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Denaturation versus pH of lyzozyme and biosynthetic human growth hormone by differential scanning calorimetry and circular dichroism: a comparative study

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Abstract

The thermal behaviour of lyzozyme and human growth hormone (hGH) in aqueous solutions versus pH was studied by DSC experiments. The temperature and enthalpy of denaturation were determined, showing a maximal stability of lyzozyme at pH 4.5, and a large range of pH (5.0–9.0) corresponding to the maximal stability of hGH.

These results are in good agreement with those obtained by circular dichroism measurements.

The hGH environment was modified by addition of linear or aromatic small molecules and their influence on the parameters of the denaturation was studied.

INTRODUCTION

The production of human growth hormone (hGH) is an example of the strides made by recombinant DNA technology in the large-scale production of proteins for therapeutic use. An important problem in this field lies in obtaining information concerning the tertiary structure in solution and its stability. Using differential scanning calorimetry (DSC), the disruption of the structure during the denaturation process can be studied. The presence of an endothermal effect indicates the tertiary structure of the hormone in solution.

However, biosynthetic molecules must be characterized by biophysical methods. Among the numerous tools permitting this evaluation, circular dichroism (CD) has been described as a method of wide interest in the

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study of structures of proteins and especially of polypeptide hormones. It gives information on the backbone conformation of the entire protein and on the environment of the aromatic residues present in the molecules.

In a previous study [1], a high-sensitivity sensor was developed to study the thermal behaviour of proteins in aqueous solution using a Mettler TA 2000 B DSC apparatus. In particular, lyzozyme and hGH have been studied at a concentration of 10 and 5.6 mg ml^{-1}, respectively.

The next step was the study of the stability of the conformation of lyzozyme and hGH under different experimental conditions of pH by means of DSC. In the case of hGH, a comparative study between DSC and CD was carried out. In addition, the interaction of small molecules, made up of pharmaceutical compounds, with hGH was studied.

MATERIALS AND METHODS

Proteins

Lyzozyme chloride (lot A7317) was purchased from Brookside.

Recombinant DNA-derived hGH, pharmaceutical grade, was prepared by Sanofi. It was secreted into *Escherichia coli* periplasmic space and prepared by a large-scale osmotic shock procedure; it was then purified to reach homogeneity. Preparations of hGH were kept in ammonium carbonate buffer (50 mM, pH 8.3) and stored at −20°C. Before the CD measurements, the hGH was analysed by UV spectrophotometry, SDS PAGE and reverse-phase HPLC.

DSC measurements

The DSC measurements were carried out using a Mettler TA 2000 B heat flow apparatus controlled with an Inetech 286 computer which measured the calorimetric signal via a Keithley 199 digital voltmeter.

The apparatus was standardized for temperature and heat flow, using the temperature and heat of melting of high purity compounds as described elsewhere, to give a relation between the calorimetric signal Δ and the thermal power dQ/dt.

Experiments were performed at a heating rate of 1°C min^{-1} in the range 25–100°C, the calorimeter being flushed with argon at 6 l h^{-1}.

For these experiments, a "home-made" sensor was used. The performance and calibration of the system (sensitivity 0.2 μW) have been previously described [1].

Conformational analysis

The CD measurements were made with a Jobin Yvon Mark III dicrograph. Spectra were recorded in cells of 0.1 cm and 1 cm path lengths in the far-UV and near-UV wavelength ranges respectively.

The concentration values used in the calculation of the CD intensities are of critical importance, especially when two different samples are to be compared. The concentrations of the hGH solutions were determined by UV spectrophotometry in comparison to an hGH standard solution whose concentration had first been measured by quantitative amino acid analysis.

In the near UV wavelength range, the CD values are expressed as a difference in extinction coefficient $\Delta\varepsilon$ (M^{-1} cm^{-1}), (molecular weight, 22100). In the far-UV wavelength range, they are expressed as mean residue ellipticity θ (deg cm^2 $dmol^{-1}$) (mean residue weight, 116).

The CD far-UV results were analysed to determine the conformation according to the method of Chang et al. [2].

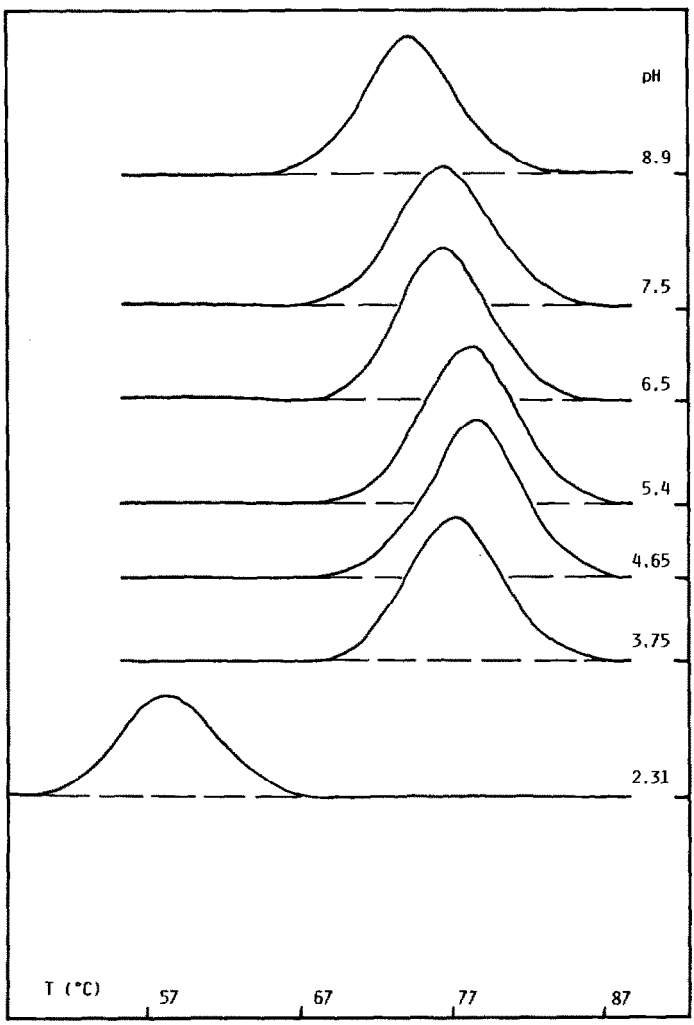

Fig. 1. DSC curves of lyzozyme versus pH.

Experimental conditions

Stability studies over a wide range of pH require carefully buffered solutions.

For CD measurements, concentrated biosynthetic hGH was diluted tenfold in 200 mM of one of the following buffers: sodium acetate, pH 3.5 and 4.7; sodium phosphate, pH 5.6, 6.7, 7.5 and 8.0; TRIS-HCl, pH 8.3 and 8.9; sodium carbonate–sodium bicarbonate, pH 9.5 and 10.5.

For DSC measurements, the following buffers were used: sodium acetate, pH 2.3, 3.3, 3.75 and 4.65; sodium phosphate, pH 7.3, 7.5, 7.8, 8.0, 8.5 and 8.9; sodium carbonate–sodium bicarbonate, pH 9.15 and 10.10.

Dilution was performed immediately before the experiments with the corresponding buffer. Concentrations of 20 and 7.6 mg ml^{-1} were used respectively for lyzozyme and hGH.

The study of the interaction between hGH and small molecules was performed with lyophilized samples each containing 1.7 mg of hGH and 44 mg of alanine. The final hGH concentration was 5.6 mg ml^{-1}.

The temperature of denaturation was determined as the top of the thermal effect of denaturation.

RESULTS

DSC experiments

Lyzozyme

Many authors [3–9] have studied the thermal denaturation of lyzozyme yet thermodynamic values such as the enthalpy of denaturation, especially

TABLE 1

Temperature and enthalpy of denaturation of lyzozyme ($M = 14450$) versus pH

Buffer	pH	T_{denat} (°C)	ΔH_{denat} (kJ mol^{-1})
Sodium acetate	2.31	57.8	287.1
	3.29	68.7	361.9
	3.75	77.0	405.1
	4.65	78.6	433.0
Sodium phosphate	5.40	77.6	422.0
	6.50	76.0	396.3
	6.9	75.8	382.0
	7.33	75.6	397.4
	8.05	74.4	388.0
TRIS-HCl	8.0	75.3	391.7
	8.5	74.3	369.0
	8.9	74.0	367.2
	7.8	75.8	368.9
	7.5	76.0	398.0
	7.3	75.6	397.0

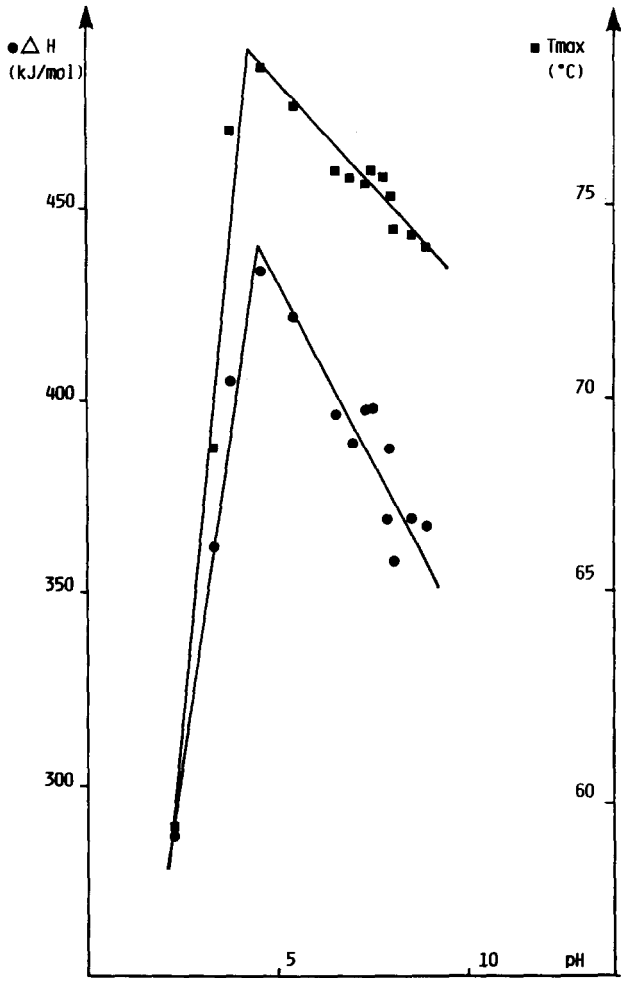

Fig. 2. Temperature and enthalpy of denaturation of lyzozyme versus pH.

versus pH, are very scarce. A correlation between the calorimetric results and the van't Hoff enthalpy, determined from difference spectral measurements [9] or optical rotation measurements [3], was needed in order to verify if the denaturation of lyzozyme can be interpreted in terms of a two-state model.

Therefore a systematic study was carried out. DSC curves of lyzozyme versus pH are shown in Fig. 1. The experimental values of temperature and enthalpy versus pH are listed in Table 1 and are displayed in Fig. 2. The evolution can be represented by two lines, as shown in Fig. 2. From pH 10.0 to 4.5, a slow increase is observed. At pH values lower than 4.5, a very fast decrease in temperature and enthalpy occurs. At pH 4.5, the maximal value of temperature and enthalpy is reached. We can conclude that, at

this value, an optimal stabilization of the tertiary structure of the molecule is obtained.

Biosynthetic human growth hormone (hGH)

The same experiments were performed on hGH, a protein with therapeutic applications.

Bewley [10] is the only author to have studied the denaturation of hGH by DSC but the experimental conditions used for the thermal analysis were not ideal: a high heating rate, 10°C min^{-1} was used; the purity of the extractive hGH was not described; and the value of the enthalpy of denaturation was imprecise (ΔH is in the range 420–840 kJ mol^{-1})

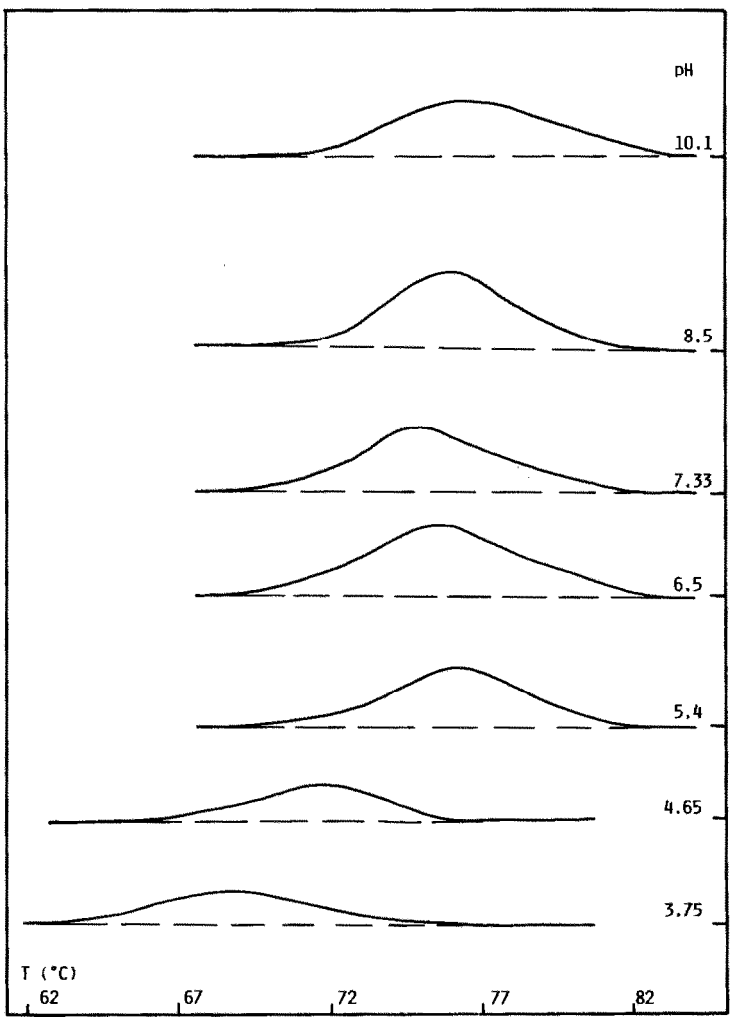

Fig. 3. DSC curves of biosynthetic hGH versus pH.

TABLE 2

Temperature and enthalpy of denaturation of hGH ($M = 22100$) versus pH

Buffer	pH	T_{denat} (°C)	ΔH_{denat} (kJ mol^{-1})
Sodium acetate	2.31	50.7	19.6
	3.29	55.9	24.1
	3.75	67.3	114.5
	4.65	70.5	128.1
Sodium phosphate	5.40	75.9	180.8
	6.50	75.4	201.9
	6.9	74.4	195.8
	7.33	74.3	201.9
	8.05	74.0	191.3
TRIS-HCl	8.0	74.4	204.8
	8.5	74.7	216.8
	8.9	76.2	218.9
	7.8	75.7	211.0
	7.5	75.7	204.9
	7.3	75.7	189.6
Sodium carbonate–sodium bicarbonate	9.15	75.9	182.3
	10.10	76.0	174.8

because of the presence of a contiguous exothermal effect attributed to the aggregation of the protein.

DSC curves of hGH versus pH are shown in Fig. 3. As previously reported [1], in our experiments a single endothermal effect is always obtained. The results are given in Table 2 and plotted in Fig. 4. From pH 10.0 to 5.0, the temperature of denaturation is constant; at pH values below 5.0, a rapid decrease is observed. A parabolic representation of ΔH_{denat} versus pH has been calculated. But considering the precision of the results ($\pm 5\%$), the following comments can be made: below pH 5.0 and above pH 9.0, a rapid decrease of the enthalpy is clearly shown; in the range pH 5.0–9.0, the highest enthalpy values are obtained, corresponding to the maximal stability of the protein.

CD experiments

Human growth hormone is a single polypeptide chain containing 191 amino acid residues. One Trp residue, eight Tyr residues, thirteen Phe residues and two disulphide bonds give the molecule a near-UV optical activity.

When analysed by the method of Chang et al. [2], the structure contents of hGH at pH 8.3 were estimated as follows: 50% α helix, 20% β structure,

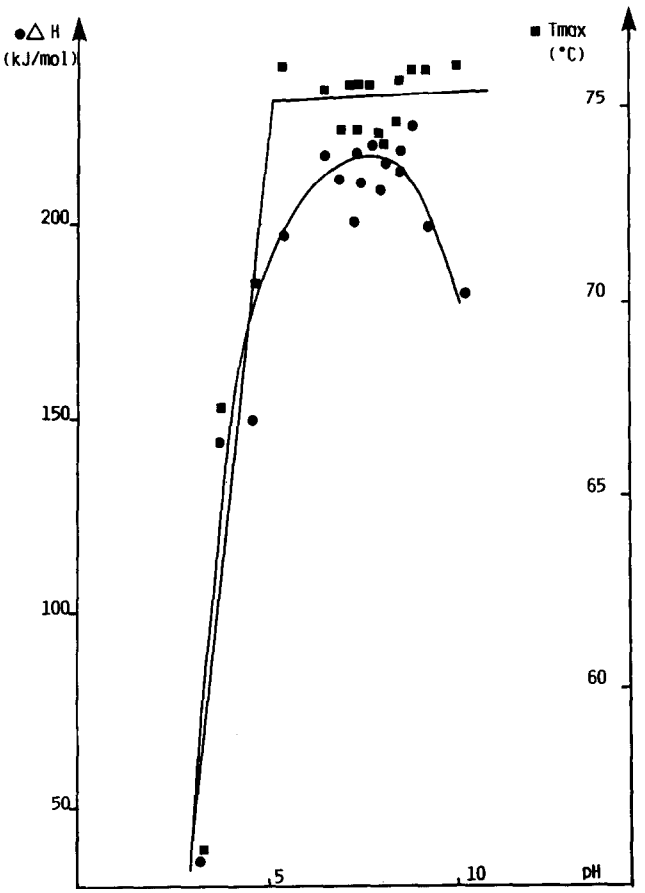

Fig. 4. Temperature and enthalpy of denaturation of hGH versus pH.

30% non-α and non-β structures [11]. These results are in agreement with those previously reported for pituitary hGH [12–14].

The CD spectra of biosynthetic hGH were studied between pH 3.5 and 10.5 (see Fig. 5). Between pH 4.7 and 8.9, the CD spectra in the near-UV and in the far-UV wavelength ranges show few, if any, variations. Below pH 4.9 and above pH 8.9, the important modifications of the near-UV spectra reflect the alteration of the aromatic residues as well as the modifications of their environment. Above pH 8.9, the titration of tyrosine groups turning to tyrosinate contributes to the observed CD change.

In contrast, the modifications in the far-UV part of the spectra were less important (data not shown), indicating that the secondary structure of the protein is slightly affected by these pH conditions. In any case, the protein conformation remains unchanged between pH 4.7 and 8.9 (Fig. 5), in good agreement with the DSC results.

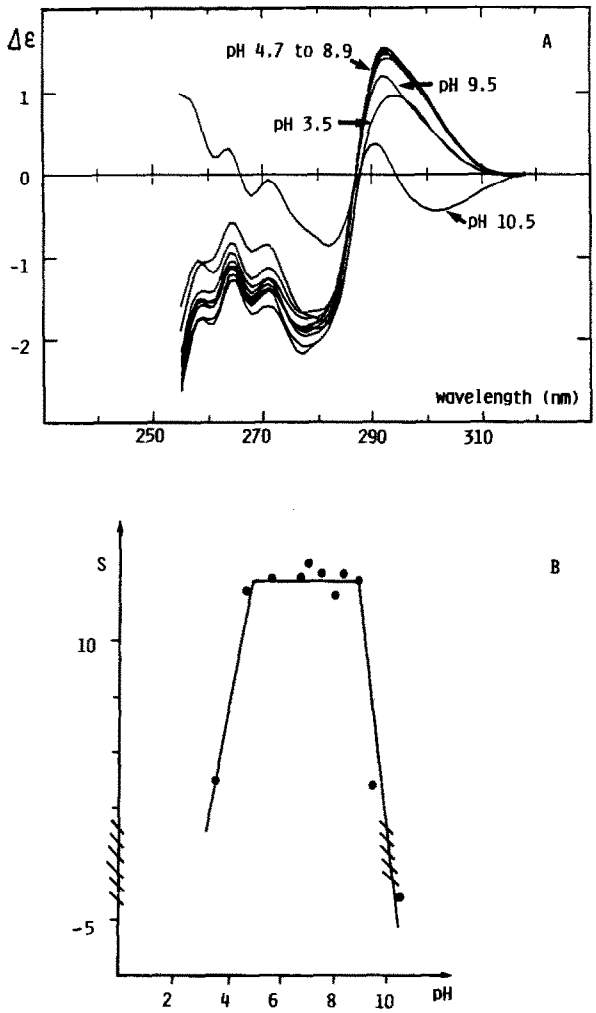

Fig. 5. The biosynthetic hGH circular dichroism spectra and the effect of pH: A, near-UV spectra (hGH concentration, 1.37 mg ml^{-1}) (m^{-1} cm^{-1}); B, CD spectrum areas between 295 and 310 nm. The pH increases from 3.5 to 10.5.

Influence of small molecules on the stability of hGH

Correlation with CD experiments has confirmed that DSC is a valuable tool with which to study the stability of proteins. Therefore small linear or aromatic molecules were used to modify the environment of hGH in aqueous solution.

DSC experiments were performed with the same experimental procedure in order to determine whether an increase or a decrease in the stability is obtained. The results are given in Table 3.

TABLE 3

Temperature and enthalpy of denaturation of hGH ($M = 22100$) in different solvents

Solvent	T_{denat} (°C)	ΔH_{denat} (kJ mol^{-1})
H_2O	79.9	174.5
Glycerol (17 mg ml^{-1})	80.4	187.4
Mannitol (10 mg ml^{-1})	80.9	169.8
Sorbitol (10 mg ml^{-1})	79.8	192.6
Tween 80 (0.3 mg ml^{-1})	79.1	196.8
Benzylic alcohol (10 mg ml^{-1})	71.8	156.8
Phenol (5 mg ml^{-1})	70.6	97.3
Urea (10 mg ml^{-1})	79.8	165.9
Metacresol (3 mg ml^{-1})	70.0	147.2 exothermal effect after denaturation
Chlorocresol (3 mg ml^{-1})	–	Denaturation at room temperature
Metacresol (3 mg ml^{-1}) + glycerol (17 mg ml^{-1})	68.5	190.7 exothermal effect after denaturation

The temperature and enthalpy of denaturation are not modified by small linear molecules such as glycerol or mannitol. However, the presence of aromatic compounds such as benzylic alcohol or metacresol, causes a dramatic decrease in the stability. With chlorocresol, the denaturation process occurs immediately at room temperature.

Of course, the importance of a slight modification of the interactions on the stability is well known, but this study shows that DSC can rapidly provide information when a suitable galenic formulation is needed.

CONCLUSION

The thermal behaviour of lyzozyme and hGH in aqueous solutions versus pH was studied by DSC experiments. The temperature and enthalpy of denaturation were determined showing a maximal stability of lyzozyme at pH 4.5 and a large pH range (5.0–9.0) corresponding to the maximal stability of hGH.

These results are in good agreement with those obtained by CD measurements.

The hGH environment was modified by addition of small linear or aromatic molecules, and their influence on the parameters of denaturation was studied.

At present, this work is in progress, in particular with other proteins, and an exploitation of the denaturation phenomenon using a kinetic evaluation is being developed.

It is now obvious that DSC equipped with a high-sensitivity sensor is a powerful tool with which to provide information on the optimal stability of proteins in solution. This is very important in the case of proteins for therapeutic use.

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