PROTON NMR STUDIES OF THE ASSOCIATION AND FOLDING OF GLUCAGON IN SOLUTION

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1. Introduction

The polypeptide hormone glucagon is an interesting system for the study of protein conformational change and folding because of the sensitivity of its structure to external conditions. In crystals, glucagon has been found to be mainly α-helical, with the individual chains related by 3-fold symmetry about two different axes [1]. The trimeric structures defined by these axes involve extensive contacts between hydrophobic regions of neighboring glucagon molecules. In dilute aqueous solution glucagon appears to have little structure [2–6], but at higher concentrations aggregates with a degree of α-helical structure have been proposed [5–7]. α-Helical structure has also been suggested for glucagon in chloroethanol [5,10] and in the presence of detergents or micelles [8,9]. Upon standing in acidic solution glucagon forms fibrils and gels thought to have mainly β-sheet structure [10–12].

The proton NMR spectrum of glucagon has been observed to depend upon concentration and solvent [10,13]. Here, we have studied in detail the dependence of the spectrum on concentration in aqueous solutions. The changes that occur support the suggestions [6,14] that the aggregated form of glucagon found in freshly prepared concentrated solutions is a trimer. Further analysis of the NMR results permits us to characterize the structure, thermodynamics, formation kinetics, and motional properties of this species and to compare it with the trimeric structures found in the crystalline state.

2. Experimental

Crystalline glucagon was obtained from Calbiochem, lyophilized from D_2O (99.8%, Bio-Rad) at pH 10.6, and used without further purification. The pH values are pH meter readings reported without correction for isotope effects. Fresh solutions for NMR studies were prepared using deuterated sodium or potassium phosphate buffer adjusted to pH 10.6. The phosphate concentration was determined for each sample by quantitative dilution of an aliquot of the prepared solution, complexation with vanadomolybdate, and an absorbance measurement [15]. A second aliquot taken from each sample was used to determine the protein concentration spectrophotometrically (ϵ_{278} = 8260, at pH 10.2) [16].

Proton FT-NMR experiments were performed using a Bruker 270 spectrometer and a Varian XL-100 spectrometer. The temperature controllers were calibrated to within ± 1°C using a standard sample of ethylene glycol [17]. The Redfield 2–1–4 pulse sequence was used for experiments in H_2O [18]. Linewidths were measured by spectral simulation, and in addition transverse relaxation time measurements were made using the Carr-Purcell sequence $(90–(\tau–180–\tau)_n)$ with the Meiboom-Gill modification [19]. Chemical shifts were measured relative to dioxane (<0.1%) as an internal standard but are tabulated here in parts per million (ppm) relative to 2,2-dimethyl-2-silapentane-5-sulphonic acid.

3. Results

The low and high field regions of the 270 MHz proton NMR spectra of glucagon observed for dilute and concentrated solutions (the latter with 0.2 M potassium phosphate) are shown in fig.1. Some resonances are shifted and broadened under the latter con-

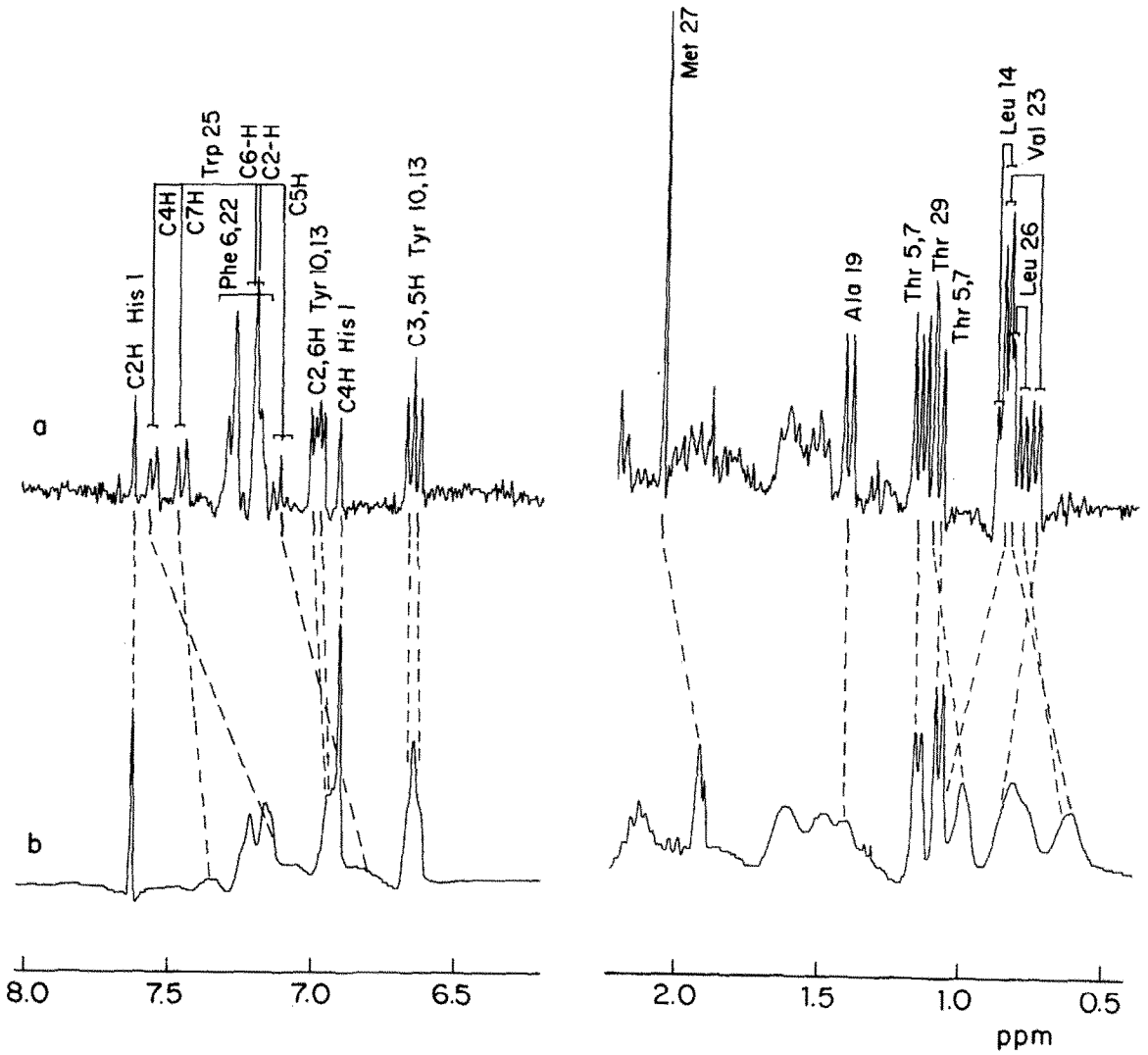

Fig.1. Low and high field regions of the 270 MHz ^{1}H NMR spectrum of glucagon in D_2O (pH 10.6, at 30°C). (a) 0.23 mM glucagon; (b) 6.9 mM glucagon with 0.2 M potassium phosphate. Resolution enhancement was accomplished by the convolution difference technique [20]. The relative intensity of the two spectra is arbitrary. The sharp resonances at 1.30 ppm and 1.89 ppm in both spectra arise from trace impurities.

ditions, which are known to promote aggregation [21], while others are essentially unchanged. Assigned resonances are listed in table 1, which also gives the sequence of glucagon.

The association behavior of glucagon was studied by measuring the concentration dependence of the chemical shifts and linewidths as a function of temperature and phosphate concentration. The shift data for the Met 27 methyl resonance are shown in fig.2. Similar results were obtained for other resonances with concentration-dependent shifts, and experiments at 100 MHz showed that the shift data are frequency independent. Fig.2 demonstrates for the methyl group of Met 27 that at low concentration the chemical shift becomes independent of temperature and phosphate concentration, both of which affect aggregation strongly. The same shift is also obtained in the presence of 8 M urea. Chemical shifts for all reso-

Table 1
Chemical shifts and linewidths for assigned resonances in the spectrum of glucagon

Assignment[a]	Monomer[b] shift (ppm)	Trimerization[c] shift (ppm)	Linewidth in[d] concentrated solution (Hz)
His 1 C2-H	7.64	0.00	2
His 1 C4-H	6.90	0.00	3
Thr 5,7 CH_3	1.08	0.01	4
Thr 5,7 CH_3	1.16	0.02	5
Tyr 10,13 C3,5-H	6.62	<0.04	6
Tyr 10,13 C2,6-H	6.96	<0.04	9
Leu 14 CH_3	0.84	0.08	17
Leu 14 CH_3	0.87	0.08	17
Ala 19 CH_3	1.40	0.00	25
Val 23 CH_3	0.74	−0.10	25
Val 23 CH_3	0.84	−0.22	25
Trp 25 C5-H	7.11	0.35	>20[e]
Trp 25 C7-H	7.46	0.15	20
Trp 25 C4-H	7.57	0.50	>20[e]
Leu 26 CH_3	0.79	0.22	30
Leu 26 CH_3	0.83	0.27	30
Met 27 CH_3	2.06	0.17	12
Thr 29 CH_3	1.12	0.18	15

The sequence of glucagon [22] is:

$^{+}H_3N$-His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–
1 5 10

Leu–Asp–Ser–Arg–Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–
15 20 25

Asn–Thr–CO_2^-
29

[a] Assignments for resonances from unique residue types were made by comparison with random coil shifts and by spin decoupling (His 1, Ala 19, Val 23, Trp 25, Met 27). Additional assignments were made by pH titration (Tyr 10,13, Thr 5,7, Thr 29) and by nuclear Overhauser effect measurements (Leu 14, Leu 26). Distinction was not made between Thr 5 and Thr 7 or Tyr 10 and Tyr 13. The assignments are consistent with the partial assignments in [3,13,23]

[b] Monomer shift values (± 0.01 ppm) are from dilute solutions at pH 10.6 in D_2O. Differences in the monomer shift values observed under other conditions are mainly attributable to changes in the protonation state of ionizable groups with pH

[c] Trimerization shift ≈ monomer shift − trimer shift (± 0.02 ppm). A positive value corresponds to an upfield shift observed on trimer formation

[d] Linewidth values (± 2 Hz) are from the spectrum of 6.9 mM glucagon in D_2O with 0.2 M potassium phosphate at pH 10.6, 30°C. In dilute solution all linewidths are <2 Hz

[e] These resonances are not well resolved in spectra of concentrated solutions

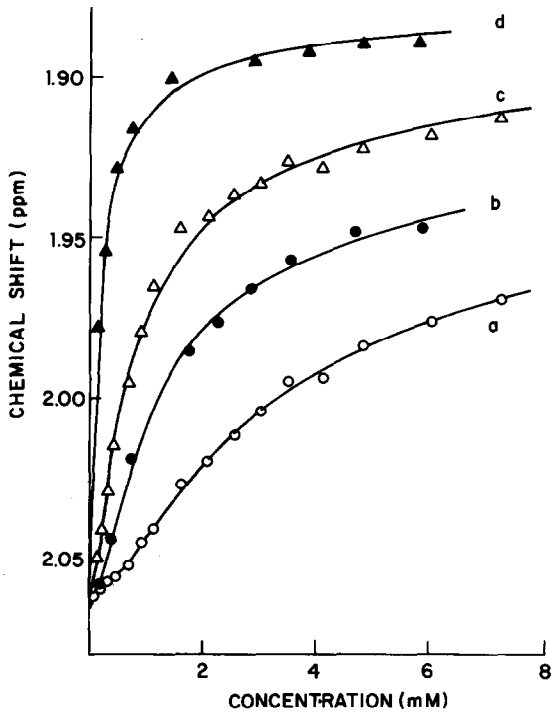

Fig.2. Concentration dependence of the chemical shift of the Met 27 methyl resonance under various conditions in D_2O at pH 10.6: (a) 0.2 M sodium phosphate, 50°C; (b) 0.2 M potassium phosphate, 40°C; (c) 0.2 M sodium phosphate, 30°C; (d) 0.7 M sodium phosphate, 40°C. Lines are unconstrained fits of data to a monomer–trimer model, except for the fit to 0.7 M phosphate data, for which the monomer shift was fixed at 2.063 ppm. These least squares fits yield a monomer shift of 2.063 ± 0.003 ppm and a trimer shift of 1.890 ± 0.015 ppm.

nances were observed to be essentially the same under varying conditions where aggregation is expected to be negligible. The set of shifts which is characteristic of monomeric glucagon is not fully identical with that expected for a random coil structure. The biggest differences are near the carboxy-terminus (Val 23, Trp 25); there may be residual structure in this part of the monomer [3].

Chemical shifts characteristic of the aggregated state of glucagon, as well as association constants, were determined by least-squares fits of the concentration dependence of the chemical shifts to various two-state equilibrium models. The monomer–trimer model provides an excellent fit to the data (see fig.2). Of the monomer–oligomer equilibrium models, only the monomer–trimer model predicts nearly the same fully associated shift when used to fit the concentration-dependent data taken at different temperatures or phosphate concentrations (fig.2). More complicated models (e.g., monomer–dimer–trimer, monomer–trimer–hexamer) give no significant improvement in the fit. Thus a two-state model, involving a well-defined trimeric species, is able to explain fully the chemical shift data. Table 1 lists the monomer chemical shifts and the changes observed on trimer formation for the assigned resonances in the glucagon spectrum.

NMR measurements have been made in H_2O with 0.2 M potassium phosphate at pH 10.6 and 30°C to compare thermodynamic constants for glucagon association obtained by NMR with those obtained by ellipticity [24] and heat-of-dilution measurements [25] under the same conditions. The association constant (K_a) for trimerization calculated for resonances with concentration-dependent shifts is 2.4 (±0.9) × 10^5 M^{-2}, in good agreement with the values in [24,25]. In contrast, for NMR data obtained in D_2O with 0.2 M potassium (or sodium) phosphate at pH 10.6 and 30°C, the calculated K_a = 1.3 (±0.6) × 10^6 M^{-2}. Thus there is a large solvent isotope effect on the thermodynamics of glucagon self-association. Similar effects on the stability of certain globular protein structures and protein complexes have been found in studies reviewed in [26].

Shift data from spectra of glucagon solutions at several pH values confirm earlier indications [16,24] that increasing the pH decreases the degree of association in the alkaline pH range; this dependence is slight above pH 10.5 and does not provide an explanation for the observed solvent isotope effect. In disagreement with [16,27] we do not find that either of the two tyrosine residues in glucagon has a concentration-dependent pK_a. The pK_a values of the tyrosine residues were measured in both dilute and concentrated solutions by fitting the dependence on pH of the chemical shift of each aromatic tyrosine resonance to the Henderson-Hasselbalch equation. All of the measured pK_a values are within 0.1 pH unit (pK_a = 10.2–10.3).

Linewidths of clearly resolved resonances measured at 30°C were essentially independent of spectrometer frequency (100 MHz and 270 MHz) and of the method of determination (spectral simulation and relaxation measurement by the Carr-Purcell sequence). The linewidths were found to increase with decrease in temperature, and at a given temperature to increase with

the degree of association as monitored by chemical shift changes. Linewidths for the assigned resonances in the spectrum of glucagon in a concentrated solution are listed in table 1; the corresponding linewidths in spectra of dilute solutions or in 8 M urea are all <2 Hz.

4. Discussion

The NMR data described in section 3 lead to a characterization of the structure and dynamics of the glucagon aggregate in aqueous solution. The concentration dependence of the chemical shifts indicates that in concentrated solution glucagon exists as a well-defined trimer. To consider the structure and internal dynamics of this trimer it is convenient to divide the glucagon chain into the amino-terminal region (His 1–Tyr 13), the carboxy-terminal region (Phe 22–Thr 29), and a central region (Leu 14–Asp 21). For all assigned resonances in the amino-terminal region, the shifts resulting from association are $< \pm 0.05$ ppm. By contrast, shifts of $> \pm 0.10$ ppm are observed for all resonances assigned to carboxy-terminal residues. Linewidths for resonances assigned to the amino-terminal residues are all <10 Hz, while linewidths of >10 Hz are found for all resonances assigned to remaining residues. This trend is most evident for methyl-group resonances (see table 1 and, e.g., the threonine resonances in fig.1). These data show that the carboxy-terminal region, which has high hydrophobic character, has a characteristic structure and low internal mobility while the amino-terminal region, which has many polar residues, is random-coil-like in that there is no persistent structure and the internal mobility is high. CD measurements [5] have indicated that there is 35% α-helical content in the solution trimer, which is consistent with the present result that only the carboxy-terminal region is highly structured.

Of the two crystal trimers [1], one (I) has heterologous contacts between the carboxy-terminal residues of one molecule and amino-terminal and central residues of another, while the other (II) has only homologous contacts between carboxy-terminal regions of each molecule. These results demonstrate that the mode of association of trimer I does not occur in solution, since it involves the segment from Phe 6 to Tyr 13 which linewidth and chemical shift data indicate is neither structured nor involved in contacts in solution. This conclusion is strengthened by the observed concentration independence of the pK_a values of the tyrosine residues, which are in this segment. By contrast the mode of association of trimer II is consistent with the solution data. The details of the structure, however, are different. The NMR data show that the helical structure from Phe 6 to Tyr 13 present in the crystal is absent in solution, presumably because of the absence of stabilizing contacts in the latter. Even in the structured carboxy-terminal region, chemical shifts calculated on the basis of the trimer II structure explain only some of the observed values, although small local alterations of the structure are sufficient to improve significantly the agreement between calculated and observed shifts.

The observed frequency independence of the chemical shifts and linewidths of the resonances studied here indicates that they are in the fast exchange region. This result has been used in the two-state equilibrium analysis of the chemical shift data and in the correlation of linewidth with mobility. Modelling the effects of two-site chemical exchange at various rates and relative populations on the NMR lineshapes places a lower limit of $\sim 500\ s^{-1}$ on the dissociation rate constant for the glucagon trimer in a 0.2 M phosphate solution (pH 10.6, at 30°C). Combining this result with the measured equilibrium constant yields a lower limit of $\sim 10^9\ M^{-2}\ s^{-1}$ for the overall effective association rate constant.

From these results the solution trimer appears to be an intermediate stage in the folding of a largely unstructured glucagon molecule to its 75–80% α-helical crystalline form. Contacts in the solution trimer stabilize some helical structure (35%), but the additional contacts that occur in the crystal are necessary to stabilize further helical structure. The solution trimer of glucagon thus has similarities to an intermediate in the folding of a globular protein. Such an intermediate, like the glucagon trimer, would be expected to exhibit both random-coil and native characteristics. Only on introducing all of the contacts present in the folded protein (here the crystalline form) would the full native structure appear. The specific details of the structured regions would also be expected to differ somewhat in the native and partially folded protein species. The association behavior of glucagon found in this work suggests that it may be analysed in terms of the diffusion–collision model which has been proposed for protein folding

[28]. More detailed studies of the glucagon system and the relevance of its association to protein folding are in progress.

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