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# Human Gene 2 Relaxin Chain Combination and Folding<sup>†</sup>

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**ABSTRACT:** Relaxin is a small 6 kD two-chain peptide member of the insulin superfamily that is principally produced in the corpus luteum of the ovary and which plays a key role in connective tissue remodeling during parturition. Like insulin, it is produced on the ribosome as preprohormone that undergoes oxidative folding and subsequent proteolytic processing to yield the mature insulin-like peptide. In contrast to the now considerable insight into insulin chain folding and oxidation, comparatively little is known about the folding pathway of relaxin. A series of synthetic pairwise serine substituted relaxin A-chain cysteine analogues was prepared, and their oxidation behavior was studied both on their own and in the presence of native relaxin B-chain. It was observed that native S-reduced A-chain oxidized rapidly to a bicyclic product, whereas individual formation of each of the intramolecular disulfide bonds between Cys<sup>11</sup> and Cys<sup>24</sup> and the native Cys<sup>10</sup> and Cys<sup>15</sup> was considerably slower. Curiously, the non-native, isomeric Cys<sup>11</sup>–Cys<sup>15</sup> disulfide bond formed most rapidly, although circular dichroism spectroscopy analysis showed this product to be devoid of secondary structure. This suggested that it may in fact be an intermediate in the subsequent formation of the native Cys<sup>10</sup>–Cys<sup>15</sup> intramolecular disulfide. Combination of the native A-chain with the B-chain proceeded rapidly as compared with the A-chain analogue that lacked the intramolecular disulfide bond suggesting that this latter element is required as a first step in the folding process. It is therefore probable that relaxin is generated from its constituent A- and B-chains in a stepwise organization manner similar to that of insulin chain combination and folding. Further studies showed that the efficiency of combination of A-chain to B-chain was not markedly influenced by reaction temperature and that a reasonable yield of relaxin could be obtained on combination of the preoxidized A-chain with the S-reduced B-chain.

More than 20 years after the determination in 1955 of the primary structure of insulin (1), the concept of the insulin superfamily of peptides was established by the identification of a similar two-chain, three cystine linkage arrangement for relaxin (2). A subsequent search for related peptides containing the same structural motif revealed a further four mammalian peptides, insulin-like growth factors (IGF) I and II, insulin 3 (also known as Leydig cell insulin-like peptide or relaxin-like factor), and insulin 4 (placentalin) (3–5). More recently, the existence of two further insulin-like peptides, insulin 5 and 6, has been predicted from cDNA sequences

(6, 7). Analysis of the recently published Celera and public domain genome databases failed to reveal the presence of additional insulin-like peptides (8) thus showing that membership of the human insulin superfamily is restricted to eight. Each of these peptides is synthesized on the ribosome as a single-chain preprohormone that consists of a signal peptide, B-chain, connecting (C-) peptide and A-chain. The C-peptide varies in length from about 30 residues in insulin to more than 100 residues in relaxin. With the exception of IGF-I and II, the preprohormones undergo cytoplasmic proteolytic conversion by prohormone convertases to yield mature two-chain peptides containing the characteristic insulin cystine cross-links and fold (Figure 1) (9, 10). The tertiary structures of insulin, IGF-I and II, and relaxin have been determined, and each is shown to possess a well-ordered hydrophobic core and three common helical segments—two within the termini of the A-chain that are joined by a loop and the third within the B-chain (11–14).

The folding and disulfide formation pathway of insulin, and more recently, IGF-I, has been the subject of intense study. Oxidative folding of both proinsulin (and proinsulin-like molecules) and the separate insulin chains each ultimately lead to the correct formation of native cystines (15–18). The latter result shows that the information required for correct folding is contained within the primary structure of the chains. Acquisition of a single stable native insulin

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<sup>1</sup> Abbreviations: CD, circular dichroism; DIC, 1,3-diisopropylcarbodiimide; DMF, *N,N'*-dimethylformamide; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); EDT, ethanedithiol; GdHCl, guanidine hydrochloride; HOBt, hydroxybenzotriazole; IGF-I, insulin-like growth factor I; RP-HPLC, reversed-phase high-performance liquid chromatography; MALDITOF MS, matrix-assisted time-of-flight mass spectrometry; PIP, porcine proinsulin precursor; [Ser<sup>A10,15</sup>] A-chain, relaxin A-chain analogue containing substitutions serine<sup>A10</sup> and serine<sup>A15</sup>; SPPS, solid-phase peptide synthesis; TIS, triisobutylsilane; TFA, trifluoroethanol.

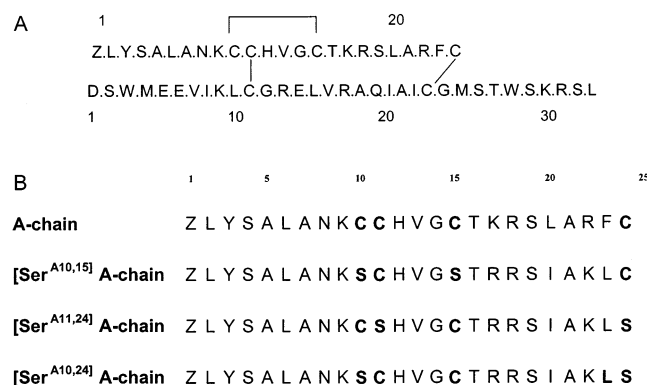


FIGURE 1: (A) Primary structure of human Gene 2 relaxin. The native peptide B-chain consists of residues 1–29, but in this study, synthetic B-chain (1–33) was used because of its improved solubility. (B) Primary structure of human Gene 2 relaxin A-chain and of Cys → Ser A-chain analogues used in this study.

structure occurs via a preferred pathway of intermediates that is typified by sequential formation of the N-terminal A-chain  $\alpha$ -helix, intramolecular A-chain disulfide, and the two intermolecular disulfide bonds followed by the remaining secondary structural elements (19, 20). In contrast, IGF-I folds via a different pathway into two disulfide isomers that have similar thermodynamic stability (21).

In most mammalian species, relaxin is produced principally in the corpus luteum during pregnancy and has a key role in the reproductive process (22). It has also been shown to stimulate chronotropic and inotropic responses in heart atria (23) and to act on the central nervous system to regulate fluid balance (24). More recently, relaxin has been demonstrated to be a potent vasodilator (25, 26) as well as an antifibrotic agent (27) suggesting that it may have potential therapeutic applications. Interestingly, in the human, there are two separate relaxin genes, designated Genes 1 and 2. The peptide encoded by the Gene 2 is the major stored and circulating form in the human (22) and consists of a 24 residue A-chain and a 29 residue B-chain (Figure 1). Remarkably, a third relaxin gene has recently been identified in the human that appears to be predominantly expressed in the brain suggesting a neuropeptide signaling role (28). Curiously, in Gene 2 prorelaxin, the connecting C-peptide is approximately 100 residues in length, as compared to the 31 residue corresponding peptide in proinsulin (22). Recombinant DNA-derived human Gene 2 prorelaxin may be readily and efficiently folded and oxidized using a mini C-peptide of length 13 residues. This C-peptide can be excised by treatment with specific endopeptidases to generate relaxin in good overall yield (29). Thus, the large length of the native relaxin C-peptide is clearly unnecessary for the correct folding of the A- and B-chains and suggests that it may perhaps have an additional function or serve as a precursor to biologically active peptides. Support for this concept has recently arisen following the surprising finding that, in pharmacological doses, human proinsulin C-peptide has an insulin-like action (30, 31). Like insulin, relaxin can be also obtained by oxidative refolding of the two constituent chains in solution at high pH although yields are variable and low due, in part, to the poor solubility of the B-chain (32, 33).

Comparatively little is known about the folding and oxidation processes leading to the *in vivo* production of

human Gene 2 relaxin or other members of the insulin superfamily from its prohormonal precursors. Consequently, this study is a first step toward determining if the disulfide bond formation and subsequent peptide folding pathway of relaxin is similar to that of either insulin or IGF-I. We have used pairwise serine substitutions of the cysteine residues in relaxin A-chain to systematically examine the mechanics of the two-chain combination process.

## MATERIALS AND METHODS

**Materials.** Solid-phase synthesis resins were purchased from Applied Biosystems Inc. (Melbourne, Australia). All Fmoc-protected amino acids were of the L-configuration, and together with *N,N*-dimethylformamide (DMF), were obtained from Auspep (Melbourne, Australia). 1,3-Diisopropylcarbodiimide (DIC) and piperidine were obtained from Aldrich (St. Louis, MO) and used without further purification. All organic solvents were of HPLC grade, and all other reagents were of the highest quality available.

**Solid-Phase Peptide Synthesis.** Continuous flow Fmoc-solid-phase peptide synthesis (SPPS) was used throughout (34). The human Gene 2 relaxin A- and B-chain peptides and analogues were assembled using a MilliGen 9050 Plus Peptide Synthesizer (Bedford, MA) and PAL-PEG-PS supports. Synthesis scale was 0.1 mmol, and Fmoc-amino acids were activated with DIC/HOBt in DMF. Amino acid side chain protecting groups were trityl (Trt) for Asn, Gln, and His; *tert*-butyl (tBu) for Ser, Asp, Glu, and Tyr; *tert*-butyloxycarbonyl (tBoc) for Lys; and pentamethylidihydrobenzofuransulfonyl (Pbf) for Arg. After each synthesis, resin-bound peptides were washed, dried, and then subjected to global cleavage and deprotection in a mixture of trifluoroacetic acid (TFA)/H<sub>2</sub>O/ethanedithiol (EDT)/triethylsilane (TIS) (94:2.5:2.5:1 v/v, 10 mL). After 2–3 h, the resin was removed by filtration and washed twice with TFA (5 mL). The total volume of the combined TFA filtrate was reduced to less than 5 mL under a stream of N<sub>2</sub>, and the peptide precipitated with cold diethyl ether and was centrifuged and triturated once more. The product was then dissolved in a solution of H<sub>2</sub>O/CH<sub>3</sub>CN/TFA (90:10:0.1 or 80:20:0.1 v/v/v) and lyophilized.

**Purification.** Crude peptides were taken up into 0.1 M Tris/6 M guanidine hydrochloride (GdHCl), pH 8.0, and subjected to S-reduction with dithiothreitol (1 h, room temperature). These were then subjected to preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C<sub>18</sub> reverse-phase column (10 × 250 mm 218TP) using a Waters 660 HPLC system. A solvent system of 0.1% aqueous TFA (buffer A) and 0.1% aqueous TFA in acetonitrile (buffer B) in linear gradient mode was used in each case. Fractions were collected and lyophilized.

**A-Chain Oxidation.** Purified S-reduced A-chains were separately subjected to oxidative folding in 0.1 M glycinate (Gly)-NaOH buffer, pH 8.3 buffer at a concentration of 0.5 mg/mL at 4 °C. 100  $\mu$ L aliquots were removed, and the oxidation was stopped with 10  $\mu$ L of 1% TFA at selected intervals. Analytical C18 RP-HPLC was used for oxidation products analysis with a gradient of 20–70% acetonitrile in 30 min. Free thiol groups were determined by the Ellman's method (35) as follows: 100  $\mu$ L of acidic sample was neutralized with 100  $\mu$ L of 0.1 M Gly-NaOH buffer, pH 10.8,

and then with 800  $\mu\text{L}$  of 0.1 M Gly-NaOH buffer, pH 8.3. A total of 100  $\mu\text{L}$  of 2 mM DTNB in 0.1 M Gly-NaOH buffer, pH 8.3 was added immediately, and the optical density at 412 nm was read on a Beckman DU-64 UV spectrometer. The  $\text{OD}_{412}$  at zero time was set as 100% reduced peptide.

**Chain Combination.** Purified A- and B-chains (2:1 w/w) were dissolved in 1 volume of 6 M GdHCl and then diluted in 5 volumes of 0.1 M Gly-NaOH buffer, pH 10.8, at a final concentration of 6 mg/mL. The mixture was incubated at 4 °C. 10  $\mu\text{L}$  aliquots were removed, and the reaction was stopped by addition of 40  $\mu\text{L}$  of 1% TFA during the course of the combination. The samples were then analyzed by RP-HPLC with conditions described above. The chain combined products were isolated, lyophilized, and further analyzed by mass spectrometry and tryptic mapping.

**Peptide Characterization.** The purity of the synthetic peptides was assessed by analytical RP-HPLC and matrix-assisted laser desorption time-of-flight (MALDITOF) mass spectrometry using a Bruker Biflex instrument (Bremen, Germany) in the linear mode at 19.5 kV.

**CD Spectroscopy.** A total of 0.4 mg of purified A-chain was dissolved in 0.9 mL of 0.1% TFA. A total of 0.1 mL of 0.5 M Gly-NaOH, pH 10.8 buffer was added to begin the oxidation at 4 °C (final pH ca. 8.3). The reaction was stopped at 1.5 h, and CD spectra were immediately recorded. The spectra were taken on a Jasco J-500C instrument at room temperature in a 2 mm path length cell. The raw spectra were converted into mean residue ellipticity ( $[\theta]_{\text{MR}}$ ) format and were smoothed. The content of secondary structure for each spectrum was calculated according to an established method (36).

**Tryptic Mapping.** HPLC fractions for combined products were pooled and dried in a Speed-Vac. For each sample of about 1  $\mu\text{g}$  of peptide, enzymatic digestion was carried out for 4 h in 10  $\mu\text{L}$  of 10 mM  $\text{NH}_4\text{HCO}_3$  at 37 °C using a trypsin/substrate ratio of 1:50 (w/w). Prior to mass analysis, 0.5  $\mu\text{L}$  of digest solution was mixed on target with 0.5  $\mu\text{L}$  of matrix (saturated  $\alpha$ -cyanocinnamic acid in 30% aqueous acetonitrile) and left to dry.

## RESULTS

A series of three human Gene 2 relaxin A-chain analogues containing specific Cys  $\rightarrow$  Ser replacements (Figure 1B) together with previously acquired (33) native A- and B-chain were readily prepared by previously optimized continuous flow solid-phase peptide synthesis. To aid the chain combination studies, a more soluble B-chain that was extended at its C-terminus by four residues was used (Figure 1A). Overall yield of purified peptides was in the range of 22–30% based on crude cleaved starting material. The A-chain analogues were used to allow determination of relative rates of disulfide bond formation both within themselves and together with B-chain. A similar approach has been previously employed in earlier studies to determine the insulin oxidative folding pathway (17). Previous work in this laboratory and by others (32, 33) indicated that the combination of the relaxin chains proceeds via preliminary oxidation of the A-chain to an oxidized intermediate of unknown disulfide disposition that then reacted with the S-reduced B-chain to generate the native product. To more thoroughly

Table 1: Secondary Structure Content of Native and Cys  $\rightarrow$  Ser Analogues of Human Gene 2 Relaxin A-Chain

relaxin A-chain	Calculated secondary structural content (%)		
	$\alpha$ -helix	$\beta$ -sheet	random coil
native A-chain	13	40	47
[Ser <sup>A10,15</sup> ] A-chain	4	26	70
[Ser <sup>A11,24</sup> ] A-chain	3	25	72
[Ser <sup>A10,24</sup> ] A-chain	3	24	73

study this aspect, each of the A-chains was separately oxidized to measure relative rates of intramolecular disulfide bond formation.

**Intramolecular Oxidation of A-Chain and Analogues.** RP-HPLC monitoring of the time course of oxidation of the A-chains showed that rapid oxidation occurred with the native A-chain (Figure 2A) with complete conversion of the four S-thiol groups into two disulfides within 2 h at 4 °C. In contrast, the formation of each of the intramolecular disulfide bonds between Cys<sup>11</sup> and Cys<sup>24</sup> and the native Cys<sup>10</sup> and Cys<sup>15</sup> was much slower, requiring up to 5 h for completion (Figure 2B,C). Curiously, formation of the non-native intramolecular bond between Cys<sup>11</sup> and Cys<sup>15</sup> was rapid and required less than 2 h (Figure 2D). MALDITOF MS analysis confirmed that the major oxidized product in each experiment (shown by the arrow in Figure 2A–D) was monomeric. Very little dimeric product was present (shown by the astericked arrows). Quantitative Ellman's test time course analysis of the free thiol groups showed that two thiol groups disappeared rapidly during the first hour of oxidation of the native relaxin A-chain (Figure 3A). Thereafter, the rate of oxidation of the remaining two cysteines was moderate. The oxidation for both Cys<sup>11</sup> and Cys<sup>24</sup> and the native Cys<sup>10</sup> and Cys<sup>15</sup> was slower (Figure 3A). The oxidative formation of the non-native intramolecular bond between Cys<sup>11</sup> and Cys<sup>15</sup> was also rapid. The Ellman's test time course monitoring data was supported by RP-HPLC data (Figures 2 and 3A). The folding process was also monitored by direct secondary structure analysis using CD spectrometry. At 1.5 h, only the native A-chain showed significant secondary structure (Figure 4), which was calculated to be approximately 13%  $\alpha$ -helix. In contrast, each of the mutant chains was largely unordered (Figure 4, Table 1).

**Combination of A-Chain with B-Chain.** The combination of native relaxin A- and B-chains in solution proceeded smoothly and seemingly via an oxidized A-chain isomer that is believed to be the double disulfide (A10–15, 11–24) bonded peptide (Figure 5A). Reaction is complete as evidenced by the disappearance of starting S-reduced B-chain within 24 h. In contrast, combination of the [Ser<sup>A10,15</sup>] A-chain proceeded much more slowly although also complete within the 24 h monitoring period (Figure 5B). The overall yield for native relaxin is about 30% based on initial A-chain peptide, while that for [Ser<sup>A10,15</sup>]-relaxin is 3–5% (Figure 5). The RP-HPLC retention time of [Ser<sup>A10,15</sup>]-relaxin is greater than for native relaxin (Figure 5) suggesting that the former has a greater exposure of surface hydrophobic residues. Ellman's thiol monitoring of the combination also confirmed the much slower rate of [Ser<sup>A10,15</sup>]-A-chain combination with B-chain (Figure 3B). Given that native relaxin B-chain alone self-oxidized very slowly (Figure 3B), this indicates that the [Ser<sup>A10,15</sup>] A-chain has only a very weak



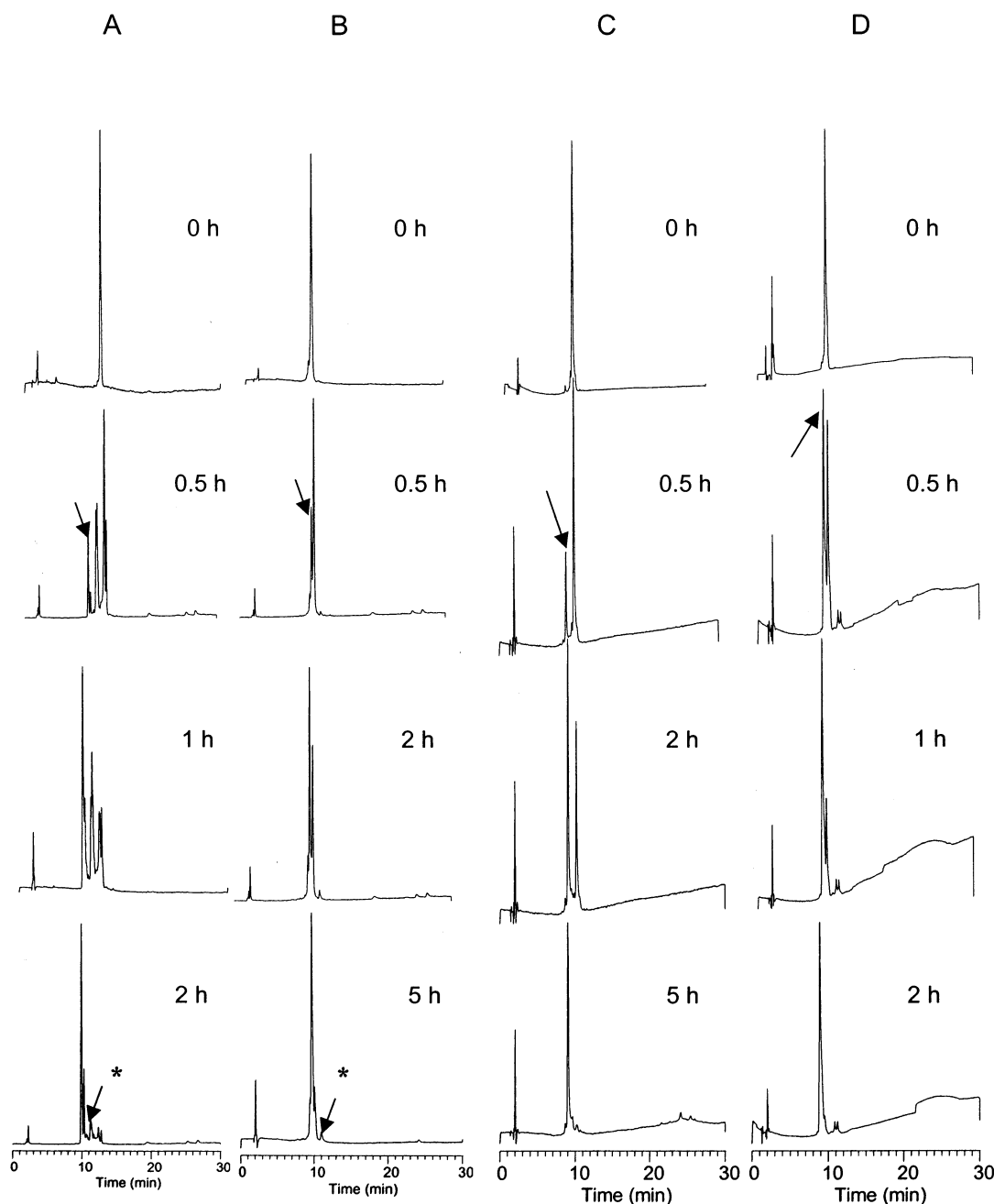


FIGURE 2: Analytical RP-HPLC time-course monitoring of oxidation of reduced (A) native A-chain, (B) [Ser<sup>A10,15</sup>] A-chain, (C) [Ser<sup>A11,24</sup>] A-chain, and (D) [Ser<sup>A10,24</sup>] A-chain. Arrow indicates oxidized product. Asterick indicates dimerized product.

interaction with the B-chain. In contrast, the thiol group oxidation was rapid for the native A- and B-chain combination in which three thiol groups were consumed within 0.5 h (Figure 3B). This also indicates that the S-reduced B-chain obviously requires the presence of oxidized A-chain for the combination reaction to occur efficiently.

**Tryptic Mapping of Combined Relaxins.** Following combination of the native relaxin and the [Ser<sup>A10,15</sup>]-relaxin, these were isolated by RP-HPLC and subjected to tryptic mapping and mass analysis. The data showed that the key tryptic fragments were those expected for chains aligned in the parallel configuration (Figure 6) confirming that they possessed the correct interchain disulfide bonds.

**Effect of Temperature on Efficiency of Chain Combination.** Duplicate oxidative folding experiments were performed with native A- and B-chains at different temperatures. After 24 h

at either 4 °C, ambient temperature, or 37 °C, RP-HPLC analysis of the products showed that best results were obtained at either 4 °C or ambient temperature (Figure 7). Increased levels of A-chain polymers were obtained at higher temperature.

**Effect of Prior Oxidation of A-Chain on Combination Efficiency.** As native relaxin chain combination follows a pathway whereby oxidized A-chain is a key intermediate in the folding process, the effect of preoxidation of A-chain followed by addition of S-reduced B-chain was assessed. At zero time, the oxidized double-disulfide A-chain (Figure 8A) was combined with B-chain. Within 24 h, consumption of starting S-reduced B-chain was complete (Figure 8B). However, the presence of relaxin was contaminated with a significant level of intramolecularly oxidized B-chain and other, later eluting, unidentified products.

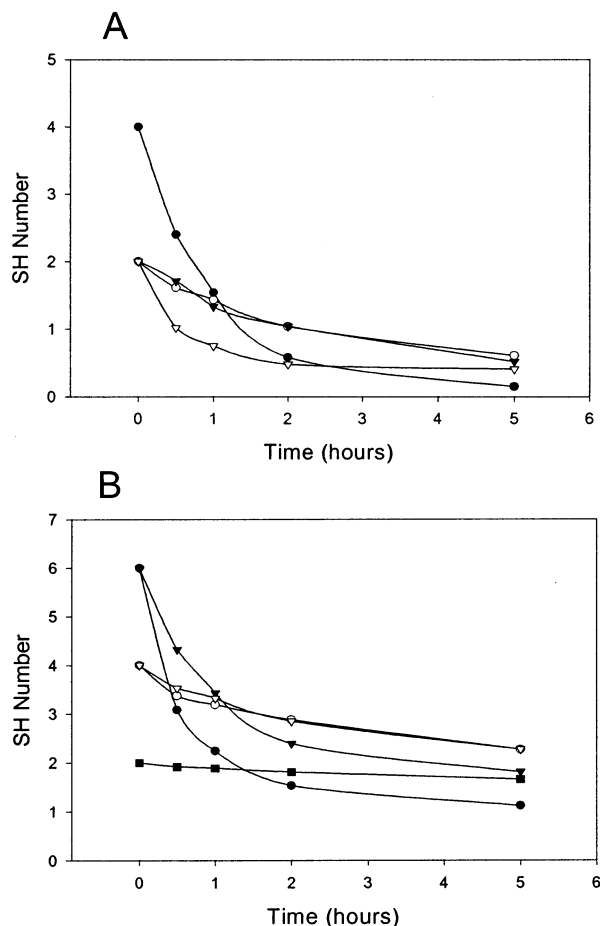


FIGURE 3: Ellman's reagent time course monitoring of oxidation of reduced relaxin chains. (A) Native A-chain and analogues. (●) Native A-chain, (○) [Ser<sup>A10,15</sup>] A-chain, (▼) [Ser<sup>A11,24</sup>] A-chain, and (□) [Ser<sup>A10,24</sup>] A-chain. (B) A-chain combination with B-chain, (●) native A-chain with B-chain, (○) [Ser<sup>A10,15</sup>] A-chain with B-chain, (■) B-chain alone, (▼) summation of oxidation of native A-chain plus oxidation of B-chain, and (□) summation of oxidation of [Ser<sup>A10,15</sup>] A-chain plus oxidation of B-chain.

## DISCUSSION

The oxidative folding pathway of insulin, both via a single-chain prohormone and its separate A- and B-chains, has been the subject of intense scrutiny. Numerous biochemical and biophysical approaches have been employed to deduce the structural and thermodynamic course of events leading to the correct disulfide pairing of the constituent chains. Of particular interest is the recognition that an apparently identical process occurs regardless of whether the individual chains are paired together or linked either via a connecting peptide of varying length or head-to-tail. It is accepted that the first folding event involves A-chain intramolecular disulfide bond formation (37). This is then followed by secondary structure formation of, first, the A-chain N-terminal  $\alpha$ -helix and then the C-terminal  $\alpha$ -helix of the same chain. In a fast step, this intermediate then combines with the B-chain to nearly simultaneously form the two intermolecular disulfide linkages. That little antiparallel combined species is observed suggests a strong primary structure influence on the orientation of combination. Indeed, earlier studies strongly indicated that initial formation of the A-chain N-terminal  $\alpha$ -helix was not the driving force for determining chain orientation (37) nor for successful chain combination

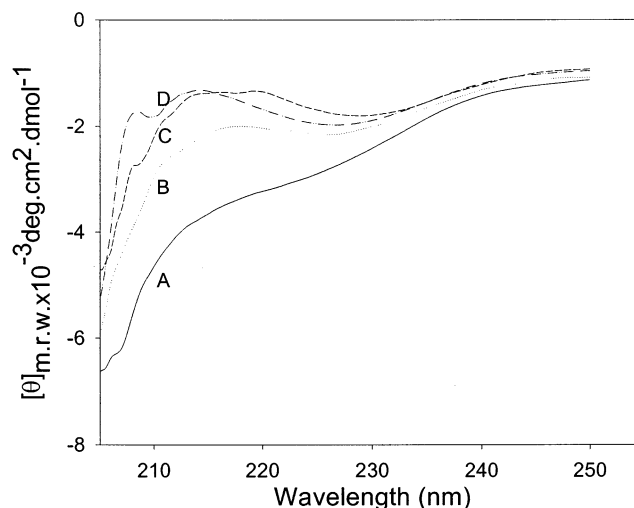


FIGURE 4: CD spectroscopic analysis of relaxin A-chain and analogues after 1.5 h of oxidative folding. (A) Native A-chain, (B) [Ser<sup>A10,15</sup>] A-chain, (C) [Ser<sup>A11,24</sup>] A-chain, and (D) [Ser<sup>A10,24</sup>] A-chain.

itself (38). Use of synthetic insulin analogues lacking the A-chain intramolecular A6–A11 cysteine to further examine the role of this bond suggests that its primary function is to tether the N-terminal A1–A8  $\alpha$ -helix as a preformed recognition element to the hydrophobic core (19, 39). It is also believed that the B-chain plays a crucial role in acting as a template for the direction of the A-chain folding process (38, 39). This primary oxidative folding pathway has been confirmed by parallel studies using a mini-C-peptide proinsulin in which the A- and B-chains are terminally linked via a short dipeptide (porcine proinsulin precursor, PIP) (20). These also indicated that an additional, but minor, pathway may exist. As with the folding of IGF-I, the (A20–B19) disulfide bond appears to form first. Further folding gives a mixture containing (A6–B7, A20–B19) and (A7–B7, A20–B19) intermediates. The (A6–B7, A20–B19) intermediate may rearrange its disulfide bond pattern before formation of the last intermolecular disulfide bond.

Recognition that relaxin has a broader spectrum of biological actions than first thought (40) has prompted us to reexamine the folding of this member of the insulin superfamily. Previous studies (32, 33) suggested that the chain combination process may be somewhat similar to that of insulin. In this study, our data showed clearly the first molecular event (e.g., Figure 5A) is the apparently independent formation of a stable bicyclic A-chain reaction intermediate in which the intramolecular (A10, 15) disulfide bond probably forms, perhaps via the (A11, 15) disulfide isomer. Small quantities of mismatched monomeric disulfide isomers of A-chain also form, but these do not appear to combine with the B-chain as indicated by the absence of change in RP-HPLC peak size during chain combination. Following completion of formation of the bicyclic A-chain as the primary intermediate, this then rapidly combines with the S-reduced B-chain to produce the native two-chain peptide that contains the characteristic insulin-like structure and relaxin activity. It was not known which of the two A-chain intramolecular disulfide bonds forms first nor which of the two subsequent intermolecular disulfides forms first. To further investigate this aspect, in the first instance, the oxidative folding behavior of A-chain and of pairwise

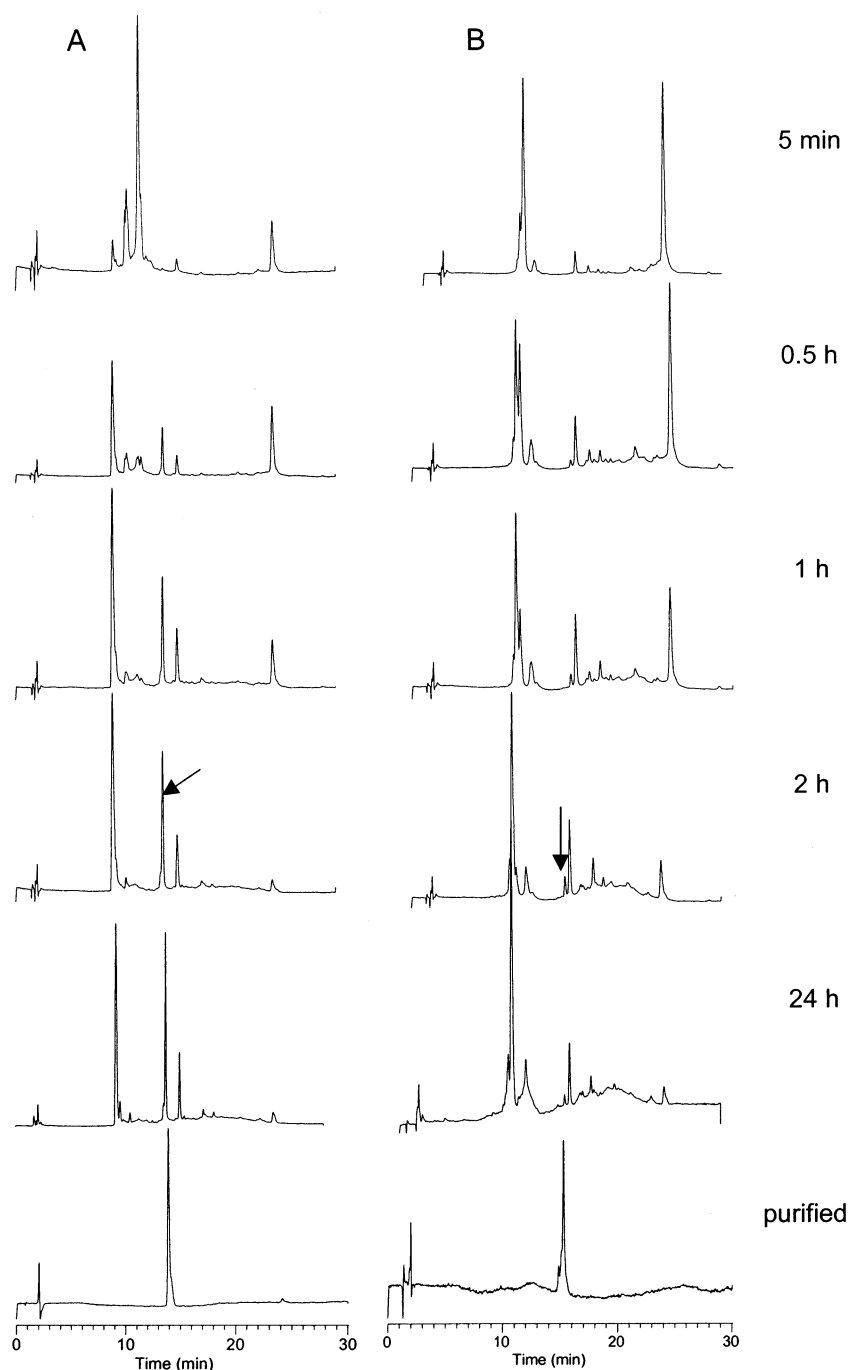


FIGURE 5: Analytical RP-HPLC time course monitoring of combination of (A) native relaxin A- and B-chains and (B) [Ser<sup>A10,15</sup>] A-chain and native B-chain. Arrow indicates chain combined product. S-reduced B-chain elutes with a retention time of ca. 24 min.

cysteine → serine analogues was monitored by both RP-HPLC (Figure 3) and thiol group measurement (Figure 3). It was observed that fastest oxidation occurred with the native A-chain in which a bicyclic peptide was obtained that lacks free thiols as indicated by the Ellman's test and which was a monomer as indicated by MALDITOF MS. In contrast, analogues lacking the capacity to form either one or the other of these two intramolecular disulfides (Ser<sup>A10,15</sup> and Ser<sup>A11,24</sup>) oxidized much more slowly but at an approximately similar rate. This suggested that the observed rapid oxidation of the native chain is dependent on the presence of all four cysteines. Curiously, however, the mismatched intramolecular disulfide bonded analogue, [Ser<sup>A10,24</sup>] A-chain, oxidized fastest of the three analogues implying that this bond may

form first during native chain combination before rearranging to the A<sup>10,15</sup> and A<sup>11,24</sup> disulfide bonded peptide. This was supported by CD spectroscopy data that showed the oxidized analogue [Ser<sup>A10,24</sup>] A-chain is devoid of secondary—and thus native-like—structure (Figure 4). NMR spectroscopy analysis is presently being employed to conclusively elucidate the cysteine pattern of the bis-cyclic A-chain. The X-ray crystal structure of native human Gene 2 relaxin shows that it possesses a cysteine pairing that is identical to that of insulin (14). Additionally, use of tryptic mapping together with mass spectrometric analysis and comparison to chemically assembled peptides confirmed that the A-chain intramolecular link is the Cys<sup>10</sup>–Cys<sup>15</sup> (41). Consequently, the observed fast formation of the mismatched disulfide (A11–15) is

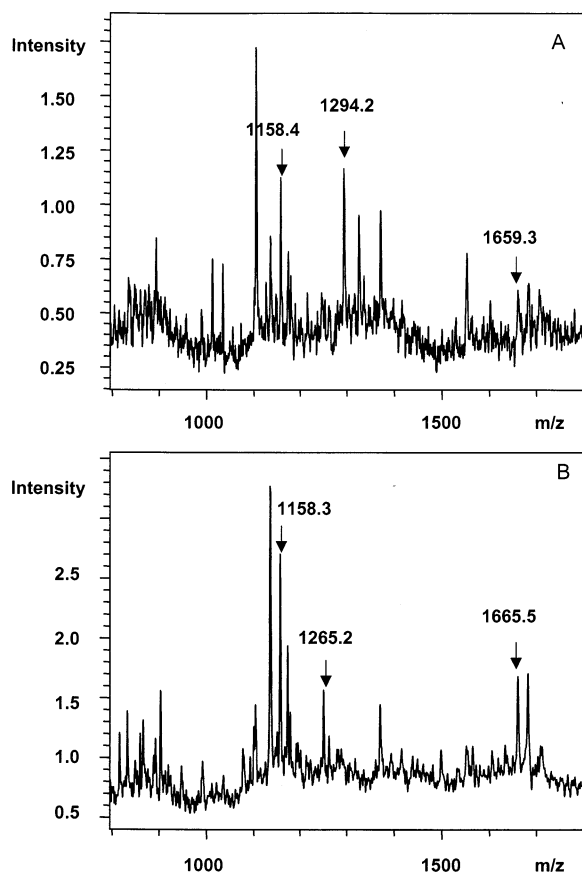


FIGURE 6: MALDITOF mass spectroscopic analysis of tryptic digest of relaxin chain combination products. (A) Native chain combined relaxin. Peak identification:  $MH^+$  1158.4, B(1–9) (theoretical  $MH^+ + Na^+$ , 1159.0);  $MH^+$  1294.2, A(10–17)/B(10–13) (theoretical  $MH^+$ , 1294.5);  $MH^+$  1659.3, A(23–24)/B(18–30) (theoretical  $MH^+$ , 1661.6). (B) [Ser<sup>A10,15</sup>] relaxin. Peak identification:  $MH^+$  1158.3, B(1–9) (theoretical  $MH^+ + Na^+$ , 1159.0);  $MH^+$  1265.2, A(10–17)/B(10–13) (theoretical  $MH^+$ , 1264.5);  $MH^+$  1665.5, A(23–24)/B(18–30) (theoretical  $MH^+$ , 1661.6).

unlikely to be anything other than an intermediate leading to the ultimate formation of the correct (A10–15) bond. In the absence of an intramolecular A-chain disulfide, pairing with the B-chain occurs much more slowly although still in the correct orientation as evidenced by tryptic mapping and mass spectrometric analysis of the resulting fragments (Figure 6). This further confirmed that the rate-limiting step for the folding of relaxin was the formation of the intramolecular A-chain disulfide bond. It is worth noting that [Ser<sup>A10,15</sup>]-relaxin had a greater RP-HPLC retention time than the native peptide. A similar effect was observed with the des-intramolecular A-chain disulfide bonded insulin (42) and suggests a higher exposure of more hydrophobic residues on the surface of the molecule. Biological assay of this relaxin analogue is presently in progress. The rate and yield of combination was largely unaffected by temperatures at or below ambient but poor at 37 °C. This differs from the situation observed for insulin where the optimum chain combination temperature is 4 °C, and the yield decreases with increasing temperature (43). Interestingly, preoxidation of A-chain followed by combination with B-chain did not necessarily result in better overall yields of relaxin. Instead, a large proportion of oxidized B-chain was obtained. As this peptide oxidizes only very slowly on its own, the presence of the bicyclic A-chain promoted both combination with the

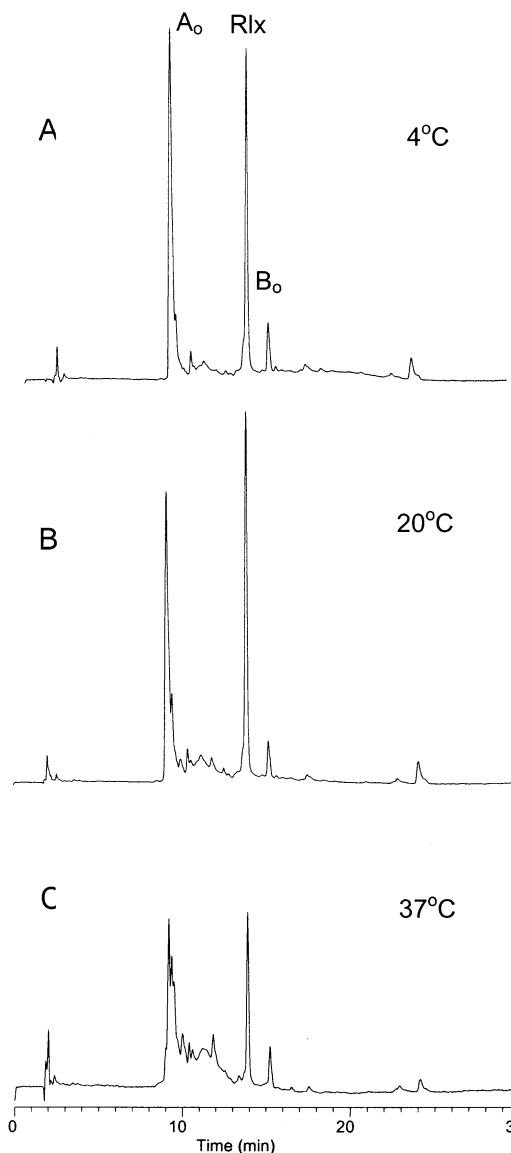


FIGURE 7: Analytical RP-HPLC assessment of effect of temperature on efficiency of relaxin chain combination after 24 h of reaction. A<sub>0</sub>, bicyclic-A-chain; Rlx, relaxin; and B<sub>0</sub>, intramolecularly oxidized B-chain.

B-chain and B-chain self-oxidation at approximately the same apparent rate. The lower yield of resulting relaxin is probably a consequence of the shortage of free thiol groups in the reaction medium.

Our results suggest that in the case of human Gene 2 relaxin, the chain folding and combination pathway is similar to that of insulin (19, 20). The necessity for the formation of the bicyclic A-chain to occur before subsequent combination with the B-chain will take place suggests that the latter peptide does not, as in the case of insulin, dictate the folding process. Whether the observed human Gene 2 relaxin pathway is also followed by human Genes 1 and 3 relaxins remains to be determined. Of interest is the observation that the N-terminal region of the A-chain of the latter peptide contains a stretch of three successive serines (28). Such a sequence is predicted to be unfavorable for the formation of an  $\alpha$ -helix that, itself, is a chain combination initiation element (37). Of interest is the finding that successful acquisition of hybrid molecules was achieved when optimized conditions were employed for the combination of



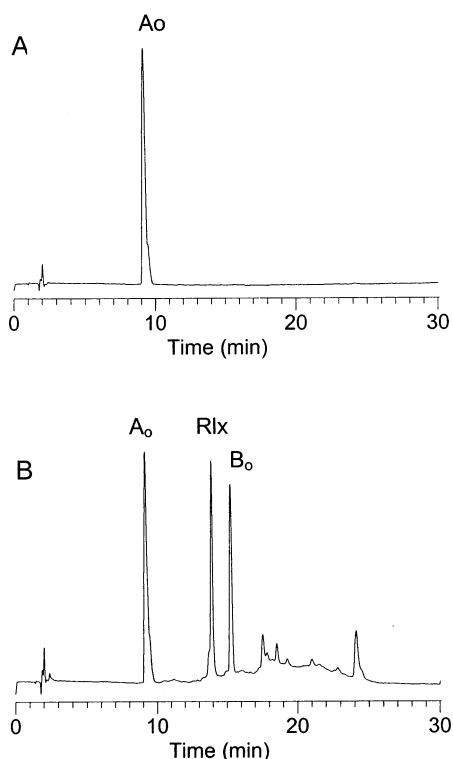


FIGURE 8: Analytical RP-HPLC analysis of effect of (A) prior oxidation of relaxin A-chain on (B) efficiency of combination with native B-chain after 24 h of reaction. A<sub>o</sub>, bicyclic-A-chain; Rlx, relaxin; and B<sub>o</sub>, intramolecularly oxidized B-chain.

relaxin B-chain with the A-chains of other insulin-like peptides including ovine and human insulin 3 (also known as relaxin-like factors) (Tang, J.-G., and Wade, J. D., unpublished). This would support the concept that, although the oxidative folding pathway may subtly differ for each of relaxin, insulin, and insulin-like growth factor I, successful oxidative folding of members of the insulin superfamily follows a hierarchic process along a redox-sensitive series of energy landscapes (19).

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