THE CONFORMATION AND MOLECULAR BIOLOGY OF PANCREATIC HORMONES AND HOMOLOGOUS GROWTH FACTORS

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I. INTRODUCTION

The ultimate goal of conformational studies of peptide hormones and growth factors must be to define the role of conformation in their biosynthesis, storage, receptor binding, and degradation. Knowledge of this kind may play an important part in the proper design of competitive inhibitors, orally administered analogues, and hormones of selectively enhanced biological activity.

The importance of the four pancreatic hormones — insulin (Table 1), glucagon (Table 2), somatostatin (Table 3), and pancreatic polypeptide (PP) (Table 4) — to diabetes, obesity, and diet control makes such studies particularly valuable. The X-ray analysis of insulin by Hodgkin and co-workers[1,2] brought together much of the earlier work on chemistry and biology[3] and was helpful not only in the design of new procedures for insulin synthesis (the use of miniproinsulin analogues for instance) but also to new insulin analogues with long half-lives and to attempts to make oral and superpotent insulins. More recent X-ray studies have detailed the conformations of insulin in different crystal environments and from species as different as the hagfish and human. In addition, computer graphics model building has been used to predict the structures of insulin-like growth factors.[4,5] At the same time X-ray studies of glucagon[6] and pancreatic polypeptide[7-9] have come to fruition and suggest important biological relationships. However, with these smaller polypeptides, and more particularly with somatostatin, the conformation is more flexible, and complementary solution studies using nuclear magnetic resonance, circular dichroism, and other spectroscopic techniques become increasingly important.

In this review we first describe methods used to define the conformations of pancreatic polypeptide hormones in the crystalline state and in solution. We then compare and contrast the conformations characterized by these techniques and discuss their dependence on the environment. Finally, we relate the conformations to the molecular biology with emphasis on themes common to the hormones including synthesis as precursors, their storage as oligomers in granules, the existence of monomers in circulation, and the importance of a particular conformer at the receptor. However, we also highlight the differences which arise, principally from differences in flexibility of the polypeptides in dilute aqueous solutions.

Table 1
THE AMINO ACID SEQUENCES OF INSULINS AND RELATED GROWTH FACTORS

A-Chains

	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Insulin																																
Bovine	—	—	G	I	V	E	Q	C	C	A	S	V	C	S	L	Y	Q	L	E	N	Y	C	N	—	—	—	—	—	—	—	—	—
Human	—	—	G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	—	—	—	—	—	—	—	—	—
Rat 1	—	—	G	I	V	D	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	—	—	—	—	—	—	—	—	—
Rat 2	—	—	G	I	V	D	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	—	—	—	—	—	—	—	—	—
Guinea pig	—	—	G	I	V	D	Q	C	C	T	G	T	C	T	R	H	Q	L	Q	S	Y	C	N	—	—	—	—	—	—	—	—	—
Casiragua	—	—	G	I	V	D	Q	C	C	T	N	I	C	S	R	N	Q	L	L	T	Y	C	N	—	—	—	—	—	—	—	—	—
Coypu	—	—	G	I	V	D	Q	C	C	T	N	I	C	S	R	N	Q	L	M	S	Y	C	N	D	—	—	—	—	—	—	—	—
Hagfish	—	—	G	I	V	E	Q	C	C	H	K	R	C	S	I	Y	N	L	Q	N	Y	C	N	—	—	—	—	—	—	—	—	—
Porcupine	—	—	G	I	V	D	Q	C	C	T	G	V	C	S	L	Y	Q	L	Q	N	Y	C	N	—	—	—	—	—	—	—	—	—
Insulin-like Growth factor																																
IGF 1	—	—	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A	P	L	K	P	A	K	S	A	—
IGF 2	—	—	G	I	V	E	E	C	C	F	R	S	C	D	L	A	L	L	E	T	Y	C	A	T	—	—	P	A	K	S	E	—
Relaxin																																
Porcine	R	M	T	L	S	E	K	C	C	E	V	G	C	I	R	K	D	I	A	R	L	C	—	—	—	—	—	—	—	—	—	—

B-Chains

	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Insulin																																		
Bovine	—	—	—	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	—
Human	—	—	—	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	T	—

Rat 1	—	—	—	F	V	K	Q	H	L	C	G	P	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	S	—
Rat 2	—	—	—	F	V	K	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	S	—
Guinea pig	—	—	—	F	V	S	R	H	L	C	G	S	N	L	V	E	T	L	Y	S	V	C	Q	D	D	G	F	F	Y	I	P	K	D	—
Casiragua	—	—	—	Y	V	G	Q	R	L	C	G	S	Q	L	V	D	T	L	Y	S	V	C	K	H	R	G	F	Y	R	P	S	E	—	—
Coypu	—	—	—	Y	V	S	Q	R	L	C	G	S	Q	L	V	D	T	L	Y	S	V	C	R	H	R	G	F	Y	R	P	N	E	—	—
Hagfish	—	—	—	R	T	T	G	H	L	C	G	K	D	L	V	N	A	L	Y	I	A	C	G	V	R	G	F	F	Y	D	P	T	K	M
Porcupine	—	—	—	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	N	D	G	F	F	Y	R	P	K	A	—
Insulin-like Growth factor																																		
IGF 1	—	—	—	—	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	C	G	D	R	G	F	Y	F	N	K	P	T	—
IGF 2	—	A	Y	R	P	S	E	T	L	C	G	G	E	L	V	D	T	L	Q	F	V	C	G	D	R	G	F	Y	F	S	R	P	A	—
Relaxin																																		
Porcine	—	S	T	N	D	F	I	K	A	C	G	R	E	L	V	R	L	W	V	E	I	C	G	V	W	S	—	—	—	—	—	—	—	—

C-Peptides

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Insulin																																			
Bovine	R	R	E	V	E	G	P	Q	V	E	A	L	E	L	A	G	G	P	G	A	G	G	L	—	—	—	—	—	E	G	P	P	Q	K	R
Human	R	R	E	A	E	D	L	Q	V	G	Q	V	E	L	G	G	G	P	G	A	G	S	L	Q	P	L	A	L	E	G	S	L	Q	K	R
Rat 1	R	R	E	V	E	D	P	Q	V	P	Q	L	E	L	G	G	G	P	E	A	G	D	L	Q	T	L	A	L	E	V	A	R	Q	K	R
Rat 2	R	R	E	V	E	D	P	Q	V	A	Q	L	E	L	G	G	G	P	G	A	G	D	L	Q	T	L	A	L	E	V	A	R	Q	K	R
Guinea pig	X	X	E	L	E	D	P	Q	V	E	Q	T	E	L	G	M	G	L	G	A	G	G	L	Q	P	L	—	—	Q	G	A	L	Q	X	X
Insulin-like Growth factor																																			
IGF 1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	G	Y	G	S	S	S	R	R	—	—	—	—	—	—	—	—	A	P	Q	T	—
IGF 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	S	R	V	S	R	R	S	R	—	—	—	—	—	—	—	—	—	—	—	

Note: A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr.

Table 2
THE AMINO ACID SEQUENCE OF PORCINE GLUCAGON

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp
16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr	

Table 3
THE SEQUENCE OF SOMATOSTATIN

H — Ala — Gly — Cys — Lys — Asn — Phe — Phe — Trp — Lys — Thr — Phe — Thr — Ser — Cys — OH
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Table 4
THE AMINO ACID SEQUENCES OF PANCREATIC POLYPEPTIDE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
aPP	Gly	Pro	Ser	Gln	Pro	Thr	Tyr	Pro	Gly	Asp	Asp	Ala	Pro	Val	Glu	Asp	Leu	Ile
bPP	Ala	Pro	Leu	Glu	Pro	Glu	Tyr	Pro	Gly	Asp	Asn	Ala	Thr	Pro	Glu	Gln	Met	Ala
oPP	Ala	Ser	Leu	Glu	Pro	Glu	Tyr	Pro	Gly	Asp	Asn	Ala	Thr	Pro	Glu	Gln	Met	Ala
pPP	Ala	Pro	Leu	Glu	Pro	Val	Tyr	Pro	Gly	Asp	Asp	Ala	Thr	Pro	Glu	Gln	Met	Ala
cPP	Ala	Pro	Leu	Glu	Pro	Val	Tyr	Pro	Gly	Asp	Asp	Ala	Thr	Pro	Glu	Gln	Met	Ala
hPP	Ala	Pro	Leu	Glu	Pro	Val	Tyr	Pro	Gly	Asp	Asn	Ala	Thr	Pro	Glu	Gln	Met	Ala

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	
aPP	Arg	Phe	Tyr	Asp	Asn	Leu	Gln	Gln	Tyr	Leu	Asn	Val	Val	Thr	Arg	His	Arg	Tyr	NH_2
bPP	Gln	Tyr	Ala	Ala	Glu	Leu	Arg	Arg	Tyr	Ile	Asn	Met	Leu	Thr	Arg	Pro	Arg	Tyr	NH_2
oPP	Gln	Tyr	Ala	Ala	Glu	Leu	Arg	Arg	Tyr	Ile	Asn	Met	Leu	Thr	Arg	Pro	Arg	Tyr	NH_2
pPP	Gln	Tyr	Ala	Ala	Glu	Leu	Arg	Arg	Tyr	Ile	Asn	Met	Leu	Thr	Arg	Pro	Arg	Tyr	NH_2
cPP	Gln	Tyr	Ala	Ala	Glu	Leu	Arg	Arg	Tyr	Ile	Asn	Met	Leu	Thr	Arg	Pro	Arg	Tyr	NH_2
hPP	Gln	Tyr	Ala	Ala	Asp	Leu	Arg	Arg	Tyr	Ile	Asn	Met	Leu	Thr	Arg	Pro	Arg	Tyr	NH_2

II. STRUCTURE DETERMINATION AND PREDICTION

Techniques which have contributed to our understanding of the conformation of polypeptide hormones include diffraction and microscopic, spectroscopic, and immunological techniques, as well as computer-based predictive methods. The complexity of the molecular structures along with their differing flexibilities and abilities to oligomerize make necessary the use of several complementary techniques.

A. Diffraction Analysis and Microscopy

The most detailed information concerning the conformation of polypeptide hormones has been derived from X-ray analysis of hormones in the crystalline state. In this respect the pancreatic hormones have been the most amenable of all peptide hormones to study, as good crystals of many different forms have been obtained (see Table 5). This may derive from the fact that crystals are formed in the endocrine cells as storage granules (see Section IV.B), and the crystals obtained in the laboratory are a reflection of this biological process. Interest in the crystals from a pharmaceutical view has also meant that they have been well characterized; for instance, crystallization of insulin is part of the industrial purification process, and several crystalline forms are sold as slow-acting insulins for the treatment of diabetics.

1. Crystallization

Many factors complicate the crystallization of hormones, the most important of these being the presence of impurities in the form of precursors and intermediates, the relatively low solubility of the hormones, their complicated patterns of self-association, and the requirement for specific metal binding.

For insulin up to 5% of proinsulin, and intermediates with some C-peptide residues, may be incorporated into the rhombohedral 2-Zn crystals, while a few per cent of a glucagon with an extension of eight residues at the C-terminus occurs in glucagon crystals. Table 5 shows that insulins of different sequences and different chemical modifications may give rise to the same crystalline lattice, although the optimal pH for crystallization, the crystal morphology,and the crystal cell dimensions may differ slightly. However, insulins with little difference in sequence may crystallize in different forms and consequently require purification; for instance, the two polymorphic forms of rat insulin, differing at only three positions in the sequence (see Table 1), must be separated using ion exchange chromatography, and they prefer different crystal habits (see Table 5 and Figure 1).

The relatively low solubility of pancreatic hormones appears to be a result of the many hydrophobic residues on the molecular surface. As a consequence, pancreatic hormones are generally dissolved at low ionic strengths in crystallization experiments, and they are brought to a state of low supersaturation by varying the pH and temperature rather than the ionic concentration. For instance, insulin (5mg/mℓ) is dissolved in citrate buffer in the presence of zinc ions at approximately pH 7, the pH is lowered carefully until precipitation begins (about pH 6.3 for porcine insulin), the solution is dissolved by gentle warming to approximately 60° C, and the crystals form on controlled cooling to room temperature over a period of several days in a hot-box (described by Blundell and Johnson[10]). Similar techniques have been used for avian pancreatic polypeptide,[11] glucagon,[6] and proinsulin.[12] However, crystals of somatostatin suitable for X-ray analysis have not been obtained.

Of critical importance to the successful crystallization of all pancreatic hormones is control of the state of self-association; this may depend on the concentration of the protein, the pH, and the presence of specific metal ions and organic compounds. For instance, at pH 6 in the presence of zinc ions, insulin forms 2-zinc insulin hexamers in rhombo-

Table 5
CRYSTALLINE FORMS OF PANCREATIC HORMONES

Hormone	Modification species	Space group	Cell constants a	b	c	α	β	γ	Resolution	Ref.
Insulin	Porcine 2-Zn	R3	82.50	82.50	34.0	90	90	120	1.2	19, 24, 26
	Porcine 4-Zn	R3	80.7	80.7	37.6	90	90	120	1.5	23, 100
	Porcine	$P2_1$	62.3	61.8	47.8	90	90	110		14
	Porcine	$I2_13$	79	79	79	90	90	90	1.7	20
	Porcine	$P2_12_12_1$	58.2	51.9	38.5	90	90	90		210
	Bovine 2-Zn	R3	82.5	82.5	33.8	90	90	120	2.3	102
	Porcine 2-Cu	R3	82.5	82.5	34.0	90	90	120	2.8	64
	Human	R3	82.5	82.5	34.0	90	90	120		224
	Hagfish	$P4_12_12$	38.4	38.4	85.3	90	90	90	2.5	21, 22
	Cod	$P2_12_12_1$	84.6	64.5	47.8	90	90	90		210
	Rat I	R3								33
	Rat II	$P4_232$	67	67	67	90	90	90		33
	Chinchilla 4-Zn	R3	80.7	80.7	37.6	90	90	120	2.8	69
	Turkey	$P2_12_12_1$	52.96	56.54	91.85	90	90	90	3.5	8
	Desamido	R3	82.45	82.41	33.69	90	90	120	1.9	108
	A1 Glu	R3	82.55	82.55	33.78	90	90	120	1.9	108
	A1 "Thiazolidine"	R3	82.5	82.5	33.8	90	90	120	2.3	52
	A1 t-Boc	R3	82.5	82.5	33.5	90	90	120	3	52
	A1-Acety	R3	82.5	82.5	33.8	90	90	120		52
	A1, B29 Diaminosuberoyl	R3	83.2	83.2	33.9	90	90	120	3.2	158
	Despenta	C2	58.7	27.9	24.0	90	100.6	90		110
	Despenta	$P2_12_12$	70.8	62.4	57.6	90	90	90		110
	Proinsulin	$P4_12_12$	50.8	50.8	148	90	90	90	3.5	12, 114, 215
	Proinsulin	$P4_12_12$	157.8	157.8	132					114, 215
Glucagon	Porcine pH 9	$P2_13$	47.91	47.91	47.91	90	90	90	3	16
	Porcine pH 6		47.1	47.1	47.1	90	90	90	3	6
	Turkey pH 9	$P2_13$	47.7	47.7	47.7	90	90	90		8
Pancreatic polypeptide	Turkey	C2	34.18	32.91	28.45	90	105.26	90	1.4	7, 11

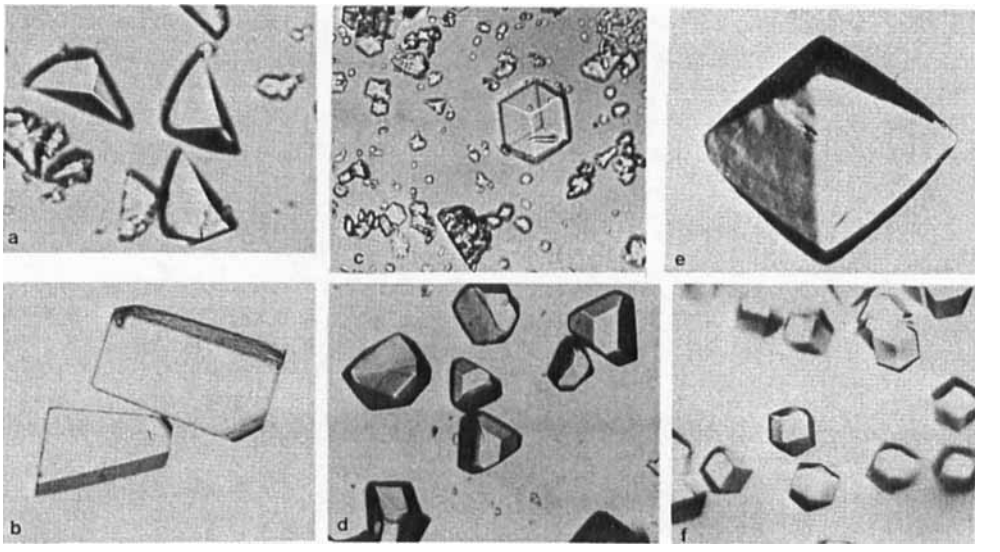


FIGURE 1. A selection of different crystal forms for PP hormones illustrating the variation in crystal morphology and symmetry from (a) avian pancreatic polypeptide (C2), (b) turkey insulin ($P2_12_12_1$) through (c) rat insulin I (R3), bovine insulin (R3) (d) to the cubic forms for rat insulin II ($P4_232$) (e) and turkey glucagon ($P2_13$) (f).

hedral (R3) crystals. The presence of a 1 *M* anion concentration (Cl^-, I^-, or SCN^-) leads to 4-zinc insulin hexamers which crystallize in a related rhombohedral form.[13] The presence of phenol or m-cresol in the solution of 2-zinc hexamers gives a monoclinic crystal form, but at the same pH the absence of zinc ions leads to a heterogeneous oligomer population and no crystals form.[13,14] At pH 2, dimers, but not higher oligomers, predominate and an orthorhombic crystal form is given[15] (see Table 5). In a similar way, glucagon crystallization gives rise to related cubic forms in the pH range 3 to 9.5 where helical trimers can form, but not at acid pH where beta-sheet fibrils exist.[6,16,17,212] Avian pancreatic polypeptide forms crystals in conditions where dimers are formed (pH 5 to 9) and in the presence of zinc ions which are responsible for cross-linking the dimers into an extended three-dimensional crystal lattice.[7,8]

2. X-ray and Neutron Diffraction

Although polypeptide hormones have been crystallized relatively easily, their structure analyses have been difficult and lengthy operations. 2-Zinc insulin crystals were first studied by X-ray techniques by Crowfoot-Hodgkin (1935)[18] although the successful structure analysis at 2.8 Å resolution came 34 years later[1] and the refinement at approximately 1.5 Å resolution is only just completed.[19] In a similar way, X-ray studies of crystalline glucagon commenced in the 1950s,[212,16] but were successfully completed only in 1975.[6] The low solvent content of the crystals and the sensitivity of the conformation of the hormones to the heavy metal ions used in the method of isomorphous replacement have led to difficulties in these X-ray studies. In fact the crystal cell dimensions of glucagon vary with the presence of different metal ions, leading to lack of isomorphism.[6]

However, once one member of a hormone family has been studied by X-rays, the conformation so determined can be used in the method of molecular replacement to determine the structure of others with homologous structures (see Reference 10 for a review). This method is the basis of the successful analyses of several crystal forms of porcine insulin and of insulins of other species such as hagfish.[20-22]

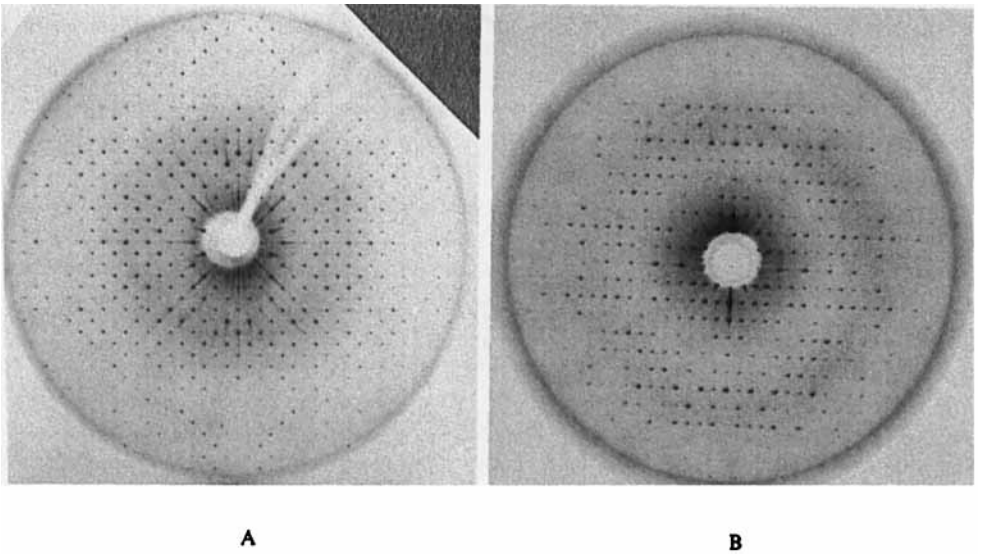


FIGURE 2. Zero layer X-ray precision photographs for (A) aPP (μ = 29°) and (B) porcine glucagon (μ = 18°) contrasting the relative order in the two crystals.

The molecular detail, to which the analysis will lead, depends on the static and thermal disorder of molecules in the crystalline lattice, as well as the size and quality of the crystals. Figure 2 shows some typical diffraction patterns. On the one hand, the analysis of avian pancreatic polypeptide (aPP) has been extended to a resolution better than 1 Å where carbon atoms are clearly resolved, hydrogens may be located, and anisotropic vibrations analyzed. The electron density map at 1.4 Å resolution (Figure 3) allows the molecular conformation to be defined with precision (0.2 Å) and the isotropic thermal parameters defined.[9] The refinement of the insulin structure by Dodson et al.[23] is at a similar resolution, and refinement of the same form but at 4°C has been reported by Sakabe et al. at 1.2 Å[24,25] resolution and at 10°C at 1.8 Å resolution by the Peking group.[26] On the other hand, glucagon molecules are flexible in the crystals limiting the formal resolution of the study to approximately 3.0 Å, although the N-terminus and some amino acid side chains are apparently more or less completely flexible even in the crystals and their position cannot be properly defined (Figure 4).

Neutron diffraction analysis of proteins provides complementary information to X-ray diffraction, distinguishing N, C, and O atoms, defining hydrogen bonds directly rather than inferring them from the location of other atoms, and providing a clearer picture of solvent structure. Very large crystals are required for these studies, and although analyses of several proteins are in progress, that for insulin has been discontinued.[27]

3. Electron Microscopy and Diffraction

Electron microscopy and diffraction have been used to study small insulin and glucagon crystals such as those which exist in vivo as intracellular storage granules. Mature granules of most mammalian insulins and glucagon from teleosts often have sharp edges and regular repeats which suggest that they are crystalline.[28] Serial sectioning and high-voltage microscopy of thick sections combined with tilting stage and electron diffraction experiments have permitted comparisons between crystal forms in vivo and those grown in vitro. For example, the teleost glucagon granules are clearly rhombic dodecahedra (see Figure 5) with cell dimensions which are consistent with some shrink-

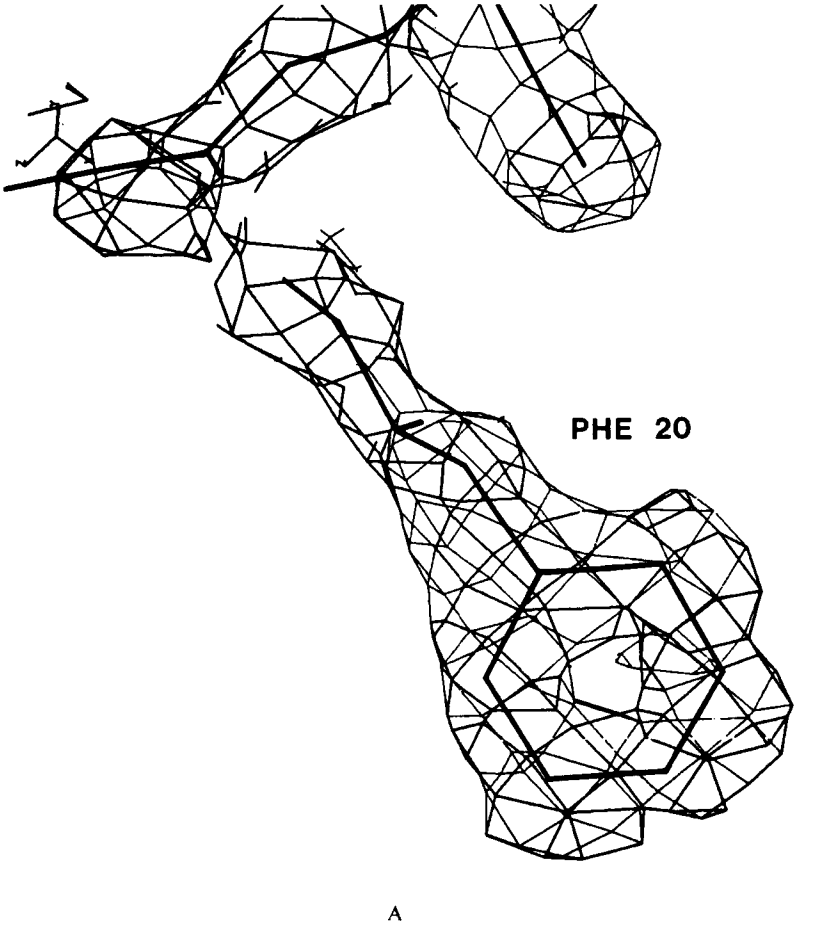


FIGURE 3. Electron density for the 1.4 Å resolution map of aPP showing the fit for residues. (A) Phe 20 (B) Asp 22—Asn 23 and compared with the 3 Å resolution map for (C) porcine glucagon.

age and distortion of those observed in the crystals.[29] However, optical transforms of the micrographs and electron diffraction of the crystals have not unequivocally identified the spacegroup as a consequence of the low resolution.[30]

For rat and mouse insulin granules, regular cross-sections have been observed,[31,32] and these have allowed the construction of tentative models for packing of zinc insulin hexamers. In this case crystals obtained in the laboratory can be used as a basis for the analysis of the granules. Purified rat insulins crystallize in two distinct space groups — rat 1 as rhombohedra and rat 2 as regular octahedra ($P4_232$, a = 67 Å).[33] The 50 Å repeats observed for hexagonal views of rat granules are compatible with the packing of zinc insulin hexamers in a rhombohedral cell — the hexamers being about 50 Å diameter — but further studies are needed for confirmation of a cubic form and to clarify whether the two insulins are packaged separately. More detailed measurements on the grass snake insulin granules have shown cubic symmetry with a = 74 Å, quite close to the values for cubic crystals $I2_13$, a = 79 Å.[34,222] However this crystal form contains dimers of insulin with no zinc. Model-building insulin hexamers in $P2_13$ with a = 67Å provide a plausible arrangement which could give rise to a 90 Å repeat, as seen in micrographs[35] (see Figure 6). Purified grass snake insulin has not been examined, but rhombic dodecahedral

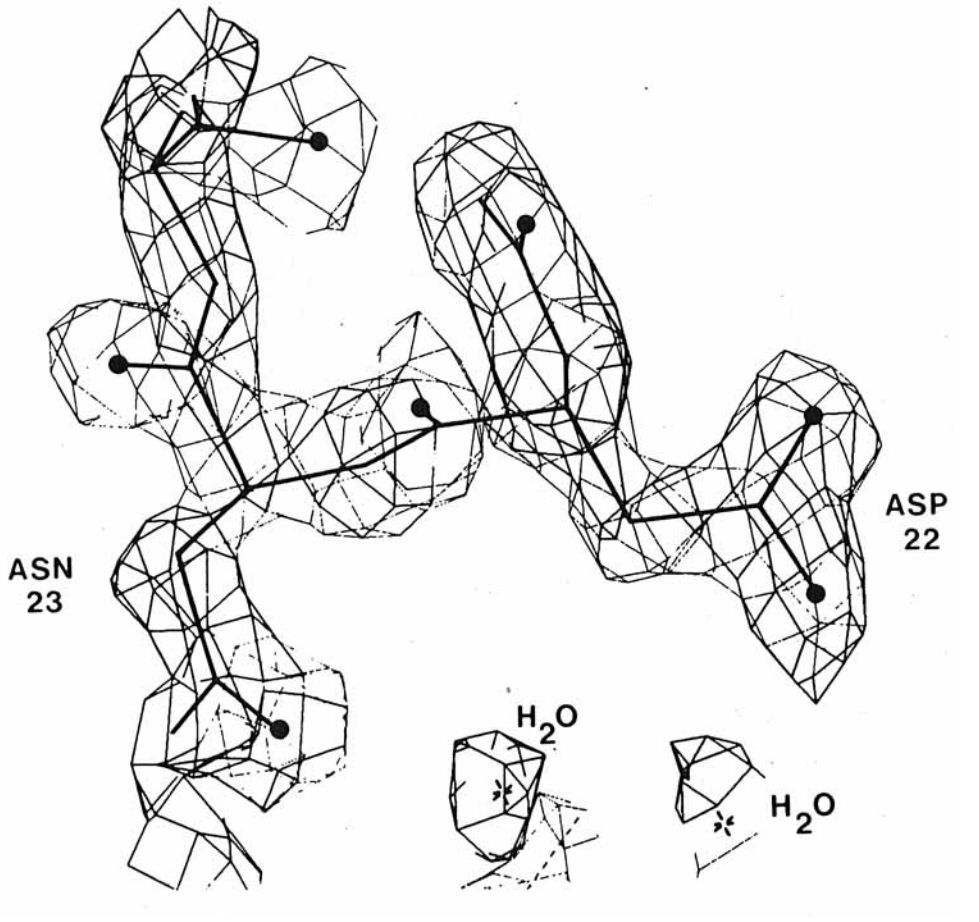


FIGURE 3B

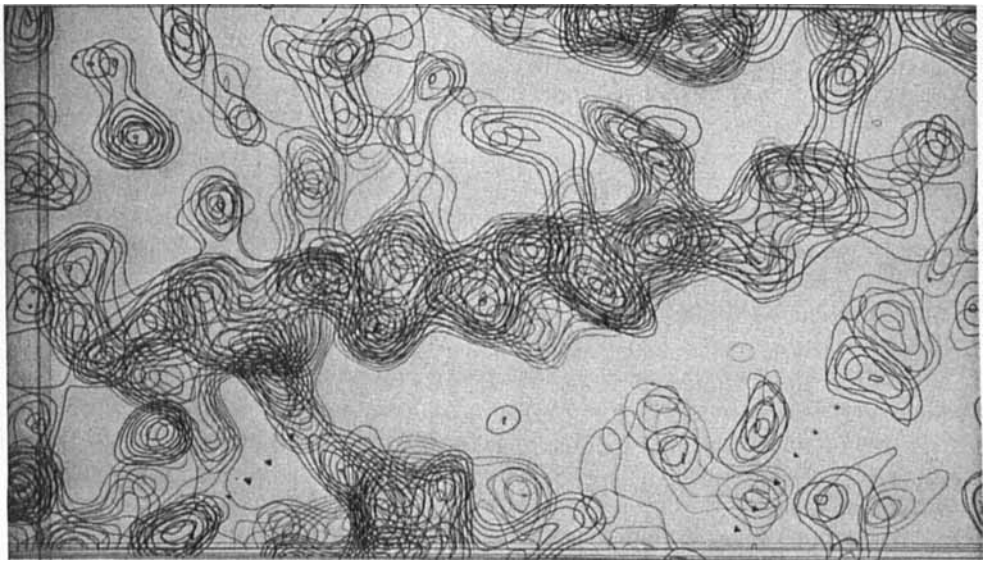


FIGURE 3C

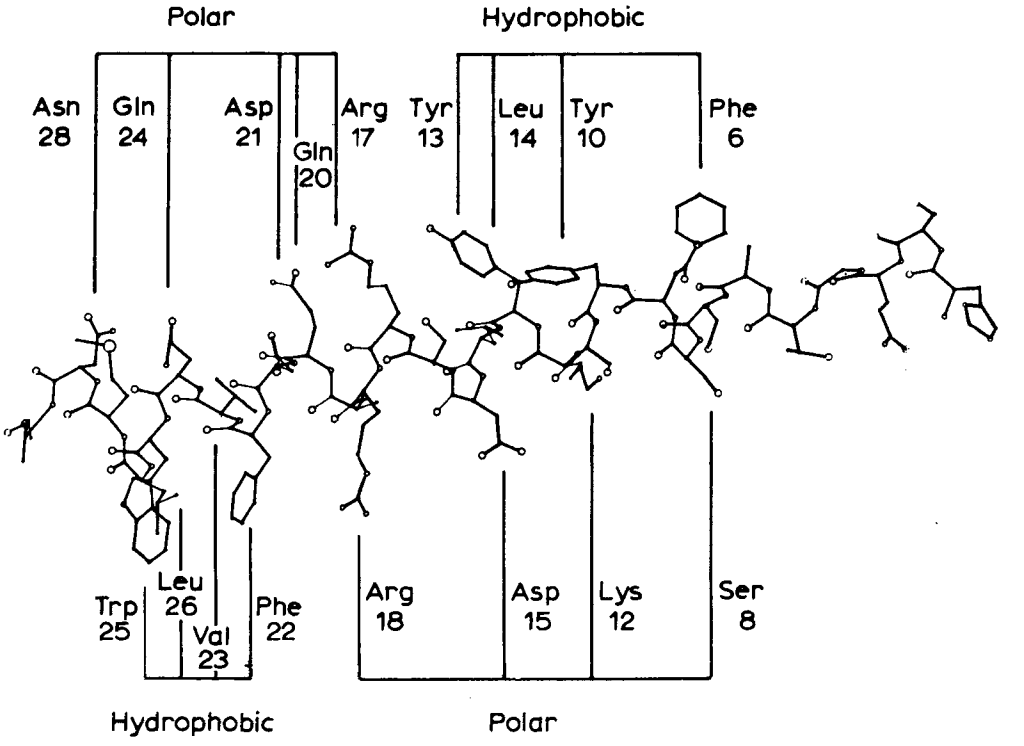


FIGURE 4. The glucagon molecule showing the distribution of polar and nonpolar amino acid side chains on the surface of the alpha-helix and the less-ordered N-terminal region.

crystals have been reported from a related snake[36] insulin and a fish insulin.[37] Crystallographic examination of these materials would be a good test of the $P2_13$ model. Although the microscopy experiments give a clear indication of the involvement of crystalline aggregates in vivo, the detail is of very low resolution.

Several authors have made attempts at electron microscopy and diffraction of small crystals in vitro for direct comparison with the granules in vivo.[38,39] However, the crystals of 2-Zn insulin crystallized at pH 6.3 have proved very difficult to image, and it appears that this is due to lack of contrast. Improved diffraction patterns can be obtained from crystals with added zinc ions, such as those used as slow-acting insulins.[39] This raises the question as to whether more metal ions are not bound to the granules in vivo, which often show well-defined repeats (see Section IV.B). Attempts to image individual molecules in the electron microscope after heavy metal coupling have not proved very convincing to the present authors.[40]

B. Spectroscopic Methods

1. Raman Spectra

The sensitivity of several amide bands to conformation makes the technique of laser Raman spectroscopy suitable for comparing conformations in proteins and for use in the determination of secondary structure. In addition, although X-ray diffraction is essentially restricted to crystals, and ORD and circular dichroism (CD) spectra to solution studies, laser Raman spectra may be obtained with equal ease in solids, solutions, and suspensions. Raman spectra have been used to study pancreatic hormone structures in the crystalline state, as powders, as fibrils, and in solution.

In the earlier work the crystalline powders of insulin were used for investigation.[41,42]

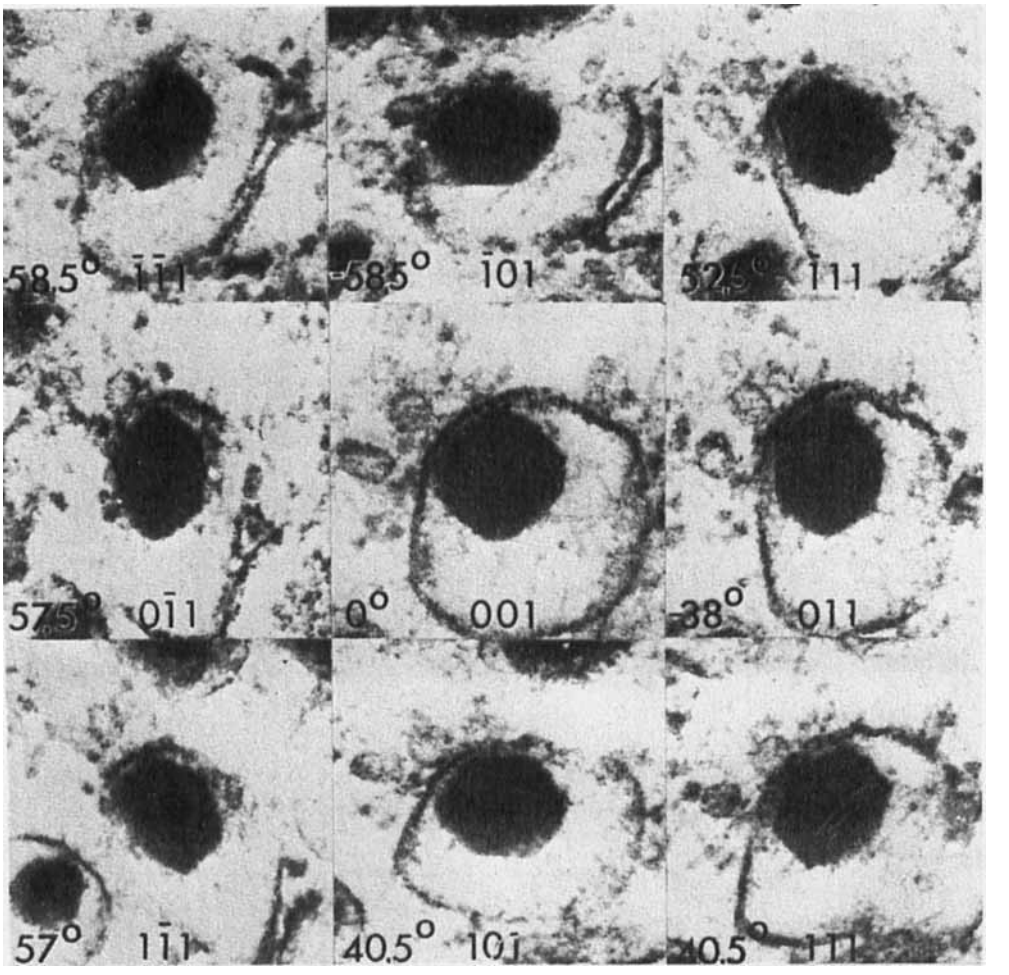


FIGURE 5. Series of tilting stage electron micrographs of rhombic dodecahedral A-cell granules from the teleost *Xiphorus helleri*. (From Lange, R. H. and Klein, C., *Cell Tiss. Res.*, 148, 561, 1974. With permission.)

However, it became evident that Raman spectra obtained under such conditions were not a true reflection of the crystalline state. Loss of order occurred in the protein crystals on air drying and due to the laser beam heating the powder surface, especially when the humidity was not controlled. The subsequent work of Yu et al.[43] on large transparent crystals of rhombohedral 2-Zn insulin took advantage of the absence of Tyndall scattering and absorption, and the crystals were kept completely immersed in the mother liquor. By comparing the spectra in H_2O and D_2O the effects of side group vibrations were eliminated and the contributions of the main chain amides isolated. Estimates of the contributions to the spectra from alpha-helix, beta-sheet, and random coil (no H-bonds) from other proteins of known structures allowed the secondary structure of insulin to be determined and shown to be consistent with that found in the crystals by X-ray analysis.[44] Comparisons with the solution structure indicated a similar conformation at pH 7, where zinc-insulin hexamers exist, but a decrease of alpha-helix of 10 to 17% at pH 2.1. However, these conformational changes may not be real, as the spectral changes may result largely from the dissociation of hexamers and even dimers at the lower pH, leading to increased solvent accessibility of the alpha-helices. Raman spectra have been used to

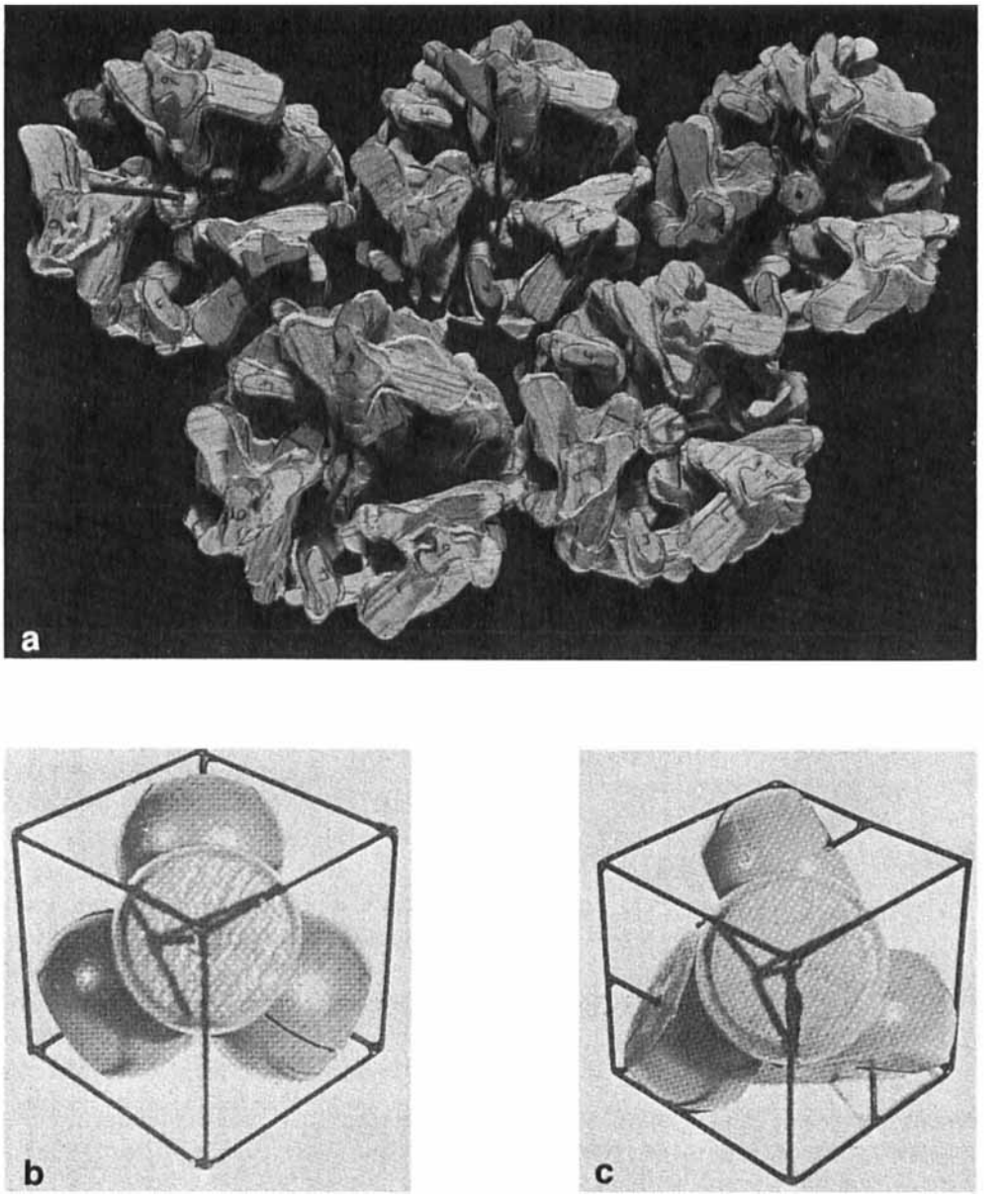


FIGURE 6. Packing of insulin hexamers in various space groups. The arrangements in (a) and (b) correspond to those found in rhombohedral (R3) crystals and cubic ($P4_232$) crystals of rat insulin I and II, respectively. The arrangement in (c) is for space group $P2_13$ and may correspond to the forms found in rhombic dodecahedral insulin granules, such as those of grass snake.

show the similarity of lyophilized insulin and proinsulin[41] and to monitor the formation of beta-sheet-containing fibrils from both insulin and glucagon in very acid solutions.[43,45]

For the smaller somatostatin molecule, laser Raman spectra have been used to estimate disulfide bond torsion angles.[46] However, the high peptide concentrations employed may lead to intermolecular interactions, which stabilize a particular conformation.

2. Nuclear Magnetic and Electron Spin Resonance

The advent of high-resolution proton nuclear magnetic resonance (NMR) spectrometers at 270 MHz allows conformational studies of insulin and glucagon at physiologically meaningful pH and concentration. Because of the low solubility of insulin at neutral pH, early proton NMR studies were carried out in solvents and under conditions where one would not expect the hormone to exist in its native state. For example, Kowalsky[47] reported 56-MHz spectra at pH 2 and 10 by using 10 and 20% aqueous solutions, while others measured spectra of insulin dissolved in trifluoroacetic acid at 56 and 220 MHz.[48] The more recent experiments of Williamson and Williams[49] have more successfully studied the transformations between different conformations in solution under conditions as near to physiological as possible. After demonstrating that the spectra are essentially the same at pH 9 for 2-zinc hexamers as at pH 8.0 where the insulin is less soluble and the spectra take longer to record, Williamson and Williams[49] were able to assign the key reference resonances due to the methyl groups of isoleucines, valines, and leucines and the aromatic protons of the histidines, phenylalanines, and tyrosines. This enabled them to follow conformational changes on the assembly of 2-zinc insulin hexamers and 4-zinc insulin hexamers and even predict a 6-zinc hexamer which has yet to be identified crystallographically. Bradbury and Brown[57] have also followed conformational changes on self-association in solution. All these workers have relied strongly on the crystal structure for interpreting the NMR spectra and have shown that the crystal structure must be largely retained in solution.

^{19}F and ^{13}C NMR measurements have been reported for insulin derivatives in which the amino groups have been trifluoroacetylated or carbamylated with ^{13}C-enriched potassium cyanate.[50,51] The observation of two closely spaced carbamyl glycine lines in the latter experiments where insulin dimers were the dominant species in aqueous solution have been held to reflect the imperfect twofold axis found in crystals (see Section III.B.1). However, the insulin molecule is known to be very sensitive to modification at the A1 glycine residue with respect to structure and aggregation, especially when this involves a change of charge.[52] This may lead to a mixed population of conformers, including monomers and dimers with slightly different structures.

The high-resolution NMR studies of glucagon by Wüthrich and his colleagues[53] and by Karplus et al.[138] have produced evidence concerning the conformation in solution which is complementary to that obtained in the crystals by X-ray analysis. For instance, Wüthrich et al.[53] have shown that although glucagon comprises a largely flexible conformation in dilute aqueous solutions, there is a local nonrandom spatial structure involving the fragment Phe 22-Val 23-Gln 24-Trp 25.[53] Of great significance for future developments is the detailed study by Wüthrich and co-workers of glucagon-bound lipid micelles using nuclear Overhauser enhancement (NOE) difference spectra.

Using per-deuterated lipid micelles containing 1 glucagon to approximately 40 lipids it was found that highly selective truncated nuclear Overhauser enhancement (TOE) difference spectra could be obtained with preirradiation times of one to several seconds due to the limited extent of proton-proton spin diffusion.[54-56] Distance geometry algorithms determine the bounds of conformational space occupied by the polypeptide. Theoretical considerations indicate that for a flexible peptide, NOE will be observed if two atoms have nuclei separated by a mean distance of less than 5 Å. Analysis of the spectra gives rise to a series of conformations which are consistent with the distance restraints defined by the TOE. These conformations are then tested to check that they do not imply TOE which is not observed. The results indicate that although the glucagon molecule adopts a conformation which is amphipathic, the side chains are not in precisely the same geometrical arrangement reported for the X-ray analysis. Whether this precludes a mainchain alpha-helical conformation depends critically on the estimates of errors in both the NMR and the X-ray models, which are difficult to quantify when there

are high thermal vibrations. Nevertheless, this method gives interatomic distances which limit the conformations which may exist in solution, and it should have important applications for studying the hormones bound to their receptors.

NMR experiments have been performed on fragments, analogues, and whole somatostatin.[58-62] Such studies have been hindered by severe line-broadening, aggregation, and signal assignment problems, and it is clear that a complete assignment of observed resonances to specific parts of the 14 amino acid molecule is still not a trivial process.

Electron spin resonance (ESR) studies have been of more limited use but have found application in the study of insulin hexamers with paramagnetic transition metal cations instead of Zn^{2+}. The earlier studies of Brill and Venable[63] on rhombohedral crystals containing Cu^{2+} gave hyperfine splitting, line-broadening, and g-values consistent with an octahedral complex with a tetragonal distortion, similar to the sixfold coordination observed for zinc ions. These results have been recently confirmed by X-ray studies of the 2-Cu insulin crystals.[64] Evidence for further binding sites of copper ions is given by ESR studies on lyophilized native and cross-linked insulins.[65]

3. Circular Dichroism

This technique has been used extensively in the study of all four pancreatic hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. Although the spectra are complex superpositions of many rather wide bands allowing estimation of only gross features of secondary structure, the ability of the technique to detect small changes has been most useful in comparing crystal structures with those in solution.

Spectra for insulin were recorded when instruments first became available,[66] and since then most insulin analogues and related molecules have been investigated. Interpretation of the spectra was complicated by the self-association of insulin and the effects this had on the spectra.[67] The insulin dimerization mechanism involves the formation of a beta-pleated sheet between molecules and restriction of the motion of several aromatic amino acids. Figure 7 shows how the spectra change as the aggregation state develops from a population containing both monomers and dimers to one comprised mainly of zinc insulin hexamers.

Estimation of the solution conformation of insulin in the monomeric state by CD has presented serious technical problems. Pocker and Biswas[225] have recently recorded CD spectra at very low insulin concentrations where a high monomer population is expected using a relatively new instrument design and a modified recording strategy. Subsequently,[226] spectra for monomeric and dimeric populations were calculated. These spectra are held to indicate a considerable loss of protein secondary structure on disruption of the insulin dimer. However, it is known that some monomeric insulins (e.g., casiragua, see Figure 15) have a rather similar CD to more concentrated bovine insulin. It is therefore important to obtain confirmation of the reliability of the spectra measured at 60 n*M* concentrations and proof that the estimates of the hormone concentration are not disturbed by absorption of the hydrophobic monomer onto the container.

CD studies have been most important in the study of insulin analogues with altered biological activity since chemical modifications or sequence changes distant from a receptor binding region could give rise to the reduced activity by altering the three-dimensional structure.[68,69] However in view of the dependence of the spectra on the level of self-association, comparisons of solution structures of insulins using CD should be carried out at equivalent levels of aggregation and not merely at the same concentration.[69]

The success of more quantitative approaches to the interpretation of CD spectra has been varied. Determination of elements of secondary structure by curve fitting combinations of model curves for helix, sheet, coil, and beta-bend to the observed spectra have

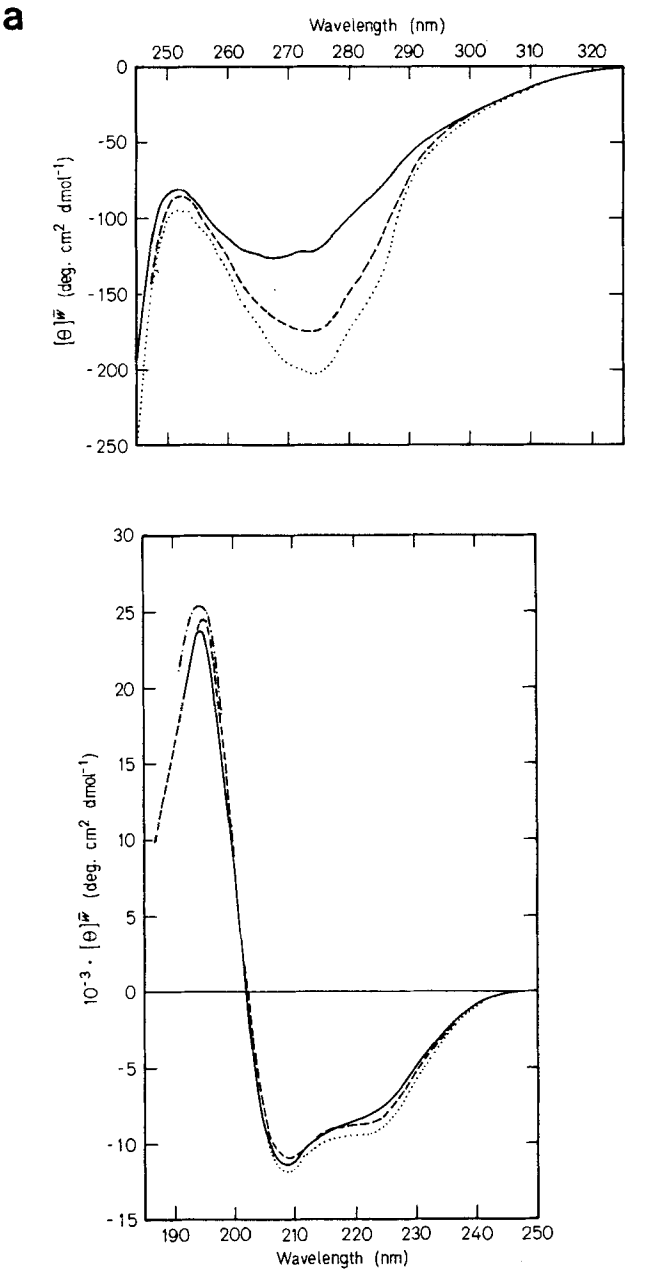


FIGURE 7. (a) CD spectrum of bovine insulin as a function protein concentration at pH 7.8 (—3.8 μM, - - - - *38* μM, - · - · 70 μM, ... *380* μM). At the lowest concentration there is a significant monomer population. (b) CD spectrum of bovine insulin as a function of zinc concentration at pH 7.8 (—0, - - - - 0.33, ... 0.5 Zn per insulin monomer). Insulin concentration 380 μM above and 76 μM below 200 nm. At the highest protein and zinc concentration — zinc insulin hexamers will predominate. (From Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R., and Neville, R. W. J., *Eur. J. Biochem.*, 55, 531, 1975. With permission.)

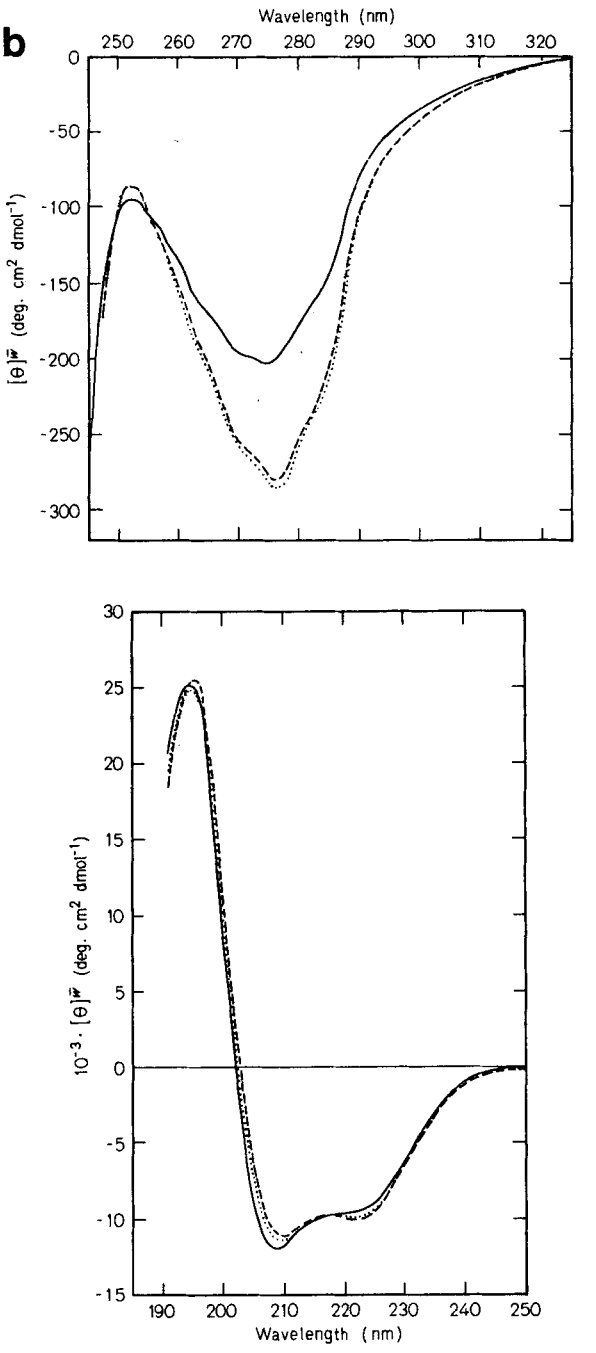
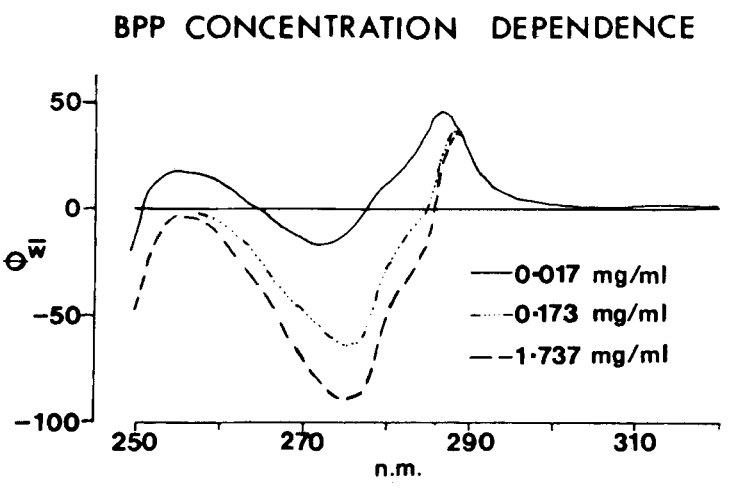


FIGURE 7B

not proved very reliable.[70] Model curves are derived either from the CD of proteins with known crystal structures or from the spectra of polylysine in defined conditions. The poor fit may be related to aromatic transitions in the far ultraviolet (UV). Analysis of near UV spectra, however, has been more successful. Strickland and Mercola[71] and more recently Wollmer and co-workers[72,73] have attempted to calculate the tyrosyl CD using theoretical approximations and the crystal atomic coordinates to compare this with the



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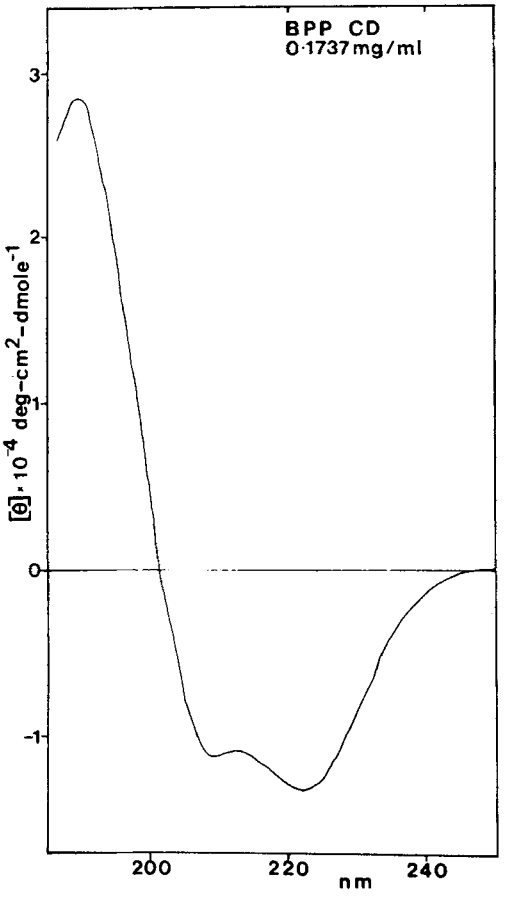


B

FIGURE 8. (A) The near UV CD of bPP at pH 8 as a function of polypeptide concentration indicating the involvement of tyrosine(s) in the association to dimers. (B) The far UV CD of bPP at pH 8 indicating a substantial helix contribution.

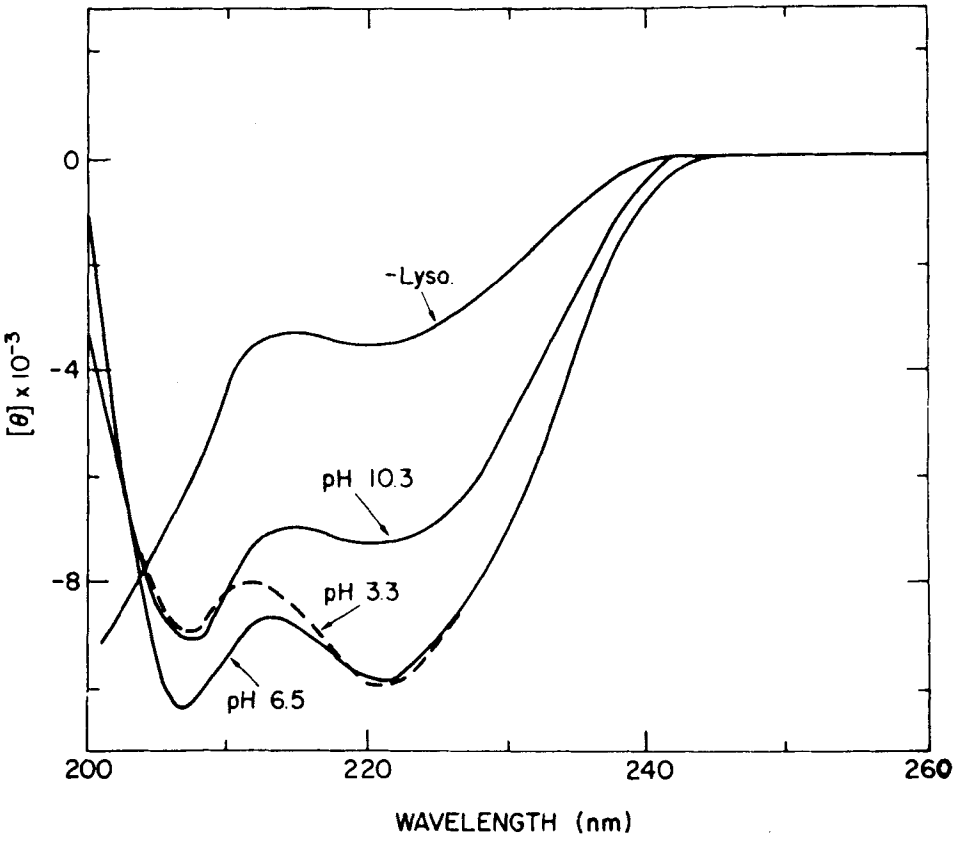


FIGURE 9. Effect of lysolecithin (193 μM) on the CD spectra of glucagon (7.2 μM in 0.002 M sodium phosphate) at three different pH values. No lysolecithin — LYSO. (From Schneider, A. B. and Edelhoch, H., *J. Biol. Chem.*, 247, 4986, 1972. With permission.)

observed spectra. The calculations generated the correct sign for the 275-nm band and provided a fairly good estimate of the magnitude in different association states. However, the rotational strength for the hexamer was dominated by a term from the interaction of B1 Phe and A14 Tyr and yet the spectrum of Des-Phe-B1 insulin was close to that of porcine insulin. Introduction of disorder terms or omission of these residues led to a close match of calculated spectra for the two insulins which was consistent with the observed disorder of A14 tyrosine in Des-Phe-B1 insulin crystals by X-ray analysis.[73] The method may also be applied to distinguishing which, of the two conformers observed crystallographically, is assumed by the monomer, but in this respect the dilute aqueous solutions required to obtain complete dissociation give rise to technical problems for the measurement of the CD spectra.

The far UV CD spectra of avian and bovine pancreatic polypeptides (aPP and bPP) indicate a considerable amount of helical secondary structure even at high dilution[8] (see Figure 8). However, as the association constant of aPP to dimers is high at neutral pH, it is difficult to measure the CD of solutions which correspond to monomers alone.[74-76] For bPP, the near UV CD shows a concentration dependence corresponding to the lower constant for self-association indicating the involvement of tyrosyl residues in dimer formation.[74] However, there is little change in the far UV which is consistent with a retention of the secondary structure in the monomer. Similar conclusions are derived from the conservation of the far UV spectrum of aPP in mildly acid conditions where the dimer is destabilized.[76] Further experiments are in progress to define the conforma-

tion of aPP at high dilutions and at acid pH, and the contributions of the tyrosyl residues to the near UV CD are being calculated on the basis of the structure determined by X-ray analysis. The bPP molecule is an especially good candidate for these calculations since there are no disulfides or phenylalanines.

In contrast to insulin and pancreatic polypeptide, the CD of glucagon has a very strong dependence on both concentration and environment in the near and far UV regions, indicating a large change of conformation on self-association or interaction with micelles[77-79] (see Figure 9). Glucagon CD spectra have been measured only at high and low pH due to the limited solubility in the neutral region. At pH 10 with low glucagon concentrations there is an indication of a small amount of secondary structure, which some have suggested is alpha-helix, but a more alpha-helical conformation is induced by concentrating to 3 mg/mℓ. The conformational change accompanies an association of molecules probably to trimers and possibly higher oligomers. This association is accompanied by changes in ellipticity and wavelength in the CD arising from the tyrosine and tryptophan chromophores. Perturbations in this region are also observed by optically detected magnetic resonance studies.[220] Modifications of the glucagon molecule to introduce a positive charge on Met27 inhibits self-association and dramatically increases the solubility at neutral pH. This facilitates spectroscopic analysis of the glucagon monomer, but the conformational consequences of the modification complicate the interpretation.[135] Although tentative models for somatostatin have been based on CD, there is disagreement on the interpretation.[80,81] Both flexible nonordered conformations, equilibrium populations of distinct conformers, and even a "hairpin" loop structure with antiparallel beta-sheet have been suggested.

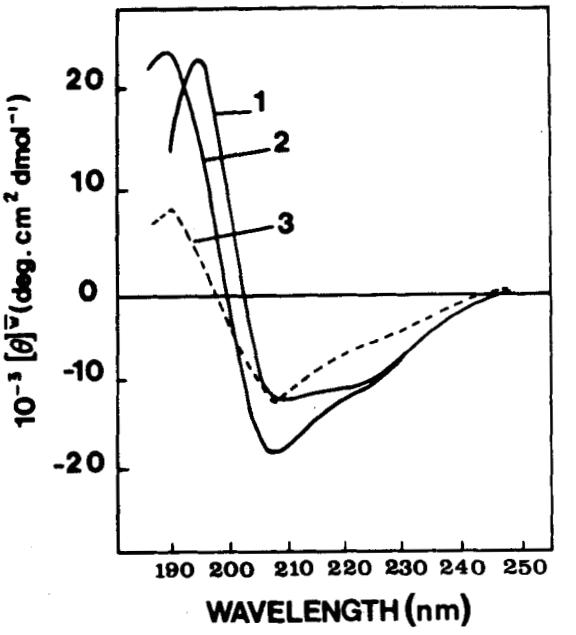
In spite of the rather general nature of comments that can be made about the molecular structure from CD, it continues to be an important complementary technique to X-ray analysis and other methods. For instance the generation of an insulin-like CD for relaxin (Figure 10) provides important confirmation for model building by insulin homology.[82] Recent advances with vacuum far UV CD may provide more reliable spectra for curve-fitting estimates of the conformation in solution.[83]

C. Immunological Techniques

Use of radioimmunoassay and immune hemolysis assays by Arquilla and co-workers[84] on a limited number of insulin analogues enabled them to define certain spatial relationships between antigenic determinants on the insulin molecule. The conclusions of these experiments regarding the folding of insulin using antisera from various strains of guinea pigs were remarkably close to those of the crystal structure analysis. The experiments highlighted the importance of tertiary structure to the integrity of determinants and explained the poor cross reactivity between antisera to isolated chains and fragments and the whole molecule.

Further studies with antisera directed towards the regions of A19 Tyr and A14 Tyr, coupled with observations on the ability of proinsulin to dimerize and hexamerize, led to the prediction that the connecting peptide of proinsulin must lie over the surface of the insulin molecule not involved in association, obscuring determinants in the region of the tyrosine residues. The connecting peptides of proinsulins vary in length and antisera have been prepared which show little cross reaction between human, beef, and pig proinsulins.[85] Presumably the variable residues provide a surface which varies from species to species, while the conserved regions make rather specific contact with the insulin surface.

Immunological experiments on glucagon and other peptides are carried out at high hormone dilutions where glucagon has little structure, and thus although N-terminal and C-terminal directed antiglucagon sera have been described, little structural information has been obtained.[8,86,87] Although pancreatic polypeptide is small, it seems to



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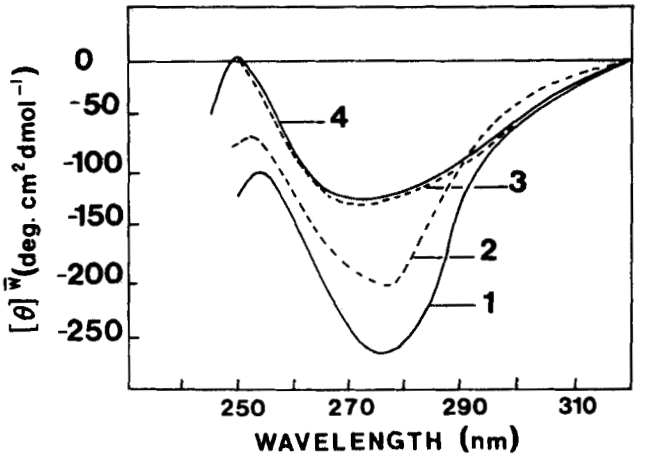


B

FIGURE 10. (A) Comparison of the far UV CD spectra at pH 7.2 for (1) zinc-free porcine insulin (50 μM), (2) porcine relaxin, and (3) guinea pig insulin (40 μM). (B) Near UV CD spectra of (1) insulin (50 μM) with zinc (25 μM) and (2) without zinc compared to relaxin (40 μM) with (3) and without (4) zinc. (From Schwabe, C. and Harman, S. J., *Biochem. Biophys. Res. Commun.*, 84, 374, 1978. With permission.)

have a well-defined structure in solution, and this is also implicated by immunological experiments using antisera to various fragments of the molecule.[88] The fragments are obtained from CNBr degradation and enzymatic digests.

D. Prediction and Model Building

1. Statistical Methods

As our knowledge of the general principles involved in stabilizing polypeptide structures increases, more efforts are being made to apply this knowledge directly to the prediction of structure. The more recent complex computer-based methods have tended to be used for a small number of test cases, but a selection of rather simpler methods have been widely applied.

Chou and Fasman,[89,211] for instance, have used their techniques of sequence-based prediction to make a conformational analysis of glucagon. They conclude that alpha-helix and beta-sheet have rather similar probabilities. This is consistent with the existence of beta sheet fibrils in acid conditions and alpha-helix in trimers, higher oligomers, and crystals at neutral pH.[78] However, as Schiffer and Edmundson[90] and more recently Lim[91] and Ptitsyn[92] have pointed out, helices normally occur on the outside of globular proteins; unlike beta-sheet they are rarely buried in the hydrophobic core. As a consequence, one side of the helix is generally hydrophobic and the other hydrophilic. This may alternatively be expressed in terms of the occurrence of hydrophobic residues at positions n, n + 1, and/or n + 3, and/or n + 4. This stereochemical feature of the alpha-helix is independent of the helix-forming potential of most individual amino acids (as derived by statistical analysis of known protein structures), and its existence has been used to further assess the probability of helix formation of polypeptide sequences. There are two regions of the glucagon helix which have this arrangement of hydrophobic residues, and this increases the probability of helix formation, especially in a nonpolar environment. The C-terminal portion of the pancreatic polypeptide sequence shows a very marked distribution of polar and nonpolar residues, and its likelihood of being helical was noted early on (Figure 11).[11] Within the helices of glucagon and pancreatic polypeptide there is a region of low helix probability, but the high potential for helix on either side ensures the continuity of the structure in the crystal. These regions however may be of significance in defining boundaries to folding intermediates in solution. The unusual distribution of proline residues in the N-terminal region of pancreatic polypeptide was noted but only to the extent that alpha-helical or beta-sheet structures were unlikely. In retrospect the prediction of a polyproline-like conformation was possible.

Analysis of sequences of C-peptides from proinsulins using the Chou and Fasman procedure indicates two regions of high helix potential at the N- and C-termini and a likely beta-bend in the middle.[93,94] These indications have prompted spectroscopic examination of C-peptide fragments and model building of the structural elements predicted to the insulin molecule using X-ray, CD, and immunological data as a guide.

The Chou and Fasman empirical prediction methods indicate that residues 6-12 of somatostatin are in beta-sheet,[80] but a method based on globular protein structures where long-range interactions with respect to sequence are likely to be dominant must be considered cautiously with such a small polypeptide containing a disulfide bridge.

2. Model Building Homologous Polypeptides

The structures of families of related proteins are of particular interest in studies of peptide hormone structure and function. In the absence of detailed structural information from X-ray analysis for each member of the family, one known member can be used to model the others on the basis of their homology.[5,95] Such an approach assumes that the tertiary structure is more conserved than the sequence and has been used to predict the tertiary structure of several insulin-like proteins, including hystricomorph insulins, proinsulin, growth factors, and relaxin.

The main chain conformation is first constructed with the torsion angles found in the polypeptide studies by X-ray analysis, i.e., porcine insulin. For insulin-like molecules this

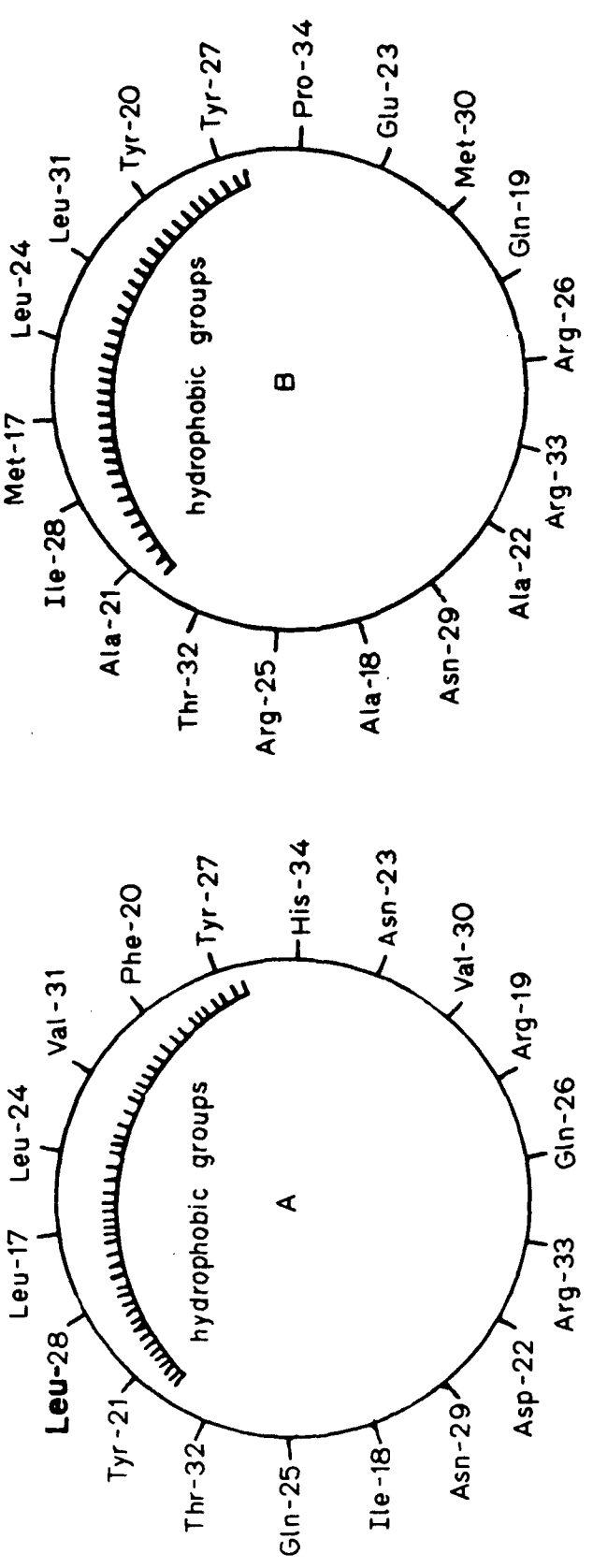


FIGURE 11. Residues 17—34 of (A) avian and (B) bovine pancreatic polypeptides represented as a Schiffer-Edmundson wheel showing a clear hydrophobic face of a proposed helical region. (From Wood, S. P., Pitts, J.E., Blundell, T. L., Tickle, I. J., and Jenkins, J. A., *Eur. J. Biochem.*, 78, 119, 1977. With permission.)

assumes the absence of insertions and deletions in the regions homologous to A1—A21 and B4—B25 which would disrupt the globular structure and disulfide bridge formation. It also implies the conservation of the critical glycines at B8, B20, and B23 which allow conformational angles not accessible to amino acids with side chains, although amino acid changes at B20 can be accommodated by making small changes in the torsion angles of B20, B21, and B22. Secondly, the core must be hydrophobic with residues which are identical or occupy the same volume. In all insulins and insulin-like growth factors (IGF), the hydrophobic core is in fact identical.[5] In relaxin there are many differences in amino acids, but these are retained as hydrophobic and may still be accommodated in the same volume.[4] For nerve growth factor (NGF), on the other hand, there are deletions in the main chain and the core has a different volume, casting doubt on the homology of this molecule with insulin.

The surface residue side chains and the chain termini are added in a way which conserves the three-dimensional structural homology but optimizes H-bond and ion-pair formation. Finally, where there are chain extensions such as the C-peptide of proinsulin or IGFs, the secondary structure is first predicted using the statistical methods described in Section II.D.1 and the chain is then added to the model in a way which optimizes hydrophobic and polar interactions.

Traditionally these model-building studies have been approached by the use of plastic or wire models. More recently, however, advances in computer technology have revolutionized such schemes. The variety of computer programs available, together with the rapid development of new and more powerful computer graphics hardware systems (such as the Evans and Sutherland Picture System) have brought model building into the computer era.[96,97] Continuous updating of coordinate sets during building, facilities for structure regularization and energy minimization, and the ease of preparation of stereo diagrams permits precise documentation of the building schedule and aids communication of the conclusions.

The test of these model-building exercises must be that they predict physicochemical and biological properties which can be tested. Thus model building of casiragua insulin[98,99] and relaxin[4,100] predicts insulin-like conformations which are consistent with the insulin-like far UV CD (see Figures 10 and 15). The model building also correctly indicates that many hystricomorph insulins will not form dimers or hexamers. Finally, for the IGFs, the relative ability to bind insulin receptors is rather well predicted (see Section IV). Model building of homologous polypeptide structures is thus shown to be useful and will no doubt play an important role in structure prediction where hormones are purified in small amounts or are not crystallizable.

III. CONFORMATION

In this section we discuss the conformation of the four polypeptide hormones. We first describe the structures of monomers which are globular for insulin, IGFs, and pancreatic polypeptide, but lacking in extensively ordered secondary or tertiary structure for glucagon and somatostatin. We then discuss the self-association which occurs through largely hydrophobic interactions in insulin, pancreatic polypeptide, and glucagon and the ordered structure which is induced progressively in glucagon on self-association. In all cases we note that the conformation and the degree of self-association depend critically on the nature of the solvent (aqueous or nonaqueous), the pH, and the presence of metal cations.

A. Monomers

1. Insulin

The first detailed information on the conformation of insulin came from the X-ray

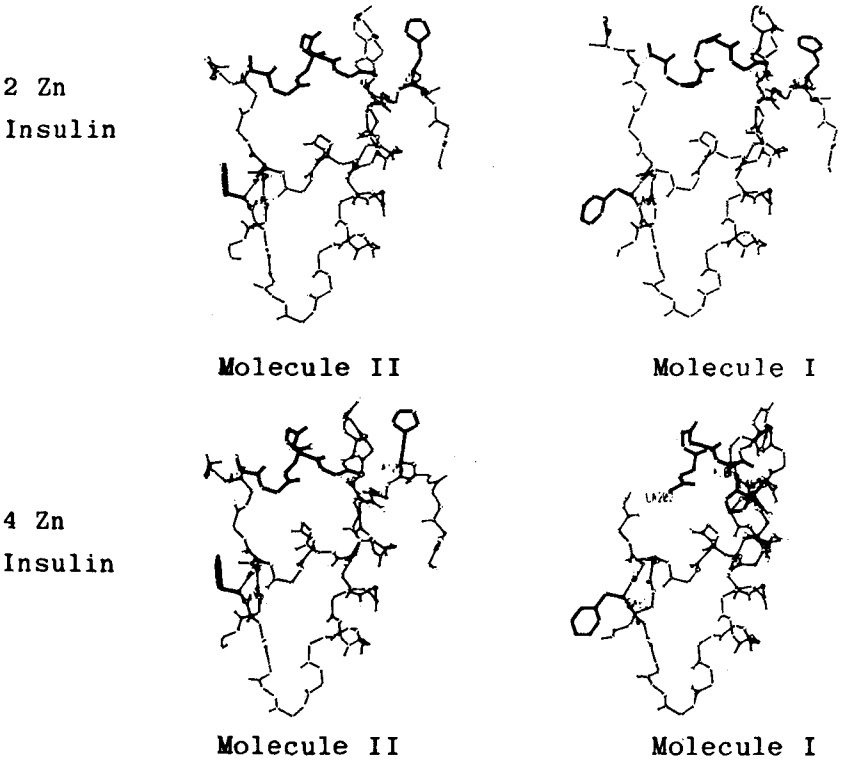


FIGURE 12. Peptide backbone conformation for molecules I and II of 2-Zn insulin and 4-Zn insulin. Both sets of diagrams are derived from the refined structures and the side chains of B5 His and B25 Phe are included to demonstrate the noncrystallographic symmetry. (From Dodson, E. J., Dodson, G. G., Reynolds, C. D., and Vallely, D. G., in *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones,* Brandenburg, D. and Wollmer, A., Eds., Walter de Gruyter, Berlin, 1980, 9. With permission.)

analysis of porcine 2-zinc insulin crystals by Hodgkin and co-workers.[1,2] In this form insulin is organized as hexamers assembled from three equivalent dimers. There are two crystallographically independent protomers, known as molecules I and II, in each dimer (note that the Peking insulin group[26] defines the molecules I and II as 2 and 1). Their conformations, which are similar but not identical, are shown in Figure 12 and involve right-handed helical segments A2—A8, A13—A19, and B9—B19. The glycines at B8, B20, and B23 allow the B-chain to fold into a more compact shape, and with the A-chain a globular molecule with a hydrophobic core is formed.

Several independent X-ray analyses of porcine 2-zinc insulin have now been completed and refined to high resolution.[19,24,26] The structures of porcine 4-zinc insulin hexamers,[23,101] porcine insulin dimers in cubic crystals,[20] and hagfish insulin dimers[21,22] (see Figures 12 and 13) have also been determined by high-resolution X-ray analysis. These show that one conformation, that of molecule II of 2-zinc insulin, occurs in all crystal structures. However in the 2-zinc and 4-zinc forms, the second crystallographically independent protomer, molecule I, is different in conformation. In 2-zinc insulin hexamers these differences presumably occur as a result of the crystal packing, and include a rotation of the helical segment A1—A5 by about 40° (see Figure 14), small relative shifts of residues A6—A9 and B1—B8, different conformations of the sidechains

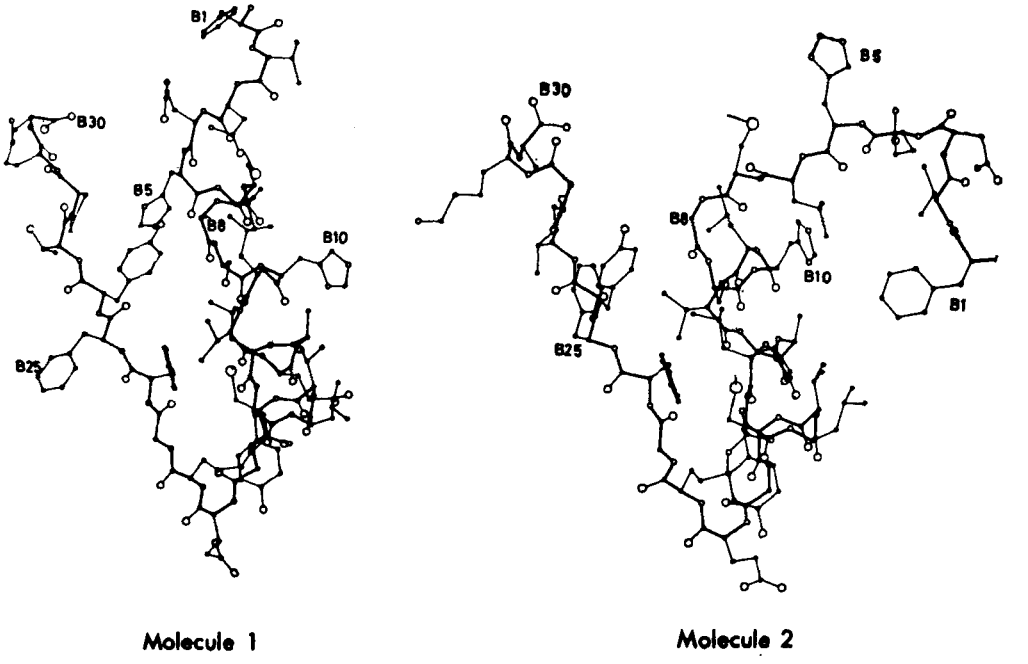


FIGURE 13. Structure of the B-chain for molecules I and II in 4-Zn insulin hexamers showing the extended B-chain helix in molecule I. (From Bentley, G. A., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Mercola, D. A., *Nature*, 261, 166, 1976. With permission.)

of His B5 and Phe B25, and a large shift of B29 and B30, which in any case are fairly flexible in the crystals. In 4-zinc hexamers the changes are larger, are induced by the presence of high chloride or other anion concentrations, and involve a rearrangement of residues B1—B8 to form an extension of the helix B9—B20 (Figure 13). There is evidence that the 4-zinc conformation also occurs in solution.[49,102,103] These observations show that, while insulin has a preferred tertiary fold, conformational changes can easily be induced.

The observations that the conformation of molecule II is observed in all crystal forms, and in particular in the cubic crystals of the dimer, indicates that it is likely to be the conformation of the dimer found in solution, but it is still possible that further conformational changes occur on dissociation to the monomer. The spectra in the tyrosyl region of the near UV CD are consistent with calculated spectra in which the aromatic groups of B1 Phe, A14 Tyr are completely disordered and the conformational flexibilities of B16 Tyr, B24 Phe, and B26 Tyr are increased compared to the crystal form. The far UV spectra reported by most workers have been thought to show only small differences on dissociation of hexamers to monomers (partly explained by the loss of beta-sheet within dimers), and so the spectra are consistent with a monomeric conformation similar to that in hexamers.[68,69] However, if the results of Pocker and Biswas[225,226] are correct (see Section II.A.), there may be much more loosening of the alpha-helices in the monomer.

X-ray and CD experiments are consistent with the existence of a similar conformation of insulins of most species including cow, human, turkey, and hagfish (although no CD spectrum has been published for the latter). However, the hystricomorph (a suborder of rodents related to the porcupine — hystrix — and guinea pig, defined anatomically by horizontal action of the jaw in mastication) insulins, with their very different sequences, provide some exceptions. Although chinchilla (only 4-Zn hexamers)[69] and casiragua (only monomers)[48,99] insulins self-associate differently from porcine insulin, CD and

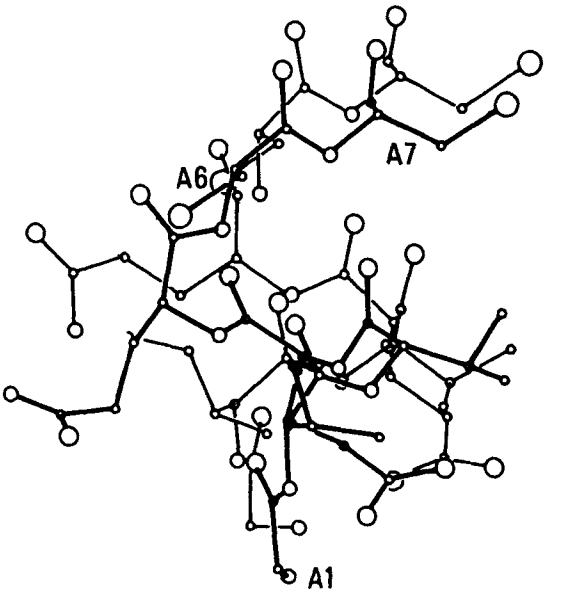


FIGURE 14. Comparison of molecules I (thin lines) and II (thick lines) in the region A1—A7 viewed down the threefold axis for porcine 2-Zn insulin. The orientation of the molecules was optimized on the main chain structures A10—A20 and B9—B19. The differences at the N-terminus were attributed to a 40° rotation about the bond between A5 and A6. (From Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Reynolds, C. D., *Can. J. Biochem.*, 57, 469, 1979. With permission.)

computer graphics model building[99] indicate that they may assume monomeric conformations very similar, if not identical, to that of porcine insulin. Figure 25 shows a model of casiragua insulin[99] in which the main chain conformation is made identical to that of porcine insulin with the exception of residues B20—B22, where B20 lysine is substituted for glycine. However, two other hystricomorph insulins, those of guinea pig and porcupine, present greater difficulties.[69,104] An insulin fold brings B22 Glu in close proximity to the terminal carboxylate at A21, which also carries a negative charge at neutral pH. These two charges would give rise to a repulsion which destabilizes the insulin fold. It is, therefore, not surprising that the CD spectra of these two insulins (Figure 15) indicate that they have rather different conformations from that of porcine insulin. However, the CD spectra of both guinea pig and porcupine insulin become more porcine insulin-like at low pH, indicating that neutralization of the negative charge allows these insulins also to assume the "insulin fold".[103,104]

Different chemical modifications of insulin, including addition, replacement, and deletion of residues, have been studied by X-ray analysis and CD.[52,105] These indicate that several parts of the insulin molecule are not essential to the general insulin fold, although small conformational changes may be induced.

Thus removal of B1—B3, replacement of B1 phenylalanine by tryptophan,[106] and addition of various groups of A1, including t-butoxycarbonyl, acetyl, glutamyl, and thiazolidine, does not prevent formation of rhombohedral 2-zinc insulin crystals, isomorphous with porcine insulin crystals.[17,52,107,108] However, for the glutamyl derivative (AO-Glu-insulin), high-resolution X-ray studies indicate that the modification gives rise to a small movement of the residues A1—A5, which have already been shown to be

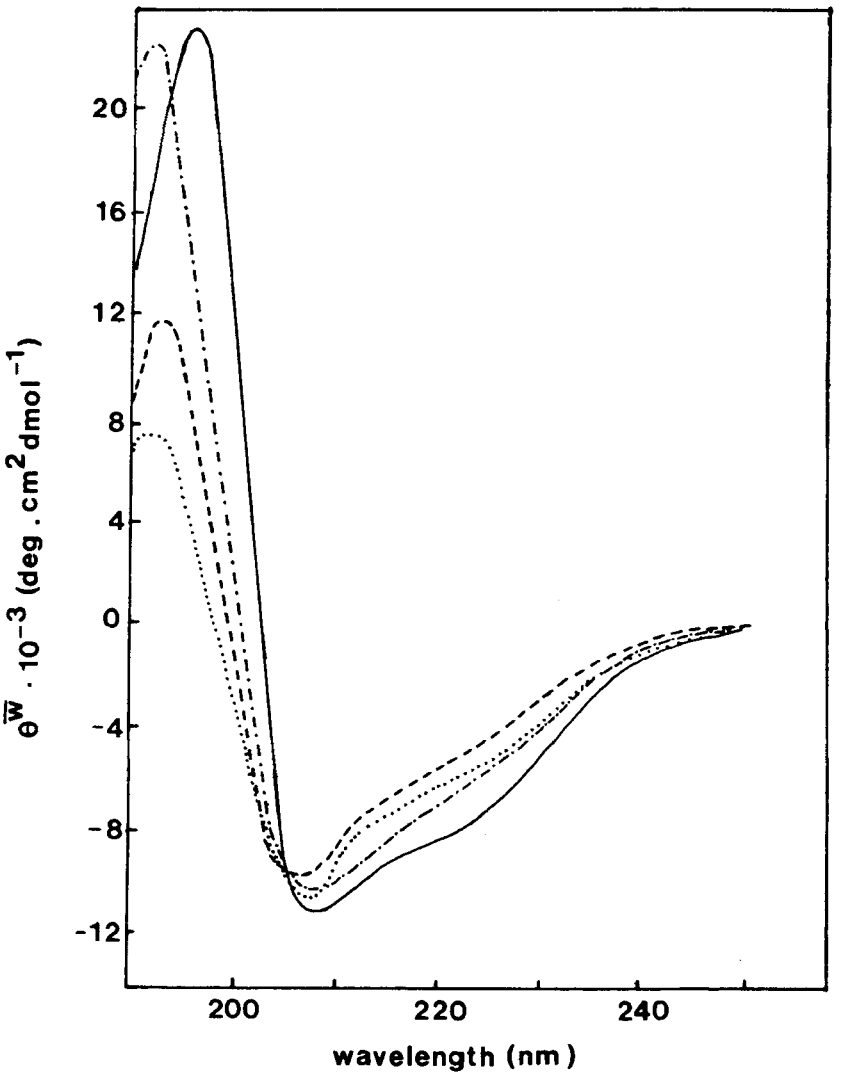


FIGURE 15. Far UV CD spectra for bovine — (3.5 μM), guinea pig ..... (40 μM), casiragua -·-·- (40 μM), and porcupine ---- (40 μM) insulins at pH 7.8. At this dilution the bovine insulin is at least 50% monomeric.

sensitive to the environment (*vide supra*), and possibly small movements of the adjacent residues B24—B26 and A19.[108] However, in the monomeric state these conformational changes may be larger, as evidenced by the CD, especially when the added group has a negative charge.

X-ray studies have also been carried out on crystals of an insulin cross-linked between A1 and B29 by diaminosuberic acid.[109] These crystals are not strictly isomorphous to native insulin, but an independent X-ray analysis at 3.2 Å resolution indicates only very small differences in conformation between the cross-linked insulin and native insulin. Des pentapeptide (B26—B30) insulin and even des hexapeptide (B25—B30) insulin may be crystallized.[110-113] Des pentapeptide insulin appears to have a conformation very similar to that of insulin.[112] CD indicates that the conformation of the insulin molecule is more sensitive to removal of the octapeptide (B23—B30), but even this material gives small twinned crystals.[113]

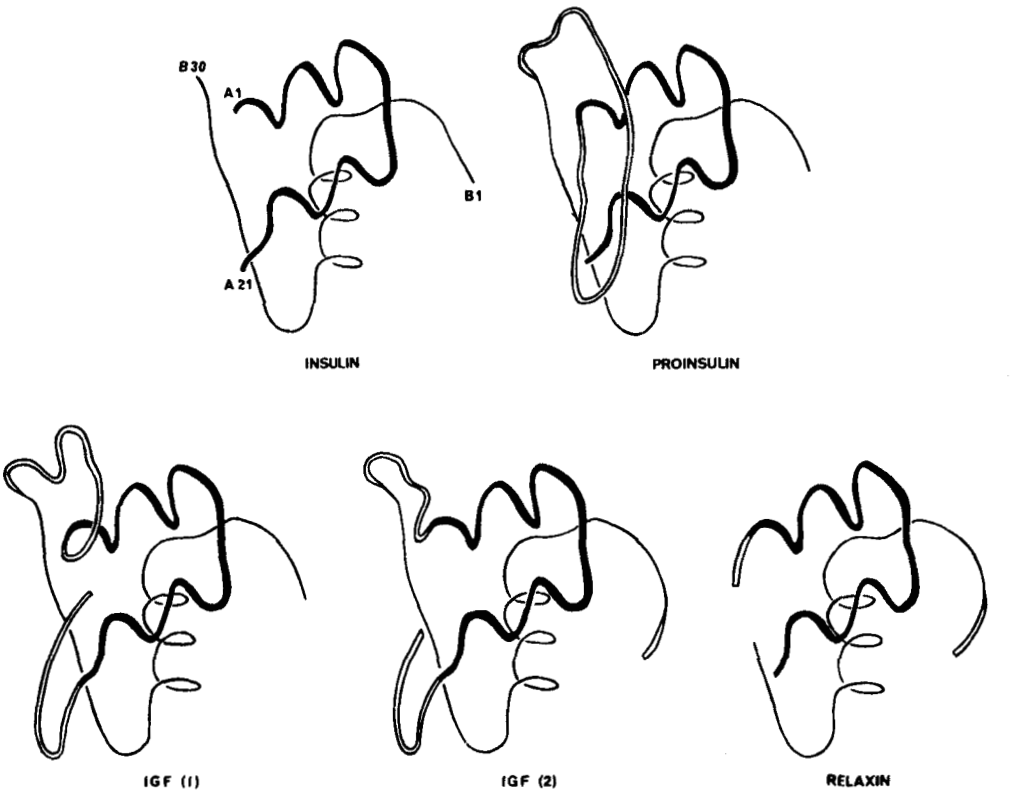


FIGURE 16. Schematic representations of the three-dimensional structure of the insulin monomer based on the X-ray analysis of rhombohedral porcine 2-Zn insulin crystals and proposed conformations based on model building for proinsulin, porcine relaxin, and insulin-like growth factors I and II.

The conformation of insulins substituted at B24 and B25 by leucine has been investigated by CD spectroscopy.[227,228] While the spectrum of the B25 analogue has much in common with native insulin, that of the B24 analogue shows marked differences which can only be partly explained by the diminished ability of the molecule to dimerize. Computer graphics model building of these analogues confirms that the B25 substitution is less readily accommodated in the insulin structure and may give rise to distortion of the conformation of native insulin.

2. Proinsulin

Figure 16 shows a schematic diagram of the probable conformation of proinsulin. This structure is based on spectroscopic and immunological data; although small crystals of proinsulin were first prepared some years ago and are now being used for a medium resolution study, detailed X-ray results have not yet been obtained.[114,115,12]

CD of the isolated C-peptide does not indicate substantial amounts of helix or beta-sheet; the ability of sodium dodecyl sulfate (SDS) to induce helix in the C-terminal fragments which have a high helix-forming potential is inhibited in the complete C-peptide,[94] although much less so at acid pH where the crystals are formed. Ultracentrifuge studies[116] of the C-fragment indicate that it has a "hairpin" type structure since the axial ratio is large, and this would place the termini of the C-peptide in positions where they could link to A1 and B30 of the folded insulin molecule. A more structured C-peptide has been proposed by Snell and Smyth[93] on the basis of the Chou and Fasman

technique for structure prediction; they suggested two helical segments. However, this is not consistent with the additivity of the CD spectra of the C-peptide and insulin to give the proinsulin spectrum, and the similarity of the Raman spectra of insulin and proinsulin.[41,94,117,118] Although the detailed conformation of the C-peptide is the subject of disagreement, most authors place the C-peptide over the face of the molecule involving the A-chain, B22, and B25.[3,85,93] This is consistent with the antibody-binding studies of Arquilla et al.[85] and the fact that proinsulin may self-associate. In any case the sequences of C-peptides of different species vary and so the conformations are probably different, although the interactions between the insulin and C-peptide may involve more conserved residues.

3. Insulin-like Growth Factors

The observation by Froesch and co-workers[119] that most insulin-like activity in human serum was not neutralizable by insulin antisera led to the eventual characterization of two insulin-like growth factors (IGF), IGF I and II, the sequences of which are shown in Table 1.[120,223] The sequences of a rat somatomedin[229] and a rat MSA multiplication stimulating activity (MSA)[230] show close homologies with IGF I and IGF II, respectively. Both IGF I and IGF II have shorter C-peptides than proinsulin and also have an extension at the C-terminus of the A-chain. The structures have been modeled assuming homology with insulin (see Figures 16 and 17).

For example, the arrangement of the cystines is identical in IGF I and insulin, and glycines 7 (B8), 19 (B20), and 22 (B23) allow the backbone to assume the same three-dimensional structure as insulin. The side chains of Leu 5 (B6), Leu 10 (B11), Val 11 (B12), Ala 13 (B14), Leu 14 (B15), Val 17 (B18), Phe 23 (B24), Ile 43 (A2), Val 44 (A3), Leu 57 (A16), and Tyr 60 (A19), which are entirely or partially buried in the hydrophobic core of insulin, are identical in IGF and allow formation of a globular structure with a hydrophobic core.

The remaining side chains of the residues 5-25 (B6—B26) and 42-61 (A1—A20) are almost entirely on the surface of the molecule and are very easily accommodated in an insulin-like structure. Most, but not all, are hydrophilic and differ between insulins and IGF. Residues 1-4 of IGF (B2—B5) differ but also lie on the outside of the molecule and can be added to the model without disruption of the tertiary structure. They were placed in a conformation similar to that of insulin in rhombohedral crystals. However, for insulin it is clear that residues B1—B3 play little role in the stabilization of the tertiary structure and the same is almost certainly true for IGF residues 1 and 2. Residues 25-29 of IGF can also be accommodated on the surface of the tertiary structure in positions approximately equivalent to residues B26—B30 of insulin; however, the rather surprising reversal of the Pro, Lys sequence in IGF leads the chain away from the main core of the molecule and makes the structure "looser" at this point. These features can be observed in the stereo view of the molecule shown in Figure 17.

It is difficult to be precise about conformations of surface polar side chains. For example, althought the side chain of Lys 27 can be folded against the side chain of Phe 25, it was placed pointing directly into solution. This decision was influenced by the finding in the X-ray refinement of insulin that Lys B29, the equivalent group in insulin, is less important structurally than was originally thought, and does not always form an intramolecular ion pair with Glu A4.[19]

The residues 30-41 comprising the connecting peptide sequence of IGF I easily span the positions of the B30 and A1 of insulin. Indeed, it has often been remarked that the connecting peptide of insulin (about 30 amino acid residues in length) need only be three residues in length to achieve the simple object of spanning the two chains and allowing the polypeptide to achieve the correct three-dimensional structure. In fact, a synthetic

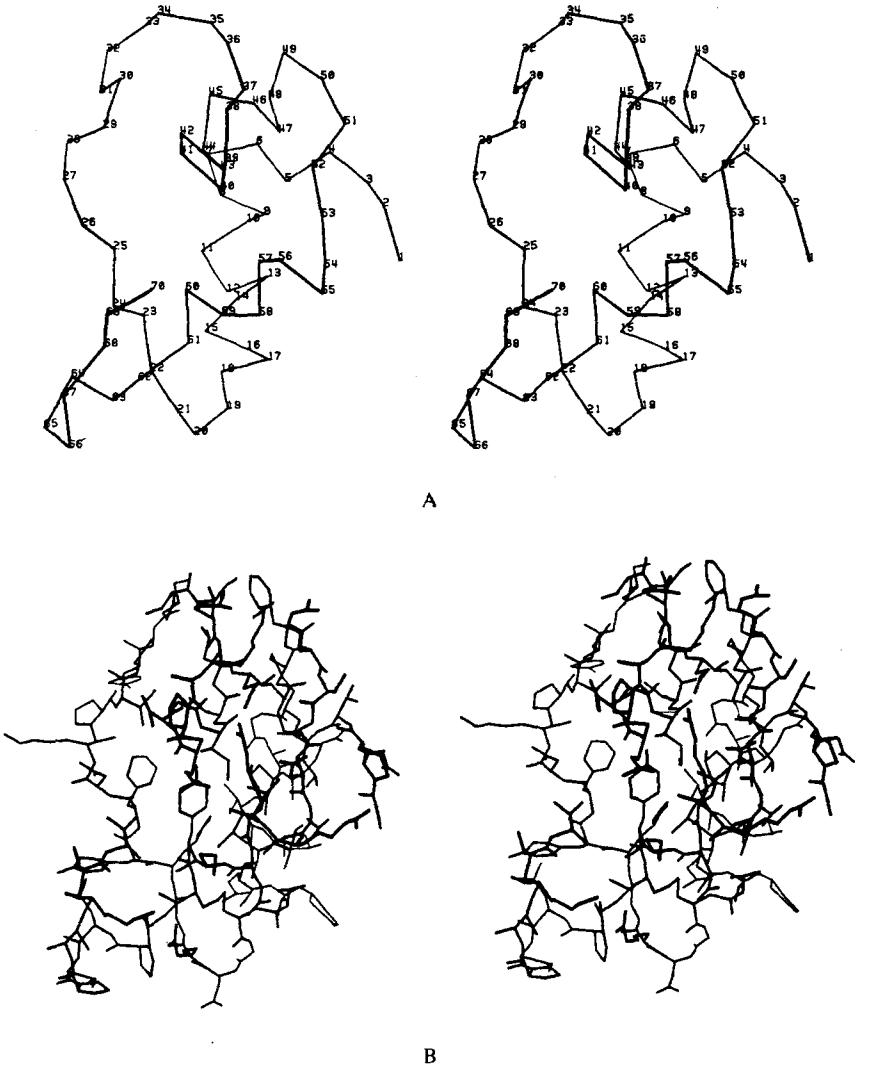


FIGURE 17. Stereo diagrams of (A) the alpha-carbon backbone conformation and (B) the complete molecule of IGF-I proposed from model building. (From Blundell, T. L., Bedarkar, S., Rinderknecht, E., and Humbel, R. E., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 180, 1978. With permission.)

bridge of about 10 Å can mimic the role of the connecting peptide in guiding chain folding and ensuring correct pairing of sulfurs in the cystines.

In the absence of an X-ray analysis of proinsulin and of any homology of the connecting (C) peptides of insulin and IGF, possible conformations were considered from first principles. Polar residues were placed exposed to the solvent or forming ion pairs or hydrogen bonds. Conversely, nonpolar groups were placed against similar groups where possible.

Aš suggested by the Chou and Fasman method, two beta-turns were built into residues 28-34 so that the hydrophilic residues 33-37 may have the side chains away from the surface. In this way Tyr 31 has its side chain towards the top of the hydrophobic surface comprising 11 (B12), 23 (B24), 24 (B25), and 25 (B26) that is involved in dimer formation in insulin. Also Arg 36 and Arg 37 have their side chains in the vicinity of the acid groups

of Asp 45 (A4) and Glu 46 (A5). The remaining residues of the connecting peptide (38-41) are then easily folded to join Gly 42 (A1).

Residues 63-70 have fewer constraints on their conformation. The presence of Pro 63 and Pro 66 and the hydrophilic groups such as Lys 65, Lys 68, and Ser 69 makes helix unlikely. However, the Leu 64 would probably pack against the more hydrophobic surface residues such as Met 59 (A18), Tyr 60 (A19), and Tyr 24 (B25) in this region. As shown in Figure 17, the residues are probably folded back on themselves: this allows the hydrophilic side chains to be in contact with solvent and brings the COOH-terminus into the region of the guanidinium group of Arg 21 (B22).

The tertiary structure described leads to a reasonably compact globular molecule. The many charged groups are distributed over the surface of the molecule, giving rise to a complicated series of ionic interactions. For instance, Arg 21 (B22) is close to Glu 58 (A17) and Ala 70 alpha-carboxylate, and an extensive arrangement of charged groups is formed.

A similar conformation is predicted for IGF II, although the shorter C-peptide and extension at the C-terminus makes the molecule more insulin-like.

4. Relaxin

Relaxin, a polypeptide hormone synthesized and stored in the corpus luteum and responsible for dilation of the symphysis pubis prior to parturition, also consists of one A-chain and one B-chain linked by disulfide bridges.[121,123,218,221] Although there is very little sequence homology (see Table 1), model building shows that this molecule may also have a similar backbone conformation to insulin. A schematic diagram of the main chain is shown in Figure 18A and a stereoview of the three-dimensional structure is shown in Figure 18B.

The mainchain A1—A20 and B6—B21 (insulin-numbering) can adopt a conformation identical to that of insulin so that intrachain and interchain disulfides occupy equivalent three-dimensional positions to those of insulin. This is made possible by glycines at B8 and B20. The hydrophobic side chains of B6, B11, B12, B14, B15, B18, A2, and A16 which contribute to the hydrophobic core of insulin may then occupy the same volume in relaxin.

B11 and 12 are conserved as in all insulins. Of particular interest is the complementary nature of many of the sequence differences. For example, in insulin B6 Leu and B14 Ala are in close juxtaposition; in relaxin their respective positions are reversed: B6 Ala and B14 Leu. In a similar way A2 Ile and A16 Leu of insulin are A2 Leu and A16 Ile in relaxin. These pairs of side chains point towards the center of the core from opposite sides (see Figures 19A and B). The B15 Trp of relaxin is beautifully accommodated almost completely buried in the core with no relative changes of the positions of the A and B chains. The close-packed occupancy of the core is thus maintained.

Addition of surface side chains leads to the suggestion of many ion pairs, in particular A5 Lys and A15 Asp of relaxin (see Figure 19d), which are A5 Gln and B15 Gln in insulin. Further possible relaxin ion pairs are A8 Glu and B5 Lys, B13 Arg and B17 Glu.

Residue A19 is Tyr in insulin and Leu in relaxin. This change is easily accommodated in the model and may be related to the extra residues at the A-chain N-terminus of relaxin which are most probably covering A19 (see Figure 19C) so that A -1 Met is in contact with Leu, leading to a further ion pair, A -2 Arg to the B-chain C-terminus. In a similar way the extra residues at the B-chain N-terminus are easily accommodated. When residue B3 Phe is allowed to lie against the residues of the hydrophobic core, the chain can be further extended to fold over A12, a position which is Ile in relaxin but always hydrophilic in insulins. However, the conformations of the B-chain N-terminal residues may be flexible in relaxin as they are in insulin, and it is possible that they contribute little to the stability

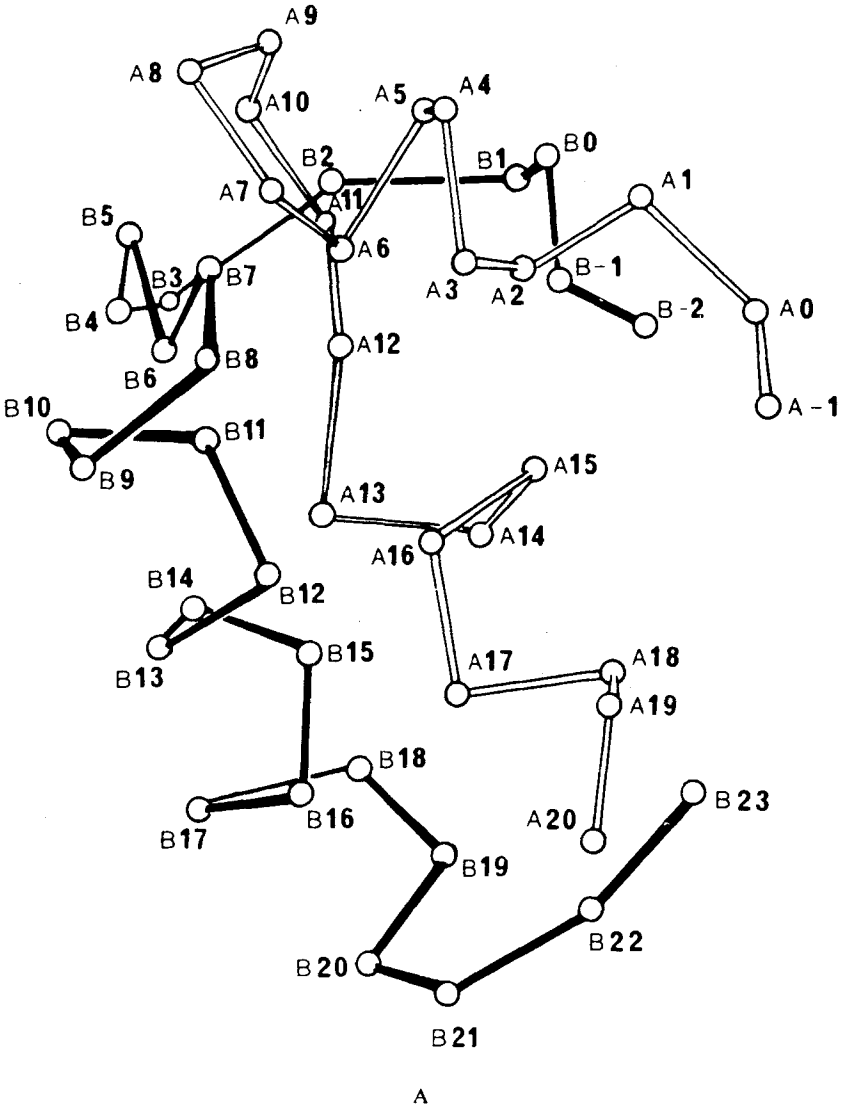


FIGURE 18. Diagram of (A) the alpha-carbon positions and (B) a stereo for the whole molecule of porcine relaxin derived from model building. (From Bedarkar, S., Turnell, W. G., Blundell, T. L., and Schwabe, C., *Nature*, 270, 449, 1977. With permission.)

of the insulin-like core. The absence of a residue at A21 might be expected to destabilize the insulin-like tertiary structure, but would not prevent its existence.

The residues at the B-chain C-terminus can be folded back on to the hydrophobic core. The tryptophan probably contributes towards stabilizing the tertiary structure. This residue may be placed so that it partially compensates for the lack of residues in relaxin equivalent to B24 Phe and B26 Tyr of insulin. Thus the tertiary structure would be expected to be more stable than that of desoctapeptide insulin (B23—B30 deleted) which has a tertiary structure rather different from that of native insulin. As a result of the shorter chain, residue A3 in relaxin — a serine residue — occurs on the surface of the molecule whereas in insulin this residue is usually a valine and is close to the B-chain C-terminus.

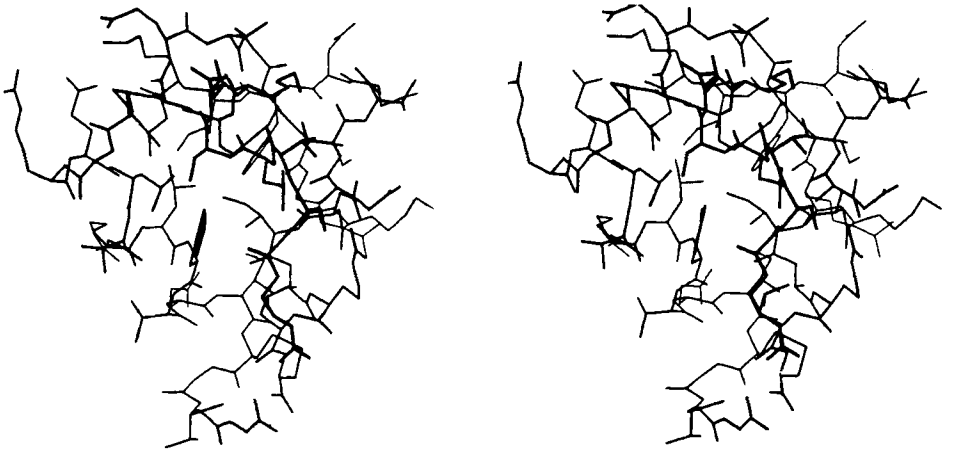


FIGURE 18B

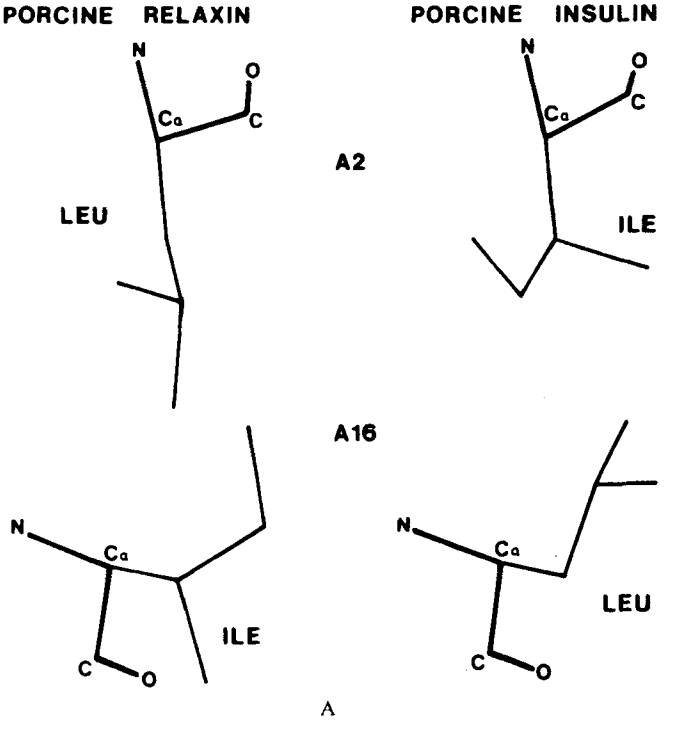


FIGURE 19. The complementary sequence changes in the hydrophobic core of relaxin are displayed in (a) and (b) compared to the equivalent areas in porcine insulin. On the surface of relaxin an ion pair (A5—A15) is possible (c) and the A-chain extension covers A19 (d).

An insulin-like structure for porcine relaxin has been proposed independently by two groups; similar conclusions were reached in both studies.[4,95] Further model-building studies using the sequences of sand tiger shark and dogfish relaxins[100,124] and rat relaxin[125] indicate insulin-like structures, although the sequences are quite different from those of porcine relaxin.

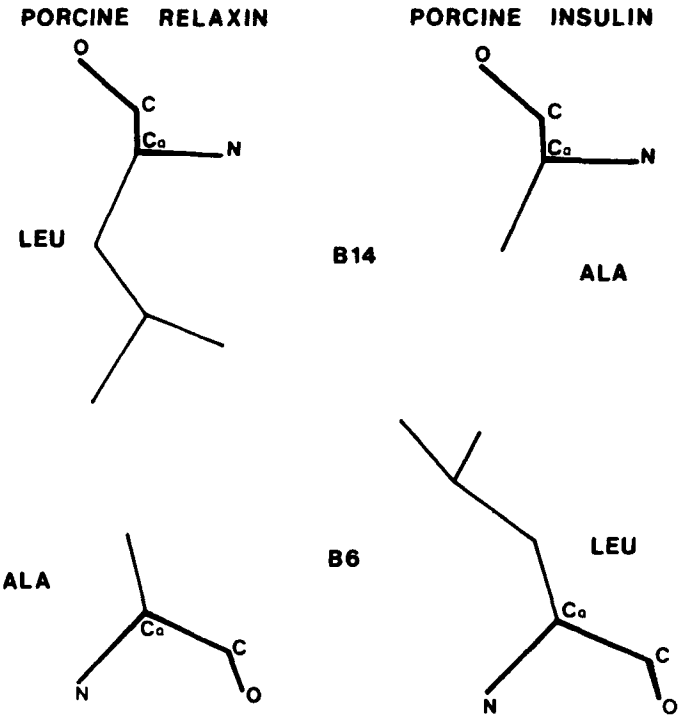


FIGURE 19B

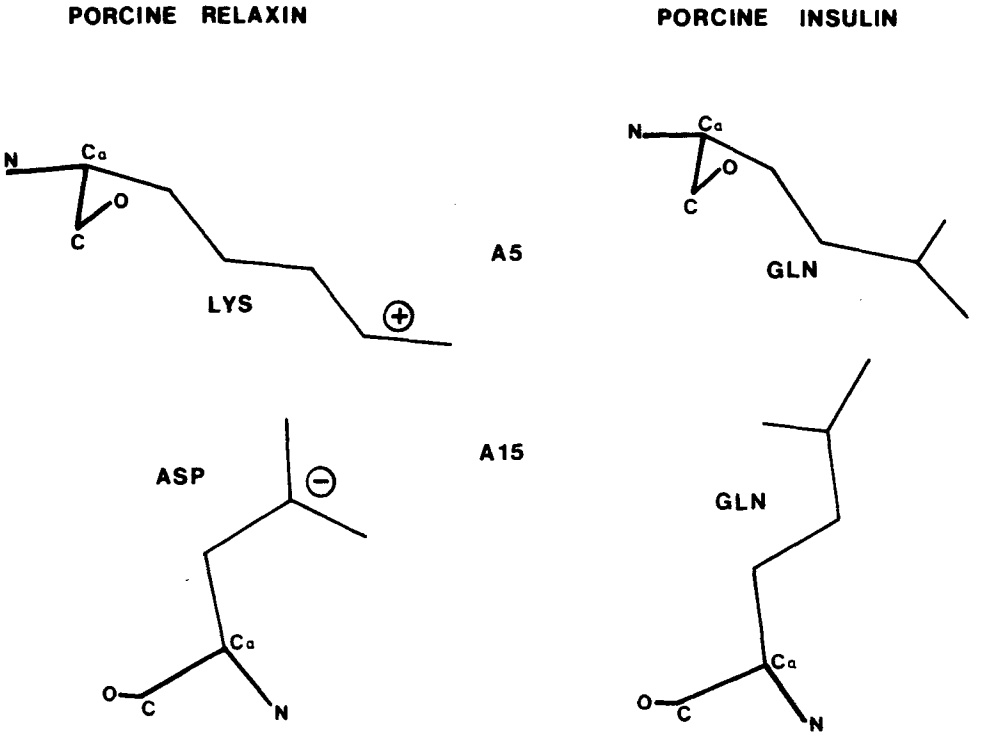


FIGURE 19C

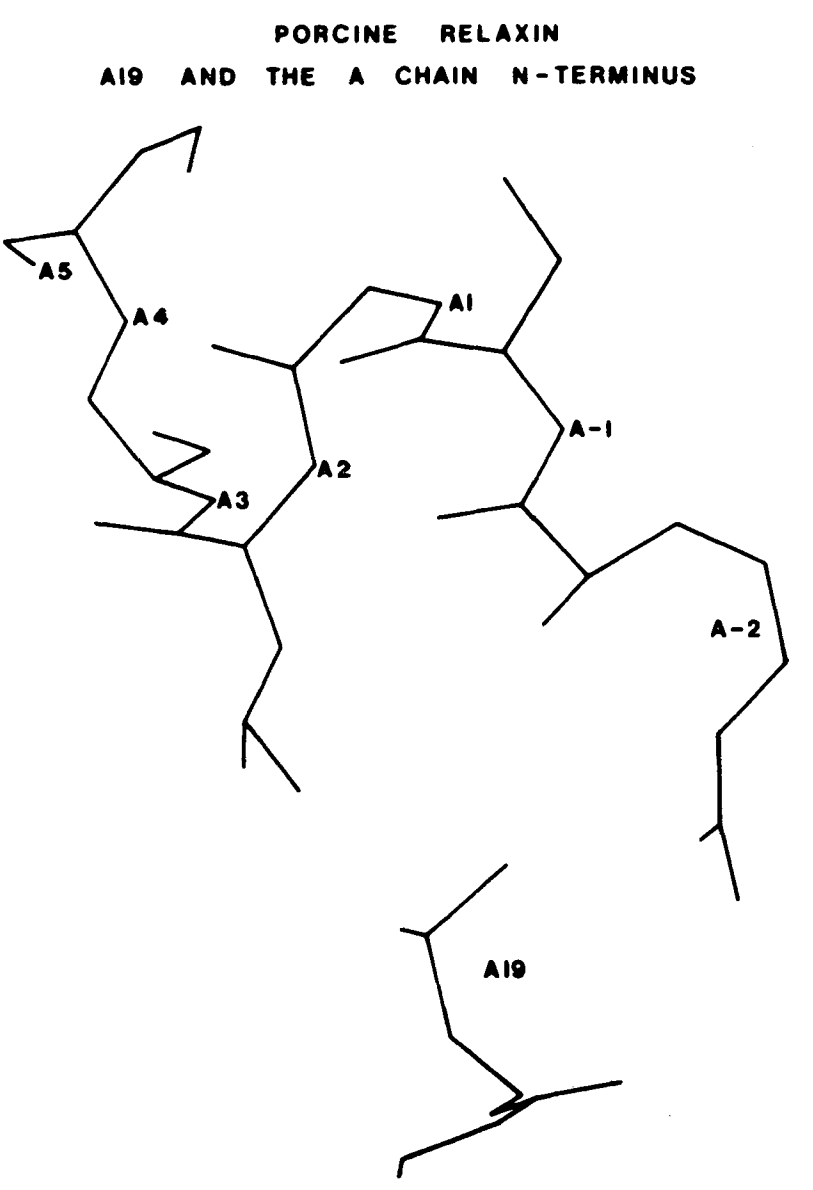


FIGURE 19D

5. Pancreatic Polypeptide

Pancreatic polypeptide (PP) is a 36 amino acid peptide[126,127] (see Table 4) with an amidated C-terminus, a feature known to occur commonly in polypeptide hormones such as gastrin, secretin, and oxytocin.

The conformation of avian (turkey) pancreatic polypeptide (aPP) has now been defined in crystals by X-ray analysis at 0.98 Å resolution.[7,9,11,128] As illustrated in Figure 20, the molecule comprises two well-defined secondary structures lying approximately antiparallel. Residues 1-8, including proline at positions 2, 5, and 8, form a polyproline or collagen-like helix with ϕ, ψ angles averaging $-70°$, 136°, respectively. This conformation with an approximate threefold repeat brings the proline residues on to the same side so that they all contribute towards hydrophobic interactions, with the side chains of the alpha-helix formed from residues 14 to 31. Residues 9-12 form a type I

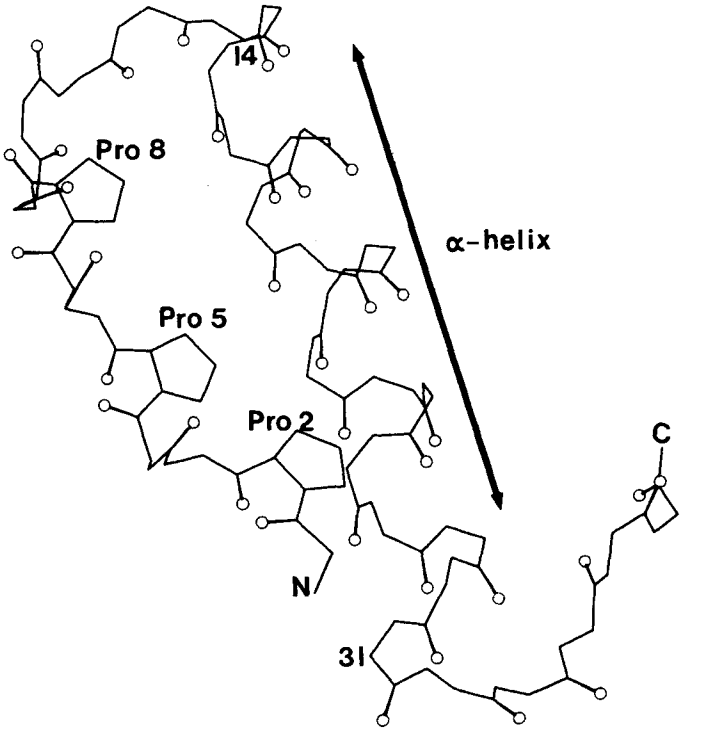


FIGURE 20. Main chain atoms of the aPP molecule showing the relationship between the polyproline-like helix (N-terminal region) and the alpha-helix.

beta-bend, and the carbonyl group of Pro 13 hydrogen bonds to the NH group of residue 16 at the N-terminus of the helix to give a type 3 turn.[129] Residues 33 and 34 also form a turn, allowing the C-terminus to be oriented away from the helix axis as shown in Figures 20 and 21.

The contacts between the polyproline-like helix and the alpha-helix are hydrophobic and are established by interdigitation of the three proline side-groups between the nonpolar side groups of one face of the alpha-helix, giving van der Waals' contacts of Pro 2 with Val 30 and Tyr 27, of Pro 5 with Tyr 27, Leu 24, and Phe 20, and of Pro 8 with Phe 20 and Leu 17. The polyproline helix appear to bend gently around the alpha helix in a way which optimizes contacts. A further hydrophobic contact occurs between the aromatic side chains of Tyr 7 and Phe 20. This arrangement of groups allows an extensive surface region to be buried and undoubtedly accounts for the surprising stability of the conformation of this peptide even at high dilutions as indicated by CD.[8,74,75]

Table 4 shows that the mammalian PP molecules are highly homologous one with another, although only 16 residues of the avian molecule (aPP) are conserved in the bovine molecule (bPP). Computer graphics model building and predictive methods show that bPP would be expected to have a helical structure between residues 14 and 31 in the same way as that observed in the aPP molecule. Further, the side chains of the alpha-helix which pack against the prolines at 2, 5, and 8 are conserved hydrophobic and allow equivalent tertiary interactions to be formed. The very conservative variation in the residues 9-12 allows formation of the beta-bend, and the Pro can easily be accommodated at position 14 at the N-terminus of the alpha-helix instead of position 13, as in aPP. More difficulty is experienced in the replacement of His 34 by proline, and this

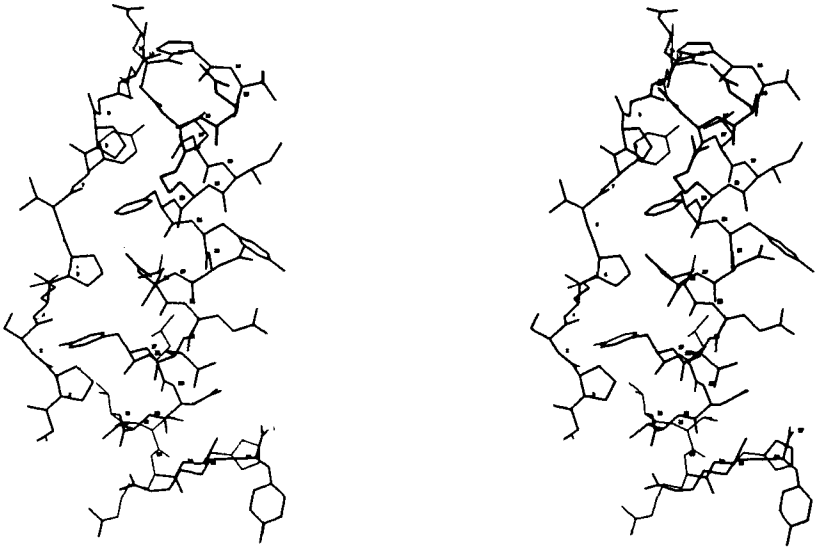


FIGURE 21. Stereo diagram of the complete aPP molecule with a similar orientation to Figure 20.

change may be expected to alter the flexibility and conformation of the C-terminal residues. Nevertheless, the model is consistent with a conserved general conformation, especially with respect to the helical content, as has been demonstrated by CD.

6. Glucagon

The small size and lack of disulfide bridges makes glucagon a flexible structure in dilute aqueous solutions. CD and NMR confirm that there is little secondary structure. A recent NMR study indicates an interaction between the side chains of Trp 25 and Val 23, which is incompatible with an alpha-helical conformer.[53] However this secondary structure probably also accounts for the ellipticity of the CD which had previously been attributed to one turn of helix.

In nonaqueous systems such as cetyltrimethyl ammonium bromide,[130] dimyristoyl glycerophosphocholine,[131] lysolecithin,[79] and glycols,[132] tryptophan fluorescence (see Figure 22) and CD indicate the formation of an ordered structure which is probably an alpha-helix. This has been attributed to the amphipathic nature of a helix formed by glucagon (see Figure 4) which would be stabilized by the presence of hydrophobic molecules in solution. More recently Wüthrich and co-workers[133,134] have shown using NMR, *vide supra,* that glucagon binds close to the surface of lipid micelles as a monomer. A well-defined conformer is stabilized in the C-terminal residues 22-27, which is amphipathic but may not be alpha-helical.

7. Somatostatin

Although somatostatin contains a disulfide bridge, considerable conformational flexibility is still possible. CD studies have been held to indicate a "hairpin" structure with several residues in antiparallel pleated sheet.[80] Similar conclusions were reached using Raman spectra.[46] However studies using infrared dichroism[136] and NMR[61,62] point to the co-existence of several different conformers, some of which may be stabilized in some analogues or fragments. For example, spectra for fragments ranging from Thr 10-Phe 11 to Lys 9-Thr 10-Phe 11 – Thr 12-Ser 13-Cys 14 indicate a ring current shift by the Phe 11 aromatic ring on Thr 10, which is maintained in whole somatostatin.[59,60] In DMSO the

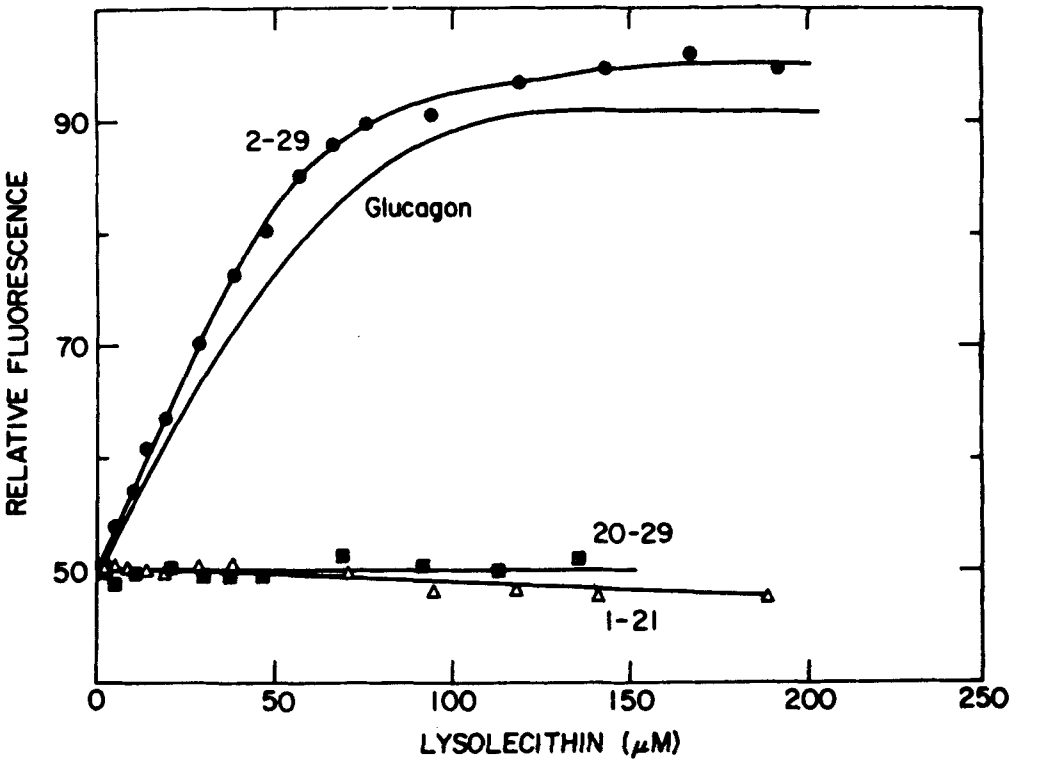


FIGURE 22. The effect of lysolecithin on the fluorescence of glucagon peptide fragments of glucagon. Glucagon (1.3 μM), and peptides 2-29 (3 μM), 20-29 (7.1 μM), and 1-21 (9.6 μM) were titrated in 0.002 M sodium phosphate pH 6.2 with lysolecithin. Tryptophan fluorescence (excitation at 280 nm and emission at 340 nm) was followed for the fragments containing this residue and tyrosine fluorescence (275 and 305 nm) was used for the 1-21 fragment. (From Schneider, A. B. and Edelhoch, H., *J. Biol. Chem.*, 247, 4986, 1972. With permission.)

inequivalence of Thr 10 and Thr 12 is more pronounced, indicating that this conformer is favored. Evidence that NH protons of 8, 9, 10 must be H-bonded or solvent shielded is consistent with a beta-turn involving residues 7-11. Although type I and III beta-turns are excluded, a type II beta-turn is possible.[61,62] Substitution of a D-tryptophan at position 8 should stabilize this conformation, and this substitution leads to an upfield shift of the gamma-methylene protons of Lys 9 due to the close approach of Trp 8.[58] Many analogues with this spectrum have enhanced ability to inhibit insulin secretion in vivo, but this may be due to inhibition of degradation rather than stabilization of the conformation at the receptor.

Veber and co-workers[231] recently have described the synthesis of conformationally constrained somatostatin analogues containing the D^8-Tryptophan substitution. Using a computer modelling system the feasibility of replacing the double bridge (Cys-Aha-Cys), where Aha is 7-aminoheptanoic acid, in the analogue Cyclo (Aha-Cys-Phe-DTrp-Lys-Thr-Cys) with various dipeptides in the conformations defined for the various types of β turn was tested. Suitable cyclic hexapeptides were synthesized, bioassayed and NMR spectra recorded. Closing the ring with Phe-Pro produced an analogue with twice the activity of somatostatin and with an NMR spectrum showing the characteristic upfield γCH_2 protons of Lys 9 found in larger D-Trp 8 analogues of high activity. Nine of the amino acids of native somatostatin were effectively replaced with a single proline residue.

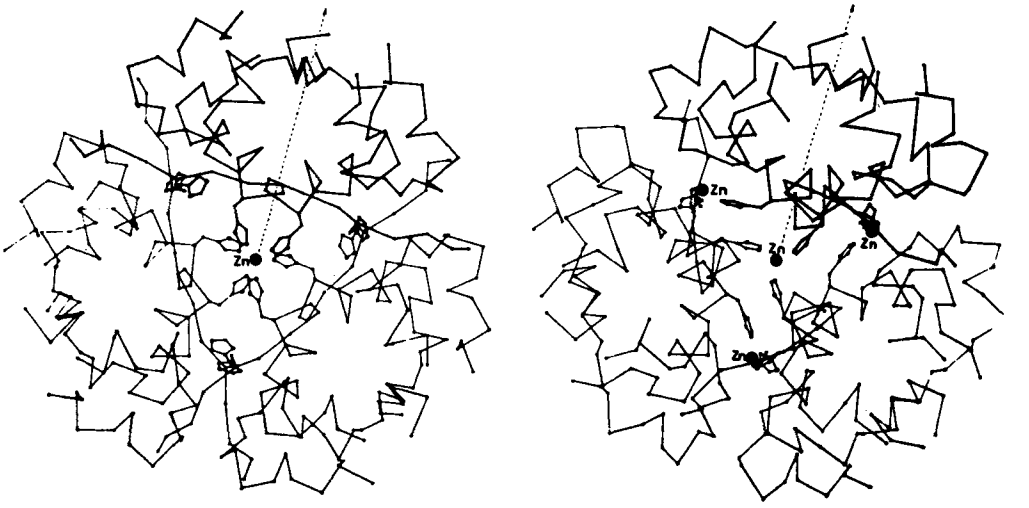


FIGURE 23. Backbone structure of 2-Zn and 4-Zn insulin hexamers. (From Bentley, G. A., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Mercola, D. A., *Nature*, 261, 166, 1976. With permission.)

The NMR spectrum of the hexapeptide indicated a high degree of molecular rigidity, suiting conformational analysis. Thus stabilization of a distinct conformer leads to enhanced receptor affinity.

B. Oligomers

The pancreatic hormones are generally quite hydrophobic in character. As a consequence they have low solubility and tend to aggregate at high concentrations. This aggregation is specific, leading to well-defined oligomers. Many features of the oligomer structure are conserved through the evolution of most vertebrates.

1. Insulin

Two surfaces of the porcine insulin monomer are largely hydrophobic. One surface is involved in isologous interactions to give a dimer, $K_D \sim 10^{-5}$ *M*, in which an antiparallel pleated sheet is formed between two equivalent regions B24—B26. Many hydrophobic groups are involved in these interactions, including B12 Val, B16 Tyr, B24 Phe, and B26 Tyr. In porcine 2-Zn insulin hexamers in rhombohedral crystals, the two molecules of the dimer are similar but not identical in conformation (see Section III.A.1); however, in the cubic crystal form the dimers appear to have a perfect twofold axis similar to that found in the hagfish dimers. It is probable that a perfect twofold axis exists in the dimers in solution, but further spectroscopic investigation is necessary.

The second hydrophobic surface involving residues B14 Ala, B17 Leu, and A14 Tyr is buried on formation of the 2-zinc insulin hexamers (see Figures 23 and 24A), in which each dimer is coordinated to two zinc ions. Each zinc ion is coordinated through B10 His imidazole groups to three insulin molecules and three water molecules. At high anion concentrations a rearrangement occurs in the dimers to give 4-zinc insulin hexamers (Figures 23 and 24) in which one zinc is coordinated as in the 2-zinc form to three B10

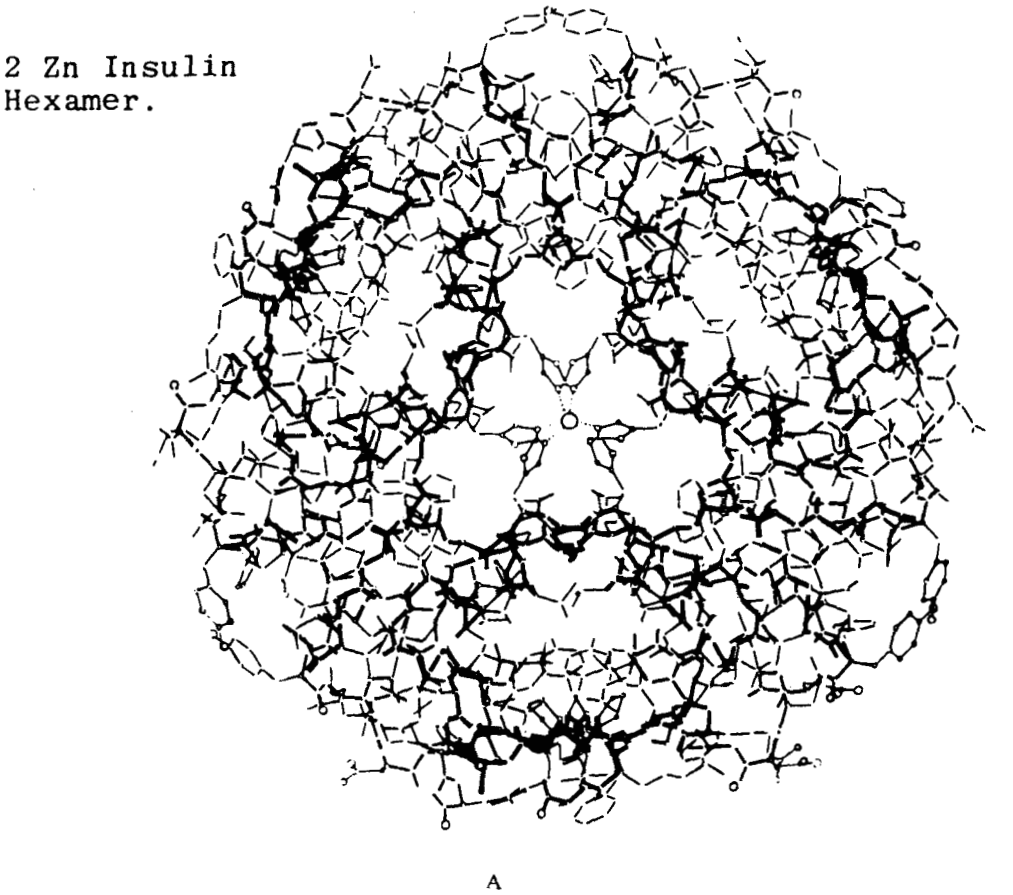


FIGURE 24. Refined structures of (A) 2-Zn and (B) 4-Zn insulin hexamers viewed down the threefold axis. (From Dodson, E. J., Dodson, G. G., Reynolds, C. D., and Vallely, D. G., in *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones,* Brandenburg, D. and Wollmer, A., Eds., Walter de Gruyter, Berlin, 1980, 9. With permission.)

imidazoles, while the three further zinc ions are each coordinated to one B10 His and one B5 His imidazole and two water molecules. NMR spectra show that the spectrum found in 2-Zn hexamers is actually assumed after titrating zinc-free insulin with 1 g atom of zinc per insulin hexamer, but that a conformational change to 4-Zn insulin is triggered by anions such as thiocyanate and not by excess zinc ions.[49] The authors have also found evidence for the adoption of a hexameric structure with 32 symmetry at high concentrations which has not been observed crystallographically but which should be able to bind six zinc ions.

Although most fish insulins appear to be able to form zinc insulin hexamers, that of the primitive vertebrate, the hagfish, forms only dimers.[137] The zinc-binding histidine at B10 found in most higher mammals is aspartate in the hagfish. Nevertheless, much of the hydrophobic surface involved in hexamer formation is hydrophobic: B6 Leu, B14 Ala, B17 Ile, B20 Gly, A13 Ile, and A14 Tyr. There appears to be no evidence that hagfish insulin aggregates to higher oligomers; this may be due to the arginine at B1. However, it implies that some biological constraint on the hagfish insulin molecule requiring this surface to be hydrophobic existed before the ability to form zinc hexamers was acquired in evolution.

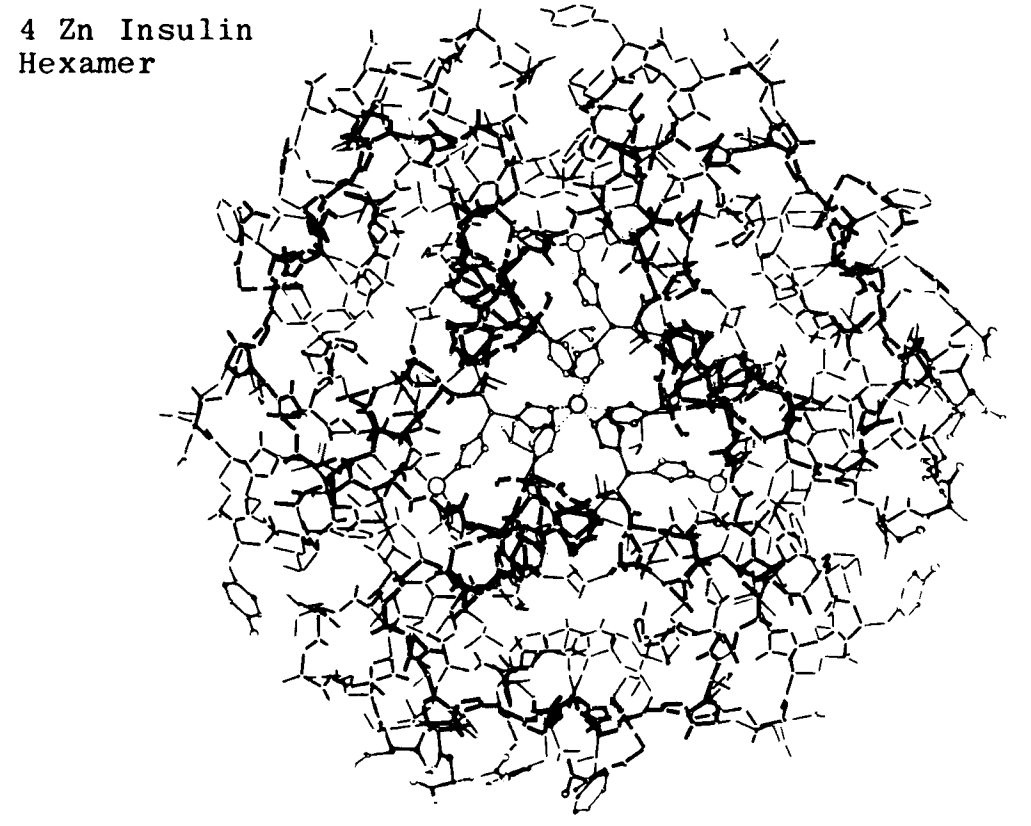


FIGURE 24B

The insulins from hystricomorph rodents such as the guinea pig,[69] coypu,[98] porcupine,[104] and casiragua[99] are characterized by a large number of amino acid substitutions, low activity, and an impaired ability to self-associate, several existing only as monomers. As described in Section III.A.1, the casiragua insulin can form an insulin-like fold (Figure 25). However the surface involved in hexamer formation is largely hydrophilic and charged as shown in Figure 26; B14 Thr, B17 Ser, B20 Lys, A13 Arg, A14 Asn; and the zinc-binding imidazole is replaced by Gln. Thus zinc insulin hexamers would not be expected to form, and binding studies with radioactive zinc confirm this. Similar amino acid substitutions also occur in guinea pig and coypu insulins.

Guinea pig, porcupine, and casiragua insulins will not form dimers. For guinea pig and porcupine insulin this is almost certainly due to the change of conformation described in Section III.A.1; all the amino acids required on the surface of the molecule for dimer formation are identical to those of porcine insulin. For casiragua insulin the inability to dimerize probably derives partly from small conformational changes in the region B24 to B26 resulting from the substitution of lysine at B20, which is usually glycine. However the main explanation for this is probably the existence of arginine at B26. Its environment in a casiragua insulin dimer would be strongly hydrophobic in close proximity to the side chain groups of A3 Val, B11 Leu, B12 Val, B16 Tyr for both molecules, and B24 Phe of molecule II (see Figure 27).[99]

2. Pancreatic Polypeptide

Although much of the surface of the aPP is polar, involving a number of charged groups, an extensive surface region is hydrophobic. This undoubtedly leads to the

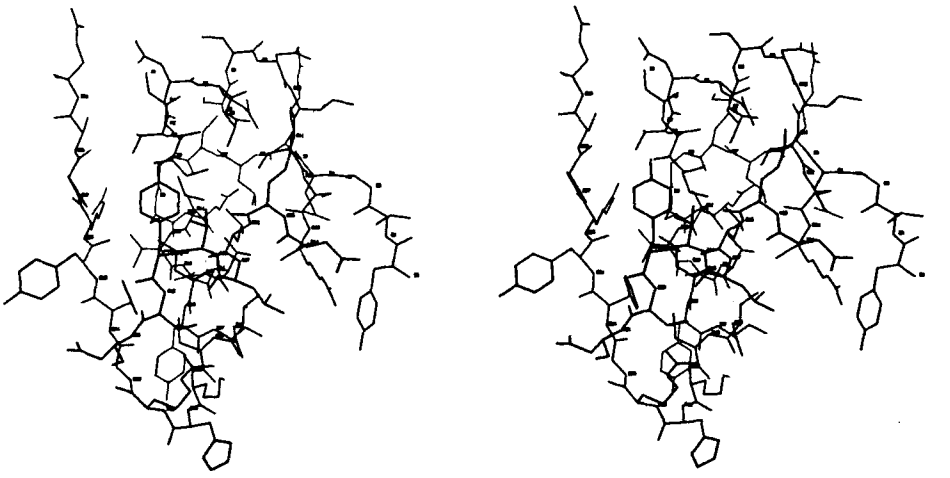


FIGURE 25. The structure of casiragua insulin model built on the basis of the structure of porcine insulin. (From Blundell, T. L. and Horuk, R., *Hoppe-Seyler's Z. Physiol. Chem.*, 362, 727, 1981. With permission.)

formation of aPP dimers, evidence for which in solution is given by centrifuge studies, gel-filtration, and CD.[8,74,76] The crystal structure indicates that dimers have a perfect twofold axis of symmetry, bringing together extensive areas involving both the alpha-helix and the polyproline-like helix (see Figure 28). The axes of the two alpha-helices in the dimer are separated by approximately 11 Å and make an angle of approximately 150°, so that the side chain of one helix fits into the grooves between the side chains of the second and vice versa. The side chains involved include Phe 20 and Tyr 21 close to the twofold axis, as well as three leucines, two valines, and one isoleucine of each helix. The aromatic rings of two equivalent Tyr 7 residues also form contacts. These very extensive hydrophobic interactions clearly account for the stability of the dimer of aPP which appears to have $K_D \sim 5 \times 10^{-8} M$ at neutral pH.[76]

In the crystal structure the aPP dimers coordinate to zinc ions, giving a stable and extensive lattice (Figure 29). Each zinc ion has five ligands, including the N-terminal nitrogen and carbonyl oxygen of Gly 1 from one dimer, the amide of Asn 23 from a second dimer, and Nε of the imidazole group from a third. The bovine molecule would also be expected to form dimers, and this has been confirmed, although zinc-linked oligomers found in crystals of aPP could not occur as the His 34 and Asn 23 are not present in bPP.

3. Glucagon

The crystal structure of glucagon demonstrated the presence of complex cubic oligomers.[6] These oligomers can be thought of as comprised of two types of trimers, each involving contacts between hydrophobic groups on the surfaces of the helical molecules. One trimer (Figure 30d) involves hydrophobic contacts between equivalent residues, including Phe 22, Val 23, and Leu 26 of each molecule. The second (Figure 30) involves contacts between Trp 25 and Phe 22 of one molecule with Phe 6, Tyr 10, and Tyr 13 of another. The formation of these two kinds of interaction is not mutually exclusive, but clearly leads to extended oligomers and precipitation or crystallization.

The crystals studied by X-ray analysis were initially prepared from glucagon at 3 mg/mℓ at pH 9.0 in phosphate buffer and the pH was then adjusted to pH 6 or pH 7. This led to a small rearrangement of the crystal packing and a cell dimension change from a = 47.7 Å (at pH 9.0) to 47.1 Å at the lower pHs. Difference Fourier procedures

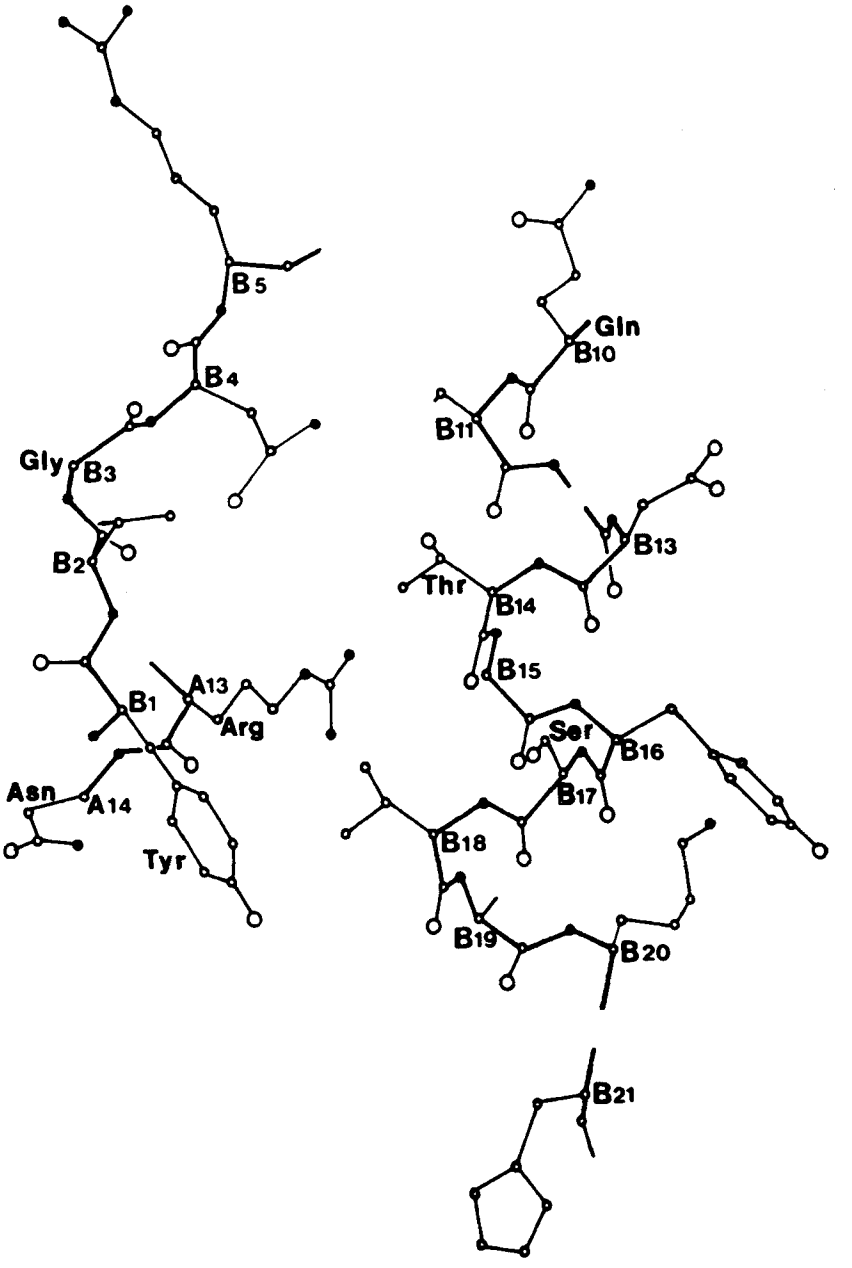
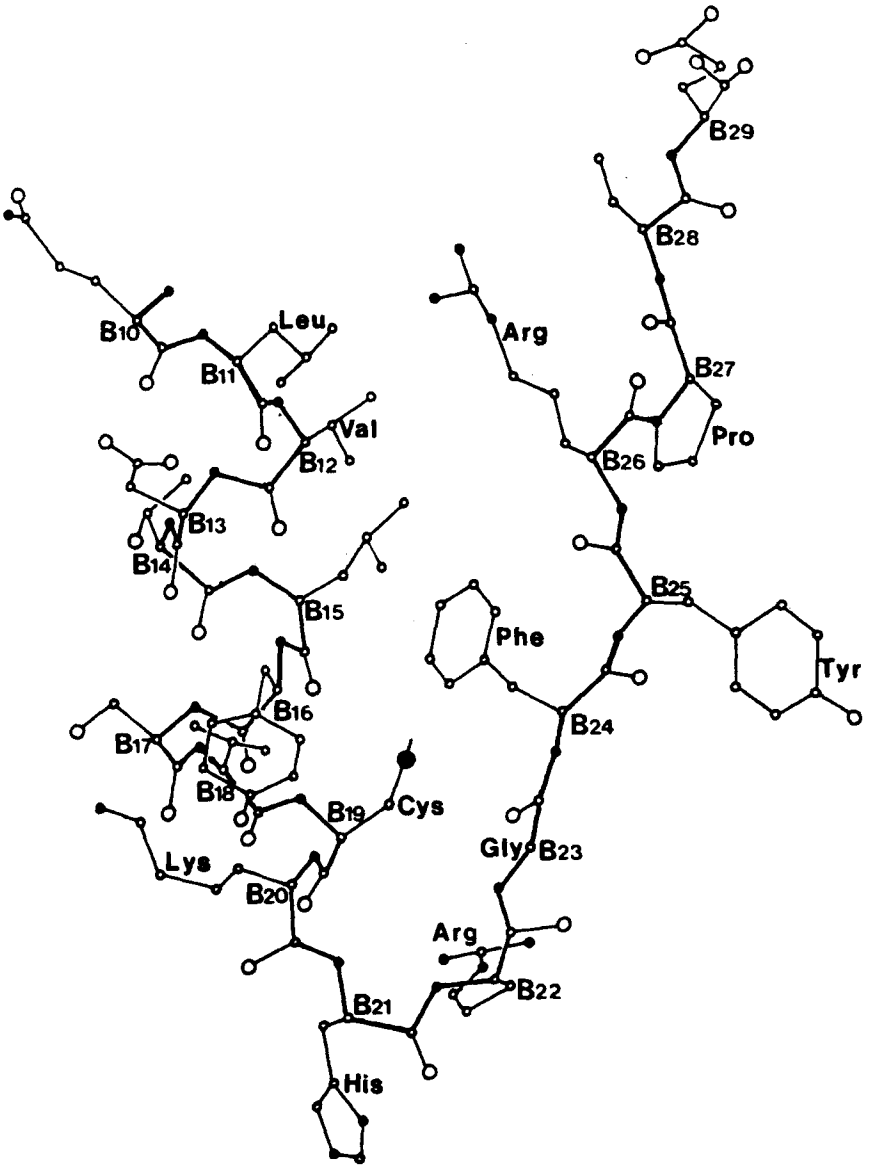


FIGURE 26. Distribution of hydrophilic side chains on the surface of the model of casiragua insulin which in porcine insulin is largely hydrophobic and involved in hexamer formation. (From Blundell, T. L. and Horuk, R., *Hoppe-Seyler's Z. Physiol. Chem.*, 362, 727, 1981. With permission.)

using the X-ray data indicate that the structure of glucagon is only a little different, but that a movement occurs in the flexible N-terminal residues on lowering the pH from 9.0. At pH 6.0 glucagon is very insoluble, but identical crystals can be obtained from glucagon solutions (1 mg/mℓ) in 0.02 *M* Tris/HCl. Similar cubic crystals (a = 48.7 Å) are also prepared at pH 3.0. These results strongly imply the existence of helical trimers in solution over the pH range from 3 to 9.5.

The glucagon molecules have no tertiary structure. They have secondary struc-



A

FIGURE 27. (A) The B-chain of casiragua insulin showing the location of B26 Arg and (B) the environment of B26 Arg in a casiragua insulin dimer would be hydrophobic and this substitution might be expected to disrupt dimer formation. (From Blundell, T. L. and Horuk, R., *Hoppe-Seyler's Z. Physiol. Chem.*, 362, 727, 1981. With permission.)

ture — a helical conformer — and quaternary structure — the trimers and oligomers of cubic symmetry. The crystal structure indicates that the helix may be stabilized by the formation of oligomers as it results in a removal of hydrophobic groups from unfavorable interactions with the aqueous solvent. These observations are consistent with the previously reported CD spectra which imply that helix is induced by self-association, although the far UV spectra are not consistent with the high percentage of helix observed in the crystal structure. More recently Wagman and co-workers have reported 270-MHz proton NMR spectra for glucagon at different polypeptide concentrations and con-

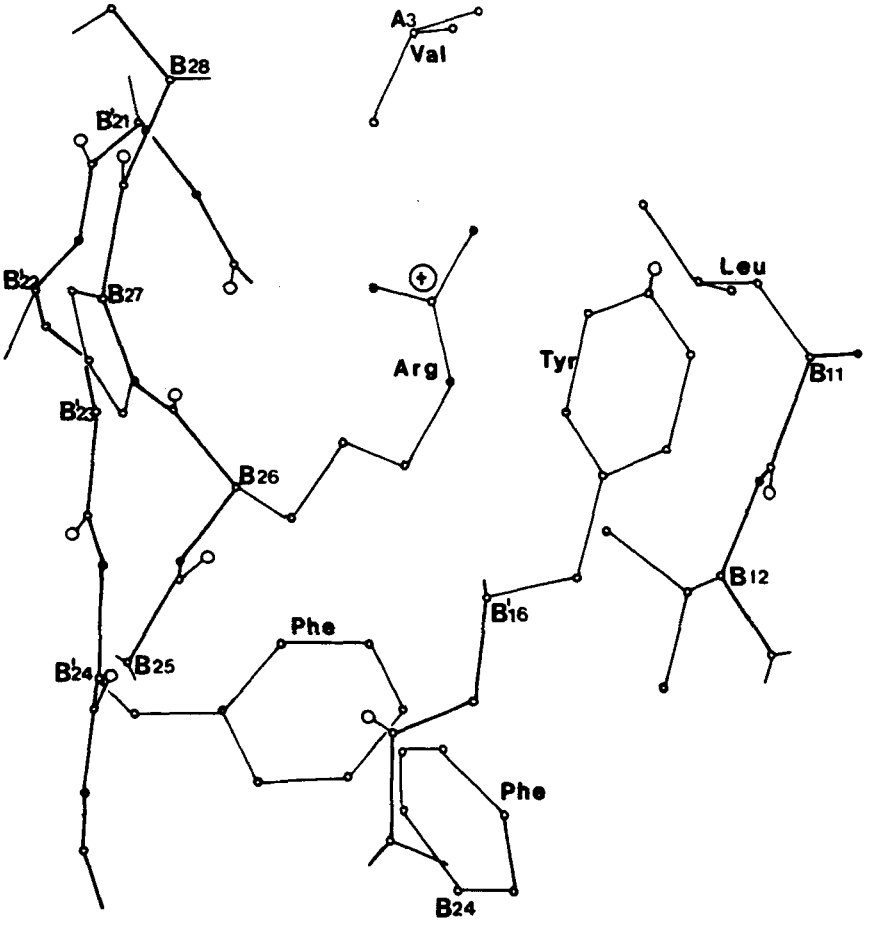


FIGURE 27B

firmed that the aggregated form of glucagon found in freshly prepared concentrated solutions is a trimer.[138] However they conclude that the trimer involving tyrosine interactions does not occur in solution at pH 10.6, since no perturbation of the resonance in segment Phe 6 to Tyr 13 was observed and there was no concentration dependence of tyrosine pKa. They conclude that the trimer involving contacts between the C-terminal residues forms first, although the alpha-helical structure is induced only in the region 20 to 27. This is consistent with the small amount (34%) of helix predicted by CD. They conclude that the further formation of alpha-helix is induced on oligomer formation through the intermolecular interactions observed in the crystal structure and characterizing the second trimer.

It is possible that lowering the pH would increase the percentage of the trimer involving the tyrosines, which would be ionized at high pHs, and that this increased formation of the trimer would account for the decrease of solubility of glucagon under these conditions.

IV. MOLECULAR BIOLOGY AND CONFORMATION

In this final section we explore the relationship between conformation and molecular biology of the four pancreatic hormones. We do not pretend to make a proper survey of the many avenues of research into the synthesis, storage, circulation, receptor binding,

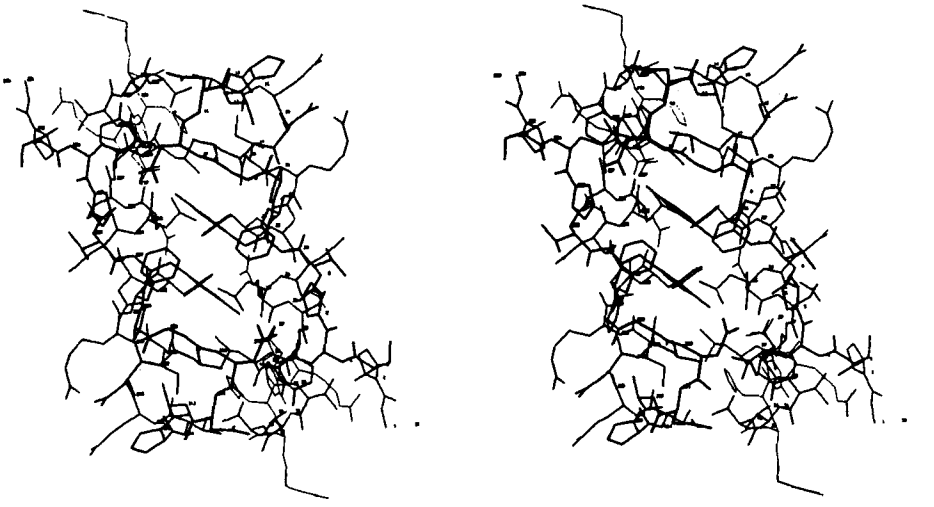


FIGURE 28. Stereo diagram of the aPP dimer with side chains viewed down the twofold axis.

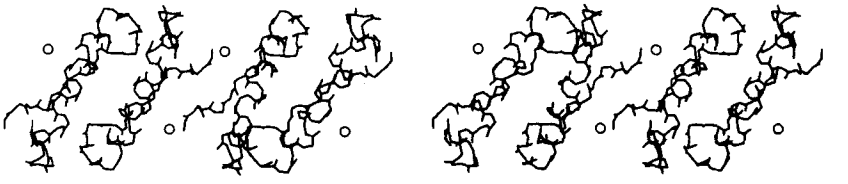


FIGURE 29. Stereo diagram of the backbone atoms of two aPP dimers illustrating the cross-linking by zinc ions.

and degradation of the hormones, but rather our intention is to discuss those aspects where knowledge of the conformation might be helpful in our understanding of the biology. For a more detailed treatise on these aspects the reader is advised to refer to the splendid reviews included in the *Chemistry, Structure and Function of Insulin and Related Hormones,* edited by D. Brandenburg and A. Wollmer.[139]

A. Precursors

There is now good evidence that all four pancreatic polypeptide hormones are synthesized as larger precursors which are processed prior to hormone storage in granules of the endocrine cells.

Of the four hormones, the insulin system has been most widely studied and is best understood. Insulin is synthesized as preproinsulin comprising a ~20 amino acid hydrophobic N-terminal extension of proinsulin.[140] Amino acid[141] and DNA[148,142-144] sequencing have demonstrated that both the sequence and the length of the extension are quite variable, but its function is conserved as a signal peptide involved in the location of the ribosomes at the endoplasmic reticulum and the extrusion of newly synthesized chain into the lumen of the endoplasmic reticulum. In the B-cell the lifetime of this precursor would be very short, and the extension may be removed before the molecule assumes an insulin-like conformation. Although the hydrophobicity of the residues of the extension might interfere with the folding process, the extension at the B-chain N-terminus would not sterically inhibit the insulin fold. It is even conceivable that the hydrophobic extension wraps itself around a largely folded proinsulin molecule, endowing it with membrane solubility.

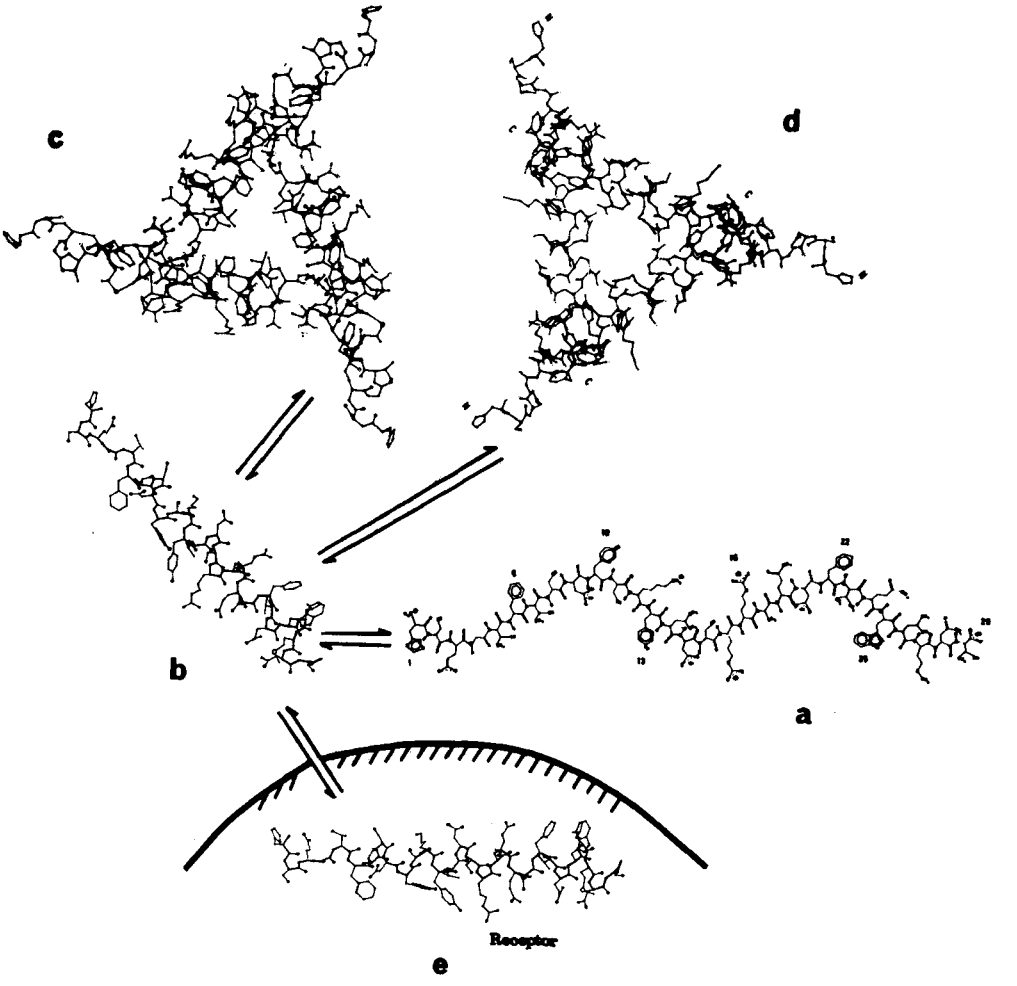


FIGURE 30. (a) The sequence of 29 amino acids in glucagon which in crystals exist as helical conformers (b) associated together in two types of trimers (c) and (d). In dilute aqueous solutions the trimers dissociate to give an equilibrium population of flexible monomers, but at the receptor a helical conformer (e) is probably stabilized.

Proinsulin is the major species involved in transport within the endoplasmic reticulum and Golgi prior to granule formation.[145] Proinsulin should assume an insulin fold leading to a correct pairing of the disulfide bridges as described in Section III.A.2 and form dimers and zinc hexamers. In the presence of zinc ions and at a neutral pH the proinsulin hexamer is more soluble than the insulin hexamer[114,146] and it would be less likely precipitated in the endoplasmic reticulum, an eventuality which would lead to obstruction of the supply of insulin to the Golgi (see Figure 31).

The sequences of the DNA for the rat insulin I and II have shown that each contains an intervening sequence of 119 base pairs in a region corresponding to the 5′ untranslated portion of the mRNA.[143,147] In addition, the rat insulin II gene contains a 499 base pair intervening sequence in the region of DNA coding for the C-peptide. More recently it has been shown that the human and chicken insulin genes resemble the rat II gene in having two intervening sequences, the second at exactly the same position in the C-peptide encoding region of the gene.[142,144] This similarity of the human, chicken, and rat II genes implies that their ancestral insulin probably contained two intervening

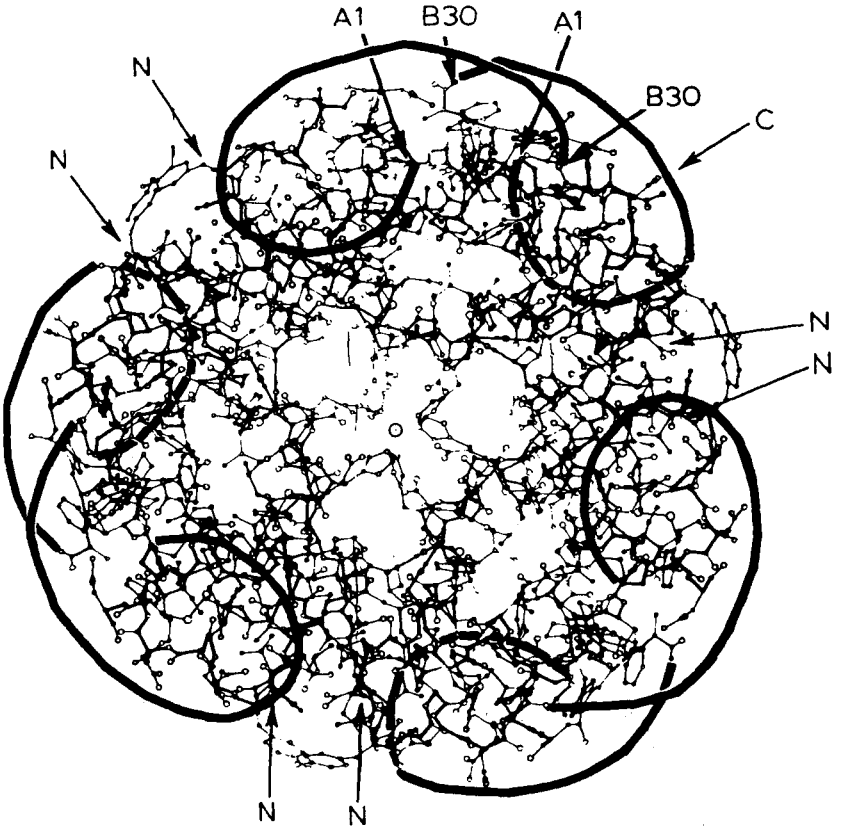


FIGURE 31. Diagrammatic representation of the accommodation of the proinsulin-connecting peptide over the surface of the 2-Zn insulin hexamer. (From Blundell, T. L., Dodson, G. G., Hodgkin, D., and Mercola, D., *Adv. Protein Chem.*, 26, 279, 1972. With permission.)

sequences. These observations are of interest with respect to the relationship of intervening sequences to the existence of "folding units" or "domains" in the tertiary structure.[143] Although the insulin molecule is itself a single "domain" in the sense that smaller entities (for example, the A and B chains) probably do not maintain their conformations independent of each other in aqueous solutions, the A- and the B-chains clearly operate as "folding units". The A-chain comprises two right-handed helices folded together in an antiparallel fashion, while the B-chain comprises two extended chain structures folded onto, and in contact with, the helical region (see Section III.A). These may represent interactions which are formed obligatorily at an early stage in the folding process. Thus the intervening sequence divides the insulin gene into two parts, which might be related to ancestral genes, coding for smaller peptides, each having a stable conformation available to it. In this respect there is a parallel with the position of intervening sequences in immunoglobulin genes in regions coding for the links between the domains, each of which must be evolved from a similar ancestral gene. Furthermore, the existence of the intervening sequences at this position coding for the insulin C-peptide may contribute towards its high variation in sequence, including additions and deletions, which finds its extreme in the much shorter C-peptides of the insulin-like growth factors.

As predicted from the analogy between relaxin and insulin, recent protein biochemistry and DNA sequencing has established the existence of a prorelaxin molecule.[149] Although only a few residues are needed to allow the B-chain C-terminus to be con-

tinuous with the A-chain N-terminus, the C-peptide is very considerably longer than even the C-peptide of insulin.[149] Rat prorelaxin has 164 amino acid residues, of which 105 constitute the C-peptide. There is little significant homology between the rat prorelaxin C-peptide and the proinsulin C-peptides. A surprising feature is the absence of a cluster of basic residues immediately adjacent to the C-terminus of the B-chain.

Proglucagon has recently been shown to contain the sequence of the glucagon extended by eight residues at the C-terminus which had previously been found in bulk crystalline glucagon preparations.[150,151] It appears to be closely related to glicentin, a 100 amino acid polypeptide from the intestine. Recombinant DNA techniques have shown that somatostatin is synthesized as a protein of 119 amino acids with somatostatin at the C-terminus,[152] consistent with the earlier observation of somatostatins with extended N-termini.[153,154]

Finally, Schwartz and co-workers[155] have identified a precursor of pancreatic polypeptide (PP) of molecular weight ~9000 from dog islets, which cross-reacts only with N-terminal-directed PP antisera; sequencing of this has confirmed the presence of PP in the N-terminal region.

Thus all pancreatic polypeptides appear to have large prohormones which exist prior to hormone storage once the signal peptide has been removed. Yet pancreatic polypeptide, glucagon, and somatostatin do not require a longer precursor to allow correct folding of the polypeptide as required of proinsulin; glucagon has little defined structure, and somatostatin and pancreatic polypeptide are single-chain conformers which should easily fold from a random chain. What then is the rationale for the existence of large precursors of these pancreatic hormones?

There are several possible arguments for a larger precursor. First, Steiner has suggested that a certain minimum length of peptide chain may be required for the signal peptide to locate its receptor on the endoplasmic reticulum and to allow the newly synthesized chain access to the lumen.[140,141] This in turn may favor the synthesis of several different hormone activities in tandem on one polypeptide chain as corticotropin-β-lipotropin exemplifies.[156] Second, the extra peptide of the precursor may allow it to assume a stable globular structure, endowing it with an increased solubility and a decreased susceptibility to enzymatic degradation. The increased solubility would be of particular importance to glucagon, which would have very low solubility in the lumen of the endoplasmic reticulum, giving rise to precipitation or binding to the lipid bilayers of the membranes. The stable globular structure should also decrease unwanted, nonspecific enzymatic degradation while at the same time enhancing the relative susceptibility of the peptide linkages which are cleaved in the process of conversion of the prohormone to the hormone. In the case of proinsulin, the relative susceptibility of the B22 Arg-B23 Gly and B29 Lys-B30 Ala bonds to tryptic-like digestion should be decreased (this will also be affected by self-association). The existence of the larger precursor of glucagon and pancreatic polypeptide may also decrease the availability to proteolytic enzymes of the sequences Arg (17)-Arg (18) of glucagon and Arg (25)-Arg (26) of mammalian PPs, which would superficially appear to be recognition sites for processing enzymes. Finally, parts of the extended precursors may reflect evolution from larger molecules with different but related biological activities, such as glicentin in the case of glucagon. The N-terminally extended somatostatin of 28 amino acids which has been characterized from porcine intestine and ovine hypothalamus[153] and is ten times more potent than the tetradecapeptide may constitute a further example analogous to the natural opiates, the enkephalins, and the β-endorphins.[156]

B. Storage Granules

Although all the amino acids of the prohormone are packaged in the granule and eventually released into circulation, in the case of insulin and glucagon at least, the

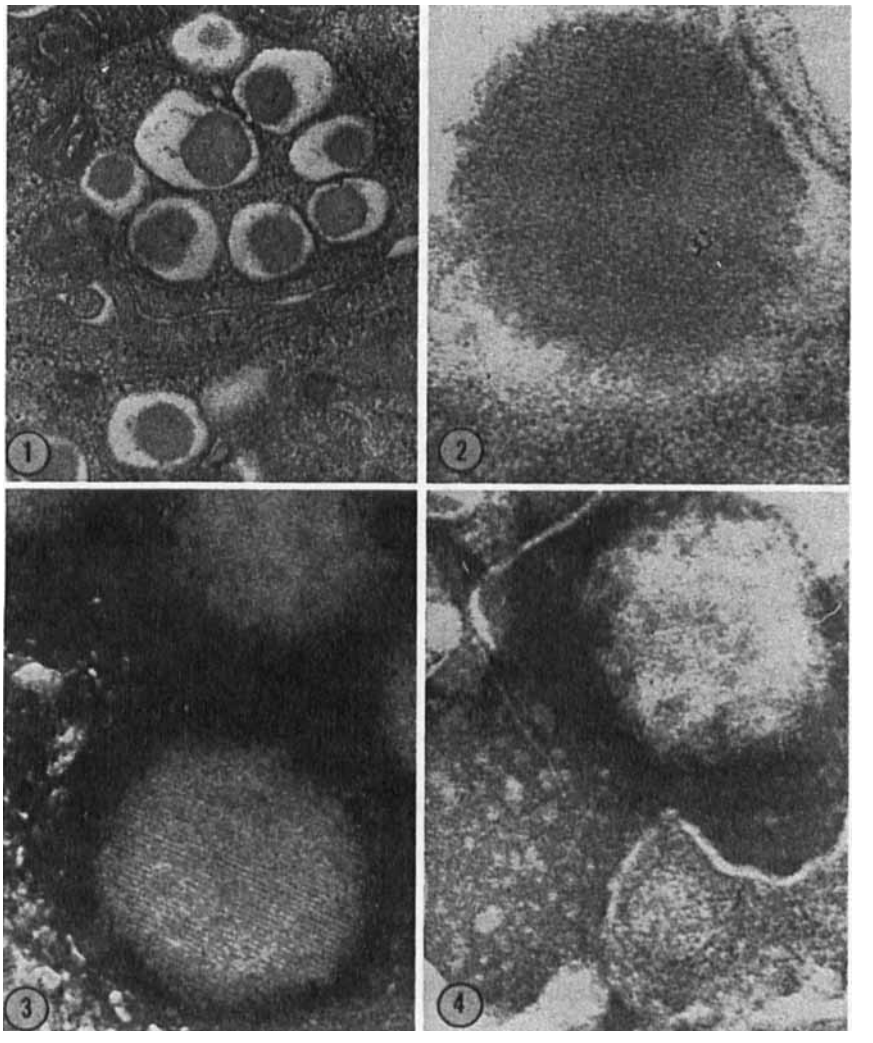


FIGURE 32. Electron micrographs of thin sections of rat beta-granules within an islet cell (1) × 48,000 and (2) × 247,000, and negatively stained beta granules (3) × 200,000 and (4) × 190,000 showing the angular profile of the granules and clear 50 Å repeats. (From Greider, M. H., Howell, S. L., and Lacy, P. E., *J. Cell Biol.*, 41, 162, 1969. With permission.)

prohormones are processed by membrane-bound enzymes before maturation of the granule.[157] For insulin and glucagon this probably leads to precipitation of an initial amorphous granule which may eventually mature to a crystalline array.

For insulin the presence of zinc indicates that zinc insulin hexamers are formed initially. This is consistent with the presence of high zinc concentrations in granules of insulins which have been shown in vitro to form zinc insulin hexamers (see Section III.B.1). The evidence from electron microscopy and diffraction that the granules (Figure 32) might be crystals of 2-Zn insulin hexamers similar to those formed in vitro has been reviewed in Section II.A.3. However, there is some uncertainty concerning the pH and metal ion concentrations in the storage granule. If the pH is ~ 7.4, the zinc insulin hexamers would bind extra zinc ions at a number of sites on the hexamer surface and possibly also at the B13 glutamate residues in the central channel, sites which have been

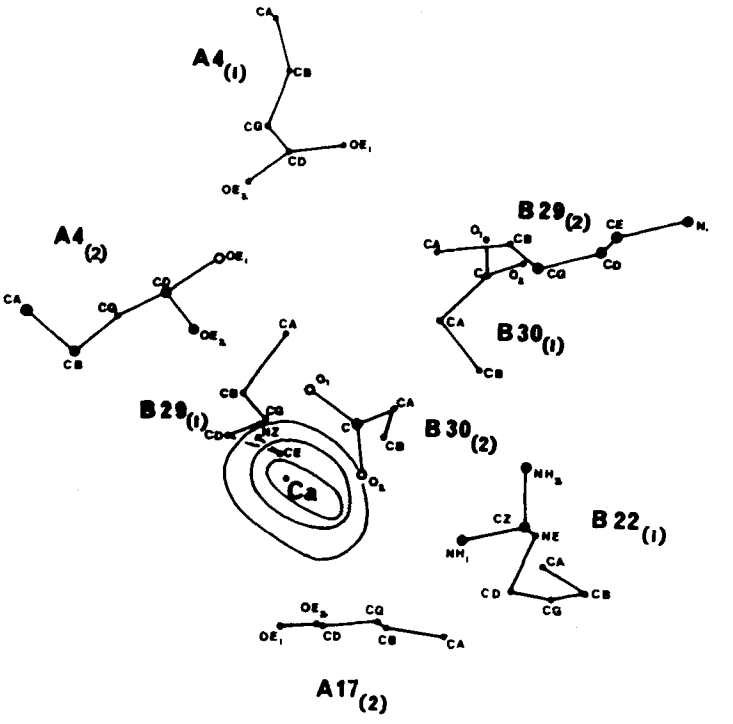


FIGURE 33. Schematic diagram of the calcium binding site of porcine insulin.

defined by X-ray analysis of the crystals.[35,158] However, under these conditions zinc insulin does not easily crystallize; a lower pH ~ 6.3 is preferred for human and porcine insulins at least (Section II.A.1). It may in fact be the presence of Ca^{2+} ions which further ensures the formation of the crystals, for it has been demonstrated by X-ray crystallography that Ca^{2+} ions can cross-link adjacent hexamers (through A4 and B30) and stabilize the crystal lattice[35] (Figure 33). It has been demonstrated that granules contain high concentrations of Ca^{2+} ions which are to some extent independent of other pools of intracellular calcium.[159,165,166]

Unlike B-cell granules, the glucagon-containing granules of the mammalian A-cell are usually amorphous. This is not surprising as in vitro experiments show that close to neutral pH glucagon normally gives an amorphous precipitate. Only under very carefully controlled conditions at relatively low concentrations can glucagon crystals be prepared at pH 7.0.[6,17] However, histochemical examination of amorphous A-cell granules using xanthydrol indicates that the crystal trimer is probably present here also.[160] This reagent interacts with tryptophan in the crystal trimer to produce an unusual grey color which is probably due to the tryptophan environment; the same environment seems to be present in the amorphous granules. The granules of several teleost A-cells are clearly rhombic dodecahedral crystals similar to those found in vitro (see Section II.A.3), confirming the presence of glucagon trimers and oligomers. Recently carp glucagon crystals in vitro have been shown to be cubic rhombic dodecahedra like those of porcine glucagon.[161] The difference may be that small changes of sequence (as yet undefined) may increase the solubility of the glucagon and allow crystals rather than amorphous granules to form in the teleosts.

Somatostatin[162] and PP[163] immunoreactive materials have been localized in electron-dense granules of pancreatic islet cells, but no examination of their contents has been reported. The possibility that PP oligomers are crosslinked by zinc ions in turkey or

chicken F (PP)-cell granules, as they are in the crystals (see Section III.B.2) is an attractive analogy with the role of zinc insulin hexamers in B-cell granules. However, while mammalian PPs may form dimers, studies in vitro show that they cannot form zinc oligomers.[164]

In summary, it appears that pancreatic hormones may be self-associated and occasionally crystalline in the storage granules of the endocrine pancreas. This appears to have several advantages. The formation of amorphous or crystalline aggregates would be an effective form of concentration of the hormones. This would, for the smaller peptides, minimize problems due to osmotic pressure. The hydrophobic surface regions of the monomers (see Section III) are inaccessible in the oligomers and unavailable for reaction with the membranes of vesicles enclosing the granules. The oligomers would also be less accessible to slow proteolytic degradation by the enzymes which are responsible for the conversion of the prohormone to the hormone.

If the formation of oligomers is selectively advantageous to the organism in evolution, why have many hystricomorph rodents, including the porcupine, guinea pig, and casiragua, lost the ability to form oligomers (see Section III.B.)? Porcupine insulin loses the ability to form dimers and zinc hexamers as a result of a change in a residue (B22) which is not directly involved in quaternary interactions but which leads to a change of tertiary structure (see Section III.B). It is possible that this change is selectively advantageous in decreasing proteolytic cleavage of the porcupine insulin (B22 is normally arginine and is susceptible to trypsin-like cleavage) even though the tertiary structure is changed and the quaternary structure is lost.[98,104] The amino acid substitutions observed in guinea pig insulin would then stabilize the monomeric form by making it more hydrophilic. The changes in the casiragua amino acid sequence may also derive from a similar advantage in protection from proteolytic degradation, although by a less obvious mechanism.[98] The diversity in the hystricomorph insulins may then be rationalized as different but related responses to a change of enzymes available in the endoplasmic reticulum or Golgi and possibly those involved in conversion of the prohormone to the hormone.[98,167] However, the observation that some hystricomorph insulins have high growth-promoting ability may indicate evolution towards a growth factor role.

The formation of amorphous or crystalline granules of oligomers may be an alternative method of storage to complexation with a large protein, such as the binding of the neurohypophyseal hormones, oxytocin or vasopressin, with neurophysins. A similar situation exists with nerve (NGF), epidermal (EGF), and insulin-like (IGF) growth factors.[123,168] This would be a more viable alternative in the case of growth factors, for which the equivalent of the endocrine gland is the circulation. The growth factors are required to be released more evenly over a longer period of time than the pancreatic hormones which have half-lives in the range of 3 to 6 min. On the other hand, it is possible that small extensions in the half-lives of insulins may be obtained by changes in the crystallinity and the crystalline forms of the granules (as observed among species), which would affect their rate of dissolution once in circulation.

C. Circulation, Receptor Binding, and Biological Activity

1. Hormones in Circulation

When released into the circulation the hormones experience a rapid and large dilution. Consideration of the known association constants for insulin, PP, and glucagon indicates that at circulating concentrations of 10^{-9} to 10^{-11} *M*, the monomeric species will predominate.[128,169,170] The only exception may be for the pancreatic polypeptide (aPP) of chickens, where circulating levels are quite high[171] and the dimer is more stable than for other species.[8,75]

Our discussion of the conformation of monomers (Section III.A) has indicated that the larger molecules, insulin, relaxin, pancreatic polypeptide, and the growth factors, are

likely to circulate as relatively stable globular structures. On the other hand somatostatin and glucagon will exist as an ensemble of conformers with some limitations on the flexibility resulting from the disulfide bridge of somatostatin and the Val 22-Trp 25 interaction of glucagon.

2. Hormone Receptor Systems

We must now consider the evidence relating to the conformer (or conformers) which binds the receptor and leads to a biological response.

In this respect it would be clearly advantageous to study directly the complexes between the hormone and the purified membrane receptor. However, in spite of the initial optimism in the period following the early receptor binding studies for insulin and glucagon on isolated cells and membrane preparations, progress has not been very impressive. Although advances in affinity chromatography have been useful,[172] the problems of dealing with membrane proteins present as a very small percentage of the total have been daunting. More particularly the loss of biological activity once the system has been disassembled has meant that the receptor can only be followed by hormone binding studies, and hormone affinity may be decreased in the isolated receptor.

The most promising developments have involved the cross-linking of the hormone to the receptor either by use of conventional crosslinking agents or by photo affinity labels which can provide a marker during purification (see Czech et al.[232] for a review). The insulin receptor appears to be a multi-subunit glycoprotein system linked by disulfide bridges rather like an immunoglobulin.

Here we consider receptor binding from isolated cells, membranes, and solubilized receptors.[139] Where possible, the receptor binding and biological response are measured on the same cell system (Figure 34), but this has often led to difficulties where the rate of degradation is high, as for insulin in liver cells. In contrast, careful receptor binding studies may be carried out on lymphocytes where the responses are of more doubtful physiological relevance but degradation is minimized.[173] For correlation of structure and function, in vivo studies are now considered less useful, as they are complicated by degradation (see Section IV.C).

The identity of the receptor-bound conformer and the amino acid residues involved directly in the hormone receptor interactions is approached by analysis of the hormone receptor affinity constant, the kinetics of the association and dissociation processes, and comparative studies of various hormone analogues.

3. Insulin Receptor Binding Region

For insulin, De Meyts and co-workers have examined the temperature and pH dependence of receptor binding on cultured human lymphocytes and presented an analysis of the thermodynamics of the interaction.[174,175] The thermodynamics were not classical since a nonlinear van't Hoff plot was obtained. However the change from an entropically driven process at low temperatures (5°C) to an enthalpically driven process at higher temperatures (37°C) and the observation of a large negative heat capacity (Figure 35) change at 25°C were interpreted as indicative of a predominantly hydrophobic interaction. Estimation of the heat capacity which might be contributed by exclusion of solvent from the surface indicated that a surface area, larger than that involved in dimerization, must be buried in receptor binding. However the analysis was based on the assumption of a simple one-step reaction. More complicated binding reactions could generate the observed nonlinear van't Hoff plot in the absence of hydrophobic effects. The pH optimum for insulin binding drops with increasing temperature to 37°C. This finding was used to correct the thermodynamic analysis for ionization enthalpies and also to define the nature of the ionizable groups from hormone and receptor involved in the interaction. Two groups were identified, one probably being an

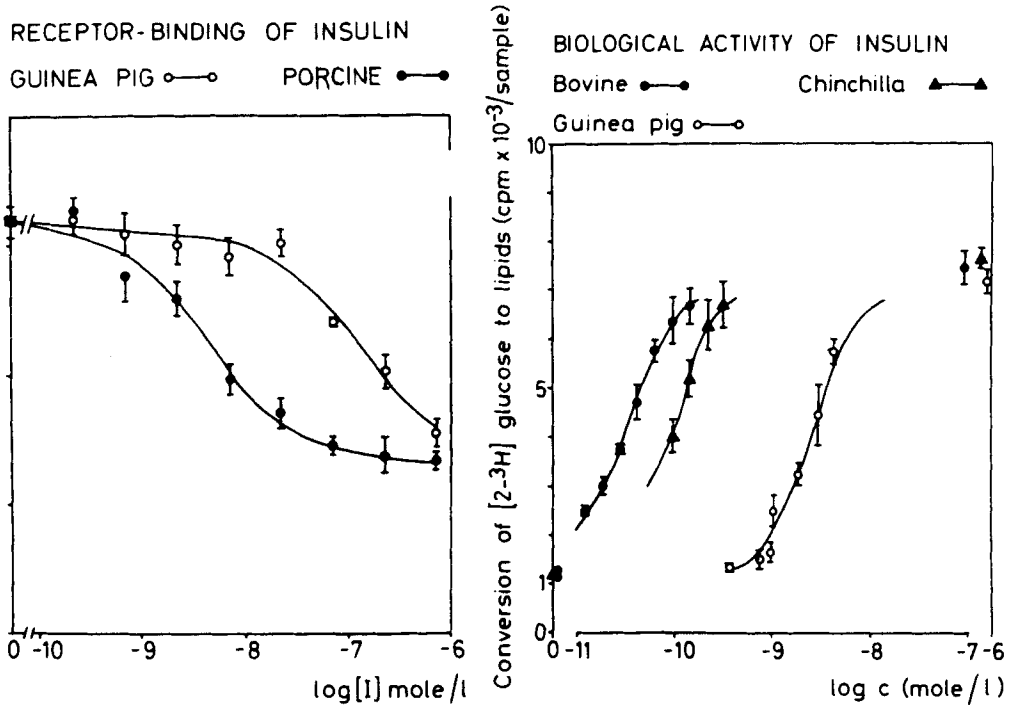


FIGURE 34. A comparison of the ability of porcine and guinea pig insulins to inhibit the binding of ^{125}I-labeled insulin to insulin receptors of fat-cell membranes. (B) a demonstration of the stimulation by bovine, chinchilla, and guinea pig insulins of the incorporation of (^{3}H) glucose into lipids by rat fat cells shown as a logarithmic function of insulin concentration in incubation. (From Pullen, R. A., *Mol. Cell. Biochem.*, 8, 1, 1975. With permission.)

unprotonated carboxyl group in a hydrophobic environment on the receptor and the other an alpha amino group (possibly A1 Gly of insulin).

The first proposal for the nature of the receptor binding region was presented in 1971 on the basis of the receptor binding and biological activity of different species of insulins and of chemically modified insulins.[2,3] The receptor binding was proposed to involve an insulin conformer similar to that defined by X-ray analysis with receptor interactions mediated by a largely invariant surface region, part of which is involved in dimer formation. The most recent model is very little changed and is shown in Figure 36.[52,169] The size of the surface region is consistent with the thermodynamic analysis, being larger than the region involved in dimer formation. It includes a central hydrophobic region (B24 Phe, B25 Phe, B26 Tyr, B12 Val and B16 Tyr, A19 Tyr) and polar side groups A1 Gly, A4 Glu, A5 Gln, A21 Asn, B13 Glu, B21 Glu, B22 Arg, and possibly B9 Ser, B10 His, and B27 Thr on the periphery.

Much of the chemical modification has been centered on A1, where maintenance of the positive charge appears to be critical, although quite large groups can be added with little loss of receptor affinity.[52,107,176,216,217] The conformation of the insulin molecule is very sensitive to modification at A1. Many of the modifications of A1, especially where there is a change of charge or a conformational change, lead to a decrease of dimerization similar to that of receptor affinity, even though A1 is not directly involved in dimer interactions.[52] This is a further parallel between receptor bonding and dimerization.

However, this parallel does not always hold for modification at A1. In the special case of the insulin cross-linked between A1 and B29 by diamino suberic acid, the conformation (see Section III.A.1) and equilibrium constant for dimer formation are close to

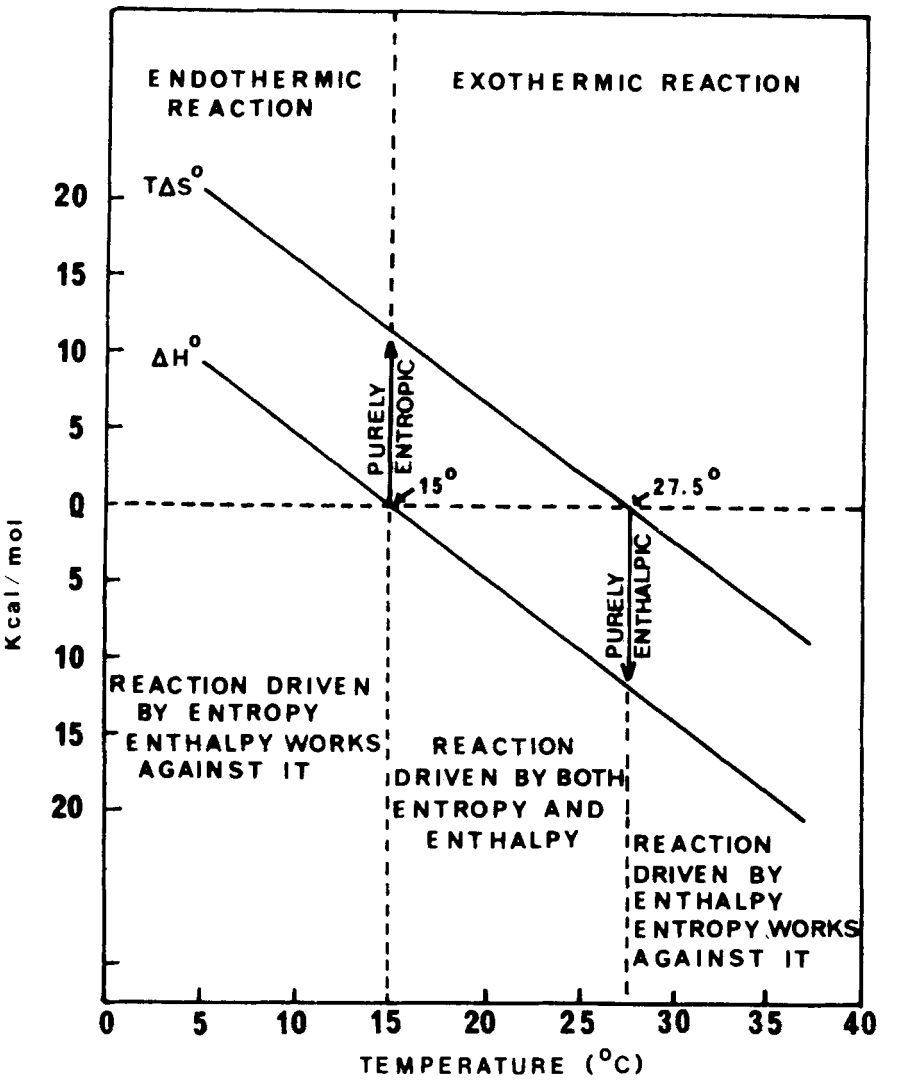


FIGURE 35. Driving forces in the insulin-receptor interaction derived from study of the temperature dependence of receptor binding. (From Waelbroeck, M., Van Obberghen, E., and De Meyts, P., *J. Biol. Chem.*, 254, 7736, 1979. With permission.)

those of insulin, although there is a reduction of receptor binding and biological potency on a fat cell to 5% that of native insulin.[109] This may result from direct interaction with the receptor of the modifying group, which will have less flexibility than groups attached only to A1. It is important to note that the model assuming a receptor binding region larger but inclusive of the region involved in dimer formation requires a decrease of receptor binding if the dimerization is reduced, but not necessarily the converse. Nevertheless, it is possible that the decrease of receptor binding is due to inhibition by the cross-link of an obligatory conformational change as suggested by Dodson et al.[109]

The hagfish insulin also has low receptor affinity but dimerization similar to that of bovine insulin.[137,177] In this case the reduced receptor affinity may relate to the presence of B9 Lys, B10 Asp, B21 Val, or B27 Asp residues which are not found in these positions (on the periphery of receptor binding region) in any fully active insulin. However, little chemical modification has been reported for these residues.

Modification of the amino acid residues in the region B22—B27 has been the subject

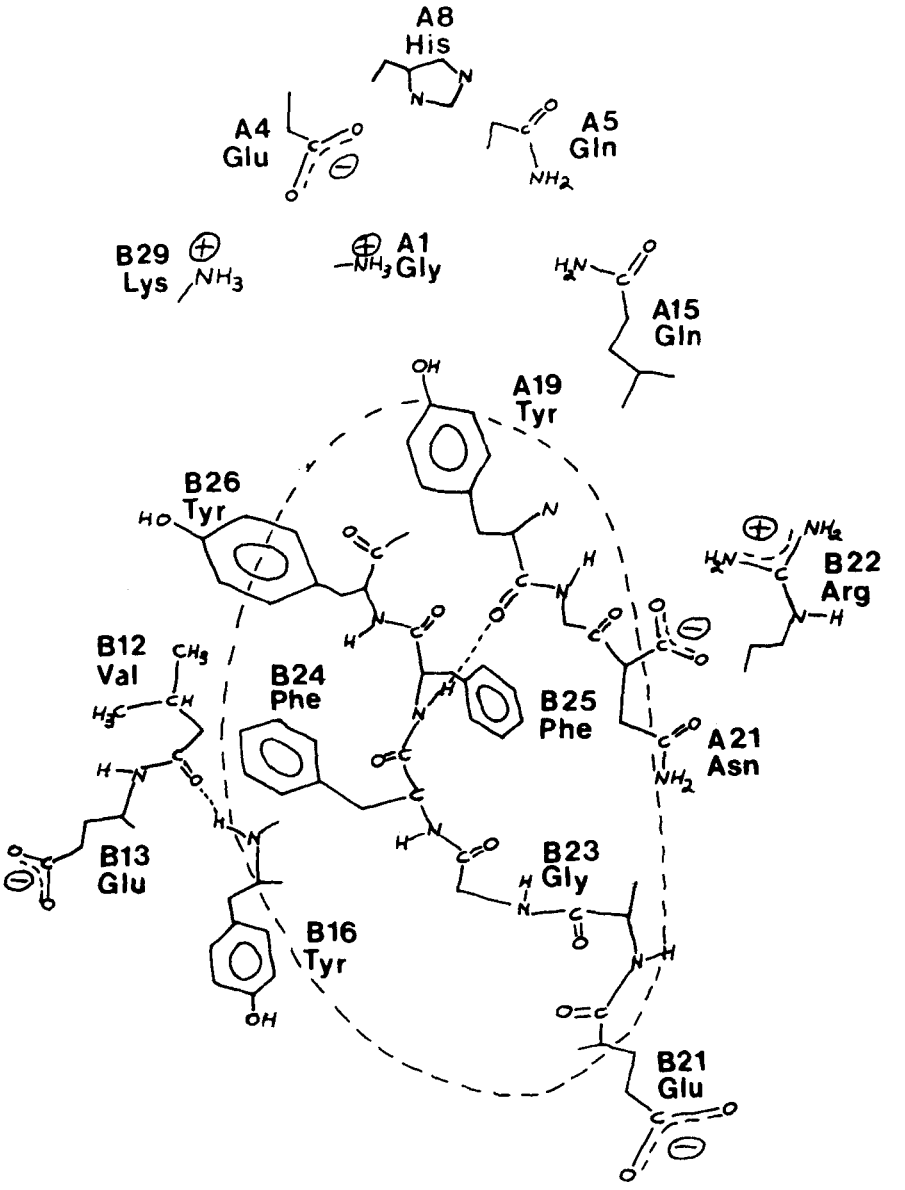


FIGURE 36. A diagrammatic representation of the arrangement of amino acids on the insulin molecular surface which are probably involved in receptor binding.

of extensive detailed studies. Natural analogues with modifications in these residues are provided by the hystricomorph insulins, of which the guinea pig, casiragua, and porcupine have been characterized in detail.[69,167,178] B22 Arg is substituted by Asp in guinea pig and porcupine, and both show considerably reduced receptor affinity,[104] but this, like the reduced dimerization, might be mediated by the conformational change. Casiragua insulin has a B25 Tyr and B26 Arg which would introduce a positive charge into an uncharged and largely hydrophobic region, and this may lead to reduced receptor affinity.[98,99]

The Shanghai, Aachen, and other insulin groups have been carrying out a series of modifications using enzymatic degradation and partial replacement. They have prepared

despentapeptide (des B26—B30),[179] deshexapeptide (des B25—B30),[113] desheptapeptide (des B24—B30),[111] and desoctapeptide (des B23—B30)[180,68] insulins which have decreasing activity with decrease in the length of the B-chain. As residues are added back to the desoctapeptide insulin, the activity increases with the addition of B23 as glycine or D-alanine, and B24 as phenylalanine restoring much of the receptor affinity.[219,224]

Interestingly, Kikuchi et al.[233] have shown that an insulin B-chain pentapeptide corresponding to residues B22-26 can at high concentrations potentiate both the biological activity and receptor binding of suboptimal concentrations of insulin (or insulin where B22-30 are removed).

Tager et al.[181] have recently described a mutant human insulin where either B24 or B25 phenylalanines was substituted by leucine. This insulin has low activity. Subsequently both B24 Leu and B25 Leu insulins have been synthesized by a combination of solid-phase synthesis and semisynthesis from enzymatically degraded insulin.[182] These analogues showed only 10% and 1%, respectively, of the activity of porcine insulin in competing for the binding of ^{125}I-iodoinsulin to both rat adipocytes and human lymphocytes. Although individually both analogues had full agonist activity, Tager et al.[182] found that the B24 Leu analogue was an active antagonist of insulin action when assayed mixed with native insulin.

This antagonistic effect was not reproducible in the hands of de Meyts et al.,[234-236] although these authors agreed with the low receptor binding of the [Leu B25] analogue, a result of particular interest in view of the lack of conformational change. B25 phenylalanine is therefore strongly implicated in receptor binding. When both B24 and B25 are substituted by alanine, larger conformational changes occur in the B24 substituted analog and in this case it has less activity than the B25 analogue.[228]

The semisynthetic route for the production of insulin analogues starting with desoctapeptide insulin and adding peptides of varying character should provide a useful selection of variations in the central part of the receptor binding region.[139,213]

In general, receptor binding and biological response at least on the fat cell system are proportional. This indicates that the region responsible for most of the receptor affinity is sufficient for biological response. However, the result described above concerning antagonistic activity of B24 Leu insulin indicates that B24 Phe may be more critical for activity than it is for receptor affinity. The lower activities of hagfish,[177] porcupine,[104] and A0 Arg[176] (arginine residue added at A1 Gly) insulins compared to their receptor binding in some assays indicate that other residues may also be more critical for biological response than for receptor affinity.

The importance of the residues involved in dimerization to full potency could be due to the fact that the dimer binds to the receptor. However, we have seen that the thermodynamic data are consistent with a monomer in solution but this does not preclude dimerization at the receptor. However, if the receptor binding region is larger than the dimerization region, it should be possible to inhibit dimer formation while still leaving some of the receptor binding region undisturbed. This appears to be the case, as guinea pig,[183] porcupine,[104] casiragua,[99] and tetranitroinsulins[184] have no measurable affinity for dimerization, although all are full agonists albeit with much reduced receptor affinity. It should also be possible to decrease receptor binding with little reduction of dimerization as in [B25 Leu] insulin[227,235] and hagfish insulin.

In summary, the present data allow the following statements concerning the receptor binding of insulin:

1. No fully potent insulin (i.e., one which is not only a full agonist but also has full receptor affinity) has a conformation very different from that of porcine insulin, a reduced ability to dimerize, or any change in the amino acids included in the putative receptor binding region of Figure 36.

2. A monomeric insulin can be a full agonist but will have reduced receptor affinity.
3. Small changes in conformation or the nature of some of the amino acids in the putative receptor binding region (Figure 36) will lead to reduced receptor affinity, although in most cases the analogues are full agonists.

These observations are consistent with the model for receptor binding proposed involving a complementarity in shape, hydrophobicity, charge, and hydrogen binding capacity between the receptor and the insulin receptor binding region. However, an important role for conformational changes and protein flexibility is not excluded. Initial recognition may be accompanied by a conformational change which is available to all the fully active insulins so far studied. The conformational flexibility of insulin, for example, between molecules I and II in the X-ray structure or between the 2-zinc and 4-zinc forms, is well established, and similar changes may very easily occur on receptor binding. However, if this were so, it should be eventually possible to trap an analogue in this conformation, which should then bind the receptor with higher affinity. The model does not exclude covalent changes involving either addition or bond breakage from the mechanism of receptor binding, although this would have to happen at a stage after initial receptor binding as whole insulin can be removed from the receptors. The ability of antireceptor antibodies to activate the receptor also makes the critical role of a specific covalently modified or degraded insulin unlikely.[185]

4. Receptor Binding of Insulin-like Growth Factors

The receptor binding model outlined in the previous section would imply that IGFs should bind to insulin receptors with reduced affinity if the models described in Section II.A.3 are correct. These models suggest that the residues of the hydrophobic region involved in dimer formation are largely accessible. In fact they are more accessible in IGF II than in IGF I, and so IGF II should bind insulin receptors more strongly, a prediction borne out by subsequent studies of Zapf et al.[186] and de Meyts and co-workers.[187] The abilities of these two growth factors to stimulate the conversion of glucose to CO_2 in adipocytes is lowered by 120 and 60 times, respectively, compared to insulin.

It is now becoming apparent that three groups of factors are closely related, if not identical, to the IGFs: somatomedin A, somatomedin C, and multiplication-stimulating activity (MSA). They all stimulate incorporation of sulfate into proteoglycans of cartilage, they have mitogenic activity in fibroblasts, their concentration in serum is regulated by growth hormone, and they share the ability to give insulin-like effects on adipose and muscle tissue (see Blundell and Humbel[168] for a review). However, the discovery that these IGFs (or somatomedins) are recognized by the insulin receptor does not explain why their potency in promoting growth effects in fibroblasts is about 300 times that of insulin. The explanation appears to lie in the existence of specific receptors which are responsible for growth-promoting effects and which can express activity even when the insulin receptor is blocked with antireceptor antibodies.[188]

These observations have prompted attempts to discern the structural features which are responsible for the growth-promoting specificity. By extension of the model described for insulin and using the knowledge that insulin binds weakly to the growth-promoting receptors, it has been suggested that the growth factor receptor is complementary to both the insulin-dimerizing surface and the adjacent A-chain extensions. Test of this hypothesis requires further receptor binding and biological activity studies which Kahn and co-workers are now carrying out.[189] Interestingly, some of the hystricomorph rodent insulins are more potent growth factors than expected from their poor ability to bind insulin receptors, and Lazarus and co-workers have speculated that the ability of the hystricomorph receptor to accommodate rather drastically altered

insulin analogues may be an indication that this receptor is linked primarily to the production of mitogenic effects.[104,190]

5. Negative Cooperativity

Scatchard analysis of insulin receptor binding data using a variety of cell membrane preparations has revealed curvilinear plots, and initially this was interpreted as indicating two receptor populations, a low-capacity high-affinity set and a much larger pool with a lower hormone affinity. De Meyts, however, suggested that the data could be interpreted in terms of negative cooperative interactions between receptor sites.[191] Insulin binding to its receptor may induce a loss of affinity of the other receptor sites due to an accelerated dissociation rate of the insulin-receptor complex. This has subsequently been demonstrated experimentally, but the interpretation of Scatchard curves and the dissociation rate experiments has been disputed.

In order to identify the regions of the insulin molecule which might be involved in triggering the site-site interactions, De Meyts and co-workers hae studied a large number of insulin analogues in a kinetic assay measuring negative cooperativity.[192] Only a small number of analogues showed impaired cooperativity, even though some of those tested had large modifications and very low activities. The loss of cooperativity became more severe with the progressive removal of residues from the B-chain C-terminus. B25 appears to be of particular importance, as both de Meyts et al.[235,236] and Olefsky et al.[237] find that (B25 Leu) insulin does not show negative cooperativity. The most extreme loss of cooperativity was observed for desoctapeptide-insulin (with eight residues removed from the B-chain) or with DAA insulin where the two C-terminal residues were removed with carboxypeptidase digestion. Although these analogues show very low activities, their inability to induce cooperativity was observed at concentrations much higher than those necessary to saturate the receptor pool. These findings led to the designation of a subsite within the receptor binding region of insulin involving principally the hydrophobic central region (B23—B26) and A21. At high concentrations of dimerizing analogues the cooperativity is diminished, but this is not the case with monomeric analogues. This implies that dimers can bind to the receptor, possibly through a "back to back" insulin dimer involving surfaces normally involved in contacts between dimers in the hexamer since the other surface is presumably buried at the receptor contact region. IGFs are fully cooperative and there is an indication that this can be reduced at high concentrations.[187] It seems therefore that the IGFs can dimerize, but the nature of this dimer is unclear (see Figure 37).

The IGFs and casiragua insulin show sequence alterations in the proposed cooperative site residues, B25 and B26 being tyrosine and phenylalanine in IGF, and tyrosine and arginine in casiragua insulin. As both molecules are fully cooperative, glycine followed by two aromatic residues would appear to be the requirement for cooperativity.

The physiological significance of negative cooperativity may be related to desensitizing receptors in the presence of excess hormone but it is hard to substantiate at present, especially since the effect is most marked at low temperatures. However, the phenomenon is proving to be a useful tool in the study of receptor-receptor interactions, especially with covalently linked insulin molecules.[187]

6. Glucagon Receptor Binding

The affinity constant for the glucagon receptor binding is $\sim 10^9$. Rodbell et al. have suggested that destabilization of the hormone-receptor complex at low temperatures and in the presence of urea is indicative of hydrophobic interactions.[193] They have further shown that tyrosyl residues are involved in receptor binding.[194] Iodinated glucagon binds the receptor with increased affinity at pH7.0, but in alkaline solutions the affinity

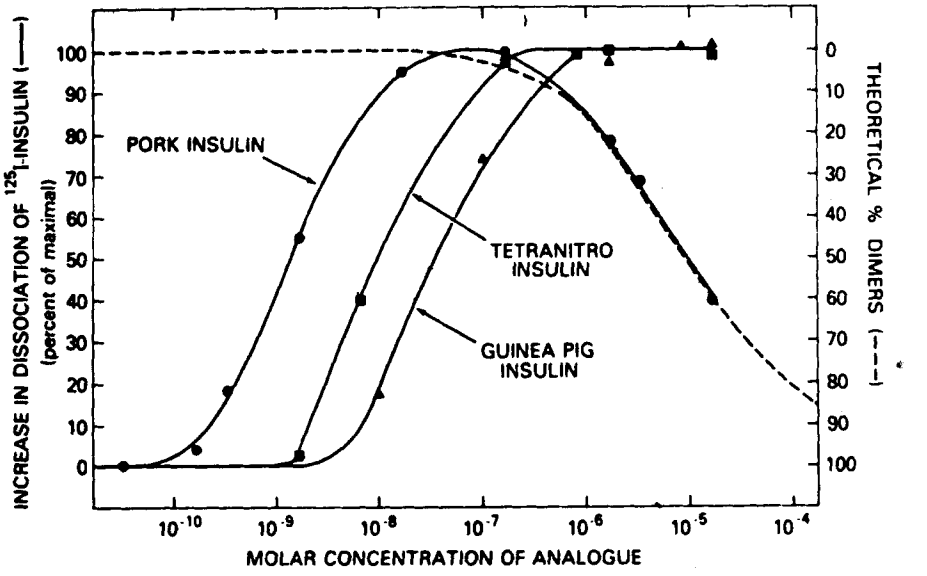


FIGURE 37. Effect of insulin dimerization on negative cooperativity. The ability of porcine insulin and two nondimerizing insulins to induce negative cooperativity measured as their ability to accelerate the dissociation of ^{125}I-insulin. The assay includes high concentrations of the analogues, where dimerization of insulin becomes significant. The dashed line represents a theoretical curve for dimerization. (From De Meyts, P., Van Obberghen, E., Roth, J., Wollmer, A., and Brandenburg, D., *Nature*, 273, 504, 1978. With permission.)

decreases strongly with ionization of the iodotyrosyl phenoxy group, which has a pK of 8.2.

The amino acid sequence of glucagon has been strongly conserved in evolution, and so little information regarding the importance of particular residues has derived from this source. However extensive chemical modification experiments have been carried out, and the results have been conveniently reviewed by Epand.[195] It seems that the entire glucagon molecule and not an isolated segment is important for full receptor binding affinity. Modifications which introduce polarity or charge into hydrophobic residues reduce binding, but where the character is maintained, binding can be increased. In contrast, modification of the polar residues generally leads to a marked loss of activity and in several cases the loss of biological activity is greater than the loss in receptor binding.

Fragments which have little ability to form helices (1 to 21, 1 to 23, 20 to 29, and 22 to 29) do not compete with glucagon at the receptor and have weak or no glucagon activity. These results are suggestive of a helical conformer at the receptor stabilized by hydrophobic interactions as indicated in Figure 30.

We have seen above that trimerization also involves a helical conformer. Edelhoch and co-workers have shown that the trimerization constant is $6.1 \times 10^6 \pm 2.5 \times 10^6$ in 0.2 *M* phosphate pH 10.0, 23 to 25°C, but that the interaction is enthalpy driven.[196] In order to explain the greater affinity for the receptor and the positive entropy change it is necessary to assume that unlike in trimers, all the hydrophobic residues are buried in the hormone-receptor complex. The N-terminal residues may initially remain flexible. Subsequent binding appears to be important in eliciting a biological response but may not give a proportional further stabilization of the hormone-receptor complex.[6,170]

Recently, however, glucagon-(1-6)-hexapeptide has been shown to be capable of activating adenylate cyclase and of displacing ^{125}I-glucagon from its receptor.[197] Modification of the sequence in apparently rather minor ways to achieve homology with

the equivalent regions of vasoactive intestinal peptide and secretin gives products which are no longer glucagon partial agonists.[198] These results suggest that virtually all of the groups required for glucagon function are contained in the N-terminal region. However the product has only 0.001% the activity of the native hormone and is a partial agonist. The possibility that this derivative is not operating solely by a glucagon-specific mechanism has been noted.[195] With some other peptide hormones, like gastrin, some 10% biological activity is maintained with a C-terminal tetrapeptide.[199]

7. Biological Potency of Pancreatic Polypeptide and Somatostatin

For PP, several sequences have been determined, and fragments have been prepared by cyanogen bromide cleavage and enzymatic degradation.[200] However the assay systems for PP are not as advanced as those for insulin and glucagon. Loss of activity has been noted on removal of the C-terminal tyrosine amide, but a synthetic C-terminal pentapeptide has been shown to be inactive.[201] These preliminary experiments indicate that while a particular residue is essential for activity, a larger part of the molecule is necessary for the expression of this activity. This would appear to be analogous to the situation with His 1 of glucagon. In both glucagon and PP the structure of the regions critical to the biological activity is less well-defined than the remainder of the molecule, and this flexibility may be important for biological activity. For insulin and glucagon the surface regions involved in self-association have been implicated in receptor binding, and it is tempting to speculate that a similar process is involved with PP receptor binding.[7]

In sharp contrast to the other pancreatic hormones, more than 150 synthetic analogues of somatostatin have been prepared.[202] Several of these exhibit a selective ability to inhibit the release of a particular hormone, but as yet the three-dimensional structural requirements for receptor binding are unclear. As noted earlier, even with the D-Trp[8] analogues where a particular conformer and target seem to be favored, the situation is confused by the changed susceptibility to degradation. It is interesting that the N-terminal extended somatostatin with 28 amino acid residues shows higher activity in pancreatic than pituitary targets.[238]

8. Degradation

The molecular biology of polypeptide hormones imposes several contradictory demands. Although hormones must be stable in storage, perhaps for days, they must be disposed of in a matter of minutes. This is an important prerequisite for hormonal control of metabolic processes.

The pancreatic hormones may be removed or degraded by one of several mechanisms. They may be directly excreted into the urine, they may be inactivated by extracellular enzymes, or alternatively they may be internalized. Recently the process of receptor-mediated endocytosis followed by lysozomal degradation has been the subject of considerable study.[205] This mechanism is consistent with the observation that degradation is decreased with reduced receptor binding,[206] and so explaining the relatively higher in vivo biological activities of many insulin analogues which have extended half-lives.[207]

Whether the degradation occurs extracellularly or after internalization, it is clear that the flexible peptides like glucagon will be more easily proteolyticly cleaved than the more globular proteins like insulin. However, although insulin has a peptide link (B22 Arg—B23 Gly) which is relatively susceptible to tryptic digestion, much of the insulin is degraded by an alternative specific mechanism involving glutathione insulin transhydrogenase.[208] In this respect it is interesting that insulin has an unusually exposed disulfide bond at A7—B7 which may allow easy scission leading to a loss of tertiary structure and subsequent proteolytic degradation.[169]

The rapid degradation of somatostatin is one of the principal limitations to the use of

the hormone in the treatment of diabetes, acromegaly, and ulcer.[202] It has been shown that endopeptidase cleavage of the Trp^8—Lys^9 bond is a major degradation route giving a half-life of 2 to 4 min in man.[209] Rat serum can be five times more active than human serum in this degradation.[210] PP, like the other hormones, has a short half-life (in the region of a few minutes) but, surprisingly, serum levels often remain elevated for long periods after feeding.[200] The degradation route for IGFs is presumably rather similar to that for insulin, although the serum carrier protein pool of bound IGF will complicate the half-life measurement.[168]

9. Models for Hormone Receptor Binding

Several models have been presented for polypeptide hormone receptor interactions, and it is instructive to review them here in light of the information available for the pancreatic hormones. Schwyzer, for instance, has highlighted ways in which a hormone message might be encoded in the hormone structure.[203] He has suggested that in the simpler, more flexible molecules the message might be present in the amino acid sequence in a linear fashion, (sychnologic model). At the other extreme, the message might be encoded in parts which are separated in the sequence, but which are brought together in the receptor binding conformation (rhegnylogic model). This latter model is most likely to be relevant where the conformation found at the receptor is stabilized in aqueous solution by tertiary interactions within the hormone. These two extremes are most closely represented on the one hand by glucagon, in which the receptor binding sequence is contiguous and appears to involve almost the whole of the molecule, and on the other hand by insulin, where A1, A19—A21 and B9—B16 and B21—B27 which are separated in the primary structure are involved in receptor binding. In the latter case these are brought together and stabilized by the tertiary interactions of the insulin structure.

Burgen et al. have pointed to two possible mechanisms for receptor interactions with flexible polypeptides such as glucagon.[204] One of these would involve selection of a particular conformer from an ensemble existing in equilibrium in aqueous solution. This is unlikely for glucagon, as the major conformers in solution involving the Val 22—Trp 25 interaction are incompatible with an amphipathic helical structure at the receptor. The second mechanism proposed is an induced-fit or "zipper" model in which part of the molecule is induced into the proper conformation on binding, and the rest of the molecule folds in stages subsequent to this. For glucagon this may involve initial induction of the helical conformation in the region 21-27, followed by helical formation in the 6-14 region and finally some firming up of the structure in the region 15-20, which has a low potential for secondary structure formation.

In most cases the mode of interaction may well be intermediate between the extremes of a rigid structure and a totally flexible one. We have already seen that the limited, but real, flexibility in the globular hormone, insulin, may be of importance. Furthermore although PP assumes a reasonably stable tertiary structure, the flexible C-terminus is important for biological activity.

Much has been made in the past of the "incomplete enzyme" model for the hormone receptor. This has been envisaged as equivalent to ribonuclease S, in which the activity is induced only when the S-peptide binds, inducing a helical conformation in the formerly unstructured peptide. However, the demonstration that the enzyme adenylate cyclase is a distinct molecule, able to move independent of the receptor, implies that this model is rather oversimplified for the glucagon system.[214] Nevertheless, it is possible, indeed probable, that large conformational changes are induced into the receptor on hormone binding. These may be responsible through allosteric effects for cooperativity and cyclase activation.

Finally the concept that the receptor and the hormone can adopt conformations which

are complementary in shape, charge distribution, hydrogen bonding capacity, and hydrophobicity is a useful model. In the pancreatic hormones the hydrophobic residues may play a rather important role in stabilizing the hormone receptor complex.

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