Proton Nuclear Magnetic Resonance Study of Glucagon

Dinshaw J. Patel

Bell Telephone Laboratories, Inc., Murray Hill, New Jersey 07974.
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ABSTRACT: A reinvestigation of the proton nmr spectra of glucagon in acid solution indicates that aggregation and not a helical segment at C-terminal end accounts for line broadening and chemical shift changes in native and urea denatured solutions. The assignment of some amino acid residues involved in aggregation are outlined.

A detailed conformational study of the 29 amino acid leniar hormone glucagon has been recently reported.[1] From an analysis of thermal difference and optical rotatory dispersion spectra in dilute aqueous media (<0.5 mg/ml) the authors concluded that glucagon in solution existed in helix $\rightleftarrows$ random coil equilibrium with the helix content estimated to be $\sim$10% at room temperature over a wide pH range.[1] A comparison of the aliphatic region in the proton nmr spectrum at 60 MHz of glucagon (20 mg/ml) at acid pH in the presence and absence of 6 *M* guanidinium hydrochloride led the authors to deduce that the short helical segment contains the long aliphatic side chains and hence surmise that the helix extends over Phe-Val-Gln-Trp-Leu-Met at the C-terminal end.[1]

Glucagon is known to aggregate in solution.[1-4] Swann and Hammes[2] have suggested a monomer–dimer–hexamer mode of association while Gratzer and Beaven[3] and Blanchard and King[4] favor a monomer–trimer mode of association as measured by physical techniques for aqueous solutions of glucagon at basic pH. The associated state(s) have been shown by X-ray studies in the crystal[5] and optical studies in solution to have helical character.

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1                5                10
His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-
                     15                20
          Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-
                               25
                Phe-Val-Gln-Trp-Leu-Met-Asn-Thr
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We have reinvestigated at 220 MHz the proton nmr spectra of glucagon in acid solution. We conclude (a) that the line broadening and shifts observed at high concentrations (20 mg/ml) of glucagon are assignable to aggregated state(s) rather than the previously proposed[1] helix at the C-terminal end,[6] and (b) that amino acid assignments to the side chain resonances of aggregated glucagon solution permits partial identification of the amino acids involved in intermolecular contact in the aggregated species.

(1) W. B. Gratzer, G. H. Beaven, H. W. E. Rattle, and E. M. Bradbury, *Eur. J. Biochem.*, **3**, 276 (1968).
(2) J. C. Swann and G. G. Hammes, *Biochemistry*, **8**, 1 (1969).
(3) W. H. Gratzer and G. H. Beaven, *J. Biol. Chem.*, **244**, 6675 (1969).
(4) M. H. Blanchard and M. V. King, *Biochem. Biophys. Res. Commun.*, **25**, 298 (1966).
(5) M. V. King, *J. Mol. Biol.*, **11**, 549 (1965).
(6) We have informed Professor Gratzer of our nmr results which contradict their previous conclusions. Since then they have suggested (ref 3, Note Added in Proof) that line broadening may be partly or largely due to an effect of association at C-terminal end of glucagon.

Experimental Section

Glucagon was purchased from Calbiochem. Samples were made up in solutions of 10% CD_3CO_2D–D_2O. Nmr spectra were run on a 220-MHz Varian nmr spectrometer equipped with a Varian variable-temperature unit. Ethylene glycol was used to monitor temperatures (±1°). A Fabri-Tek computer of average transients with 1024 channels was used to improve signal to noise. All nmr spectra were taken on freshly prepared nonviscous samples.

Results

We have studied glucagon solutions at concentrations 7.0×10^{-3}, 7.0×10^{-3}, + 8 *M* urea and 1.4×10^{-3} *M* in 10% CD_3CO_2D–D_2O. The proton nmr spectra of these three solutions in the aliphatic and aromatic regions are shown in Figure 1.

On comparison of the nmr spectrum of 7×10^{-3} *M* glucagon solution in the presence and absence of 8 *M* urea, one observes that the broad resonances sharpen up and chemical shift changes occur on addition of urea. These changes can arise (a) due to breakage of aggregation by urea and/or (b) due to breakage of secondary structure resulting in greater freedom of movement for side chains on addition of urea. If aggregation is responsible for line width and chemical shift changes then there should be a concentration dependence. Comparison of the line widths and chemical shifts of the 7×10^{-3} and 1.4×10^{-3} *M* glucagon solutions (Figure 1) indicate a concentration dependence. Further the 1.4×10^{-3} *M* glucagon solution resembles the 7.0×10^{-3} *M* glucagon + 8 *M* urea solution. This rules out the earlier proposal[1] based on nmr evidence that the chemical shift changes with guanidinum chloride in the aliphatic region are due to secondary structure represented by a helix extending over Phe-Val-Gln Trp-Leu-Met at a concentration of 6×10^{-3} *M* glucagon solution. The data do not rule out the possibility of a helix in this region but do provide evidence against the arguments previously put forward[1] to prove its presence.

Since the primary sequence of glucagon is known,[7] an attempt can be made at identifying residues involved in aggregation by their increased line widths in 7×10^{-3} *M* glucagon solution. The region 0.7–1.1 ppm shows broad resonances corresponding the two leucines and one valine. The two doublets at 1.25 ppm indicate that two out of three threonines in glucagon have sharp methyl resonances. The alanine methyl doublet has broadened considerably or shifted from its position at 1.45 ppm in the aggregated glucagon solution. The

(7) W. W. Bromer, L. G. Sinn, A. Staub, and O. K. Behrens, *J. Amer. Chem. Soc.*, **78**, 3858 (1956).

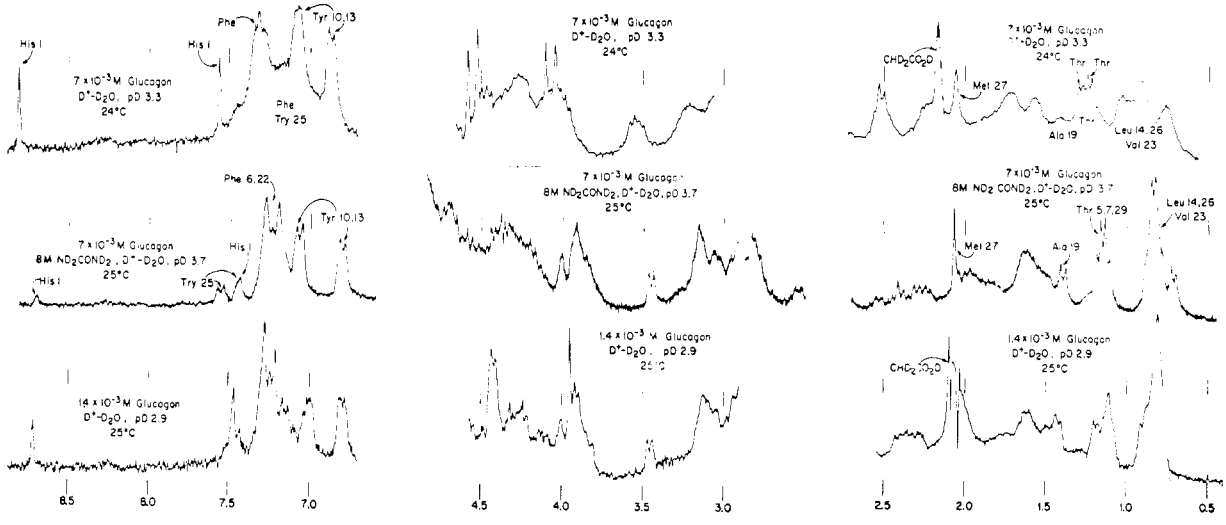

Figure 1. Proton nmr spectra (500-Hz sweep width) at 220 MHz of glucagon in 10% CD_3CO_2D–D_2O. The 7×10^{-3} M glucagon solution was time averaged over 16 scans (100 sec each), 7×10^{-3} M glucagon solution + 8 M urea was a single scan (2500 sec), and 1.4×10^{-3} M glucagon solution was time averaged over 128 scans (100 sec each). The aromatic region of 7×10^{-3} M glucagon spectrum was scaled twofold over aliphatic (0.5–4.5 ppm) regions. Side bands of water are observed at 4.0 and 4.5 ppm.

methionine singlet at 2.05 ppm shows some broadening as compared to sharp threonine doublets. In the aromatic region the histidine protons are sharp as are the doublets at 6.8 and 7.1 ppm corresponding to the two tyrosines. The resonance at 7.3 ppm corresponds to one of the two phenylalanine residues in glucagon and its area of five protons compares favorably with area of four protons each for tyrosine resonances at 6.8 and 7.1 ppm. The remaining phenylalanine resonance and tryptophan resonances make up the broad envelope centered at 7.2 ppm. The broad nature of the tryptophan resonances is verified by the absence of sharp tryptophan doublets of 1 proton each at ~7.4 and ~7.6 ppm and also the large line width of tryptophan indole NH at 10.05 ppm compared to line width of histidine protons (Figure 2). The spectrum of 7×10^{-3} M glucagon in 10% CH_3CO_2H–H_2O contains sharp peptide NH doublets of one proton each at 9.0 and 8.8 ppm (Figure 2). From our studies of the related hormone secretin and its fragments[8] these doublets can be assigned to the peptide NH of second and third amino acids from the protonated free N-terminal end, namely Ser 2 and Gln 3 peptide NH resonances.

We summarize below the line widths of identifiable resonances in the aggregated 7.3×10^{-3} M glucagon in

(8) D. J. Patel, M. Bodansky, and C. Ondetti, unpublished results.

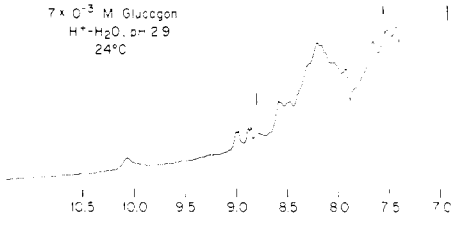

Figure 2. Proton nmr spectrum (1000 Hz sweep width) at 220 MHz of 7×10^{-3} M glucagon in 10% CH_3CO_2H–H_2O time averaged over 250 scans (100 sec each).

10% CD_3CO_2D–D_2O solution. The sharp resonances are observed for His (1), Ser (2), Glu (3), Tyr (10), and Tyr (13), two threonines, and one phenylalanine residues. Broad resonances are observed for Leu (14), Ala (19), Val (23), Try (25), Leu (26), Met (27), one threonine, and one phenylalanine.

From the above data one can postulate that aggregation in glucagon may involve the residues extending from leucine 14 to threonine 29. If the aggregation in acid solution involves predominantly dimers, the glucagon aggregate can be visualized as a tail-to-tail dimer.

The analysis of the secondary structure of glucagon in dilute aqueous solution in its nonaggregated form must await elucidation of the peptide NH and CH resonances as these and not the side chain reasonances are sensitive to structural changes.