CONFORMATIONAL STATES OF GLUCAGON

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Glucagon is a polypeptide hormone of 29 residues with a known sequence (Bromer *et al.*, 1957). It has been reported to form cubic crystals (King, 1959), and King (1965) has presented a crystallographic model, in which the glucagon molecules pack as rods with dimensions compatible with those of α-helices. Thus far no polypeptide of comparably short length, other than the synthetic homopolymers, has ever been found to possess a large proportion of α-helical structure in water, and in dilute aqueous solution glucagon appears to be no exception (Fig. 3; see also Blanchard and King, 1966). Blanchard and King have reported, however, that at high concentrations at pH *ca.* 10, changes in optical rotatory dispersion are observed, the value of the Drude constant rising rapidly with concentration. We have now observed an association process also in acid solution, and we describe here an investigation of the conformation of glucagon as a function of its aggregation states.

Methods Crystalline glucagon (Novo Research Institute; Lilly Research Laboratories Ltd.) was used. It was homogeneous by paper and starch gel electrophoresis at several pH values. The molar absorptivity was derived on the basis of the formula weight by nitrogen determination, using the ninhydrin method (Jacobs, 1962); the molar absorptivity (pH 2) at the absorption maximum at 278 mμ was found to be 8310; E(1 mg/ml, 1 cm.) = 2.38. Infrared spectra were examined in Perkin Elmer 21 and 237 instruments. For solutions in D_2O cells of

0.025 mm path and with BaF$_2$ windows were used, and films were cast on BaF$_2$ discs. Ultraviolet absorption spectra and difference spectra were measured on Beckman DK-2A and Hilger Ultrascan spectrophotometers, and optical rotatory dispersion in a Bellingham and Stanley Polarmatic 62 polarimeter, using cells of path lengths from 0.01 to 4 cm. Sedimentation coefficients were determined with a Spinco model E ultracentrifuge at 42,040 r.p.m., using the schlieren optical system.

Results In freshly prepared acid, neutral and alkaline solutions glucagon shows a small Cotton effect minimum at about 234 mμ, with a reduced mean molar residue rotation, $[m']$, in the range -2500 to -3500°, indicating that little α-helical structure is present. When a glucagon solution at pH 2 is allowed to stand for some days at room temperature, it becomes progressively more viscous, and sedimentation coefficients become very large, typically of the order of 35*S* in a 0.5% solution after

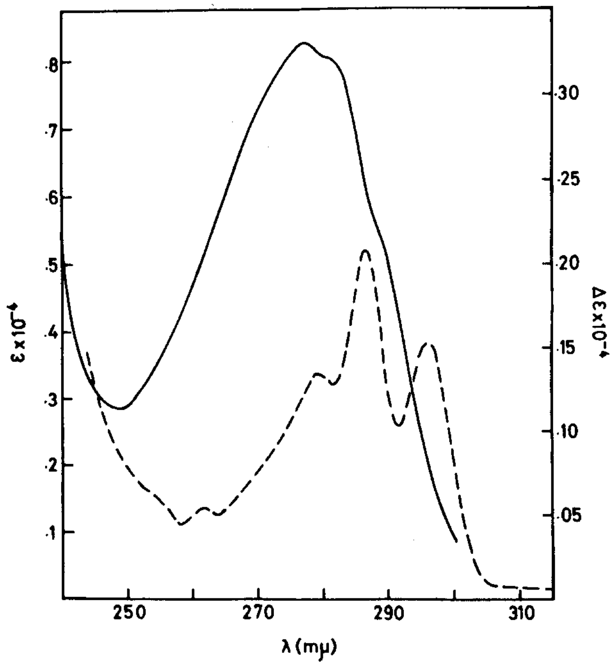

Fig. 1 Ultraviolet absorption spectra of glucagon at pH 2 :

———— Fresh solution (left-hand scale)

– – – – Difference spectrum of solution after 20 days at room temperature (sample) *vs* fresh solution at same concentration (reference) (right-hand scale).

a few days, the boundary being hypersharp. On further standing a gel is formed, which shows marked positive birefringence in the polarising microscope. These gels cannot be used to measure meaningful optical rotations. After a longer period at room temperature, the gel gives way to a fibrillar precipitate. The early stages of gel formation are associated with prominent changes in the absorption spectrum associated with the tyrosine and tryptophan residues, with generation of a large time-dependent difference spectrum (Fig. 1).

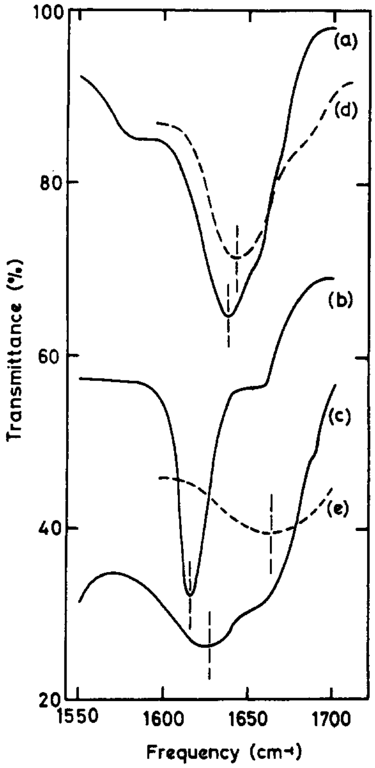

Fig. 2 Infrared spectra of glucagon in different states :
(a) freshly prepared solution in D_2O/DCl, pD 2;
(b) gel formed from (a) on standing (uncompensated);
(c) dried film of precipitate from acid solution after standing (hydrogen form);
(d) solution in phosphate buffer, pD 10.4;
(e) deposited crystals, dried down from phosphate mother liquor (hydrogen form).
Note that especially in (e) there was a high scattering background and a very broad band. The shoulder near 1660 cm^{-1} in (a) and (b) arises from the presence of some hydrogen form, since not all the D_2O can be removed from the sample without the possibility of adventitious changes in state.

Infrared spectra of freshly prepared glucagon solutions in D_2O/DCl, gels from the same solutions and precipitates from these and from H_2O/HCl solutions, as well as solutions in 0.2M phosphate buffer at pD 10.2, are shown in Fig. 2, and it is evident that the acid aggregated states of glucagon are substantially in the β-conformation. In D_2O the amide I band lies at 1613 cm^{-1}, and in the hydrogen form of the fibrils it appears about at 1630 cm^{-1}, both positions being characteristic of β-conformations (Bamford et al., 1956). In the latter, moreover, one can discern a shoulder at 1685 cm^{-1}, associated with the $\nu(O,\pi)$ mode, shown by Miyazawa (1960, 1961) to be characteristic of the antiparallel β-form. No such effect occurs in films of crystals formed at pH 10.2, where the amide I band is at 1665 cm^{-1} (hydrogen form), or in suspensions of crystals in their mother liquor, pD 10.2, where it is at 1635 cm^{-1} (deuterium form). We cannot by this means distinguish between α-helix and a random conformation, but it is possible to state

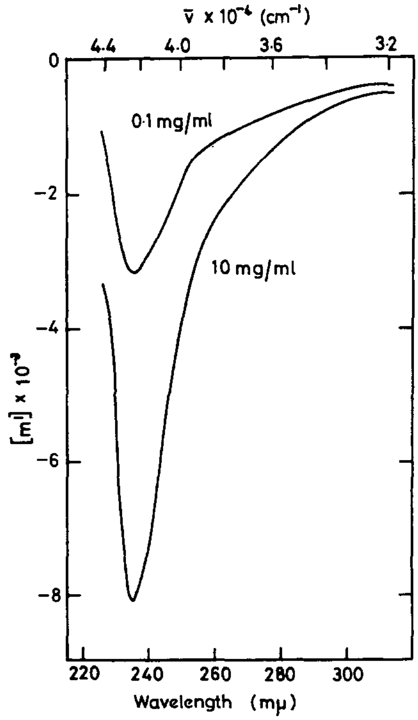

Fig. 3 Optical rotatory dispersion curves of glucagon in phosphate buffer, pH 10.2, at low and at high concentrations (0.1 and 10 mg/ml) measured in 1 cm and 0.01 cm path lengths.

with certainty that the β-form does not occur in the crystalline material at the alkaline pH, or in solution under these conditions.

In order to distinguish between an α-helical and a random conformation, we have measured optical rotatory dispersion curves through the region of the longest wavelength polypeptide Cotton effects. Fig. 3 shows such curves measured in 1 cm. and 0.01 cm. path lengths, at concentrations of ca. 0.1 mg/ml and 10 mg/ml. It is clear that the increased concentration leads to enhancement of the Cotton effect at 234 mμ, which is most simply interpreted in terms of the formation of α-helical structure to the extent of some 40-50% (see Yang and McCabe, 1965).

Discussion Our results on the alkaline solution lend support to the suggestion of Blanchard and King (1966) that aggregation under conditions of incipient crystallisation is accompanied by the progressive formation of α-helical structure. We are not of course able to say whether the molecule ultimately becomes fully helical and then crystallises, nor is the precision of such data adequate to distinguish between the trimerisation process suggested by Blanchard and King and other modes of association. Their results indicate that crystallisation will proceed also at pH 8 (where the solubility is very low), and it is in any case unlikely that it should depend on the partial ionisation changes in the single lysine and the two tyrosine residues at pH 10.

In acid solution the molecule loses three negative charges from the aspartic acid residues, and this is evidently sufficient to displace the conformational equilibrium in favour of the β-form. It may be noted that glucagon is rich in serine and threonine (seven of the 29 residues), and that such polymers - notably silk fibroin (Iizuka and Yang, 1966) and poly-L-serine (Bohak and Katchalski, 1963; Fasman and Potter, 1967) - display a strong tendency to enter the β-form even in solution.

Glucagon appears to present a striking example, so far paralleled only by a homopolypeptide, poly-L-lysine (Rosenheck and Doty, 1961; Sarkar and Doty, 1966; Townend et al., 1966), of a conformational polymorphism, which enables it with only minimal adjustment of the environment to exist in aqueous solution in an α-helical, antiparallel β, or a largely random state.

Summary Glucagon, a polypeptide of 29 residues, has been found

to exist in a largely random form in dilute solution. At acid pH, aggregation occurs, with formation first of a gel and then a fibrillar precipitate; both these forms are found to consist of antiparallel β-chains. In mildly alkaline solution, prior to crystallisation, the molecules again appear to associate, but this time with the progressive formation of α-helices.

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