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^{1}H Nuclear-Magnetic-Resonance Studies of the Molecular Conformation of Monomeric Glucagon in Aqueous Solution

Chris BOESCH, Arno BUNDI, Max OPPLIGER, and Kurt WÜTHRICH

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Zürich-Hönggerberg

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Dilute aqueous solutions of glucagon were investigated by high-resolution ^{1}H nuclear magnetic resonance at 360 MHz. Monomeric glucagon was found to adopt predominantly an extended flexible conformation which contains, however, a local non-random spatial structure involving the fragment –Phe-22–Val-23–Gln-24–Trp-25–. This local conformation is preserved in the partial sequence 22–26 and could thus be characterized in detail. Two interesting conclusions resulted from these experiments. One is that the local spatial structure in the fragment 22–25 of glucagon is identical to that observed in the fragment 20–23 of the human parathyroid hormone. Secondly, the backbone conformation in the C-terminal fragment of glucagon in solution must be different from the α-helical structure observed in single crystals of glucagon. These new structural data are analyzed with regard to relationships with glucagon binding to the target cells.

Glucagon is a polypeptide hormone which consists of one linear peptide chain with 29 amino acid residues [1]. The biological function of glucagon was found to be related with specific binding to a plasma membrane receptor, which results in stimulation of adenylate cyclase [2]. To gain more detailed insight into the structure-function relations, the molecular conformation of glucagon was extensively investigated, both in single crystals and in solution. In the single crystal X-ray structure, glucagon trimers were observed, where the individual molecules adopt a mainly α-helical conformation [3]. Glucagon in solution was investigated by various spectroscopic techniques, such as circular dichroism [4–7], optically detected magnetic resonance [8] and nuclear magnetic resonance (NMR) [9–11]. Two observations stand out among the results of the solution studies. One is that the conformational properties of glucagon manifested in the spectral parameters depend strongly on the solution conditions. Thus it was reported that glucagon in freshly prepared dilute aqueous solution adopts predominantly a flexible 'random coil' form, while under different solution conditions or simply after prolonged standing of the solutions more highly structured aggregates were observed [4–8]. Conformational changes are apparently also induced by interactions of glucagon with lipids or detergents [12, 13]. Secondly, the descriptions of the solution conformations are throughout in the terminology of circular dichroism measurements, i.e. random coil structures and those with varying α-helix of β-pleated sheet contents were distinguished [4–9].

In view of the pronounced conformational polymorphism shown by numerous investigations under different experimental conditions, it appears particularly important to complement the single-crystal X-ray structure analysis by detailed conformational studies in well-defined solutions. High-resolution NMR is the method of choice for obtaining a many-parameter characterization of the solution conformation of polypeptides [14]. In the earlier NMR studies of glucagon [9–11] the interpretation of the spectral data was, however, complicated by aggregation in the relatively concentrated peptide solutions or by additives used to prevent aggregation. In the present study the high sensitivity of Fourier transform ^{1}H NMR spectroscopy at 360 MHz was used to investigate dilute aqueous solutions of glucagon.

At the outset of this investigation we noticed that the amino acid sequences of glucagon [1] and human parathyroid hormone [15] both contained the pentapeptide fragment -X-Val-Gln-Trp-Leu, with X standing for phenylalanine in glucagon and for arginine in the parathyroid hormone. An extensive investigation of the human parathyroid hormone had shown

Abbreviations. NMR, nuclear magnetic resonance; Z, benzyloxycarbonyl protecting group.

that this particular pentapeptide fragment adopts a local non-random spatial structure, which is preserved also in the pentapeptide partial sequence [16,17]. Here the pentapeptide Phe-Val-Gln-Trp-Leu was synthesized, its solution conformation determined by ^{1}H NMR and compared with the spatial structure of the corresponding pentapeptide fragment in the intact glucagon molecule. This strategy for the present study appeared promising with regard to both comparison with the corresponding experiments with parathyroid hormone [16,17] and the implication from earlier investigations that the above peptide fragment is essential for binding of glucagon to its receptor [18].

MATERIALS AND METHODS

Synthesis of Phe-Val-Gln-Trp-Leu

The *N*-protected tripeptide Z-Gln-Trp-Leu (Z, benzyloxycarbonyl) was obtained as a gift from Dr W. Rittel (Ciba-Geigy AG, Basel). The corresponding free tripeptide was obtained by hydrogenation with palladium on coal. The protected dipeptide Z-Phe-Val was purchased from Bachem AG (Liestal). The two peptides were coupled by the *N*-hydroxysuccinimide active ester technique [19]. The solvent was then removed at room temperature in the high vacuum and methanol was added to the residue. The precipitate was filtered off and washed several times with 2-propanol followed by drying in the vacuum. The protected pentapeptide was homogeneous by the criteria of thin-layer chromatography in different systems, with R_F = 0.81 in 1/1 chloroform/methanol. The free pentapeptide was obtained through removal of the benzyloxycarbonyl protecting group by hydrogenation with palladium on coal with methanol as the solvent. The catalyst was filtered off and the filtrate evaporated to dryness. Subsequently the peptide was twice lyophilised from water. The product was homogeneous by the criteria of thin-layer chromatography in different systems, with R_F = 0.31 in 6/4 chloroform/methanol. ^{1}H NMR at 360 MHz showed that the product contained the expected amino acid composition.

Preparation of the NMR Samples

The synthetic partial sequence Phe-22—Val-23—Gln-24—Trp-25—Leu-26 of glucagon was studied in 0.05 M solution in ^2H_2O or in a mixed solvent of 90% H_2O/10% ^2H_2O.

Bovine glucagon was obtained from Calbiochem and used without further purification. In view of the previously described pronounced dependence of the glucagon conformation on the solvent medium [4—13], particular care was taken with the sample preparation. To prevent aggregation, the lowest possible peptide concentration for high-resolution ^{1}H NMR was employed, i.e. either 0.1 mM or 0.05 mM, and the ionic strength was kept low. For chemical shift measurements, some experiments were repeated with and without addition of an internal reference. The p^{2}H values were adjusted by the addition of a trace of KO^{2}H or ^{2}HCl solution. pH-meter readings in ^2H_2O solution are reported without correction for isotope effects [20]. p^{2}H and temperature were selected so that according to previous reports monomeric forms of glucagon should prevail in both basic [6] and acidic [21] solution (Fig. 1). All the measurements were completed within 24 h after sample preparation. With these precautions, identical ^{1}H NMR spectra could be reproduced in different experiments.

Besides preventing aggregation of glucagon, special care in the sample preparation was also required with regard to optimizing the ratio of the relative intensities of the solute and solvent resonances. ^2H_2O of isotope purity of 99.979% was obtained from the Eidgenössisches Institut für Reaktorforschung (Würenlingen). For the crucial experiments, glucagon was twice lyophilized from ^2H_2O to replace the labile protons with deuterium, and the final solution was handled under a dry nitrogen atmosphere, carefully degassed and sealed for the NMR measurements.

It should be pointed out that the high-resolution ^{1}H NMR spectra present a sensitive means to monitor the equilibrium between monomeric and aggregated forms of glucagon. In solvent media where aggregation is expected [4—7], pronounced line broadening was observed in the NMR spectra, e.g. after prolonged standing of the solutions [7].

NMR Measurements

360-MHz Fourier transform ^{1}H NMR spectra were recorded on a Bruker HX 360 spectrometer. Under steady-state conditions the solvent resonance in the carefully deuterated and degassed samples could be almost completely suppressed [14]. An acceptable signal-to-noise ratio in 0.1 mM glucagon solutions was thus obtained with accumulation of 5000—10000 scans. For spin decoupling experiments, double-resonance difference spectra were recorded as described previously [22]. Chemical shifts are relative to internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate.

RESULTS

Selected ^{1}H NMR parameters of the synthetic partial sequence 22—26 of glucagon are listed in Table 1. The parameters which are not shown in the table were found to be essentially identical to the corresponding random coil values [20]. In particular,

Table 1. *^1H NMR parameters for valine and tryptophan in the partial sequences 22–26 of glucagon and 20–24 of human parathyroid hormone [15] and in intact glucagon*
The assignment of the methyl resonances is arbitrary; γ^A is always the resonance at higher field. Glucagon 1–29 values were measured for monomeric glucagon at two different p^2H values, i.e. $p^2H = 2.4$ and 10.8, $t = 37\,^\circ C$. Chemical shifts δ are given $\pm$ 0.01 ppm, spin-spin coupling constants $J \pm 0.5$ Hz. Glucagon 22–26 and parathyroid hormone values were measured at p^2H 7.0, $t = 30\,^\circ C$, $\delta \pm 0.002$ ppm, $J \pm 0.2$ Hz

Peptide	δ			$^3J_{\alpha\beta}$	$^3J_{\alpha\beta A}$	$^3J_{\alpha\beta B}$
	$\gamma^A CH_3$	$\gamma^B CH_3$	αCH			
	ppm			Hz		
Glucagon 1–29	0.75	0.84	3.88	7.9	–[a]	–[a]
Glucagon 22–26	0.683	0.802	3.942	8.0	5.1	8.8
Parathyroid hormone 20–24	0.742	0.845	4.065	7.7	5.3	8.8
Random coil value [20]	0.942	0.969	4.184	6.9	6.0	7.8

[a] Not measured.

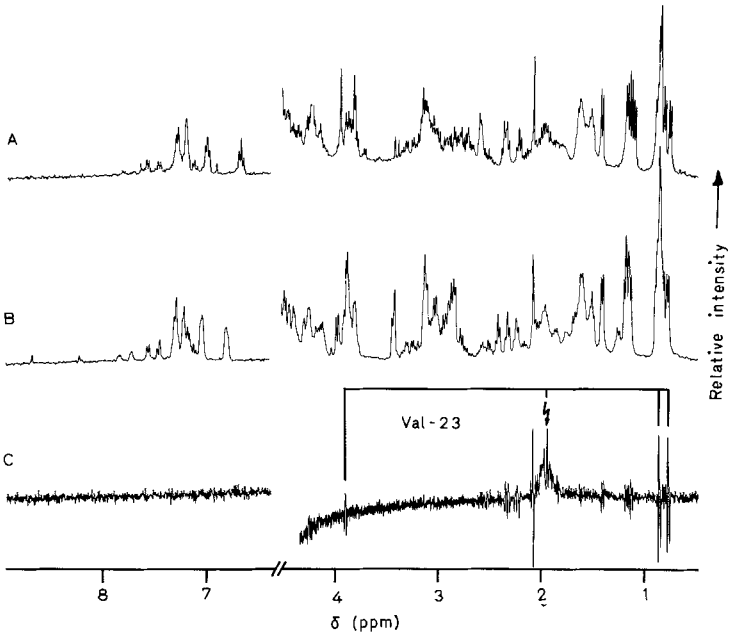

Fig. 1. *360-MHz Fourier transform ^1H NMR spectra of 0.1 mM solutions of glucagon in ^2H_2O, $t = 37\,^\circ C$.* (A) $p^2H = 10.8$. (B) $p^2H = 2.4$. (C) Double-resonance difference spectrum obtained as the difference of two spectra recorded with and without spin-decoupling irradiation at 1.93 ppm. The chemical shifts of the spin system of Val-23 are also indicated

there was no evidence for intramolecular hydrogen bonding in the pH dependence of the amide-proton chemical shifts in H_2O solution of the pentapeptide [23].

Outstanding among the conformation-dependent spectral features of the glucagon fragment 22–26 (Table 1) is the high-field shift of one of the γ-methyl resonances of Val-23. In the ^1H NMR spectrum of glucagon, a high-field-shifted methyl doublet resonance is also readily seen (Fig. 1). The spin decoupling experiment of Fig. 1C, where irradiation at 1.93 ppm caused the simultaneous collapse of two methyl doublets at 0.75 and 0.84 ppm and a weaker doublet resonance at 3.88 ppm, unambiguously showed that this high-field methyl line was part of a valine A_3B_3MX spin system [14]. Since glucagon contains only a single valyl residue in position 23 [1], the ^1H NMR parameters of Val-23 had thus been obtained also for intact glucagon (Table 1).

In Fig. 1 spectra of glucagon at two different p^2H values are presented to show that solutions of monomeric species were obtained at basic and acidic p^2H. The spectra at p^2H 10.8 and 2.4 both contain narrow lines characteristic of the monomeric peptide. The high-field-shifted methyl doublet at 0.75 ppm is seen at both p^2H values, and a result similar to that of Fig. 1C was also obtained at p^2H 10.8. While small differences between the molecular conformations at the two p^2H values cannot be excluded, it should be pointed out that most of the differences between the spectra A and B in Fig. 1 are a direct consequence of

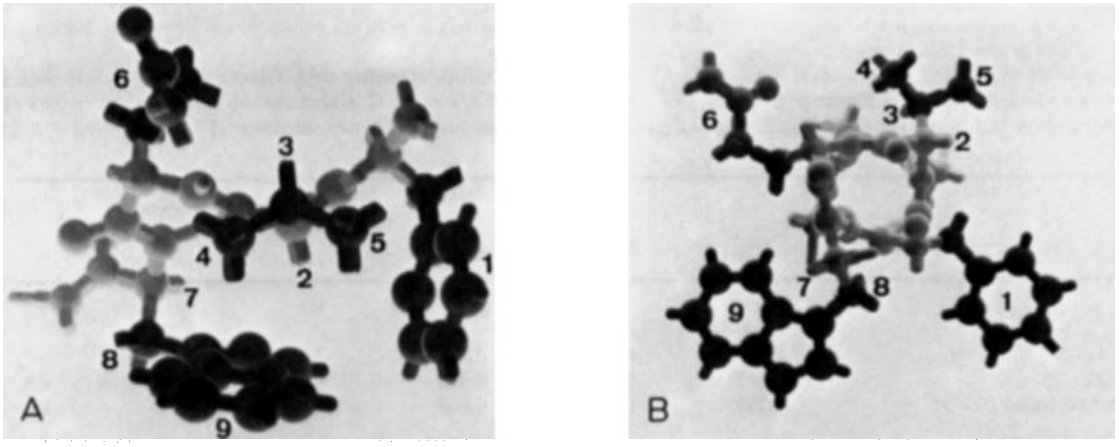

Fig. 2. *Two spatial structures of the fragment —Phe-22—Val-23—Gln-24—Trp-25— in glucagon constructed with Labquip molecular models.* (A) Conformation proposed on the basis of the NMR data for glucagon in aqueous solution. For the residues 23—25, the structure is identical to that of the corresponding fragment 21—23 in human parathyroid hormone [17]. (B) α-Helical conformation. Different parts of the structure are identified with the following numbers: 1, Phe-22; 2, α-proton of Val-23; 3, β-methine proton of Val-23; 4 and 5, γ-methyls of Val-23; 6, Gln-24; 7, α-proton of Trp-25; 8, β-methylene group of TrP-25; 9, indole ring of Trp-25

the protonation of the ionizeable groups in glucagon [1]; e.g. in the low field region, the titration shifts of the histidine and tyrosine resonances lead to a somewhat different appearance of the two spectra.

DISCUSSION

Comparison of the partial sequences glucagon 22—26 and parathyroid hormone 20—24 in Table 1 shows that the two peptides have nearly identical NMR parameters, except that the resonances of valine are shifted to even higher field in the glucagon fragment. The structural implications of the high-field shifts of the valine resonances and the vicinal spin-spin couplings in Table 1 were previously discussed in detail, and it was shown that the NMR parameters define a unique spatial arrangement of the tripeptide fragment -Val-Gln-Trp- [17]. The NMR data now show that this tripeptide sequence must adopt very similar spatial structures in glucagon and in human parathyroid hormone.

A molecular model of the structure proposed for the fragment 22—25 of glucagon in solution is shown in Fig. 2A. For the residues —Val-23—Gln-24—Trp-25—, the structure is identical to that in the corresponding sequence 21—23 of human parathyroid hormone [17]. The α proton and the γ-methyl groups of Val-23 are located close to the indole ring plane of Trp-25, which explains why they are shifted to high field relative to the random coil positions (Table 1). The spatial arrangements of the Val-23 and Trp-25 side-chains relative to the peptide backbone correspond to those predicted by the vicinal coupling constants in Table 1. The arrangement of the Phe-22 side-chain in the model of Fig. 2A is compatible with the observed relative chemical shifts of corresponding proton resonances in the parathyroid hormone and glucagon peptides. In the partial glucagon sequence 22—26, where Phe-22 is at the N-terminus and hence quite mobile, it produces relatively small upfield ring current shifts for the α proton and both methyls of Val-23 (Table 1). That only the α proton of Val-23 is sizeably shifted upfield relative to the position in parathyroid hormone (Table 1) would then seem to imply that in intact glucagon Phe-22 is more rigidly fixed in the position shown in Fig. 2A than in the partial sequence 22—26. In addition to its compatibility with the NMR data, the structure of Fig. 2A is also satisfactory in that it includes extensive contacts between hydrophobic groups, which appear to be the main factor for stabilizing this conformation [17].

Besides the non-random structure of the fragment 22—25 (Fig. 2A), the population of which was estimated to be approximately 20% at ambient temperature (see the preceeding paper [17] for details of the procedures used to estimate populations), the NMR data indicate that monomeric glucagon in aqueous solution adopts primarily a flexible extended 'random coil' form. This is in agreement with earlier spectroscopic observations which indicated the occurrence of 'random coil glucagon with some ordered structure near Trp-25' [8,9]. When comparisons with these earlier studies are pursued in more detail, it should also be considered that different solvent systems were used and that the optical detection of magnetic resonance studies were done in frozen solutions at low temperatures.

An intriguing result of the NMR experiments is that the NMR parameters are incompatible with the helical glucagon structure in single crystals [3]. This

is illustrated by the α-helical structure in Fig. 2B. It is readily apparent that the $C^{\alpha}-C^{\beta}$ bonds of Val-23 and Trp-25 are located on the surface of the helix at an angle of approximately 180°. For geometric reasons it would thus be impossible in a helical structure that the resonances of Val-23 could be affected by the ring current field of Trp-25. [That the high-field shifts of the Val-23 resonances (Table 1) are mainly due to the indole ring current field was previously demonstrated by comparison with a peptide analogue, where tryptophan was replaced by octahydrotryptophan [17].] As was discussed in the preceding paper [17], it is also unlikely that the solution conformation of Fig. 2A occurs in an equilibrium with a sizeably populated helical structure.

To relate the present results on the solution conformation of glucagon with the biological function we have to consider a situation where glucagon occurs in an aqueous medium at physiological concentrations (approx. 0.01 nM), from where it has access to the target cells. On the basis of the X-ray structure of glucagon it was previously argued that while the hormone must, from the available evidence, occur as a monomeric random coil in the dilute solution, a helical conformation might be stabilized by hydrophobic interactions with the binding sites on the receptor [3]. In this model glucagon in solution would retain no spatial structures which might function as recognition sites for the receptor, and binding would thus probably occur via an induced-fit mechanism. The NMR data described in the present paper now show that a local, quite rigid non-random spatial structure is contained in the solution conformation of monomeric glucagon, and there is no indication that this structure should not be preserved at physiological concentrations. Interestingly, this local non-random structure is formed by a peptide fragment which was long ago found to be essential for binding of glucagon to its receptor [18]. The following would then appear to be an attractive scheme for receptor binding of glucagon.

Solution

Monomeric random coil forms ⟷ Monomeric structure of Fig. 2A

Receptor

(Glucagon conformation stabilized by receptor interactions (helical structure [3] or as yet unknown conformation)) ⟷ glucagon structure of Fig. 2A bound to target cell

This scheme is based on the experimental observation that the dynamic ensemble of molecular structures, which constitute the solution conformation of glucagon [24, 25], contains a sizeable population, approximately 20%, of the molecules in the form of Fig. 2A. The local rigid spatial structure of the fragment 23–25 would function as the recognition site for binding to the target cells. While the initial binding of glucagon would thus be governed simply by the vertical equilibrium reaction in the above scheme, the overall reaction mechanism might be more intricate and involve successive conformational rearrangements of the bound polypeptide hormone. Such secondary reaction steps might present an effective regulatory mechanism; e.g. glucagon could be accumulated on the target cells in the form of Fig. 2A followed by structural rearrangements of the bound hormone, which would result in a reactive receptor · glucagon complex. It was previously reported that interactions with lipids or detergents induce global changes of the glucagon conformation [12, 13]. To get more precise data on the structural properties of glucagon in environments resembling more closely that of the receptor-bound hormone, work is in progress to use high-resolution NMR for studies of the polypeptide conformation in lipid/water interphases.

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