with that of insulin in the presence of PDI. Time courses of both disulphide bond formation for reduced A chain, A plus B chains and proinsulin, and helix structure formation for the reduced A chain were also investigated.

MATERIALS AND METHODS

Materials

Tetrathionate was prepared as described previously [32]. Dithiothreitol was from Serva. Carboxypeptidase B with an activity of 180 units/mg of protein, and trypsin with an activity of 10900 units/mg of protein, were from Sigma Chemical Co. PDI with an activity of 810 units/mg of protein was a gift from Dr. C. C. Wang (Institute of Biophysics, Academia Sinica, Beijing, People's Republic of China) [27]. Resource[™] Q (1 ml) and HiTrap[™] (5 ml) columns were from Pharmacia Biotech.

Preparation S-sulphonates of insulin A and B chains

The procedures for expression and purification of human [A6, A11-Ser] proinsulin, conversion of mutant proinsulin into [A6, A11-Ser] insulin by carboxypeptidase B and trypsin, and purification and identification of mutant insulin were as described previously [33,34]. The preparation of S-sulphonates of native A and B chains was carried out as described elsewhere [27,35]. The sulphonation of [A6, A11-Ser] insulin was carried out under the same conditions as that for native insulin.

Recombination of S-sulphonates of insulin A and B chains

The recombination reaction was carried out in 0.1 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA. The molar ratio of S-sulphonates of A and B chains was 1:1, and the final protein concentration was 0.6 mg/ml. The reaction mixture containing 375 μ M dithiothreitol with a thiol to S-sulphonate ratio of 1.2, and 0.68 mg/ml of PDI was incubated at 4 °C for various time periods [27]. Isopropanol was added to aliquots to 40% (v/v) and the pH was adjusted to 8.3 to terminate the reaction for FPLC analysis. PDI was precipitated during this treatment. For PAGE analysis the pH of the aliquots was adjusted to pH 3.0 to terminate the reaction and changed back to pH 9.0 before loading on to the gel. The recombination of S-sulphonates of mutant A and native B chains was carried out under the same conditions except that 250 μ M dithiothreitol was employed with the same thiol to S-sulphonate ratio of 1.2.

Analysis of recombination product

The aliquots, terminated at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 24.0 h, were loaded on to an anion FPLC column-Resource[™] Q (1 ml). The recombination product was eluted with 0.1 mM Tris/HCl

buffer, pH 8.3, containing 40% (v/v) isopropanol, with a NaCl gradient of 0–1 M in 10 min at a flow rate of 1 ml/min. Meanwhile, the recombination products were also analysed on a native 15% (w/v) polyacrylamide gel. The refolded insulin contents were determined both by calculating from the FPLC profiles and by thin-layer densitometric scanning of the gels.

Oxidation of reduced insulin chains

S-sulphonate of insulin A chain, 1 mg in 0.5 ml of 0.05 M Glycine/NaOH buffer, pH 10.8, was reduced by a 20-fold (in terms of thiol groups) excess of dithiothreitol. After a 1 h incubation at 37 °C, the reaction mixture was loaded on to a desalting FPLC HiTrap[™]-column (5 ml) to remove excess dithiothreitol, and eluted with 0.05 M Glycine/NaOH buffer, pH 8.3. The protein concentration of the eluted peak was adjusted to 0.15 mM and incubated at 4 °C for various time periods. The determination of the thiol groups of the aliquots was carried out with 5,5'-dithio-bis-(2-nitrobenzoic acid) according to [36]. The procedures for the oxidation of reduced insulin A and B chains, and reduced proinsulins were the same as those for the oxidation of reduced insulin A chains.

CD spectra of the oxidation of reduced insulin A chains

The oxidation of reduced insulin A chains was carried out as described above. Oxidation was terminated by adjusting the pH to 3.0 at various time periods. CD measurements were performed on a Jasco J-500C CD spectropolarimeter in a 0.2 cm path length cell. All spectra were recorded at 25 °C and scanned from 250 to 190 nm. The content of secondary structure for each spectrum was calculated according to an established method [37].

RESULTS

The recombination reaction was carried out under the same conditions as described previously [27]. The recombination products were analysed by FPLC as shown in Figure 1(A). Peaks 1, 2, and 3 are S-sulphonated B chain, A chain and refolded native insulin respectively. Peaks 2' and 3' are S-sulphonated mutant A chain and refolded mutant insulin respectively. The corresponding peak of insulin was identified by retaining time, and the insulin contents of the products were determined from the FPLC profiles. There was no significant difference between the retaining time for native insulin and its mutant on anion exchange chromatography. Mutant A chain, which contains two S-sulphonated groups, showed a shorter retaining time than native A chain with four S-sulphonated groups. The reaction for the recombination of S-sulphonates of native insulin chains was close to 80% completion in approx. 2 h. The final yield of insulin was approx. 22%, as we reported previously [27]. Meanwhile, the final yield of [A6, A11-Ser] insulin was only approx. 7%. Both have a similar hyperbola curve for the time course of recombination. Native PAGE was also employed to analyse the recombination products as shown in Figure 1(B). The bands for PDI, S-sulphonated B chain and insulin can be clearly identified. The A chain was too soluble to be detected by Coomassie Brilliant Blue staining. It was also observed that native insulin and mutant insulin had a very similar mobility rate during native PAGE, as we have reported for peptide mapping of proinsulin and intra-A chain disulphide bond deleted proinsulin by native PAGE previously [33]. The insulin contents were

Figure 1 Recombination analysis of insulin A and B chains

(A) FPLC profiles of the recombination products of both native insulin (top) and [A6, A11-Ser] insulin (bottom) from their S-sulphonated A and B chains. Peak 1 represents position of the S-sulphonated B chain. Peaks 2 and 2' represent the positions of the S-sulphonated A chain and mutant A chain respectively. Peaks 3 and 3' indicate the positions of the refolded native insulin and its mutant respectively. The peaks are identified by their retaining time. a, b, c, d, e, f, g, h, and a', b', c', d', e', f', g', h' represent recombination products at 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0 and 24.0 h respectively, for native insulin and [A6, A11-Ser] insulin. (B) Electrophoretic analysis of the recombination products of S-sulphonated A and B chains of native insulin and [A6, A11-Ser] insulin on a 15% polyacrylamide gel. a, b, c, d, e, f, g, h and a', b', c', d', e', f', g', h' represent recombination products at 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0 and 24.0 h respectively, for native insulin (a–h) and [A6, A11-Ser] insulin (a'–h'). Both insulin and PDI standards are indicated.

quantified by thin-layer densitometric scanning of the gels. These data agree well with the results obtained from FPLC analysis.

The time course of the oxidation of reduced insulin A chains (Figure 2A) showed that two thiol groups disappeared rapidly during the first 2 h of oxidation for the native A chain, followed by a slow oxidation. However, no rapid oxidation of thiol groups was observed for the intra-chain disulphide bond deleted A chain. Figure 2(B) shows the time course of the oxidation of reduced insulin A and B chains. Similar to the A chain alone, two thiol groups disappeared rapidly during the first 2 h of the native A chain experiment, which was followed by slow oxidation. No rapid oxidation was observed for the proinsulin sample. A similar result was again observed for the proinsulin case as shown in Figure 2(C). Two thiol groups oxidized rapidly during the first 2 h for native proinsulin, while no rapid oxidation of thiol groups was observed for mutant proinsulin. After formation of the first disulphide bond, the oxidation of the other four thiol groups was accelerated for native proinsulin, while the slow process was observed for mutant proinsulin.

The time course of A chain helix-structure formation was monitored by a CD spectropolarimeter. As illustrated in Figure 3, great changes of helix structure formation for the native A chain were observed during the first 2 h of oxidation, while only small changes were observed for the mutant A chain. Calculations of secondary structures from these CD traces indicate that the β -sheet content remained close to 32% in both experiments, whereas the α -helix content increased from 27% to 53% in the

Figure 2 Time course of the oxidation of reduced insulin chains

(A) Time course of the oxidation of reduced native A chain (●) and its mutant chain (■). (B) Time course of the oxidation of reduced native A chain (●) and its mutant chain (■) in the presence of reduced native B chain. (C) Time course of the oxidation of reduced proinsulin (●) and mutant proinsulin (■).

wild-type and only marginally to 34% in the mutant chain (Table 1).

DISCUSSION

Cecil and Weitzman have electrolytically reduced the specific disulphide bonds in insulin and showed that the two inter-chain disulphide bonds are more easily reduced than that of the intra-A chain, indicating the importance of inter-chain disul-

Figure 3 CD spectroscopic analysis of the secondary-structure formation during oxidation of reduced native A chain and its mutant chain

Time course of CD spectra for the mutant chain (upper spectra) and the native chain (lower spectra). From left to right are samples terminated at 0.5, 1.0, 2.0 and 3.0 h respectively.

Table 1 The calculated secondary-structure contents at different refolding time periods for both mutant and native A chains

Refolding time (h) ...	Calculated secondary-structure content (%)			
	0.5	1.0	2.0	3.0
Mutant A chain				
α-Helix	26	31	30	34
β-Sheet	28	33	32	34
Random	46	36	38	32
Native A chain				
α-Helix	27	31	47	53
β-Sheet	30	33	32	32
Random	43	36	21	15

phide bonds in maintaining the three-dimensional structure of insulin [30]. The intra-A chain disulphide bond is also important for the stability of the native molecule. The structural analysis of an intra-A chain disulphide bond deleted mutant shows that there are not many structural changes, except regional unfolding in the A chain N-terminal segment, and that this intra-A chain disulphide bond of insulin is important for its activity [33,34,38]. A ¹H NMR determination of the solution structure of the oxidized insulin A chain shows that significant populations of the peptide exist with a 3₍₁₀₎-helical conformation over residues 12–17. This region corresponds to the A chain C-terminal helix in the crystal structure of the two Zn insulin hexamers [39]. When mixing S-thiomethyl A and B chains, the chains fold gradually and the secondary force interactions are thought to bring about pairing of the chains [40]. It is conceivable that the intra-A chain disulphide bond may pair and form at first, to help the A chain form a native-like conformation. When the B chain moves into contact with the A chain, the hydrophobic core then promotes the pairing of the other two inter-chain disulphide bonds [41]. This could provide a good explanation for the low refolding yield of [A6, A11-Ser] insulin and for the lack of an intra-A chain disulphide bond as shown in Figure 1. The importance of the intra-A chain disulphide bond could also be elucidated by comparing the number of insulin isomers refolded from experimental data with that from theoretical calculations. For

random formation of disulphide bonds of A₁B₁ isomers, there are 12 A₁B₁ isomers for native insulin, but only two A₁B₁ isomers for [A6, A11-Ser] insulin. When the number of disulphide bonds drops from three to two, the probability of mispairing of disulphide bonds should decrease five times for [A6, A11-Ser] insulin. Consequently, the yield of [A6, A11-Ser] insulin should increase five times. However, the opposite result was observed. The recombination yield for [A6, A11-Ser] insulin was only approx. 7%, much lower than that of the native insulin. This indicates that the pairing of disulphide bonds is not a random process, but a highly organized one. The formation of the intra-A chain disulphide bond may help the A chain form a stable helix structure to finish the correct pairing with the B chain.

Unlike that of IGF-I, formation of the intra-A chain disulphide bond of insulin is a fast process during its folding, as was confirmed by the time course of the oxidation of the insulin-chain free-thiol groups. In the presence of the A chain alone, one disulphide bond was formed in the first 2 h of oxidation for the native A chain, while few changes were observed for the intra-A chain disulphide bond deleted A chain. This indicates that during the oxidation of the reduced A chain, the intra-A chain disulphide bond forms first. In fact, it has been shown that the two thiol groups disappear very quickly during the oxidation of the reduced A chain, but the oxidation is slow for the reduced B chain [42]. In the presence of both A and B chains, one disulphide bond

again was observed to form quickly for the native A chain experiment, but not for the mutant A chain. The presence of B chain had little influence on the rate of one disulphide bond formation. This provides further evidence that the intra-A chain disulphide bond forms first. As IGF-I studies show that disulphide bond Cys47–Cys52 (corresponding to the insulin intra-A chain disulphide bond) forms late, it is necessary to employ the insulin precursor to verify this result. In the proinsulin experiment, we again showed that one disulphide bond formed quickly within the first 2 h for native proinsulin, and a very similar curve of disulphide bond formation was observed for both the reduced A chain and the reduced A plus B chains. We therefore propose that unlike IGF-I, the intra-A chain disulphide bond forms first rather than later during the folding of the insulin precursor. The folding pathway of the insulin precursor seems quite different from that of IGF-I. The C-peptide in proinsulin has been shown to serve as a connecting peptide in proinsulin folding, and this has been demonstrated by single chain des-B30 insulin [27], cross-linked insulin [29], as well as double C-peptide proinsulin [26]. The C-peptide showed no influence on the formation of the first disulphide bond, but it did have some effects on the formation of other disulphide bonds, as shown in Figure 2(C) for rapid formation of the other two inter-chain disulphide bonds. After formation of the intra-A chain disulphide bond, C-peptide may help to bring the disulphide bond stabilized A chain close to the B chain to form the other two inter-chain disulphide bonds.

The time course of helix structure formation during the oxidation of reduced A chains also supports our suggestion. The A chain in insulin forms two helix structures (A1–A8, A12–A18) [31] and the N-terminal helix structure is stabilized by the intra-A chain disulphide bond [38]. Our CD spectra showed that little difference in α -helix content for the intra-chain disulphide bond deleted A chain was observed during the folding, while a large increase of α -helix content was seen for the native A chain within the first 2 h (Figure 3 and Table 1). The net α -helix content increase for the native A chain reached 20% from 0.5 to 2 h, while only a 4% net α -helix content increase for the mutant A chain was observed during the same period of time. In the reduced state both native and mutant A chains contain high contents of random structure. In the oxidized state the mutant A chain still shows quite high random structure (Table 1). This indicates that formation of the intra-A chain disulphide bond may stabilize the partially folded A chain helix structure. Since the 222 nm band on the CD spectra is not very visible, there is a possibility that the spectral changes observed may not be entirely due to secondary structural changes as is the case for the dissociation of insulin dimers to monomers [43]. Still it should be noted that the rate for intra-A chain disulphide bond formation is almost the same as that for helix structure formation as shown in Figures 2 and 3. Also, the rate for intra-A chain disulphide bond formation is almost the same as that for insulin molecule formation as shown in Figures 1 and 2. This suggests that formation of the intra-A chain disulphide bond is a rate limiting step during insulin precursor folding. It is reasonable that the deletion of the intra-A chain disulphide bond is the predominant reason for mispairing of A and B chains, which results in the low recombination yield. The formation of the intra-A chain disulphide bond may stabilize the A chain folding intermediate and accelerate the correct pairing with the B chain in the recombination process. Structural differences among insulin family members have been widely reported. The predicted structural homology of relaxin to insulin is high, but there are still significant differences in the terminal regions of the B-chain [18]. The structure of bombyxin-II is more closely related to relaxin than

to insulin [20]. The tertiary structure of mini-proinsulin again differs from that of mini-IGF-I, despite strict conservation of non-polar residues in their respective hydrophobic cores [44]. Furthermore, oxidative folding of IGF-I is shown to yield two major products, IGF-I and IGF-swap, which have similar thermodynamic stability [45], while no disulphide bond isomers have been isolated for insulin precursor during its folding. It is thus reasonable to expect that the folding pathway of the insulin precursor may be different from that of IGF-I, rather than IGF-I-like as has been suggested [15,38]. The intra-A chain disulphide bond forms first during insulin precursor folding, whereas in the case of IGF-I the Cys47–Cys52 disulphide bond (corresponding to the insulin intra-A chain disulphide bond) forms late.

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