J. Sci. Soc. Thailand, 3 (1977), 34-41

EFFECTS OF HEAT AND SOLVENTS ON THE CIRCULAR DICHROISM OF DISUCCINYLATED GLUCAGON

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(Received 31 January 1977)

Summary

Disuccinylated glucagon was studied by circular dichroism, at different temperatures, in the presence of urea, lithium chloride and three organic solvents. Ethanol, 2-chloroethanol and 2,2,2-trifluoroethanol, in order of effectiveness, promoted helix formation. Urea enhanced the positive band at 227 nm, whereas lithium chloride caused an increase in the negative band at 222 nm. Increase in temperature caused a negative inversion of $(\theta)_{230\ nm}$ *in high urea (and guanidine hydrochloride) concentrations and a positive* $(\theta)_{230\ nm}$ *inversion in lithium chloride. Results obtained with glucagon are used for comparison.*

Introduction

Disuccinylated glucagon (DS-glucagon) is a good natural model for studying polypeptide conformations. It can be prepared pure by reacting succinic anhydride with the two primary amino groups belonging to the histidine-1 and lysine-12 residues of glucagon[1]. Glucagon is a 29-amino acid polypeptide hormone of known sequence and devoid of disulphide linkage[2]. DS-glucagon is much more soluble than glucagon from pH 5 upward. Proton magnetic resonance (p.m r.) studies have shown that DS-glucagon is a random coil[1,3]. Circular dichroism (c.d.) spectrum of DS-glucagon, above pH 5, is not dependent on either pH or concentration up to 20 mg/ml[3].

Guanidine hydrochloride (GuHCl), urea, inorganic salts, acids and organic solvents have been used as perturbants of synthetic and natural peptides and polypeptides in solution with the aim of characterizing their conformational states[4,5]. Using chiroptical probes, it is generally accepted that organic solvents tend to induce helix formation, as indicated, for example, by the prominent negative c.d. extrema at 222 nm and 207 nm. On the contrary, there have been controversies centring on the effects of salts and "denaturants", especially, guanidine hydrochloride and urea, on polypeptide conformations. The positive c.d. band near 220 nm found in a large number of charged and uncharged homopolymers and copolymers has been attributed by some to the unordered form[6-8] and by others to the extended helical form similar to that of poly-L-proline II[9,10]. Hence, the diminution and sign inversion of this c.d. band by perturbants have been interpreted variously as evidence for the conversion from a more unordered to a more helical form or from extended helical to unordered form. With these two opposing views in mind, DS-glucagon and also

glucagon were studied by the circular dichroism technique in the presence of urea, lithium chloride and organic solvents at different temperatures. The intention was to obtain more data for further consideration in resolving the above-mentioned problems. The choice of polypeptides here is interesting because glucagon is a natural polypeptide of biological importance and both glucagon and DS-glucagon have the same sequence of amino acids, but differ in the native conformation and charge in the absence of perturbants. Only data from DS-glucagon studies will be shown, but comparison with glucagon will be made throughout.

Materials and Methods

DS-glucagon was prepared and isolated as previously described[1]. Glucagon was the crystalline material obtained from Sigma and used without further purification. Screening for purity by gel electrophoresis was routinely done to reject samples having too high degree of impurity. C.d. spectra were recorded on a Cary-61 spectropolarimeter standardized with camphor-10-sulphonic acid. Temperature was maintained by using a thermostatted cell block with circulation of glycol. A thermocouple probe was used in conjunction with a Wheatstone bridge to monitor the solution temperature which was read from a previously calibrated curve. Baselines were constantly run and straightened to give better ellipticity readings and localizations of peaks and troughs. Solution concentration was determined by using $E^{1\%}_{278\ mn}$ of 2.38[11]. Guanidine hydrochloride (BDH) and Urea (Arista) were of spectroscopic grades and dried before use. LiCl was obtained from BDH (Analar). Ethanol, 2-chloroethanol and 2,2,2,-trifluoroethanol (Sigma) were all of spectroscopic quality.

Results

At pH 8, c.d. of DS-glucagon was without prominent features down to 220 nm (Fig. 1). Lithium chloride affected the c.d. spectrum in the opposite direction to urea. Whereas high concentrations of urea, especially at low temperatures, enhanced the positive band at 227 nm, LiCl promoted the negative features. As shown, the 227 nm positive band is clearly visible in the c.d. of DS-glucagon in 9 M urea at 7°C and remains discernible even in 4 M urea. At 40°C, in 8–10 M urea, the residual of this band is still observable also. This band escaped detection in a preliminary study on the unmodified glucagon[12]. The c.d. curve in 8 M GuHCl is shown for comparison. At higher concentrations of LiCl, the negative feature at 222 nm became more prominent, especially at low temperatures. Fig. 1 shows a clear c.d. extremum in the presence of 3 M LiCl. Upon raising the temperature of the solutions, the positive bands (in urea) and the negative bands (in LiCl) became smaller and less distinct and the c.d. spectra tended towards that of DS-glucagon in dilute salt concentration (low salt). Fig. 2 shows the effects of temperature on DS-glucagon in urea and LiCl. The transition curves in low salt and in 8 M GuHCl are shown for comparison. With increasing temperature, the molar residue ellipticity at 230 nm, $(\theta)_{230\ nm}$, of DS-glucagon in low salt remained constant, whereas that in > 1.5 M urea and > 0.8 M GuHCl decreased and that in LiCl increased. The c.d. of DS-glucagon showed enhanced negative amplitudes at the

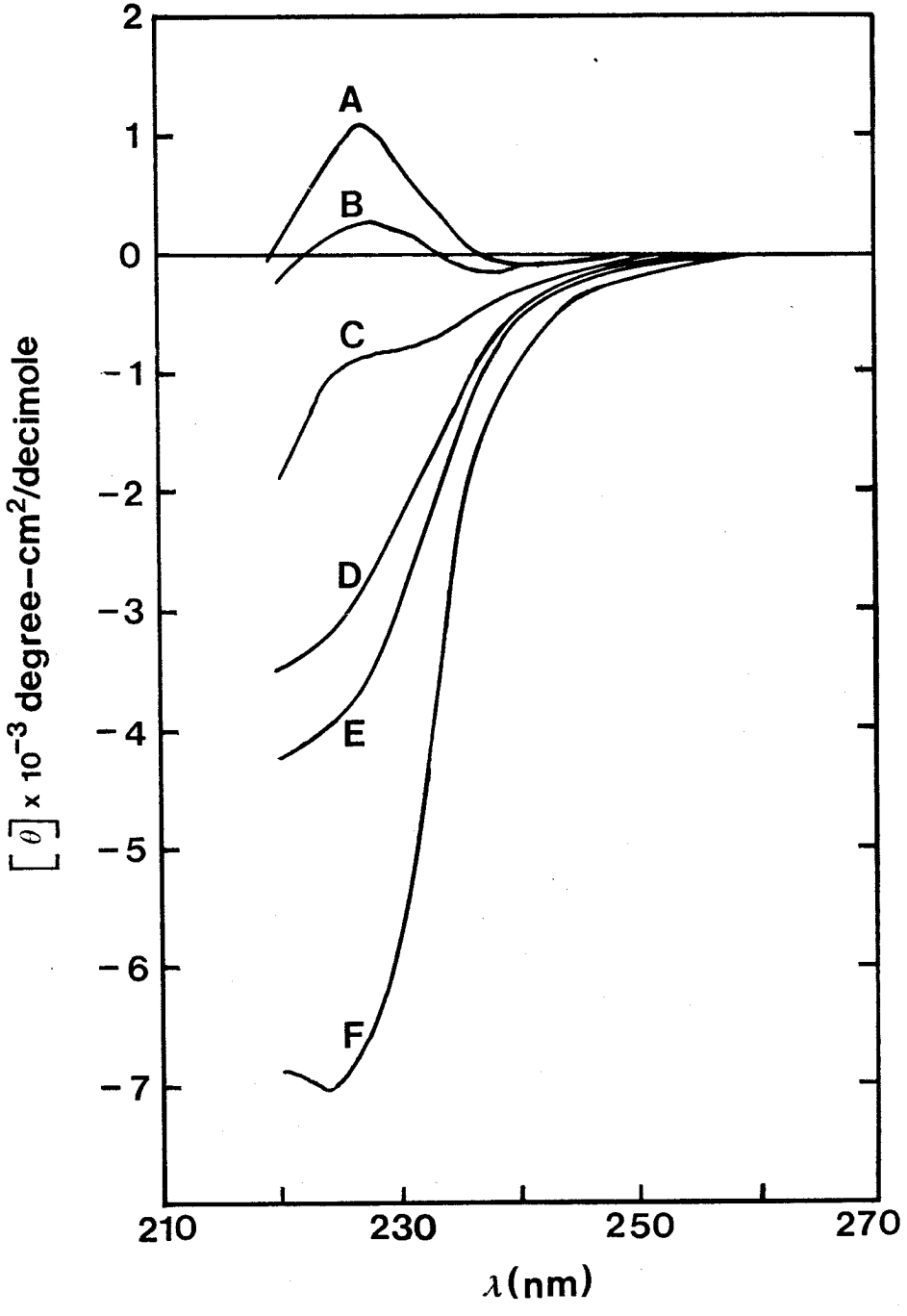

Fig. 1. C.d. spectra of disuccinylated glucagon, 1×10^{-4} M, pH 8.0, 7 °C, in various solvent media. A — 8 M GuHCl, B — 9 M urea, C — 4 M urea, D — 0.01 M phosphate, E — 1 M LiCl, F — 3 M LiCl. $[\theta]$ is the molar residue ellipticity.

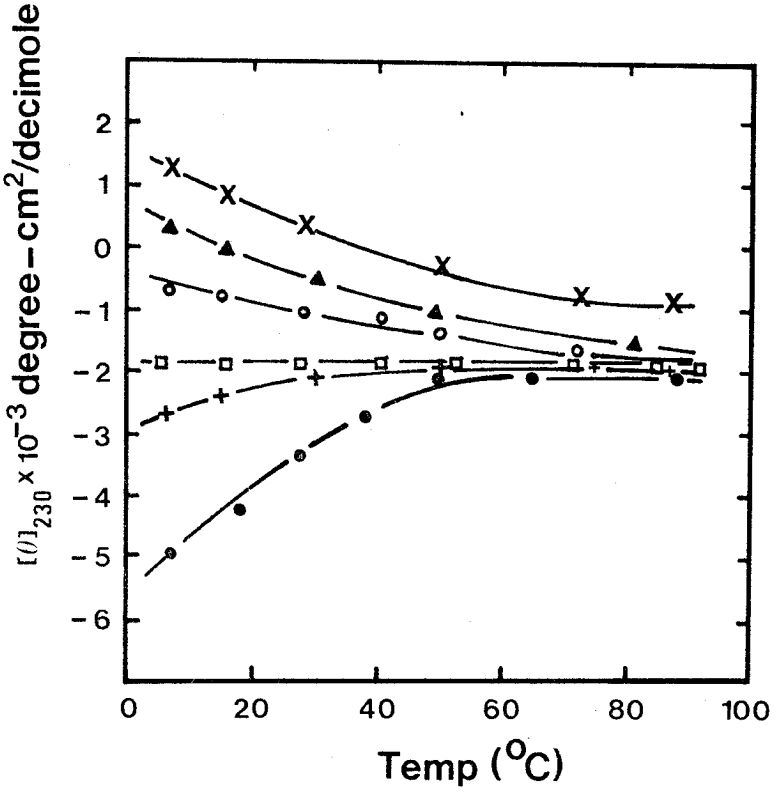

Fig. 2. The dependence of $[\theta]_{230\,nm}$ of disuccinylated glucagon, 1×10^{-4} M. pH 8.0, on various solvents. ×——× 8 M GuHCl, ▲——▲ 9 M urea, O——O 4 M urea, □——□ 0.01 M phosphate, +——+ 1 M LiCl, ●——● 3 M LiCl.

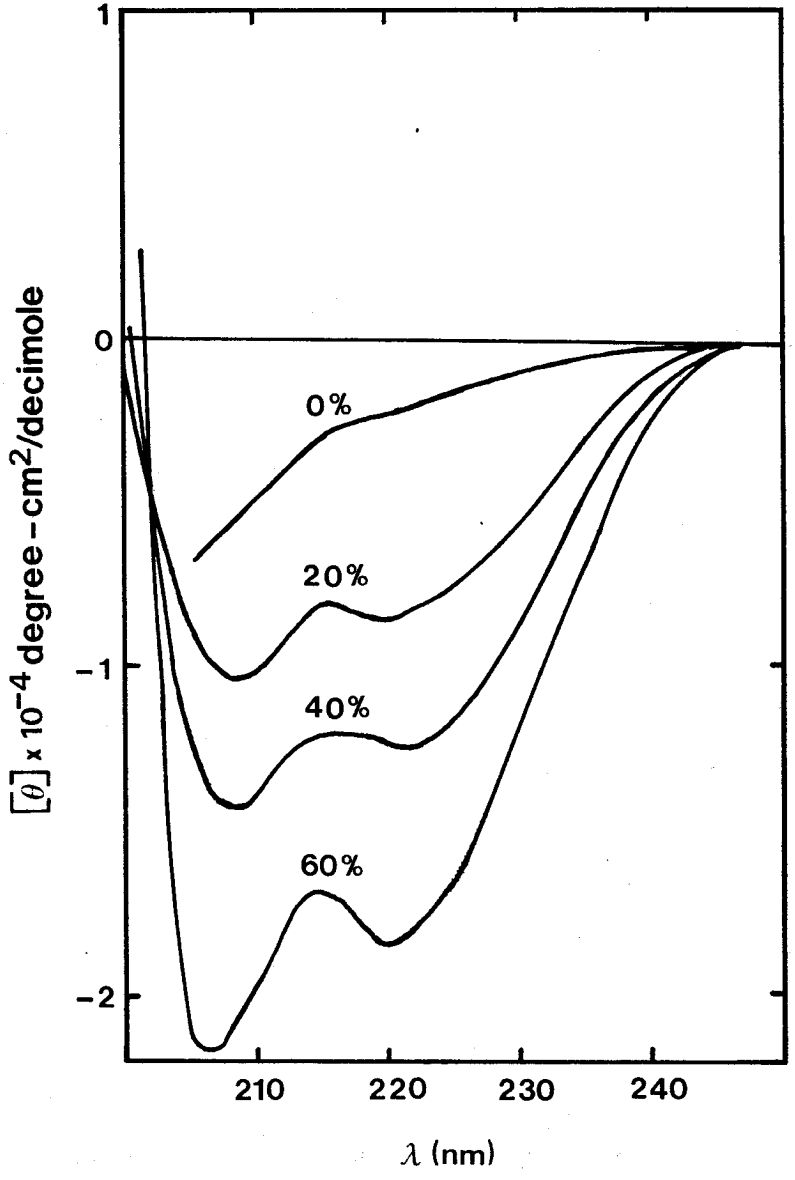

Fig. 3. C.d. spectra of disuccinylated glucagon, 1×10^{-4} M, pH 8.0, 25 °C, in the presence of increasing percentage of 2, 2, 2-trifluoroethanol (v/v) in the solvent.

two prominent features, 222 nm and 208 nm, in the presence of increasing concentration of organic solvents: ethanol, 2-chloroethanol and 2,2,2-trifluoroethanol. The extent of the changes was in the order ethanol < chloroethanol < trifluoroethanol for the same concentration of organic solvents. Fig. 3 shows a series of c.d. curves of DS-glucagon in the presence of 0–60% trifluoroethanol. Heating reduced the amplitude of the two ellipticity bands.

Experiments with glucagon, mostly at 1×10^{-4}M, under the same conditions gave nearly quantitatively and certainly qualitatively the same results as those obtained with DS-glucagon. Two exceptions are worth mentioning here: (i) $(\theta)_{230\ nm}$ of glucagon in low salts, in < 3 M urea and in < 2 M GuHCl showed positive temperature inversion; (ii) $(\theta)_{230\ nm}$ of glucagon became quite invariant with temperature in about 3.5 M urea and 2 M GuHCl.

It is emphasized here that experiments were conducted over a wide range of pH's and polypeptide concentrations and the same general results were observed. All changes up to 90°C at all perturbant concentrations have all been found reversible by c.d.

Discussion

Despite some structural and charge differences, both DS-glucagon and glucagon are flexible enough to undergo helix formation easily in the presence of organic solvents. The change in solvent composition and dielectric constant of the medium in the presence of trifluoroethanol, chloroethanol and ethanol certainly promotes hydrogen bondings in the backbone and interactions among the side chains[4]. Indeed, the c.d. spectrum of DS-glucagon in 60% trifluoroethanol shows clear features of an α-helix and the $(\theta)_{222\ nm}$ indicates a 70% α-helical content[6]. Heating apparently reduces the helical content by causing greater chain motion and changed solvent-polypeptide interactions.

Although glucagon trimerizes at high concentrations, e.g., at 10 mg/ml or 3×10^{-3} M at pH 10.2, it exists essentially as a monomer at 1×10^{-4} M[3]. DS-glucagon is a monomer and a random coil up to 20 mg/ml or 6×10^{-3}M. Thus we were studying monomers. At high concentrations of "denaturants", urea and GuHCl may affect the polypeptide conformation by binding to the polypeptides and by lowering the water activity[4,24]. Their binding to the polypeptide conceivably causes the backbone to assume an ordered structure showing a positive band at 228 nm here[13], or they may interact with and perturb the peptide chromophore and thus the c.d. spectrum[14-17]. This latter possibility is supported by our previous results. It was found that while c.d. spectra of DS-glucagon in 8 M GuHCl and in low salt are different (see Fig. 1), all features of the amino acid peaks in their p.m.r. spectra are identical and the line-widths correspond to those obtained from calculations of contribution from individual amino acids in a random coil DS-glucagon. Heating changes the c.d. spectra of DS-glucagon, in the presence of urea, GuHCl and LiCl, toward that in low salt, whose c.d is temperature, pH (above 5) and concentration invariant. At high "denaturant" concentrations (Fig. 2), increase in temperature may shift the equilibrium of binding between the denaturants and water, and between

the denaturants and polypeptides to affect the polypeptide conformation and peptide chromophore perturbation at the same time. At the particular low denaturant concentrations, where the c.d. of DS-glucagon is invariant with temperature, the effects of heating on denaturant binding and conformational equilibrium are probably minor and there is no residual helical structure in glucagon. The positive temperature inversion of glucagon in low salt, or in < 3 M urea, or in < 2 M GuHCl, may reflect mainly certain amount of unfolding of its residual ordered structure by thermal energy. Values of $(\theta)_{230\ nm}$ at high temperatures approach, but do not coincide with, that of DS-glucagon in low salt, probably because of difference in the solvents used.

It is worth emphasizing that the c.d. characteristics and changes discussed above and similar effects of salts, solvents and denaturants have been observed in a number of peptides and polypeptides[1,5,13,18]. The interpretations of these observations are usually not without some uncertainty[7,9,14,15]. For example, similarity exists between the temperature effects on poly (N^5-ω-hydroxypropyl-L-glutamine) in the absence and presence of $CaCl_2$ and KCl[19] and the effects on glucagon in the absence and presence of urea (or GuHCl) and LiCl. The explanations may not be the same for both cases, however, even though LiCl is similar to KCl in many ways and $CaCl_2$ has been known as a polypeptide structure breaker similar to urea and GuHCl. In any event, it is certain that the "random coil" c.d. curves of charged poly-L-lysine and poly-L-glutamic acid cannot be used as general standards for all random coils[7,20,21]. Neither does the positive band around 220 nm necessarily indicate extended helical structures maintained by charge repulsion[14,22,27]. Results obtained from c.d. studies show that the 220 nm positive extremum can arise from polypeptides without charged side chains, charged polypeptide backbones or charged molecules bound to the polypeptides. The explanations for the basis of the 240 nm negative band are almost pure speculations[23] and thus will not be discussed here, even though it is observable in glucagon and DS-glucagon in high concentrations of denaturants and at low temperatures (Fig. 1 and 2).

The effects of LiCl on the c.d. of both DS-glucagon and glucagon are similar and these are just the opposite to those of urea and GuHCl. Although LiCl has been known to break ordered structures, the conventional interpretation here would be the promotion of helix formation in the presence of increasing concentration of LiCl. The melting profiles seem to support this interpretation, since heating can cause ordered structures to unfold and finally produce a random coil. The c.d. spectrum of DS-glucagon in LiCl at high temperatures is similar to that in the presence of low salts. But one also has to take into consideration the effects of LiCl on water activity and the binding of Li^+ ions to the peptide groups, especially at high salt concentrations[24-26]. LiCl probably does not promote helix formation by screening charge interaction between side chains because both DS-glucagon and glucagon seem to be affected similarly by it. Li^+ binding to the peptide group may change the peptide dipole moment enough to affect the c.d. spectrum and this salt binding effect may be temperature dependent since heating changes the binding equilibrium and probably chain conformation.

The central problem of the relationship between c.d. spectrum and conformation is still far from totally resolved. More extensive studies with model compounds and natural polypeptides plus theoretical calculations will be needed before any generalizations about the effects of perturbants on polypeptides can be made.

Acknowledgements

I wish to thank T. Vadjarodaya and S. Bangthamai for their skilful assistance in the preparation of the manuscript. Spectrophotometric facilities provided and arranged by Dr. Walter B. Gratzer are greatly appreciated.

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บทคัดย่อ

จากการศึกษาอนุพันธ์ disuccinylated glucagon โดยใช้วิธี circular dichroism ที่อุณหภูมิต่างๆ ในสารละลายมี urea หรือ lithium chloride หรือสารทำละลายอินทรีย์ปนอยู่ด้วย พบว่า ethanol, 2-chloroethanol และ 2, 2, 2-trifluoroethanol ทำให้เกิด helix มากขึ้นตามลำดับ urea ทำให้เกิดยอดบวกที่ 227 nm แต่ lithium chloride ทำให้เกิดแอ่งลบที่ 222 nm การเพิ่มอุณหภูมิทำให้มีค่า ellipticity ที่ 230 nm วกกลับไปทางลบ เมื่อสารละลายมี urea หรือ guanidine hydrochloride เข้มข้นปนอยู่ แต่เมื่อมี lithium chloride อยู่ ค่า ellipticity ที่ 230 nm จะเปลี่ยนไปทางบวก ผลการทดลองกับอนุพันธ์ใช้เปรียบเทียบกับกลูคากอนด้วย