Coypu insulin

Primary structure, conformation and biological properties of a hystricomorph rodent insulin

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Insulin from a hystricomorph rodent, coypu (*Myocaster coypus*), was isolated and purified to near homogeneity. Like the other insulins that have been characterized in this Suborder of Rodentia, coypu insulin also exhibits a very low (3%) biological potency, relative to pig insulin, on lipogenesis in isolated rat fat-cells. The receptor-binding affinity is significantly higher (5–8%) in rat fat-cells, in rat liver plasma membranes and in pig liver cells, indicating that the efficacy of coypu insulin on receptors is about 2-fold lower than that of pig insulin. The primary structures of the oxidized A- and B-chains were determined, and our sequence analysis confirms a previous report [Smith (1972) *Diabetes* 21, Suppl. 2, 457–460] that the C-terminus of the A-chain is extended by a single residue (i.e. aspartate-A22), in contrast with most other insulin sequences, which terminate at residue A21. In spite of a large number of amino acid substitutions (relative to mammalian insulins), computer-graphics model-building studies suggest a similar spatial arrangement for coypu insulin to that for pig insulin. The substitution of the zinc-co-ordinating site (B10-His→Gln) along with various substitutions on the intermolecular surfaces involved in the formation of higher aggregates are consistent with the observation that this insulin is predominantly 'monomeric' in nature. The c.d. spectrum of coypu insulin is relatively similar to those of casiragua insulin and of bovine insulin at low concentration.

INTRODUCTION

The insulins of the hystricomorph rodents exhibit many novel changes in their structures when compared with other mammalian insulins (Smith, 1972; Zimmerman & Yip, 1974). These insulins represent a class of 'natural variants' that have been characterized by both biochemical and biophysical techniques to further our understanding of the structure–function relationship of this protein hormone (Wood *et al.*, 1975; Horuk *et al.*, 1979, 1980a).

Alterations in the primary structures of these rodent insulins have given rise to certain unusual properties, such as non-cross-reactivity with anti-(bovine insulin) serum (Neville *et al.*, 1973), failure to self-associate (Wood *et al.*, 1975; Horuk *et al.*, 1980b) and a relative elevation of growth-promoting effects (King & Kahn, 1981; King *et al.*, 1983).

The invariant nature of asparagine-A21 in all known insulin sequences together with enzymic modification studies of this residue (Slobin & Carpenter, 1963; Horuk, 1980) suggest that this region of the A-chain may play a crucial role in the maintenance of the tertiary structure of the molecule (Blundell *et al.*, 1972). The C-terminal region of the A-chain has been implicated in the 'putative receptor-binding' region of the hormone responsible for eliciting its metabolic effects (Pullen *et al.*,

1976; De Meyts *et al.*, 1978). More recent evidence suggests that this region of the molecule also plays a role in promoting the growth effects of insulin (King *et al.*, 1982). Earlier primary-structure analysis of coypu insulin suggested the presence of an A22 residue (aspartate). In order to determine the effects of this extra residue on the biological activity and three-dimensional structure of the molecule, we have isolated and further characterized coypu insulin. In the present paper we report a confirmation of the primary structure of coypu insulin, conformational analysis through the use of interactive computer-graphics model-building and c.d. spectra. The biological potency and receptor-binding affinity of this hystricomorph insulin is compared with that of bovine insulin.

MATERIALS AND METHODS

Materials

Collagenase (*Clostridium histolyticum*) was obtained from Boehringer, Mannheim, Germany. Trypsin [L-tosylphenylalanylchloromethane ('TPCK')-treated] and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [3-³H]Glucose was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and pig [A14-¹²⁵I]monoiodoinsulin

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A-chain																																	
	-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	
Pig			G	I	V	E	Q	C	C	T	S	I	C	S	<u>L</u>	<u>Y</u>	Q	L	E	N	Y	C	N										
Bovine										A	V																						
Rat 1					D																												
Guinea pig					D						G	T		T	R	H			Q	S													
Chinchilla					D																												
Casiragua					D						N				R	N			L	T													
Coypu					D						N				R	N			M	S					D								
Porcupine					D						G	V							Q														
Cuis					D						R			T	S				R														
IGF-1					D	E			F	R	S			D		R	R			M			A	P	L	K	P	A	K	S	A	-	
IGF-2					E				F	R	S			D		A	L			T			A	T	-	-	P	A	K	S	E	-	
B-chain																																	
Pig			<u>F</u>	<u>V</u>	N	<u>Q</u>	H	<u>L</u>	C	G	S	<u>H</u>	L	V	E	<u>A</u>	L	V	<u>L</u>	V	C	<u>G</u>	E	R	G	F	F	Y	T	P	K	A	
Bovine																																	
Rat 1					K						P																						S
Guinea pig					S						N					T				S		Q	D	D					I			M	D
Chinchilla						K									D																		
Casiragua			Y		G		R				Q			D	T							K	H					Y	R	P	S	E	-
Coypu			Y		S		R				Q			D	T							S						Y	R	P	N	D	-
Porcupine																																	
Cuis				F	N	R					N				D				V			K	D						S	R	D	-	
IGF-1			-	G	P	E	T				E				D				F									Y	F	N	K	P	T
IGF-2	A	T	R	P	S	E	T				G	E			D	T		Q	F			D					Y	F	S	N	K	P	A

Fig. 1. Comparison of the amino acid sequence of coypu insulin with those of pig insulin, other hystricomorph rodent insulins and the insulin-like growth factors

The sequences of bovine (Ryle *et al.*, 1955), rat 1 (Clark & Steiner, 1969), guinea-pig (Smith, 1966), chinchilla (Wood *et al.*, 1975), casiragua (Horuk *et al.*, 1979), porcupine (Horuk *et al.*, 1980a), coypu (Smith, 1972) and cuis (Bajaj, 1984) insulins are shown. For comparison, sequences of the insulin-like growth factors (IGF-1 and IGF-2; Reinderknecht & Humbel, 1978) are also included. The residues involved in formation of porcine insulin dimers are overlined, those involved in formation of hexamers are underlined, those involved in binding the receptor are in solid boxes, and those on the periphery of the receptor-binding region are circled.

from NOVO Research Institute, Bagsvaerd, Denmark. Polyacrylamide-gel electrophoresis was performed as described by Ornstein (1964).

Methods

A 300 g batch of coypu pancreas was subjected to standard acid/ethanol extraction (Peterson *et al.*, 1975) and diethyl ether precipitation (Zimmerman & Yip, 1974). The crude insulin precipitate was further purified by a combination of molecular-sieving chromatography on Sephadex G-50 (superfine grade) equilibrated with 1M-acetic acid and cation-exchange chromatography. The latter step was performed on CM-52 CM-cellulose equilibrated with 0.04 M-sodium citrate buffer, pH 3.3, developed with a gradient to 0.3 M-NaCl (for further details see Horuk, 1980).

Coypu insulin (1 mg) was subjected to performic acid oxidation by the method of Hirs (1956). The resulting chains were separated by h.p.l.c. on a Waters Associates μ Bondapak C₁₈ column as described previously (Bajaj *et al.*, 1983).

Amino acid analysis, Edman degradation and analysis of the amino acid phenylthiohydantoin derivatives were performed as described (Gowan *et al.*, 1981).

The rat epididymal fat-cell assay for coypu insulin was performed at 37 °C in parallel with standard pig insulin (Moody *et al.*, 1974). The binding affinity of coypu insulin was measured by its ability to bind to rat epididymal fat-cells (Gliemann & Sonne, 1978), rat liver plasma membranes (Cuatrecasas, 1971) or pig liver cells (Gammeltoft *et al.*, 1978) in comparison with pig insulin.

The c.d. spectra were recorded on a Cary model 61 circular-dichroism spectrometer, the instrument being calibrated with an aq. 0.1% solution of (+)-10-camphorsulphonic acid. The spectral band width was 1.5 nm and the samples were maintained at 27 °C.

In the absence of structural information from X-ray analysis we used an interactive computer-graphics model-building approach to obtain information of the tertiary structure of coypu insulin. This approach assumes structural similarity of the molecule under study (coypu insulin) with a functionally related or identical molecule (such as pig insulin). Thus the proposed three-dimensional structure of coypu insulin was constructed with torsion angles identical with those of molecule 2 of the insulin dimer, as defined by the high-resolution refinement of pig insulin (Dodson *et al.*, 1980). Substitutions where necessary were obtained by imposing side-chain residue changes in the amino acid sequence of pig insulin by using the computer program FRODO (Jones, 1978) modified for the Evans and Sutherland Picture System 2 (J. A. Jones & I. J. Tickle, unpublished work). By using the interactive facilities of the system, adjustments were made to optimize the molecular structure in terms of molecular geometry and bond lengths. Pictorial representations of several regions of interest were generated by using the MIDAS computer program (I. J. Tickle, unpublished work).

RESULTS

The amino acid sequence of coypu insulin is presented in Fig. 1, and data to substantiate the presence of an aspartate residue at position A22 are presented in Table 1. A sequence of Asn-Asp can be difficult to establish because of partial deamidation of asparagine to aspartate during purification and sequence procedures. The problem is enhanced when this sequence occurs at the C-terminus of the peptide, where extractive losses are increased and the effects of carry-over from previous cycles become more pronounced. The amount of aspartate contaminating cycle 21 as a deamidation

Table 1. Amino acid compositions of native coypu insulin, of the isolated A-chain and of a fragment released during digestion of coypu insulin A-chain with trypsin

Values are all calculated relative to Leu = 4 (native insulin) and to Leu = 1 (insulin fragments).

Amino acid	Amino acid composition (residues/molecule)		
	Native coypu insulin	Isolated A chain	C-Terminal fragment of A-chain
Asx	8.14 (8)	5.47 (5)	3.13 (3)
Thr	1.54 (2)	0.89 (1)	— (0)
Ser	3.78 (4)	1.67 (2)	0.86 (1)
Glx	4.12 (4)	2.27 (2)	1.03 (1)
Pro	0.91 (1)	— (0)	— (0)
Gly	3.10 (3)	1.36 (1)	— (0)
Ala	— (0)	— (0)	— (0)
Cys*	6.16 (6)	4.05 (4)	0.38 (1)
Val	4.02 (4)	0.99 (1)	— (0)
Met	0.88 (1)	0.99 (1)	0.94 (1)
Ile	1.89 (2)	1.75 (2)	— (0)
Leu	4.19 (4)	1.16 (1)	1.01 (1)
Tyr	3.63 (4)	0.65 (1)	0.94 (1)
Phe	0.78 (1)	— (0)	— (0)
His	1.02 (1)	— (0)	— (0)
Lys	— (0)	— (0)	— (0)
Arg	5.13 (5)	1.00 (1)	— (0)
Trp†	N.D.	N.D.	N.D.

* Determined as cysteic acid.

† Not determined (N.D.).

product was typical of the relative amount of aspartate seen in the asparagine residues at positions A9 and A14 (results not shown). However, the shift in the relative intensities of the Asn/Asp peaks in cycle 22 is evidence that a new residue of aspartate appears in cycle 22. Significant asparagine remains in cycle 22 as a result of carry-over from cycle 21.

The proposed sequence of the A-chain predicts that the amino acid compositions of native coypu insulin, the isolated A-chain and the C-terminal fragment arising from trypsin digestion of the A-chain should contain eight, five and three residues of aspartic acid respectively. The compositions of these peptides are presented in Table 1 and offer supporting evidence for the sequence. In addition, the C-terminal fragment has been sequenced and the pattern of asparagine/aspartate in cycles 8 and 9 for the peptide is similar (results not shown) to that observed in cycles 21 and 22 of the sequencing of the A-chain. Our analysis also confirmed the sequence of the B-chain (Smith, 1972) and indicated that the B-chain terminates at position B29.

The biological activity of coypu insulin was compared with that of pig insulin in the lipogenesis assay with rat epididymal adipocytes. The same maximal response was obtained with the two insulins, and the dose-response curves were parallel (results not shown). The relative potency of coypu insulin was $3.2 \pm 0.3\%$ (mean $\pm$ S.D.) as determined in four independent experiments.

Receptor-binding affinities relative to those of pig insulin were measured at 37 °C in rat fat-cells, rat liver plasma membranes or pig liver cells, and as shown in Fig. 2 the binding curves for coypu and pig insulins were

parallel. The binding affinities were determined as 3–8% of those of pig insulin (Table 2), which is 2–3-fold higher than the relative biological potency. In separate experiments on adipocytes (results not shown) we found that coypu insulin did not antagonize the effect of pig insulin.

The minute quantities of material available restricted the c.d. studies to single measurements at low concentrations. Pronounced sequence similarity suggested comparing the c.d. spectrum of coypu insulin with that of casiragua insulin (see Figs. 3 and 4). In the far-u.v. region, the two spectra are not only nearly superimposable, but they also both show a blue-shifted cross-over point and positive band as compared with the spectrum of bovine insulin. As the patterns of aromatic residues for the hystricomorph insulins are the same, we cannot offer a straightforward explanation for the somewhat larger differences in their near-u.v. spectra. It should be mentioned that the concentration of the hystricomorph insulins was calculated on the basis of their absorption by using the coefficient $A_{276}^{1\text{cm}, 0.1\%} = 1.06$ for bovine insulin, which seems justified by the presence of the same number of tyrosine residues in corresponding environment. [In an earlier publication (Horuk *et al.*, 1980b) the casiragua insulin spectrum was calculated with the concentration based on weight. It therefore differs from Figs. 3 and 4 by a constant factor.]

Model-building studies suggest that coypu insulin can attain a spatial arrangement similar to that of bovine insulin with no difficulty. Glycine residues at B8 and B23 with positive torsion angles are involved in tight bends of the peptide backbone of the B-chain of pig insulin. These residues have been conserved in coypu insulin.

Fig. 2. Inhibition of receptor binding of [A14-¹²⁵I]monoiodoinsulin to pig liver cells by pig (○) and coypu (●) insulins

Data are expressed as the fraction of maximum receptor-bound with tracer alone. The points represent means $\pm$ S.D. for five experiments.

Table 2. Biological activity and receptor-binding affinity of coypu insulin

Values are given as means $\pm$ S.D., for the numbers of determinations given in parentheses.

Assay . . .	Activity or affinity relative to pig insulin (%)			
	Fat-cell lipogenesis	Fat-cell receptors	Liver plasma-membrane receptors	Pig liver-cell receptors
	3.2 $\pm$ 0.3 (4)	8.0 $\pm$ 0.8 (4)	5.0 $\pm$ 0.4 (4)	7.2 $\pm$ 1.1 (5)

Fig. 3. Comparison of the c.d. spectra of bovine, casiragua and coypu insulins in the far-u.v. region

—, Coypu insulin (3.8 μ M); ----, casiragua insulin (4.0 μ M); —·— bovine insulin (3.5 μ M). All solutions were zinc-free.

Fig. 4. Comparison of the c.d. spectra of bovine, casiragua and coypu insulins in the near-u.v. region

—, Coypu insulin (3.8 μ M); ----, casiragua insulin (4.0 μ M); —·— bovine insulin (3.5 μ M).

Arg-B20, which replaces the Gly-B20 of pig insulin (Fig. 1), can be accommodated in coypu insulin by allowing for slight changes in the torsion angles of neighbouring residues. The presence of Pro-B27 involves slight changes in the main-chain conformation at the C-terminal region of the B-chain. A stereo view of the proposed three-dimensional structure of coypu insulin is shown in Fig. 5.

The molecular surface forming the dimer interface in pig insulin is shown in Fig. 6. The presence of the charged B26 guanidinium side chain in an hydrophobic environment should prevent dimer formation.

The region equivalent to the second hydrophobic patch on the surface of the pig insulin monomer involved in hexamerization is shown in Fig. 7. Several substitutions, such as Tyr-B1, Ser-B3, Gln-B10, Thr-B14 and Ser-B17, in coypu insulin predict aggregation to be an unlikely event.

The Asp-A22 was added and an ion-pair formation between the negatively charged terminal carboxylate group of the A-chain and the positively charged guanidinium side chain of residue B22 is predicted as shown in Fig. 8.

DISCUSSION

Like the other well-characterized hystericomorph rodent insulins, coypu insulin has diverged greatly in its primary structure from mammalian insulins in general (Fig. 1). As a consequence, the general features observed in mammalian insulins (Dayhoff, 1978; Blundell & Wood, 1982; Blundell *et al.*, 1982) have not been observed in coypu insulin. The invariant cystine residues are conserved and the hydrophobic core (B6, B11, B15, B18, B24, A2, A16) is identical with that of pig insulin. Some residues involved in dimer formation (B12, B16, B24) are identical, but several important changes with

Fig. 5. Stereo view of the proposed three-dimensional structure of coypu insulin

Fig. 6. Residues involved in dimerization in pig insulin shown for coypu insulin

regard to the dimerization (B21, B25, B26) and hexamerization (B10, B14, B17, B20, A13, A14) interfaces of pig insulin have occurred. The effect of these substitutions on various physiological properties of coypu insulin is discussed below.

All of the evidence of primary-structure determination

Fig. 7. Residues involved in hexamerization in pig insulin become more hydrophilic in cowpu insulin

is consistent with the presence of Asp-A22 in coypu insulin; there has been no evidence to suggest that the chain ends at Asn-A21, as expected for insulins. The ion-pair formation between the negatively charged carboxylate group of Asn-A21 and the positively charged Arg-B22 side chain has been implicated in stabilizing the tertiary structure of the bovine insulin monomer (Blundell *et al.*, 1972). Computer-graphics model-building studies (Fig. 8) show that an ion-pair formation can be attained between the B22 guanidinium side chain and terminal carboxylate of Asp-A22 in coypu insulin without any drastic disruption of the conformation of the molecule. A similar interaction between Lys-B22 and the carboxy group of Gln-A22 has been predicted for dogfish insulin (Bajaj *et al.*, 1983). To date, only the two examples cited above exhibit a single-residue extension at the C-terminus of the A-chain.

Fig. 8. Close association of B22 and A22 residues is predicted in coypu insulin

An interesting feature of coypu insulin as far as the primary structure is concerned is the sequence similarity observed with casiragua insulin (Horuk *et al.*, 1979). This is not surprising, since both these rodents are classified anatomically in the same Family, Octadontoidea. However, such a sequence homology is not observed between any other pair of hystricomorph rodent insulins (Fig. 1).

Coypu insulin exhibits a low biological potency, about 3% of that of pig insulin, in the lipogenesis assay with rat epididymal adipocytes, a feature common to most insulins of this group, such as guinea-pig (Zimmerman & Yip, 1974), casiragua (Horuk *et al.*, 1979) and porcupine (Horuk *et al.*, 1980a) insulins. In addition, we observed a moderate discrepancy between the biological potency (3%) and the receptor-binding affinities (5–8%) relative to those of pig insulin in three different binding assays with whole cells or plasma membranes. Similar results have been reported for hagfish (Emdin *et al.*, 1977) and porcupine insulins (Horuk *et al.*, 1980a) and for some synthetic insulins such as the covalently linked insulin dimers (Willey *et al.*, 1980), [asparagine amide-A21]insulin (Burke *et al.*, 1980) and the insulins elongated at Gly-A1 by one to three basic amino acid residues, arginine or lysine (Rösen *et al.*, 1980). This indicates that the efficacy of the insulin–receptor complex with these naturally occurring and synthetic analogues is decreased. Because of the variable and highly substituted nature of the animal insulins, a single amino acid substitution is unlikely to be responsible for the discrepancy between biological potency and binding affinity as well as the loss in biological activity. A crucial point to note is whether

a substitution is directly responsible for the resultant biological effects, or whether conformational changes resulting from these substitutions might be responsible for the observed low bio-activity.

Although coypu insulin differs from bovine insulin in as many as 24 residues (Table 3), model-building studies suggest that coypu insulin can attain a conformation similar to that of bovine insulin. This was confirmed by the similarity of the c.d. spectrum of coypu (as well as that of casiragua) insulin with that of the bovine insulin spectrum. The major factor that militates against dimer formation in coypu insulin is the presence of arginine at B26. Although the long side chain of the guanidinium group can be accommodated, the presence of a charge in a relatively hydrophobic environment (Val-A3, Leu-B11, Val-B12, Tyr-B16 of molecule 1 and Phe-B24 of molecule 2) will hinder dimer formation. A similar situation is also observed in casiragua insulin (Blundell & Horuk, 1981). The disruption in the dimer interface could partly explain the low biological activity exhibited by coypu insulin, since this molecular interface (B24–26) along with several surrounding residues has been implicated in the ‘putative receptor-binding’ region of the molecule, and hydrophobic interactions analogous to dimer formation have been suggested as the driving force of hormone–receptor interaction (Blundell *et al.*, 1972; Pullen *et al.*, 1976). In such a case, the presence of a charged residue at B26 would certainly make any hydrophobic contact with the receptor less favourable.

The ability of coypu insulin to promote thymidine incorporation leading to DNA synthesis in human fibroblasts (King & Kahn, 1981) and Swiss NIH3T3 cells (Bajaj, 1984) suggests that this insulin is a better growth promoter than a metabolic regulator, in comparison with bovine insulin. Interestingly, coypu insulin showed a binding affinity of about 50% relative to that of pig insulin in rat brain cortex (Gameltoft *et al.*, 1984). The higher growth-promoting activity and superpotency exhibited by coypu insulin (also observed by other hystricomorph rodent insulins) in human fibroblasts (King *et al.*, 1983) suggest that another molecular surface (distinct from the surface responsible for metabolic activity) might be responsible in eliciting this physiological effect of insulin. Coypu insulin shows some similarity with the insulin-like growth factors (IGF) in its primary structure at Asp-A4, Ser-B3, Asp-B13, Thr-B14, Ser-B17 and Tyr-B25. The extension at the C-terminus of the A-chain can be compared with the six- and eight-residue extensions seen in IGF-1 and IGF-2 respectively (Fig. 2). However, none of the other insulins studied from this suborder of rodents shows an

Table 3. Difference matrix for amino acid sequence changes in insulin

	Pig	Bovine	Rat 1	Guinea pig	Chinchilla	Casiragua	Coypu	Porcupine	Cuis
Pig	2								
Bovine	4	6							
Rat 1	18	19	18						
Guinea pig	6	8	7	17					
Chinchilla	21	23	21	21	21				
Casiragua	22	24	23	20	22	7			
Coypu	7	6	7	15	9	22	23		
Porcupine	17	19	17	16	14	20	21	17	
Cuis									

extension of the A-chain, and the high rate of substitution maintained between them makes it very difficult to pinpoint any specific residue or molecular surface that might have evolved at a higher rate to optimize this function of the protein hormone. In light of these properties, hystricomorph rodent insulins have been suggested to represent another class of insulin-like growth factors (King & Kahn, 1981).

In conclusion, we have reconfirmed the existence of an Asp-A22 in coypu insulin. In spite of this addition and the large number of additional substitutions relative to bovine insulin, coypu insulin can attain the three-dimensional structure of bovine insulin with no difficulty. The very low biological activity of this insulin is characteristic of the hystricomorph rodent family of insulins, and cannot be attributed to the addition of Asp-A22. The most likely reason for the diminished activity of this insulin must lie with the large number of substitutions observed in the receptor-binding region as well as with substitutions that might perturb the conformation of the molecule.

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