Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 374-380

A COMPARATIVE CIRCULAR DICHROISM STUDY OF RELAXIN AND INSULIN

Christian Schwabe and Sue J. Harmon
Department of Biochemistry
Medical University of South Carolina
Charleston, South Carolina 29401

Received August 10, 1978

SUMMARY: The far UV CD spectra of insulin and relaxin have been compared and shown to possess essentially similar features. Where differences occur, such as in the 222 nm band, they can be attributed to the tendency of insulin to form dimers. The ratio of the 195/208 nm bands is 1.54 for porcine relaxin and thus intermediate between the value of 0.79 for insulin of hystricomorphs and 2.05 for porcine insulin. Comparison of porcine relaxin with desAsn-desAla insulin suggests that the relaxin structure is similar to the structure of insulin in solution and thus compatible with the models previously constructed on the insulin coordinates.

The primary structure of the ovarian peptide hormone relaxin (1, 2, 3, 4) can be accommodated without strain in the previously established three-dimensional structure of insulin (5). This insulin-like conformation might, however, be only one of several structures the relaxin chain could assume in solution and the ultimate proof of the similarity of relaxin with insulin must be derived from further experimental evidence. The well-known structure of insulin and its CD spectra (6, 7) invited a comparative study between the two hormones. The results of this study of CD spectra of insulin, insulin derivatives, and relaxin leads one to postulate that the proposed relaxin model is correct (8).

MATERIALS AND METHODS: The relaxin used in this study was purified from an ovarian acetone extract (NIH) by a method similar to that of Sherwood and O'Byrne (9). The relaxin thus obtained was previously used for the primary structure determination in our laboratory (10). Porcine insulin has been donated by Dr. W. Bromer (Eli Lilly Co.) and was freed from zinc by gel chromatography on Sephadex G-50 fine in 1 M acidic acid. Minor impurities were removed from the insulin by chromatography on Sephadex G-50 superfine, using 6 M guanidine·HCl as eluant. The salt was subsequently removed on a Sephadex G-25 column equilibrated with 0.05 M ammonium bicarbonate. All other chemicals used were analytical grade.

The proteins were dissolved in 0.01 M Tris-HCl (pH 7.2). Stock solutions of zinc-free insulin and relaxin were prepared and aliquots of 100 µl were withdrawn and hydrolyzed in 6 N HCl for 24 hr at 117°C for subsequent amino acid analyses. From the amino acid analysis data the concentration of each

0006-291X/78/0842-0374$01.00/0

stock solution was calculated in order to allow evaluation of the CD spectra. The solutions prepared for CD analysis contained 0.005% sodium azide as preservative. Circular dichroism measurements were made using a 0.02 and a 0.05 cm cell with a scale expansion of 0.01° or 0.04° full scale. For the near UV measurements a 1 cm cell and a scale expansion of 0.02° or 0.04° (full range) was used. All measurements were performed on a Cary model 60·CD spectrometer. The continuous spectra obtained were redrawn such that the buffer blank represents the base line. To obtain the Zn-insulin or Zn-relaxin spectra $ZnSO_4$ was added to the samples in concentrated form (5 µl/ml of hormone solution). Since mainly comparative data to the well-known insulin spectra were sought, no attempt was made to accurately measure the pathlength of the cells employed. The CD spectrometer was standardized with *d*-10-camphosulfonic acid. The results are reported as mean residual ellipticities according to the expression

$$[\theta]_\lambda = \frac{(\Psi)\ (MRW)}{10\ (C)\ (\ell)}$$

where Ψ is the observed rotation; MRW, the mean residual weight; C, the concentration in g/ml; and ℓ, the pathlength of the cell in centimeters (11).

RESULTS AND DISCUSSION: Although the primary structures of relaxin and insulin are homologous (or conservatively substituted) to only about 40% of their amino acid residues the disulfide links in the two hormones are identical (4). The surprisingly perfect fit of the relaxin sequence into the insulin coordinate system was in part due to the complimentary replacement of amino acids located in opposing positions and through retention of what constitutes the hydrophobic core in insulin (5, 6, 8). To test whether the assumptions made to construct the relaxin models, based on insulin coordinates, were indeed realistic, the circular dichroic spectra of both molecules were compared (Fig. 1). The result suggests strongly that porcine relaxin is more closely related to porcine insulin than guinea pig insulin is to porcine insulin. It is known that guinea pig insulin has a perturbed structure which may assume insulin conformation only while interacting with a receptor (6), and therefore show a lower biological activity. The slightly more intense signal elicited by relaxin in the far UV region of the spectrum is shifted to a shorter wavelength and the ratio of the ellipticities at 209 nm and 195 nm of porcine relaxin is less than that of porcine insulin but much higher than the same ratio for guinea pig insulin (6) (Table I). This would support the contention that the two structures are essentially alike. The complete absence of insulin activi-

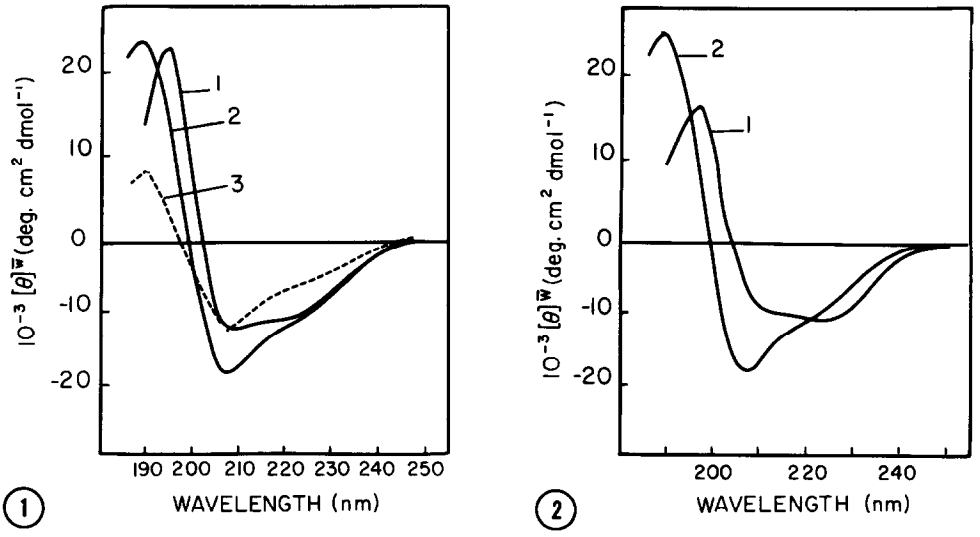


Fig. 1 Comparison of relaxin (line 2) with zinc-free porcine insulin (line 1) (50 μM) and a 40 μM solution of guinea pig insulin (line 3) (from ref. 6). The slight blue shift of the relaxin spectrum compared to that of insulin is consistantly observed. The porcine relaxin and insulin samples were dissolved in 10 mM Tris·HCl at pH 7.2.

Fig. 2 CD spectra of insulin and relaxin containing 0.5 moles of Zn per mole of protein monomer. The spectrum of relaxin (2) remains unchanged while insulin (1) shows a strong shift in relative intensity of the extremes of its spectrum.

Table I

COMPARISON OF MINIMA AND MAXIMA OF THE RELAXIN AND INSULIN SPECTRA

Hormone*	Atom Zn / Monomer	$[\theta]_{195}/[\theta]_{208}$	$[\theta]_{208}/[\theta]_{222}$
Insulin (Bovine)	0	2.05	1.38**
Insulin	0.5	2.28	1.29**
Insulin (Guinea Pig)	0	0.49	1.79**
Relaxin	0	1.54	1.66
Relaxin	0.5	1.54	1.65

*All hormone solutions were 40 μM

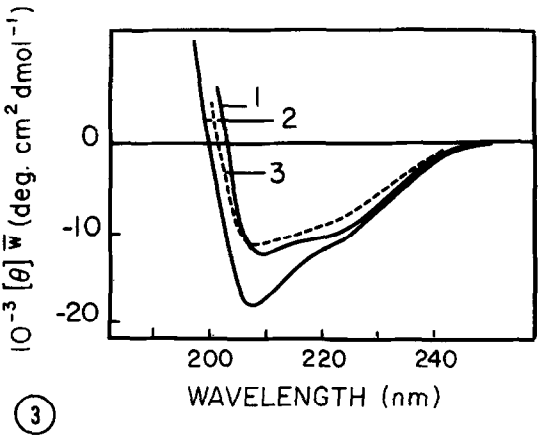
**From reference #6

ty in the relaxin molecule is therefore most likely not attributable to conformational differences but rather to side chain replacements and differences at the C and N termini.

The large differences in the 209/222 nm ratios are probably due to the tendency of insulin to form dimers in the absence of zinc (8). At greater dilutions (Fig. 2) the insulin spectrum at 222 nm changes such as to more closely resemble that of relaxin. The addition of $ZnSO_4$ (2.5×10^{-4} in a 5×10^{-4} M solution of insulin) significantly strengthened the 222 nm band; zinc addition to relaxin had no effect in the far UV spectrum (Fig. 2). Conversely, the addition of benzopurpurine 4-B, a compound that enhances the activity of exogenous relaxin in mice (12), induces a more prominent 222 nm band in the relaxin spectrum and thereby leads to a more insulin-like appearance. In analogy to insulin the dye might provide a hydrophobic surface to induce dimer formation in relaxin. Conversely benzopurpurine could induce a conformational change into the C-terminal portion of relaxin that is otherwise free to interact with the solvent (Fig. 3).

The importance of this C-terminal region for the insulin structure and its biological activity is supported by experiments with desAsn-desAla and desoctapeptide-insulin (14, 13). The desAsn-desAla-insulin cannot form an ion pair between Asn (A_{21}) and Arg (B_{22}). The structural disturbance created is evident from the CD data depicted in Fig. 4, where relaxin is compared to desAsn-desAla-insulin with and without zinc. The spectrum of the desoctapeptide-insulin is similar to that of the desAsn-desAla-insulin. Again the main difference between relaxin and insulin appears to be due to the structure in the C-terminal region. Modifications that do disturb the insulin structure sufficiently to prevent or diminish dimer formation also cause the CD spectra to shift to a relaxin-like appearance.

The near UV spectra of insulin and relaxin differ in accordance with the chromophores in each hormone. Relaxin contains two residues of tryptophan and one phenylalanyl residue compared to tyrosyl and phenylalanyl residues in insu-



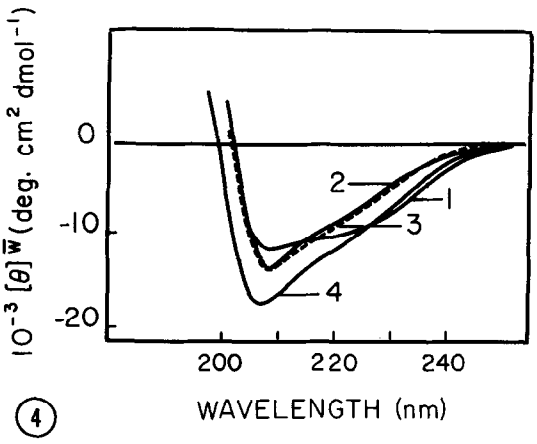


Fig. 3 The effect of benzopurpurine on the CD spectrum of relaxin. Curve 1, zinc-free insulin (50 M); curve 2, relaxin (50 μM); and curve 3, relaxin (50 μM, 25 μM in benzopurpurine 4-B).

Fig. 4 The CD spectra of insulin (1), desAsn-desAla-insulin without zinc (2), with zinc (3), and relaxin (4), are depicted. The hormone concentrations were 40 μM for relaxin and native insulin, 50 μM for desAsn-desAla-insulin, and 25 μM for $ZnSO_4$. Curve 2 and 3 are redrawn from ref. 13.

lin. Fig. 5 is included to demonstrate the lack of effect of zinc ions on the relaxin spectrum compared to the well-known effect in insulin.

The display of the backbone structure of the two hormones in Fig. 6 was generated from the scale models (7) and shows the features (helices) likely to be responsible for the spectral similarity (see 195/208 nm ratio). It is also clear that the two C-terminal tails of insulin are longer than those in relaxin and therefore more likely to make significant structural contributions during dimer formation, a fact not observed with relaxin (208/222 nm ratio). In addition, a missing C-terminal residue in the A chain of relaxin and the lack of a basic residue in the position corresonding to insulin B_{22} (conditions that allow for a stabilizing saltbridge formation in insulin) are sufficient to account for the differences in ellipticity although the bulk of both molecules is alike. A minor condition contributing to possible differences in helix energies might be due to the presence in relaxin of two Arg residues in positions B_9 and B_{13} (this designates the second and sixth residue after the

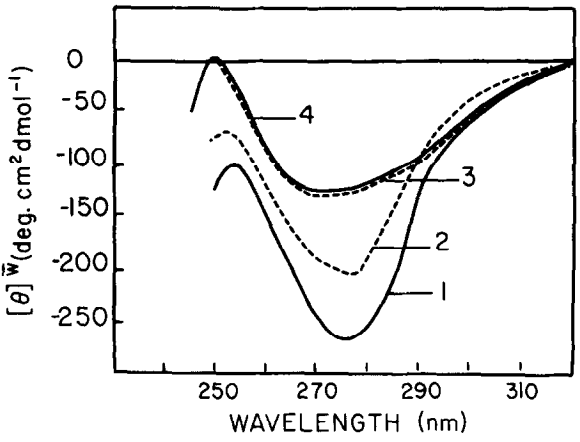


Fig. 5 New UV CD spectra of insulin (50 μM) with zinc (25 μM) (1) and without zinc (2) as well as relaxin (40 μM) with and without zinc (3 and 4).

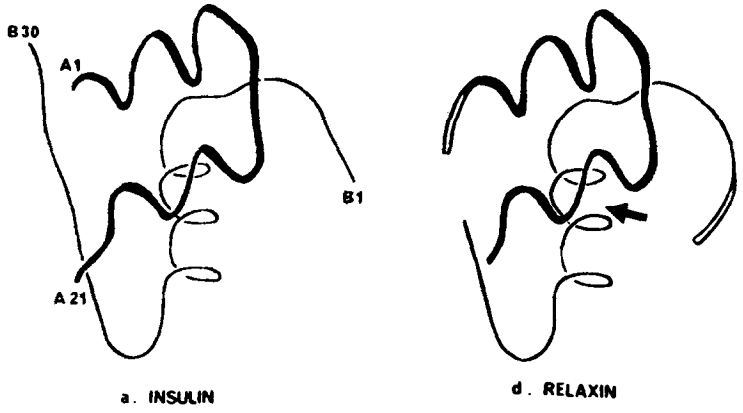


Fig. 6 Comparison of the backbone structures of insulin and relaxin. The open line at the N terminus of the relaxin A chain designates an extension not present in insulin while the open line at the C terminus of the B chain designates missing residues compared to the insulin. The arrow points to the region of the B chain helix that contains the two juxtapositioned Arg residues (see text).
(Drawings from ref. 6 with permission by Dr. T. L. Blundell)

first Cys residue according to the insulin numbering system). Although this helix-breaking effect is offset by saltbridges formed between Glu (B_{17}) and and Arg (B_{13}) as well as Arg (B_9) and Glu (A_4), the restriction in degrees of freedom of the guanidino groups could lead to a higher energy content in the relaxin helix compared to the analogue structure in insulin.

From these studies it appears that the bulk of insulin and relaxin assume a similar conformation in solution and that differences are sufficiently small to be commensurate with differences in chainlength and chromophores of the two hormones. This leads to the conclusion that the model of relaxin built on insulin coordinates is likely to be correct (8, 15).

Relaxin thus joins the "insulin group" of hormones with respect to disulfide bond distribution and conformation. Unlike the insulin-like growth factor or proinsulin, relaxin has no insulin activity nor does it share antigenic sites with insulin and thereby constitutes further evidence that quite unrelated functions may originate from duplicate genes.

ACKNOWLEDGMENT

The helpful suggestions offered by Dr. T. L. Blundell and the technical assistance of Mr. J. Collawn are gratefully acknowledged.
We thank Dr. W. Fish for the use of his Cary 60 CD spectrometer.
This research was supported in part by NICHD Grant HD-10540 and by the South Carolina state funds for research.

REFERENCES

1. Schwabe, C., McDonald, J. K. and Steinetz, B. G. (1976) Biochem. Biophys. Res. Comm. 70: 397-405.
2. Schwabe, C. and McDonald, J. K. (1977) Biochem. Biophys. Res. Comm. 74: 1501-1504.
3. Schwabe, C., McDonald, J. K. and Steinetz, B. G. (1977) Biochem. Biophys. Res. Comm. 75: 503-510.
4. Schwabe, C. and McDonald, J. K. (1977) Science 197: 914-915.
5. Blundell, T. L., Dodson, G. G., Hodgkin, D. C. and Mercola, D. A. (1972) Adv. Protein Chem. 26: 279-402
6. Wood, S. P., Blundell, T. L., Lazarus, N. R. and Neville, W. J. (1975) Eur. J. Biochem. 55: 531-542.
7. Blundell, T. L., Bedarkar, S., Rinderknecht, E. and Humbel, R. E. (1978) Proc. Natl. Acad. Sci. USA 75: 180-184.
8. Badarkar, S., Turnell, W. G., Blundell, T. L. and Schwabe, C. (1977) Nature 270: 449-451.
9. Sherwood, O. D. and O'Byrne, E. M. (1974) Arch. Biochem. Biophys. 160: 185-196.
10. Schwabe, C., Steinetz, B. G., Weiss, G., Segaloff, A., McDonald, J. K., O'Byrne, E. M., Hochman, J., Carriere, B. and Goldsmith, L. (1978) Rec. Prog. in Hormone Res. (in press).
11. Adler, A. J., Greenfield, N. J. and Fasman, G. D. (1973) Methods in Enzymology (C. H. W. Hirs and S. N. Timasheff, eds.) Academic Press 27, 675-735.
12. Steinetz, B., Beach, V. L. and Kroc, R. L. (1969) Methods in Hormone Research (R. Dorfman, ed.) Academic Press, New York 2A: 481-508
13. Goldman, J. and Carpenter, F. H. (1974) Biochem. 13: 4566-4574.
14. Brugman, T. M. and Arquilla, E. R. (1973) Biochemistry 12: 727-732.
15. Isaacs, N., James, R., Niall, H., Bryant-Greenwood, G., Dodson, G., Evans, A. and North, A. C. T. (1978) Nature 271: 278-281.