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Original article

Structural requirements of the N-terminal region of GLP-1-[7-37]-NH_2 for receptor interaction and cAMP production☆

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Abstract

A series of GLP-1-[7-36]-NH_2 (tGLP-1) and GLP-1-[7-37] analogs modified in position 7, 8, 9 and 36, have been designed and evaluated on murine GLP-1 receptors expressed in RIN T3 cells for both their affinity and activity. Ten of the synthesized peptides were found full agonists with activities superior or at least equal to that of the native hormone. Five of them were investigated for their plasmatic stability and the most stable, [a^8-desR^{36}]GLP-1-[7-37]- NH_2 (Compound 8), evaluated in vivo in a glucose tolerance test which confirmed a clearly longer activity than that of the native hormone. We also performed circular dichroism study and propose a hypothetical structural model explaining the most part of observed activities of GLP-1 analogs on RIN T3 cells.

Keywords: Glucagon-Like Peptide-1; Structure-activity relationships; Plasma stability; Glucose tolerance test; Conformations; Circular dichroism

Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosyl mono-phosphate; CD, circular dichroism; DMEM, Dubelco's modified Eagle's medium; DPP IV, dipeptidyl peptidase IV (EC 3.4.14.5); Fmoc, fluorenemethoxy-carbonyl; EC_{50}, concentration required for half maximal stimulation; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; HPLC, high performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthane; IC_{50}, concentration causing 50% inhibition of maximum binding; KRB, Krebs-Ringer bicarbonate buffer; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

☆ Here, we have employed the convention whereby the first amino acid in GLP-1 (His) is designated number 7, reflecting the fact that the peptides arises by truncation of a larger molecule : GLP-1-[1-37]. D-amino acids are written in lower case, for example e refers to D-glutamic acid. Additional abbreviations used herein are:

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1. Introduction

Preproglucagon molecule [1] is proteolytically processed in intestinal L-cells to GLP-1-[7-37] and GLP-1-[7-36]-NH_2 [2] (tGLP-1) which are secreted into the bloodstream after oral glucose or mixed meals [3] and act in vivo as incretins. An incretin is a gut peptide that promotes the secretion of insulin from pancreatic ß-cells [4] via activation of specific receptors [5]. Furthermore, unlike sulfonylureas, the effect of tGLP1 is glucose dependent and remains effective even under conditions in which sulfonylureas fail to control blood glucose adequatly [6]. Numerous studies have underlined the interest of tGLP-1 itself or agonists for its receptor as new alternative treatment for diabetes and obesity [7].

Despite these very attractive potentialities, therapeutic use of tGLP-1 is hampered by the very short half-life of the peptide which is rapidly metabolized in vivo mainly by DPP IV [8], an aminodipeptidase leading to the almost inactive GLP-1-[9-36]-NH_2. The design of active analog resistant to the proteases is therefore of the greatest interest.

In this paper, we investigated the incidence on affinity, activity and in some cases metabolic stability, of various substitutions performed on the three first amino acids (His^7-Ala^8-Glu^9-) of GLP-1-[7-37] sequence as well as the role of Arg^{36}. It has been suggested [9], that a fold or turn of the peptide backbone may cause a close steric vicinity of the N-terminus and C-terminus of tGLP-1 in solution. This structure could be stabilized by an ionic bond between the side-chains of Arg^{36} and Glu^9 (or Asp^{15}) as supported by the fact, that replacement of Arg^{36} with an alanine induced a significant decrease in both affinity and activity (IC_{50} = 4.6 ± 0.6 nM and EC_{50} = 7 ± 5 nM for [A^{36}]GLP-1-[7-36]-NH_2 versus 0.25 ± 0.09 nM and 2.6 ± 0.4 nM for tGLP-1 itself) [10].

In addition, we have attempted to correlate the observed activity with structural parameters deduced from CD spectra and NMR data and, proposed a hypothetical model of tGLP-1 N-terminal part.

2. Results

All the results are reported in Table 1. As previously reported [11], GLP-1-[7-37] and its COOH-terminal amidated analog GLP-1-[7-37]-NH_2 displayed strictly the same activity (Table 1). Consequently, we decided to consider in this work, analogs with the C-terminal carboxylic group systematically amidated in order to protect them against degradation by carboxypeptidases.

Surprisingly, the deletion of Arg^{36} improves slightly the potency, since **1** is more active than the native hormone. This could indicate that, if a turn or fold was required for full biological activity, Arg^{36} was not implicated in its stabilization.

2.1. Substitution of His^7

His^7 was described as essential since its deletion, modification or substitution results in an almost complete loss of biological activity [12]. We found that His^7 can be replaced by a phenylalanine since **2** is a full functional agonist as potent as the native hormone. The aromatic character of the residue in N-terminal position could be the determinant factor for affinity and activity rather than the charge. However, note that the deletion of Arg^{36} in **2** decreased the potency since **3** was significantly less active than the parent peptide. Replacement of His^7 by a bulky aromatic amino acid such as tryptophan (compounds **4** and **5**), results in a clear decrease in affinity and activity (ten times for affinity and more than hundred times for activity) compared to GLP-1-[7-37]-NH_2. We have also confirmed that the mutation $His^7 \rightarrow Tyr^7$ (compound **6**) reduced significantly the potency as previously described in the literature [9,10].

From our results and those previously reported by others, it appears that the N-terminal amino acid in GLP-1-[7-37] must be aromatic, less bulky than tryptophan and without any polar substituent on the aromatic ring.

2.2. Substitution of Ala^8

As previously reported [13], analogs of tGLP-1 with D-Ala or small amino acids (Gly and Aib) in position 8, bind to the receptor with high affinities and are active in the isolated perfused pancreas. Here, we confirm that the stereochemistry has no importance at this position, as **7** and **8** display the same activity than GLP-1-[7-37]-NH_2. On the contrary, replacement of Ala^8 by a serine found at the same position in several peptides of the GRF superfamily, reduced the affinity for the receptor by a factor 10 and the production of cAMP by about a factor 20 (**9** and **10**). This decrease in activity might be related to an increase in polarity rather than in volume since compouds **11** and **12** in which Ala^8 was replaced by a valine more voluminous than a serine, retain substantial biological activities. However, beyond a certain value, the incidence of the side-chain volume is critical since **13** and **14**, containing a leucine more voluminous than a valine, display a ~ 200-fold reduction in receptor activation.

These results highlight the incidence of both steric hindrance and polarity associated to the amino acid side-chain located in position 8.

2.3. Substitution of Glu^9

With the exception of glucagon which possesses a glutamine, all the peptides belonging to the GRF superfamily have aspartic or glutamic acid in position 9. This suggests that the presence of an amino acid with an acidic side-chain could be required at position 9 in this superfamily.

The minor change $Glu^9 \rightarrow Asp^9$ enhanced significantly both IC_{50} and EC_{50} since **15** and **16** are quite as active as exendin-4, the most potent tGLP-1 agonist known today [14]. Surprisingly, the analogs with Leu (**17** and **18**) or Met (**19** and **20**) in place of Glu^9 display roughly the same activity as GLP-1-[1-37]. This could suggests that Glu^9 is not implicated in the formation of an ionic bond with basic residues

Table 1
Sequence, in vitro activities and plasmatic stability of the peptides

Compound	Sequence	IC_{50} (nM)[a]	EC_{50} (nM)[b]	Stability (%)[c]
GLP-1-[7-36]-NH_2	$H^7AEG^{10}TFTSD^{15}VSSYL^{20}EGQAA^{25}KEFIA^{30}WLVKG^{35}R$-$NH_2$	0.28 ± 0.01	0.8 ± 0.1	
GLP-1-[7-37]	$H^7AEG^{10}TFTSD^{15}VSSYL^{20}EGQAA^{25}KEFIA^{30}WLVKG^{35}RG$	0.31 ± 0.01	0.9 ± 0.1	
GLP-1-[7-37]-NH_2	$H^7AEG^{10}TFTSD^{15}VSSYL^{20}EGQAA^{25}KEFIA^{30}WLVKG^{35}RGNH_2$	0.26 ± 0.01	0.8 ± 0.1	10
1	H AEG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	0.18 ± 0.02	0.5 ± 0.1	28
2	**F** AEG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.32 ± 0.06	0.9 ± 0.4	
3	**F** AEG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	1.6 ± 0.2	7 ± 0.3	
4	**W** AEG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	3.3 ± 0.6	127 ± 35	
5	**W** AEG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	4.6 ± 0.8	152 ± 41	
6	**Y** AEG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	2.7 ± 0.4	5.4 ± 0.5	
7	H **a**EG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.15 ± 0.01	0.8 ± 0.4	
8	H **a**EG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	0.33 ± 0.1	1.4 ± 0.6	100
9	H **S**EG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	2.9 ± 0.3	15 ± 6	
10	H **S**EG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	3.8 ± 0.3	17 ± 8	
11	H **V**EG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.47 ± 0.04	2.5 ± 0.8	
12	H **V**EG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	1.4 ± 0.1	3.5 ± 0.2	
13	H **L**EG TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	5.7 ± 1.7	180 ± 62	
14	H **L**EG TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	16.8 ± 1.7	218 ± 31	
15	H A**D**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.09 ± 0.03	0.4 ± 0.3	
16	H A**D**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	0.15 ± 0.01	0.22 ± 0.1	59
17	H A**L**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.4 ± 0.1	0.8 ± 0.6	
18	H A**L**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	0.24 ± 0.03	0.5 ± 0.1	40
19	H A**M**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	0.09 ± 0.01	0.7 ± 0.4	
20	H A**M**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	0.19 ± 0.03	0.8 ± 0.3	18
21	H A**S**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	1.5 ± 0.4	3.5 ± 0.4	
22	H A**S**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	4.9 ± 0.2	6.4 ± 0.2	
23	H A**V**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	9.29 ± 0.33	102.7 ± 20	
24	H A**V**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	19.51 ± 1.70	97.9 ± 10.9	
25	H A**K**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	216 ± 21	126 ± 26	
26	H A**K**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	231 ± 12	354 ± 43	
27	H A**e**G TFTSD VSSYL EGQAA KEFIA WLVKG $RGNH_2$	9.29 ± 0.33	102.7 ± 20	
28	H A**e**G TFTSD VSSYL EGQAA KEFIA WLVKG - GNH_2	19.51 ± 1.70	97.9 ± 10.9	
Exendin-4	H GEG TFTSD LSKQM EEEAV RLFIE WLKNG GPSSG APPPS	0.22 ±0.01	0.3 ± 0.1	

[a] IC_{50} expressed as mean ± SEM (n = 5 or 47 in the case of GLP-1 [$^{-}$7 – 37]-NH_2).
[b] EC_{50} expressed as mean ± SEM (n = 5 or 37 in the case of GLP-1 [$^{-}$7 – 37]-NH_2).
[c] Percentage of peptide remaining unchanged after 1 hour of incubation in human plasma.

belonging to the receptor or the sequence of the hormone. It must be noticed that the mutations $Glu^9 \rightarrow Ser^9$ (**21** and **22**) and $Glu^9 \rightarrow Val^9$ (**23** and **24**) reduce significantly the activity.

Introduction of a positive charge via a Lys^9 (**25** and **26**) or substitution of Glu^9 by its D stereoisomer (**27** and **28**) clearly reduced the potency. Whereas the receptor affinity remains to a significant level for **27** and **28**, it is strongly reduced for **25** and **26**.

From the results reported above, it appears that position 9 can accommodate acidic, polar or hydrophobic amino acids, but it cannot accept basic residues or D-Glu.

2.4. Plasma stability

Compounds **1, 8, 16, 18** and **20**, as well as GLP-1-[7-37]-NH_2were incubated with human plasma and the stability evaluated as the percentage of peptide remaining unchanged after one hour of incubation (Table 1). Surprinsingly, the deletion of Arg^{36} slows down the proteolysis since **1** is significantly more stable than GLP-1-[7-37]-NH_2 (28% versus 10% of peptide remaining unchanged). Compound **20** is approximately in the same range of stability (20%). Both **16** and **18** are clearly less degraded (59% and 40%, respectively). Therefore the nature of the residue in position 9 has a marked incidence on the activity of the proteases (presumably DPP IV) responsible for degradation in plasma. The presence of Asp^9 or a bulky hydrophobic residues such as Leu^9 reduces the rate of degradation, whilst Met^9 has less incidence.

As expected, **8** proved by far to be the most stable derivative, remaining totally unchanged after the 60 min incubation in human plasma.

2.5. In vivo evaluation

Compound **8** was therefore evaluated in vivo in an intravenous glucose tolerance test in rats at the dose of 400 ng/kg given intravenously and compared to GLP-1-[7-37]-NH_2 at

Table 2
Glucose tolerance (ΔG) and insulin response to glucose (ΔI, ΔI/ΔG) in rats treated with a single i.v. administration of GLP-1 [7-37]NH_2 or [a^8-desArg^{36}]GLP-1[7-37]NH_2 (400 ng/kg) 5 or 10 min before glucose injection.

Time (min)		ΔG	ΔI	ΔI/ΔG
		g/l	(*μU/ml*)	
5	Control rats	36.54 ± 2.23	3079 ± 243	1116 ± 133
	GLP-1[7-37]NH_2 treated rats	26.95 ± 2.09*	3205 ± 190	1550 ± 104*
	[a^8-desArg^{36}]GLP-1[7-37]NH_2 treated rats	28.71 ± 2.29*	5282 ± 318*	2389 ± 132*
10	Control rats	34.20 ± 0.91	1272 ± 40	481 ± 21
	GLP-1[7-37]NH_2 treated rats	33.31 ± 1.08	1201 ± 115	466 ± 47
	[a^8-desArg^{36}]GLP-1[7-37]NH_2 treated rats	23.33 ± 2.25*	2631 ± 349*	1509 ± 200*

Values are means ± SEM of 6-10 cases. ΔG, incremental plasma glucose values integrated over 30 min after the glucose load; ΔI, incremental plasma insulin values integrated over 30 min after the glucose load; ΔI/ΔG, insulinogenic index. *p<0.05; significantly different from control rats. The significance of differences was evaluated by ANOVA followed by a Fisher's test

the same dose (Table 2). A single intravenous administration of GLP-1-[7-37]-NH_2 five minutes before glucose loading improved glucose tolerance but had no effect on insulin secretion. No significant effects were observed when GLP-1-[7-37]-NH_2 was administered intravenously ten minutes before glucose injection. By contrast, a single injection of **8** five or ten minutes before glucose injection improved both glucose tolerance and insulin secretion. This improvement of the in vivo activity is in agreement with the high activity and the increased plasmatic stability observed in vitro.

2.6. Circular Dichroism Measurements

CD spectra of GLP-1-[7-37]-NH_2 and analogs were registered first in 10 mM sodium phosphate buffer (pH 7.35) and then in 10 mM sodium phosphate buffer (pH 7.35) containing various percentages of TFE (Fig. 1).

In phosphate buffer, the conformation of GLP-1-[7-37]-NH_2 is a combination of unordered structure (58%), β sheet (10%) and α-helix (32%). This latter percentage was roughly similar to that previously measured (35%) by Adelhorst et al. [10]. The CD spectra of all the analogs studied in this work were found to be very similar .

In TFE-water mixture, the proportion of α-helix for GLP-1-[7-37]-NH_2 increased up to 70% for a concentration in TFE close to 30% (above this limit no further change was observed). There again, all the peptides studied displayed identical CD spectra.

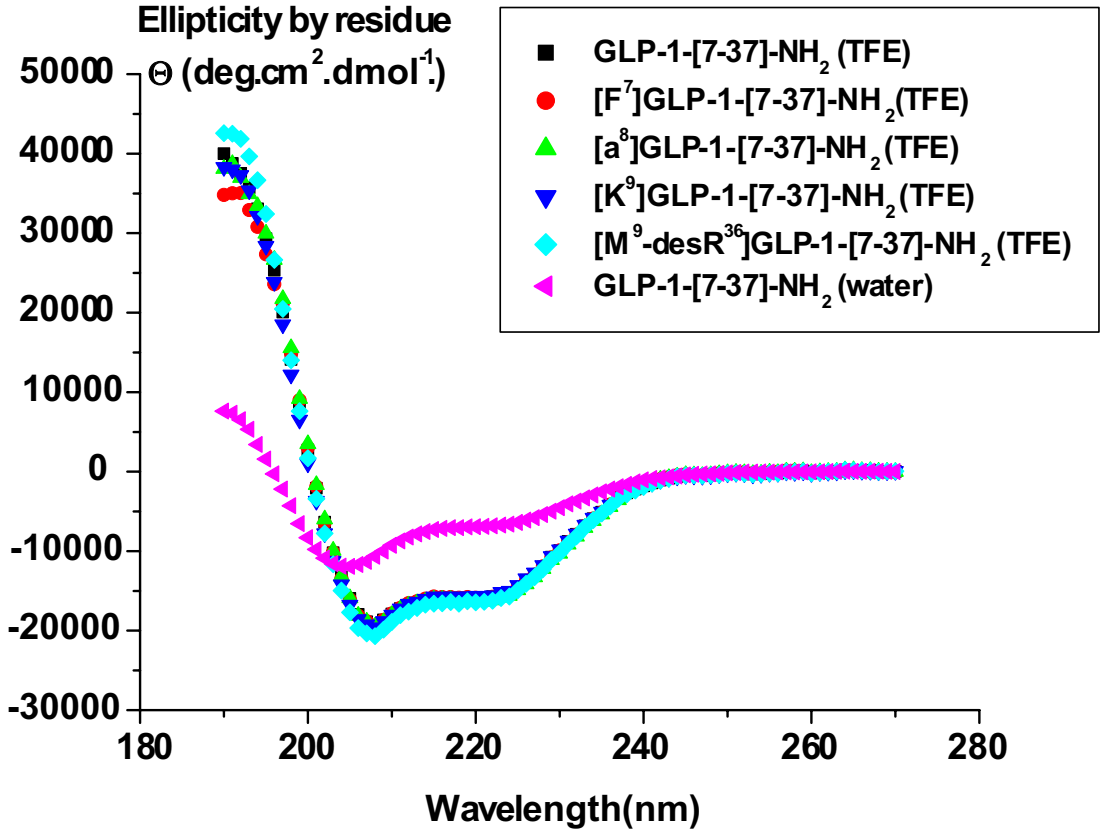

Fig. 1. CD of GLP-1-[7-37]-NH_2 and some analogs in phosphate buffer (10 mM, pH 7,35) and in phosphate buffer (10 mM, pH 7,35) containing 30% TFE.

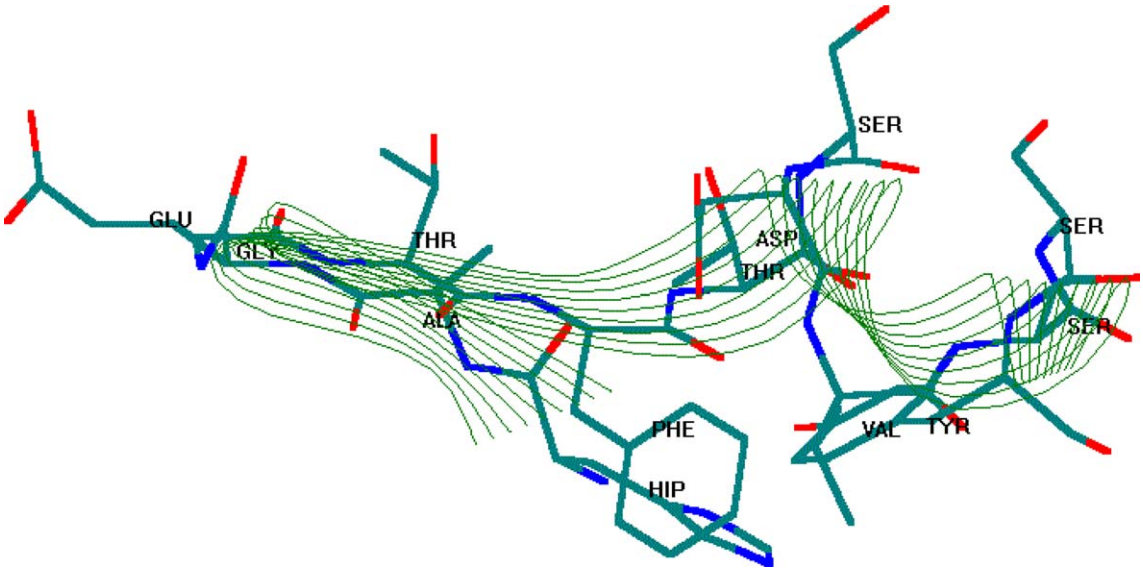

Fig. 2. Schematic model for the N-terminal part of GLP-1-[7-37]-NH_2. This model is based on the work of Thornton and Gorenstein (see ref. 15).

3. Discussion

In phosphate buffer GLP-1-[7-37]-NH_2 and analogues modified in position 7, 8, and 9 with or without deletion in 36, displayed a low percentage of helicity (about 32%). This latter increases up to about 70% in TFE-water, a membrane mimetic environment (Figure 1).

The helical percentage found here for all the GLP-1 analogs in TFE-water mixtures, is similar to that deduced (73%) from the structure adopted in deuterated dodecyphosphocholine micelles (pH 6, 50 mM sodium phosphate buffer) by [Q^{15}]GLP-1-[7-36]-NH_2 [15] that is : a N-terminal random coil segment (residues 7-12), two α helices (residues 13-20 and 24-36) and a conformationally labile linker region (residues 21-23).

Indeed, in the conformation adopted in membrane-like environments, the N-terminal part displays a great conformational lability [15], so that it is possible that the positively charged imidazolyl group of His^7 could be attracted to the negatively charged carboxylic group of Asp^{15} as assumed in glucagon [16]. This latter hormone and GLP-1 possess highly homologuous N-terminal sequences (H^1SQGTFTSD^9- and H^7AEGTFTSD^{15}-, respectively) in which the first histidine is separated from an aspartic acid by seven residues. In GLP-1-[7-37]-NH_2, an ionic interaction could occurred between His^7 and Glu^9. However, this is unlikely since the replacement of Glu^9 by uncharged amino acids (Met or Leu) gives analogues as active than the native peptide (Table 1). Consequently, we may assume that in the GLP-1 receptor-bound state, if an ionic interaction exists, it has to occur between His^1 and Asp^{15} as in glucagon [12,16].

Starting from a model based on the Thornton and Gorenstein results [15], in which the residues 13-20 and 24-36 belong to two separated helices, we have attempted to fold the N-terminal part so that the side chain of His^7 and Asp^{15} get closer. The formation of a large loop between residues 7 and 15 certainly implies the formation of a type II β-turn at Ala-Glu-Gly-Thr level. Indeed, it is well established that Gly^{10} displays an important role in GLP-1 activity. Its replacement by other residues, even small as an alanine, gives a dramatic reduction in both affinity and activation. This strongly suggests that Gly^{10} must occupy the position i+2 of a type II β-turn. In this kind of secondary structure, there is severe steric hindrance between Oi+1 and the side-chain Ri+2 so that a Gly was required at position i+2 [17].

First, the sequence Ala-Glu-Gly-Thr was built as type II β-turn (Glu $\phi = -60°$ and $\Psi = 120°$, Gly $\phi = 80°$ and $\Psi = 0°$, Figure 2). The turn is stabilized by a hydrogen bond from the carbonyl of Ala^8 to the NH of Thr^{11}. Then, without modifying the ϕ and Ψ angles for Glu^9 and Gly^{10}, the structure of the backbone was adapted so that the protonated imidazolyl group of His^7 might interact with the side-chain of Asp^{15}.

Note that in the structure obtained (Figure 2) the side-chains of Tyr^{19} and Phe^{12} as well as the imidazolyl group of His^7 and the COOH of Asp^{15}, are in close vicinity. Consequently, when His^7 is replaced by a phenylalanine, the aromatic group of this residue may interact with the phenyl groups belonging to Tyr^{19} and Phe^{12}, thus stabilizing the large N-terminal loop. This could explain the high activity displayed by **2**. In addition, the proximity of the aromatic moiety of His^7 and Tyr^{19} allows to interpret the fact that the N-terminal amino-acid in GLP-1 must be a histidine (or a phenylalanine), but not a tyrosine; in this latter case, the presence in the same region of the space of two OH groups induces a destabilizing electrostatic repulsion.

If the structure required for favorable $His^7 \rightarrow Asp^{15}$ interactions must be close to that of a type II β-turn, it is obvious that mutations performed on the two ″corners″ residues: Glu and Gly, could stabilize or destabilize the turn, according to the nature of the side-chains. It is highly probable that the biological activity is related to the ease of the formation of this reverse turn. As discussed above, this is in good agree-

ment with the receptor activity of the analogs having in position 10 other residues than Gly. In addition, the incidence on biological activity of mutations carried out at position 9 reinforces the hypothesis of the presence of a turn. Indeed, in the native hormone the side-chain of Glu^9 adopts a pseudo-equatorial arrangement around the cyclic hydrogen-bonded turn structure (Figure 2). Replacement of this residue with a D glutamic acid disposes the side-chain in a pseudo-axial configuration, fewer stable because of the occurrence of non-bonding interactions with the carbonyl of Ala^8. This explains the strong decrease in potency observed for **27** and **28**.

According to Chou and Fassman analysis [18], the frequency of occurrence in a reverse turn (f_t) for Glu (0.12) is roughly similar to that of Leu (0.14) and Met (0.18) and smaller than that of Asp (0.33). Taking into consideration these values, it is possible to interpret the biological activity of some GLP-1 analogs modified in position 9. This activity parallels to f_t [18]: whereas **15** was more potent than GLP-1-[7-37]-NH_2, **17** and **19** displayed a similar behavior in regard to receptor activation. In addition, it must be noticed that for **23** the production of cAMP was reduced by about a factor 3. In this peptide, the position i + 1 of the type II β turn, was occupied by a residue whose the f_t is small (0.08) [18].

However, note that **21** and **25** were less potent than GLP-1-[7-37]-NH_2, despite the fact that serine and lysine displayed reverse turn propensities (f_t = 0.41 and 0.27 respectively) [18] higher than that of glutamic acid found in the native hormone. This is not surprising. For **25**, we observed that both affinity and activity were dramatically reduced. This could mean that Lys^9 modified the whole structure of GLP-1 by forming an ionic bond with acidic residues such as Asp^{15}, Glu^{21} or Glu^{27}. Under these circumstances, conformations of both the C-terminal region responsible for affinity, and the N-terminal responsible for activation were altered. In the same hand, Ser^9 could destructure the loop by forming an H-bond with neighbor CO groups belonging to the backbone.

In addition to the ease of β-turn formation, the behavior on RIN T3 cells of analogs, modified in position 7 and 8, may be interpreted on the basis of stabilizing or destabilizing interactions originating inside the N-terminal loop. Thus, the fact that **4** was less potent could be related to the bulk of the first amino-acid. Despite its aromaticity, the volume of the indole nucleus is too important to insert between Tyr^{19}, Phe^{12} and Asp^{15} without modifying the configuration of the loop.

From the structure drawn in Figure 2, it appears that the methyl group of Ala^8 is directed towards the inside of the loop, so that mutations at this place, carried out with residues having long side-chain such as leucine, destabilize the structure by interacting with the amino acids facing it (Thr^{11}). Under these circumstances, position 8 can accommodate valine without dramatic change in activity but not leucine. In the case of **9**, electrostatic repulsions between the hydroxyl and the carbonyl groups belonging to Ser^8 and His^7, respectively destabilize the N-terminal structure and explain the loss in biological activity. The change in chirality: L-Ala^8 → D-Ala^8, by positioning the methyl group towards the outside of the loop, makes it more stable, so that the analog **7** is, at least, as active as the native hormone.

Such an analysis based on the consideration of interactions occurring in the large planar loop formed in the N-terminal region of GLP-1 molecule in the receptor-bound state, allows to interpret some observations previously reported in the literature too. Goke et al. [19] have shown that positions 11, 12, 13, 14 and 15 in GLP-1-[7-36]-NH_2 were very sensitive to stereochemistry. The analogs with D-residues at these positions displayed dramatic reduction in intracellular cAMP production. From Figure 2, it is clear that the replacement of Thr^{11}, Thr^{13} and Ser^{14} by D-Thr and D-Ser, respectively, generates steric hindrance between the side-chains and the CO groups of the peptide backbone. In the case of mutation L-Phe^{12} → D-Phe^{12}, the phenyl group of the analog is directed towards the inside of the loop destabilizing this latter. For the replacement of L-Asp^{15} by D-Asp, the Cα-Cβ bond of the side-chain of the unnatural amino-acid eclipse the carbonyl group. Consequently, the stereochemical changes carried out at positions 11, 12, 13, 14 and 15 generate steric constraints which modify the conformation of the N-terminal loop and reduce mainly their capacity to activate the specific receptor.

4. Conclusions

The in vitro activities of numerous GLP-1 analogs may be interpreted on the basis of the existence, in the receptor bound state, of a large loop between His^7 and Asp^{15}, with the sequence Ala^8-Glu-Gly-Thr^{11}- adopting a type II β-turn motif. This structure allows the interaction between three aromatic nuclei belonging to His^7, Phe^{12} and Tyr^{19}. Consequently, it is possible to assume that it exists at the receptor level an hydrophobic pocket able to accept this aromatic cluster and responsible for its activation.

It appears that important residues for binding and activation are non-contiguous in sequence and in space. Therefore, it has been suggested that it is unlikely that a peptide significantly smaller than GLP-1 itself, will be able to mimic its biological activity [20]. Consequently, it appears that the possibilities to make a GLP-1 agonist are restricted to either analogs with improved pharmacokinetic properties as [a^8-desR^{36}]GLP-1-[7-37]- NH_2 and others substituted analogs [21], or to derivative analogs with long fatty acid chains [22] or albumine conjugate [23].

However, it is possible that heterocyclic derivatives, mimicking the structure of the cluster formed by the side-chains of His^7, Phe^{12} and Tyr^{19} could act as antagonist by binding to the hydrophobic pocket present on the receptor. A small molecule which interacts with the tryptophan in position 33 of the human GLP-1 receptor has been recently described as an antagonist [24].

In conclusion, some of the derivatives described in this work are at least as active in vitro as GLP-1-[7-37]-NH_2 itself

but with a much higher plasmatic stability. This is the case for [a^8-desR^{36}]GLP-1-[7-37]- NH_2 which remains totally unchanged after a 60-min plasmatic incubation and exhibits a clearly longer in vivo activity than GLP-1-[7-37]-NH_2 in an glucose tolerance test in rats. This compound is currently under complementary pharmacological evaluations.

5. Experimental section

Reagents. Tyr^1-cAMP, IBMX, bacitracin and BSA (fraction V) were purchased from Sigma Chemical Co (St Quentin Fallavier, France). Na[^{125}I] (2,000 Ci/mmol) was from DuPont NEN (Mechelen, Belgium). Mono-[^{125}I]-labeled GLP-1-[7-37]-NH_2 and mono-[^{125}I]Tyr1-cAMP were obtained by the chloramine-T procedure and purified by reverse phase HPLC on μBondapak C18 column (Waters, Milford, MA). All the compounds used for solid-phase peptide synthesis (resins, coupling reagents and protected amino acids) were from PerSeptive Biosystems (Framingham, MA, USA).

5.1. Peptide Synthesis

Peptide synthesis was carried out at a 0.1-mmol scale using a continuous flow apparatus (PerSeptive Biosystems, model 9050 PepSynthesizer) starting from Fmoc-PAL-PEG-PS-resins. All N-α-Fmoc amino acids in 6-fold excess, were assembled using diisopropylcarbodiimide and 1-hydroxybenzotriazole as coupling reagent. Peptides were cleaved from the resin and deprotected using K reagent [25]. Each crude peptide was purified by semi-preparative HPLC on Δpack column (20 x 250 mm, Waters) using various acetonitrile gradients in aqueous 0.1% TFA. The purity of the collected fractions was established by analytical HPLC on Lichrosphere-100 RP-18 columns (Merck, Darmstadt, Germany) and by electrospray ionization mass spectrometry (Trio 2000, Fisons Instruments, Manchester, UK). For all the peptides synthesized, amino acid analyses and mass spectra were in complete agreement with the proposed structures.

5.2. Cell Cultures and Membrane Preparations

RIN T3 cells (obtained from Dr. L. Pradayrol, INSERM U151, Toulouse, France) were grown in DMEM containing 5.5 mM glucose (Life Technologies, Eragny, France), and supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin [26]. Plasma membranes from RIN T3 cells were prepared according to a previously described method [26] and stored at -80°C until use. Membrane proteins were measured by the Bradford assay with BSA as standard [27].

5.3. Binding Studies

Binding experiments with RIN T3 membranes were performed in 60 mM Tris-HCl buffer (pH 7.5) containing 4% BSA and 750 μg/ml bacitracin. Membranes (20-30 μg) were incubated in a final volume of 500 μl with ~15 fmol of ^{125}I-GLP-1-[7-37]-NH_2 (50.000 cpm) and unlabeled competitor ligands or analogues for 45 min at 37°C, as described previously [26]. The reaction was stopped by addition of 750 μl ice-cold KRB (pH 7.5) containing 3% BSA, centrifuged at 4°C for 5 min at 12.000 g. The pellet was rinsed with 1 ml of ice-cold KRP, sedimented by another centrifugation step, and counted for radioactivity. Unspecific binding was determined in the presence of 1 μM GLP-1-[7-37]-NH_2 and was subtracted from each value. Binding data were analyzed using a version of the Ligand program (release 2.0, Biosoft). IC_{50} values were deduced by fitting the experimental data according to the Hill equation.

5.4. Cyclic AMP Production

RIN T3 cells were grown in 24-well plates for 6 days and the medium was changed 1 day before experiments. Cells ($0.30 \pm 0.03\ 10^6$ cells/well; n = 10) were washed twice with DMEM before the addition of 0.5 ml DMEM supplemented with 1% BSA and 0.2 mM IBMX and containing the test peptides. After a 20-min incubation at 25°C, cellular cAMP was extracted, succinylated and quantified by radioimmunoassay [28]. The data are presented as a percent of maximal stimulation. 100% cAMP production corresponds to the maximal production above basal (5-8 fold) obtained by 10^{-8} M GLP-1-[7-37]-NH_2 and 0% cAMP production corresponds to the basal production in the absence of GLP-1-[7-37]-NH_2 or analogs. The EC_{50} values were calculated from the Hill equation.

5.5. Glucose Tolerance Tests

Male Wistar rats (Iffa-Credo, L'Arbresle, France) weighing about 300g were used. The animals were housed in a room with a 12-hr fixed light-dark schedule at 21 ± 2°C. Food was withdrawn for 4 hours before starting the experimentation. Glucose was dissolved in 0.9% saline and given by the saphenous vein route (0.5g/kg) to rats under pentobarbital anesthesia (60mg/kg, intraperitoneal; Sanofi, Libourne, France). Blood samples were collected sequentially by the tail vessels before and 2, 5, 10, 15, 20 and 30 min after the injection of glucose. They were then centrifuged, and the plasma was separated. Plasma glucose concentration was determined immediately in a 10μl aliquot and the remaining plasma was kept at –20°C until radioimmunoassay of insulin (Dia Sorin, Antony, France). A single injection via the saphenous vein (400 ng/kg) of either GLP-1 or **8** was performed in rats 5 or 10 min before the injection of glucose.

5.6. Degradation of GLP-1-[7-37]-NH_2 and Analogues in Human Plasma

The peptides were dissolved in 50ng/ml BSA in 1% TFA-water to give a nominal 1mg/ml concentration. Aliquots of 50μl of the 1mg/ml solution of each peptides were added to 940μl of 0.1 M TRIS (pH 8) to give 50μg/ml. These stock

solutions of each peptides were added to heparinised human plasma in gold grade borosilicate glass. The sample were maintained at 37°C and the peak heights of each peptides were determined at T=0 and at 60min. The stability of the peptides in plasma was determined by HPLC-florescence. Two columns were used: a HISEP, (20X4.6mm,5µm) and a ISRP column (Supelco, Dorset) for the elimination of protein allowing direct plasma injection. This was coupled via a switching valve to a Waters Symmetry Shield, (100X4.6mm, 3.5µm) RP8 column (Watford, Herts). The mobile phase comprised an acetonitrile/water gradient with a constant 0.05% concentration of TFA throughout. A flow rate of 1ml/min and a temperature of 40°C were used. Detection was by fluorescence at 280nm (excitation) and 340 nm (emission).

5.7. Circular Dichroism Measurements

CD measurements were carried out on a Jobin-Yvon CD6 dichrograph at a scan speed of 20 nm/min using quartz cells with 1 mm pathlength. The peptide concentration used was 0.05 µM in 10 mM sodium phosphate buffer (pH 7.35) and in 10 mM sodium phosphate (pH 7.35) containing various TFE percentages. All spectra correspond to an averaging of five separate experiments and were corrected by the base line obtained for peptide free preparations. The amounts of the various structures were estimated using the Dicroprot software (V 2.3 by G. Deléage, IBCP, Lyon, France).

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