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Synthesis and Biological Activity of Five D-Cys Analogs of Human Insulin[1]

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Five analogs of human insulin with D-Cys in different positions (A^6, A^7, A^{11}, A^{6+11}, B^7) have been synthesized by the fragment condensation approach, combined with selective disulfide formation. All of them have physicochemical properties noticeably different from those of human insulin. They possess very low biological activity (0.03–1.2%, glucose oxidation in rat fat cells). In contrast, the potency for antibody binding ranges from 7 to 70% of that of insulin. The two analogs with D-Cys in positions A^6 and A^7 have been obtained in crystalline form.

INTRODUCTION

Some years ago, we reported a total synthesis of human insulin in which the three disulfide bridges of the hormone were formed selectively in separate chemical reactions (*1*, *2*). This scheme of synthesis imposes higher demands on the choice and application of protecting groups than earlier approaches in which the formation of the disulfide bonds from the two separately synthesized peptide chains was not controlled. (For a review see (*3*).) The prerequisites for our synthesis were met through the elaboration of new methods of ensuring selective reactions at the side chains of cysteine residues (*4*, *5*) and the *N*-terminals of peptide chains (*6*). In the last step of the selective disulfide method, insulin is produced in high yield by oxidation with iodine from a precursor molecule containing cysteine residues protected by Acm[2] in positions A^7 and B^7 (**11** in Fig. 1). The method also gives access to new types of insulin derivatives, e.g., isomers with cystine bridges in unnatural positions (*7*). This paper reports the syntheses and biological activities of five analogs with natural cystine pairing, but with D- instead of L-half-cystine residues in positions A^6, A^7, A^{11}, and B^7. The six half-cystine residues, serving as bridgeheads in the intramolecular crosslinks of insulin, are of special importance for the three-dimensional shape of the molecule. Furthermore, there are indications that degradation of insulin under physiological conditions is brought about by enzymatic attack at the disulfide bonds (*8*). It was therefore of interest to replace some of the half-cystines by their D-stereoisomers and to investigate the influence of

[1] Dedicated to the memory of Professor George W. Kenner, in deep gratitude for his exemplary work and for many years of friendship.

[2] Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature. In addition: Acm, acetamidomethyl; Boc, tertiary butyloxycarbonyl; tBu, tertiary butyl; OtBu, tertiary butyloxy; Bpoc, 2(biphenyl-4-yl)-isopropyloxycarbonyl; Trt, triphenylmethyl; tlc, thin-layer chromatography; TFA, trifluoroacetic acid.

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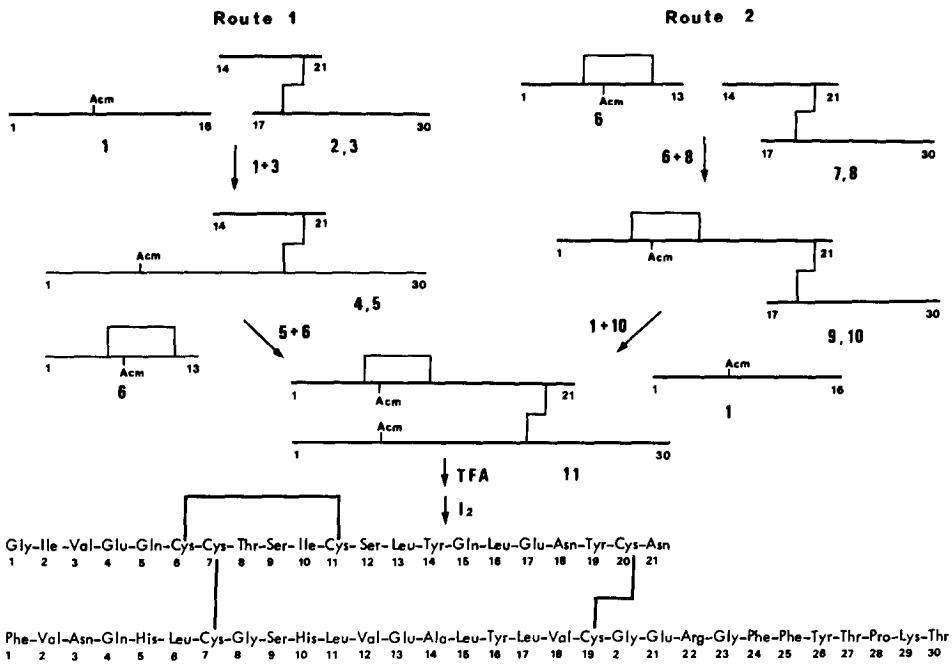

FIG. 1. Scheme of synthesis of human insulin and analogs. In addition to Acm, and with the exception of the specific groups indicated, intermediates **1** to **11** are protected by Boc (*N*-terminals and Lys), tBu (Ser, Thr, Tyr), and OtBu (C-terminals and Glu). Specific groups: **1**, B^{16}–COOH free; **2**, Bpoc in A^{14}, Trt in B^{17}; **3**, Bpoc in A^{14}, B^{17}–NH_2 free; **4**, Bpoc in A^{14}; **5**, A^{14}–NH_2 free; **6**, A^{13}–COOH free; **7**, Trt in A^{14}, Bpoc in B^{17}; **8**, A^{14}–NH_2 free, Bpoc in B^{17}; **9**, Bpoc in B^{17}; **10**, B^{17}–NH_2 free.

these substitutions on both the physicochemical and biological properties of the insulin analogs.

SYNTHESES

As with insulin itself, the analogs were built up on the classical principle, i.e., peptide fragments of variable chain length were coupled in solution to larger units. The permanent protecting groups of the tertiary butyl type were removed at the end by trifluoroacetic acid, and the side chains of His and Arg remained unprotected. The synthesis of the new peptide fragments with D-Cys in different positions is not described here in detail, as it followed the methods already published in earlier papers on the synthesis of human insulin (*2*, *4*, *9*, *10*). The most characteristic steps of the synthesis are:

1. Formation of a key intermediate containing the cystine bridge A^{20}–B^{19} by reaction of the sulfenyl-thiocarbonate derivative of Cys B^{19} with the free side chain of Cys A^{20} (*11*).

2. Formation of the cystine bridge A^{6}–A^{11} from the Trt-protected cysteine residues by oxidation with iodine, in the presence of the Acm-protected cysteine A^{7} (*4*).

3. Completion of the peptide chains from the key intermediate. The differential acid sensitivity of Trt and Bpoc in N^{α} permits the separate prolongation of the two chains from their amino ends (*2*).

4. Closure of the last cystine bridge (A^7–B^7) from Acm-protected cysteine residues (*2*).

The last steps of the synthesis are illustrated schematically in Fig. 1. The synthesis of the analogs with D-Cys in positions A^6, A^7, A^{11}, and A^{6+11} followed the pathway used in the original synthesis of human insulin itself (route 1). Since we were also interested in exploring the possibilities of another order of the fragment couplings, route 2 was employed for the D-Cys B^7 analog. In this variation, sequence A^{1-13} (**6**) is first coupled to the unsymmetrical cystine peptide (**8**), followed by sequence B^{1-16} (**1**). Fragment **7** is identical with **2**, but the positions of the two *N*-terminal protecting groups are reversed, i.e., Trt is in A^{14} and Bpoc in B^{17}. The two routes converge in the identical intermediate **11**. Table 1 summarizes the tlc data of fragments **7**–**10** of the new pathway (all-L peptides), as well as those of intermediates **1**, **6**, and **11**. In these latter cases, the all-L peptide is included for comparison with the compounds containing D-Cys in different positions. The values were obtained from chromatograms with all peptides on the same plate.

TABLE 1

R_f VALUES OF INTERMEDIATES IN tlc ON SILICA GEL

	Compound	Solvent I[a]	Solvent II[b]	Solvent III[c]
1	All-L	0.32	0.16	0.24
	D-Cys B^7	0.34	0.17	0.24
6	All-L	0.57	0.71	0.67
	D-Cys A^6	0.52	0.60	0.65
	D-Cys A^7	0.63	0.80	0.72
	D-Cys A^{11}	0.56	0.70	0.66
	D-Cys A^{6+11}	0.58	0.72	0.68
7	All-L	0.64	0.88	0.76
8	All-L	0.50	0.49	0.50
9	All-L	0.61	0.83	0.68
10	All-L	0.51	0.65	0.51
11	All-L	0.50	0.49	0.31
	D-Cys A^6	0.51	0.51	0.31
	D-Cys A^7	0.51	0.50	0.34
	D-Cys A^{11}	0.48	0.46	0.31
	D-Cys A^{6+11}	0.49	0.48	0.32
	D-Cys B^7	0.49	0.49	0.33

[a] 2-Methyl-2-butanol/isopropanol/H_2O (51 : 21 : 28).

[b] Ethyl acetate/pyridine/acetic acid/H_2O (62 : 21 : 6 : 11).

[c] 1-Butanol/acetic acid/H_2O (75 : 7.5 : 21).

Some of the smaller fragments could be crystallized. In other cases, purification was performed by chromatography on silica gel columns or, especially with all the compounds shown in Fig. 1, by countercurrent distribution. Our experience in the original synthesis of insulin was again confirmed: Certain by-products of the protected intermediates were difficult to separate and therefore partially carried over to the

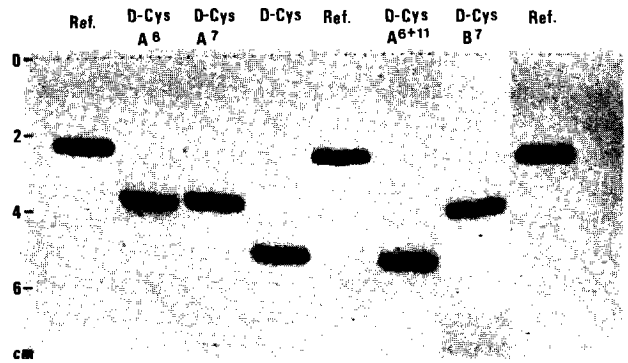

FIG. 2. tlc of insulin analogs. Adsorbent = cellulose (Merck). Solvent system IV (cf. Table 2), solvent front at 17 cm from origin. Reference = synthetic human insulin. Each sample contains 4 μg of insulin or analog, stained with acid violet 6B. (The D-Cys B^7 analog is inserted from another plate.)

following steps. However, after deprotection and oxidation of compounds **11** to the insulin analogs, purification by countercurrent distribution presented no problems. Thus, the contaminating D-Tyr B^{16} peptides, originating from partial racemization during coupling of fragment **1**, were separated with higher K values (*2*). Another case of partial racemization involved Cys B^7: About 8% of the D-stereoisomer was produced

TABLE 2

PHYSICOCHEMICAL DATA

	tlc		EP			
	Solvent IV[a,b]	Solvent V[a]	pH 1.9[c]	pH 8.6[c]	IEP[d]	K[e]
Human insulin	1.0	1.0	6.9	5.8	5.7	0.84
D-Cys A^6	1.59	1.51	6.5	5.5	5.6	0.96
D-Cys A^7	1.52	1.58	6.6	5.5	5.6	1.0
D-Cys A^{11}	2.02	1.90	5.9	5.5	5.55	1.36
D-Cys A^{6+11}	2.04	1.95	5.9	5.3	5.55	1.34
D-Cys B^7	1.60	1.41	6.4	5.3	5.6	1.05

[a] tlc on cellulose plates. Values indicated are migration distances relative to human insulin. Solvent IV = 1-pentanol/pyridine/H_2O/methyl-ethyl-ketone/formic acid (40:28:15:11:5), solvent V = 1-butanol/pyridine/H_2O/formic acid (44:24:20:2).

[b] See Fig. 2.

[c] Migration distances in centimeters during electrophoresis on cellulose acetate foils at pH 1.9 (90 min) and pH 8.6 (120 min), 20 V/cm.

[d] Isoelectric point, determined in sheets of polyacrylamide gel containing LKB Ampholites (pH 3.5–10) and 5 M urea.

[e] K values obtained in countercurrent distribution in the solvent system 1-butanol/pyridine/0.1% aqueous acetic acid (5:3:11).

from the L-peptide (and vice versa) during alkaline hydrolysis of the methyl ester of **1** (*10*). Here again, the D- and L-diastereoisomers were separated in the countercurrent purification. This means that in the synthesis of the D-Cys B^7 analog, a small amount of insulin as a by-product had to be separated.

All the analogs were purified to chromatographic homogeneity in the final countercurrent purification, except D-Cys A^6-insulin, which still contained about 3–5% of an unknown impurity with a slightly higher R_f value (Fig. 2). They were further characterized by electrophoresis on cellulose acetate foils and by determination of the

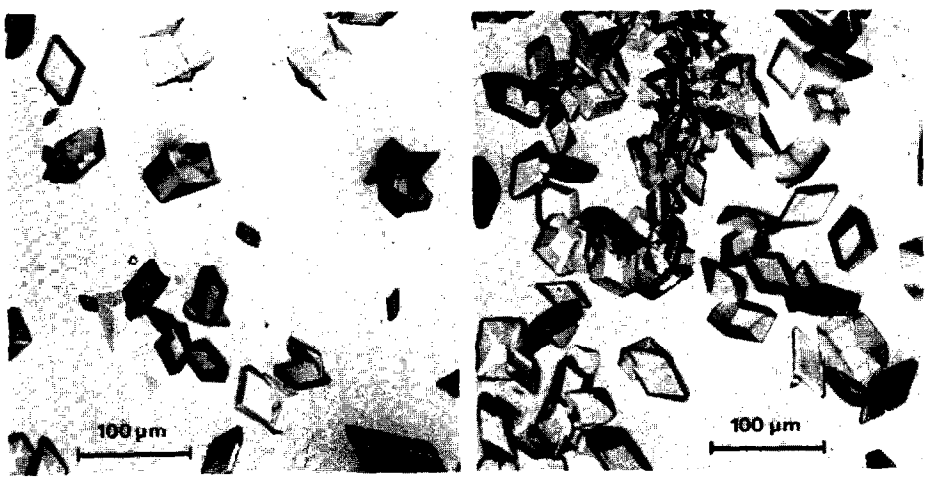

FIG. 3. Crystals of D-Cys A^6 human insulin (left) and D-Cys A^7 human insulin (right). Crystals were obtained from 0.3% solutions of the insulin analogs in 0.095 *M* citric acid–NaOH buffer (pH 6), containing 0.03 *M* NaCl, 0.012 *M* $ZnCl_2$, and 5 vol% tertiary butanol.

isoelectric points (Table 2). Attempts to crystallize the various analogs as Zn complexes were successful with D-Cys A^6- and D-Cys A^7-insulin. These compounds formed clear, rhombohedral crystals from the usual buffer solution after a few hours (Fig. 3), whereas the others formed amorphous precipitates. The five analogs were obtained in amounts ranging from 20 to 40 mg.

BIOLOGICAL ACTIVITY

The biological activity of the D-Cys analogs was evaluated, in comparison with synthetic human insulin, on the basis of glucose oxidation in isolated rat fat cells and binding to anti-bovine insulin antibodies. The assays were performed as described previously (*7*). The results are shown in Table 3. Glucose oxidation was stimulated by all analogs. Separate studies showed that they have the same intrinsic activity as insulin and are full agonists. Potencies are very low and range from 0.03 to 1.2% of that of insulin. All analogs bind to anti-bovine insulin serum from guinea pigs with potencies between 7 and 70% of that of insulin.

TABLE 3

	Glucose oxidation, rat fat cells, *in vitro* EC_{50}[a]	Anti-insulin serum binding, *in vitro* IC_{50}[b]
Human insulin	0.443 ± 0.008 (7)	0.872 ± 0.028 (16)
D-Cys A^6	192 ± 8 (3)	1.29 ± 0.05 (5)
D-Cys A^7	274 ± 44 (4)	1.80 ± 0.06 (5)
D-Cys A^{11}	60.7 ± 6.8 (3)	9.06 ± 0.33 (6)
D-Cys A^{6+11}	1666 ± 34 (3)	11.91 ± 0.39 (6)
D-Cys B^7	36.3 ± 4.6 (4)	6.88 ± 0.22 (5)

[a] Concentration for half-maximal stimulation, ng/ml. Mean ± SEM, number of experiments in parentheses.

[b] Concentrations for 50% displacement of ^{125}I-insulin in the radioimmunoassay, ng/ml. Mean ± SEM, number of experiments in parentheses.

DISCUSSION

The preparation of the analogs with D-Cys in A^6, A^7, A^{11}, and A^{6+11} followed exactly the pathway (Fig. 1, route 1) described earlier for human insulin (*2*, *4*, *9*). No new synthetic aspects emerged, except that some precursors of intermediate **6**, containing a D-Cys, were more soluble in organic solvents than the corresponding all-L peptides. For the synthesis of D-Cys B^7-insulin, a new approach (route 2) was taken. As expected, the selective acidolytic cleavages of Trt and Bpoc necessary for the separate completion of the chains of the unsymmetric disulfide **7** proceeded again without problems. The intermediates **9** and **10** had good solubility properties and showed less tendency toward self-association than the corresponding compounds **4** and **5** of the original pathway (*2*). The formation of the last cystine bridge is performed from the two Acm-protected cysteine residues A^7 and B^7 of intermediate **11** through oxidation with iodine. High dilution of the precursor molecule is needed in order to prevent the occurrence of polymer by-products. The formation of this last bridge is seemingly entirely directed by the chemical reactivity of the Acm-protected side chains and does not depend upon the presence of an insulin-like conformation in the precursor molecule. This assumption is supported by the fact that the ring closure proceeds in high yield (approximately 70%) with all the analogs synthesized so far, including the two isomers of insulin with disulfide bridges in unnatural positions (*7*).

A comparison of R_f (Table 1) and K values in countercurrent distribution of compounds **1**, **6**, and **11**, either all-L or with D-Cys in different positions, did not reveal unusual variations. However, after deprotection of intermediates **11** and closure of the third disulfide bridge from the two Acm-proteced cysteines, striking differences between insulin and the various analogs became evident (Table 2). This may be explained by the circumstance that the third bridge forces the molecules into a rather rigid conformation, which is probably very sensitive to steric changes at particular positions in the peptide chains.

Examination of a model of the insulin molecule, as elucidated by X-ray analysis (*12*), shows that replacement of the different half-cystine residues by the D-enantiomer may have a different effect on the folding of the peptide chains. While it appears that some of

these residues may be replaced by the unnatural D-stereoisomer without exerting a major influence on the peptide backbone, it is evident in other cases that such a replacement must be accompanied by a pronounced change in the steric arrangement of the peptide chains. As the model suggests, the two analogs with D-Cys in A^6 or A^7 are examples of the former, and that with D-Cys in A^{11} is an instance of the latter situation. D-Cys in B^7 seems to cause a moderate steric change in the molecule. The fact that of the five analogs synthesized only those with D-Cys in A^6 and A^7 could be obtained in the crystalline state might be explained by their conformation differing only slightly from that of natural insulin. Yet upon tlc analysis even these two analogs differ markedly from insulin (Fig. 2). Increased R_f and K values (Table 2) may both be considered as a measure of an increase in the lipophilic properties of the insulin analogs. The two compounds with the highest values both contain a D-Cys in position A^{11}. On cellulose-coated tlc plates, they migrate twice as fast as the all-L compound. This must be due to a distinct deviation of their spatial structure from that of normal insulin, in such a way that some of the lipophilic regions of the molecule are more exposed toward the surface. Conformational changes also influence the ionogenic properties of the insulin molecule, since on isoelectric focusing all of the five analogs exhibit somewhat lower isoelectric points than human insulin itself. The shorter migration distances of the analogs during electrophoresis, however, are not due to charge differences, but rather to the lipophilic retardation on the cellulose acetate carrier.

The biological activity of all five analogs in the glucose oxidation assay in fat cells is remarkably low (Table 3). This is especially unexpected in the case of the two analogs with D-Cys in A^6 or A^7; they have been obtained in crystalline form and may exist in a conformation not too different from that of natural insulin. Yet they have a potency in the order of only 0.2% of that of insulin. A still lower potency (0.03%) is observed with the analog containing D-Cys in positions A^6 and A^{11}. In these cases it cannot be excluded that the presence of traces of the all-L isomer (i.e., insulin) might be responsible for part or all of the biological activity observed. The remaining two analogs, with D-Cys in A^{11} or in B^7, have potencies in the range of 1% of insulin. This level of activity cannot to a major extent be due to the presence of insulin, since amounts exceeding 0.1% can be excluded on the basis of the final purification by countercurrent distribution.

The capacity of the five D-Cys analogs for binding to anti-bovine insulin serum from guinea pigs was found to be considerably higher than that for stimulation of glucose oxidation in fat cells. This finding is in accord with unpublished observations with other analogs, indicating, as expected, that antibody binding and biological activity are not correlated. Interestingly, the two crystalline analogs with D-Cys in A^6 and A^7 have the highest activities in the antibody-binding test (50–70%).

The low biological activity of the five analogs presented here contrasts with the comparatively high activity of the two disulfide isomers of insulin studied previously (*7*). Although it is likely that the conformation of these isomers, each of them with two of the three cystine bridges in unnatural positions, differs considerably from that of natural insulin, the activity of the isomers in the glucose oxidation assay was rather high, approximately 15% of that of insulin. These results are difficult to reconcile with the current hypothesis regarding the molecular mechanism of action of insulin, which assumes that a three-dimensional conformation similar to that in the crystalline state is

necessary for receptor binding and biological activity (*12*, *13*). On the other hand, there is evidence that membrane-bound sulfhydryl groups and disulfide oxidoreductase(s) may be essential for the mediation of biological activity (*14*). This finding—if confirmed—could imply that the activity of an insulin analog would depend on, in addition to conformation at the receptor, its capacity to react with the oxidoreductase(s). Thus the relatively high biological activity of the insulin isomers—as opposed to the low activity of the analogs containing D-Cys—would appear plausible. Further experiments are clearly needed to corroborate this hypothesis.

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