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A Central Composite Design to Investigate the Thermal Stabilization of Lysozyme

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Purpose. The formulation and processing of protein drugs requires the stabilization of the native, biologically active structure. Our aim was to investigate the thermal stability of a model protein, lysozyme, in the presence of two model excipients, sucrose and hydroxypropyl-β-cyclodextrin (HP-β-CD).

Methods. We used high sensitivity differential scanning calorimetry (HSDSC) in combination with a central composite design (CCD). As indicators of protein thermal stability, the measured responses were the unfolding transition temperature (T_m), the onset temperature of the denaturation (T_o), and the extrapolated onset temperature ($T_{o,e}$).

Results. A highly significant (F probability <0.001) statistical model resulted from analysis of the data. The largest effect was due to pH (over the range 3.2–7.2), and the pH value that maximized T_m was 4.8. Several minor but significant effects were detected that were useful for mechanistic understanding. In particular, the effects of protein concentration and cyclodextrin concentration on T_m and $T_{o,e}$ were found to be pH-dependent. This was indicative of the partially hydrophilic nature of protein-protein interactions and protein-cyclodextrin interactions, respectively.

Conclusions. Response surface methodology (RSM) proved efficient for the modeling and optimization of lysozyme thermal stability as well as for the physical understanding of the protein-sugar-cyclodextrin system in aqueous solution.

KEY WORDS: protein stabilization; high-sensitivity differential scanning calorimetry; central composite design.

INTRODUCTION

A critical aspect in the development and processing of protein drugs is stabilization of the native, biologically active structure. Proteins are structurally complex and physicochemically fragile materials that readily denature *via* disorganization of the naturally compact structure (1). Proteins are stabilized generally by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions (2). Sugars and polyols are often used as protein stabilizers because they tend to inhibit transition from the native (N) to the denatured, or unfolded (U) state (3). In contrast, cyclic oligosaccharides, or cyclodextrins (CDs), favour protein thermal denaturation (4). However, cyclodextrins have been reported to stabilize proteins against other protein degradation pathways, such as aggregation (5) and precipitation (6), and as a result improve protein folding reversibility (7). Cyclodextrins were also shown to protect proteins from freeze-drying-induced inactivation (8). Cyclodextrins are thought to have an impact on protein behaviour through weak interactions between the oligosaccharide hydrophobic core and nonpolar protein groups such as the moieties of tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues. Since sugars/polyols and cyclodextrins can inhibit different protein degradation pathways, we wished to probe the influence of combinations of these two types of additives on protein stability.

High-sensitivity differential scanning calorimetry (HSDSC) is ideally suited to the study of protein thermal denaturation in solution since it allows to measure the forces stabilizing the globular structure. In this study, we used HSDSC to monitor the heat capacity (C_p) profile of a model protein, lysozyme (EC 3.2.1.17), and, as indicators of stability, the measured responses were the unfolding transition temperature (T_m), the onset temperature of the denaturation (T_o), and the extrapolated onset temperature ($T_{o,e}$).

Hen-egg lysozyme (HEL) is a 10.7-isoelectric point (pI) enzyme comprising two structural lobes. Lysozyme polymerizes through disulfide linkages. Starting with dimerization after the loss of a proton from each of the monomers, polymerization is favoured by increasing pH. The dimer is the predominent species between pH 5 and 9. However, provided that protein concentration is less than 10 $mg.ml^{-1}$, dimerization is unimportant even at pH 6.5 (9–12).

As an alternative to the one-factor-at-a-time, classical experimental approach, the experimental design method was used, in which the levels of all factors were varied for each experiment. The advantages included a reduction in the number of trials, the ability to cover a large number of factors, the detection of interactions between factors, the detection of optima, a higher precision of the response data, and the empirical modeling of the data (13).

The experimental design method has been used in protein denaturation studies (14–16), but these have generally favoured the classical approach. For example, a study of the influences of pH and ionic strength on methionine repressor protein thermal stability (17) did not allow the possibility of detecting any interaction effects. In contrast, Tomicki *et al.* (18) recently used a central composite design to monitor the thermal stability of metmyoglobin. This design proved useful for estimating the combined effects of protein concentration, salt concentration, and pH, because these effects are unable to be assessed in isolation of one another. Here, we adopt a similar approach; however, in addition to the above factors, we have studied

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ABBREVIATIONS: α, distance of the axial points from the center point in the CCD; ANOVA, analysis of variance; β_i, regression coefficients; CCD, central composite design; CD, cyclodextrin; C_p, heat capacity; ΔC_p, heat capacity change upon protein denaturation; ΔH_{cal}, enthalpy change upon protein denaturation; ΔH_I, enthalpy change upon ionization; ΔH_{vH}, Van't Hoff enthalpy; F, ratio of the mean square of the regression by the population variance; HEL, hen egg lysozyme; [HEL], lysozyme concentration; HP-β-CD, hydroxypropyl-β-cyclodextrin; [HP-β-CD], hydroxypropyl-β-cyclodextrin conentration; HSDSC, high sensitivity differential scanning calorimetry; k, number of factor variables; N, protein native state; Phe, phenylalanine; pI, isoelectric point; R^2, coefficient of determination; RSM, response surface methodology; [Sucrose], sucrose concentration; T_o; onset temperature of the denaturation; $T_{o,e}$, extrapolated onset temperature of the danaturation; T_m, unfolding transition temperature; Trp, tryptophan; Tyr, tyrosine; U, protein denatured state.

the influence of carbohydrate excipients. Hydroxypropyl-β-cyclodextrin (HP-β-CD) was chosen as a model cyclodextrin because of its high aqueous solubility, and its pharmaceutical relevance as a potential excipient for parenteral and oral routes (19). Sucrose is commonly used as a protein stabilizer and was chosen because of its good glass forming abilities.

The choice of an experimental design affects the quality of the data (20). In particular, there are possible pitfalls in the use of a design of inadequate resolution in identifying the significant variables influencing a complex process. For example, the Plackett-Burman design is useful in estimating main variable effects provided they are not confounded with two-factor interaction effects. Our aim was to exploit the higher resolution of another design, the central composite design (CCD), so that both interaction and higher-order effects could be detected.

MATERIALS AND METHODS

Chemicals

Hen-egg lysozyme hydrochloride (lot 14103921-96) was purchased from Boehringer Mannheim GmbH (Germany), hydroxypropyl-β-cyclodextrin (batch 07B-271/1) was obtained from Janssen Biotech N.V. (Olen, Belgium), and sucrose (batch 980441OF) from BDH Chemicals Ltd (Poole, England). Citric acid anhydrous (lot K22852036 616) and disodium hydrogen orthophosphate dihydrate (lot K22761079 623) were also from BDH Chemicals Ltd and were AnalaR grade. Double distilled deionized water was used for preparation of all solutions.

Sample Preparation

According to the structure of the experimental design used (described below), five buffer solutions (pHs 3.2, 4.2, 5.2, 6.2, and 7.2, with an uncertainty of ±0.05 pH unit) were prepared by mixing in different ratios a 100 mM citrate stock solution and a 200 mM phosphate stock solution (21). This buffer covers a sufficiently wide pH range and has a small enthalpy of ionization (ΔH_I), which minimizes heat effects due to protonation changes during the denaturation reaction (17). The cyclodextrin and the sugar were dissolved at concentration levels of 0, 5, 10, 15, and 20% (w/w). Lysozyme was dissolved at concentrations of 2, 6.5, 11, 15.5, and 20 $mg.ml^{-1}$ (which correspond to 0.14, 0.46, 0.77, 1.08, and 1.40 mM, using a molecular weight value of 14.3 kDa for lysozyme). The solutions were centrifuged for 10 minutes at 13,000 rpm to remove any insoluble matter. The pH measurements were done using a Mettler Delta 340 pH meter.

Calorimetric Measurements

Thermal denaturation profiles were generated using the Micro Calorimetry System differential scanning calorimeter (MCS DSC, MicroCal Inc., Northampton, MA, USA) as described elsewhere (17). The experiments were carried out over the temperature range 25–90°C at an optimal scan rate of 1 $K.min^{-1}$ (22). The transitions were reversible as observed by rescanning the samples after allowing them to cool down to base temperature.

Experimental Design

The central composite response surface design (CCD) was chosen for its ability to estimate second-order and third-order effects, to detect interrelationships between the factors, to model the response data, to locate response optima, and thus to contribute to the physical undertanding of protein stability in solution. Disadvantages of the CCD, when compared to a three-level factorial design, include the inability to estimate certain interactive terms (i.e. quadratic by quadratic), but this is usually considered minor in comparison to the small number of trials required to obtain a second-order or third-order model (23).

A four-factor ($k = 4$), five-level CCD was used (24). This design comprises 25 points and 30 actual experiments including replicates. The CCD is made up of a $n_f = 16$ point two-level full factorial $2^{k=4}$ design (-1 and $+1$ being the coded levels for each factor and 0 for the $n_{cf} = 4$ centre points), with an additional axial block comprising $n_a = 2$ experimental points per factor at a distance $\alpha = (n_{cf})^{0.25} = 2$ from the design centre and $n_{ca} = 2$ centre points. Selection of the value of α facilitates control of the properties of the design. The four-factor design that was used is both orthogonal and rotatable, since $\alpha = 2$. Replication allowed to determine experimental error and to increase the precision of estimates. The order in which the runs were performed was randomized, as a requirement for the obervations to be independently distributed random variables and as a means to prevent effects of unknown nuisance variables.

The statistical analysis was carried out on the coded data sets in order to simplify the interpretation of the results. The coding was as follows:

$$X = 2\alpha\ (x - x_{mean})/(x_{max} - x_{min})$$

where x and X are the natural and the coded factor level, and $\beta_{natural}$ and β_{coded} the natural and coded regression coefficients respectively.

Linear regression, analysis of variance (ANOVA), and residual analysis were performed with the Excel package (version 5.0, Microsoft Inc., Redmond, WA, USA). The coefficient elimination procedure was as follows. First, all highly nonsignificant coefficients (t-test p value ≥ 0.2) were removed. The analysis was run once more, and then the less significant coefficients were removed one by one and the analysis done again until all remaining coefficients had p values <0.1. The overall analysis by Excel was confirmed by the output of two other packages, the Statistical Analysis System package (SAS, version 6.04, SAS Institute Inc., Cary, NC, USA), using the General Linear Models Procedure (PROC GLM), and the Statistical Package for Social Scientists (SPSS, version 4.0, SPSS Inc., Chicago, IL, USA). The data were initially fitted using a second-order polynomial, but a third-order model improved analysis because of the pH effect:

$$y = \beta_0 + \Sigma\ \beta_i x_i + \Sigma\ \beta_{ii} x_i^2 + \Sigma\ \beta_{iii} x_i^3 + \Sigma\Sigma\ \beta_{ij} x_i x_j + \Sigma\Sigma\Sigma\ \beta_{ijk} x_i x_j x_k$$

where y was the estimate of the response (i.e. dependent variable), x_i the factors (i.e. independent variables), β_0 the overall mean response, and β_i the regression model coefficients.

RESULTS AND DISCUSSION

Typical calorimetric recordings of the heat absorption for six independent samples are shown (Fig. 1). The endothermic transition observed was indicative of the melting of the hydrogen bonded structure, in which water successfully competes with backbone and side chain groups in the macromolecule. The observed change in excess heat capacity resulted primarily from the change in the magnitude of protein hydration (25). Thermal transitions were fitted to a single peak, non two-state unfolding model. For centre point experiments (0. 77 mM protein, 10% (w/w) cyclodextrin, 10% (w/w) sucrose, pH 5.2), the ratio of the calorimetric enthalpy (ΔH_{cal}), measured from the surface area of the normalized peak, to the van't Hoff enthalpy (ΔH_{vH}), calculated from the shape of the peak, was found to be 0.9. This deviation from unity indicated intermolecular interactions (26, 27). This may be explained by the specific experimental conditions of the centre point experiment since in other conditions, especially low concentration and acidic conditions, hen-egg lysozyme is generally found to undergo a two-state transition with a $\Delta H_{cal}/\Delta H_{vH}$ ratio very close to unity (22). In our case, the deviation from unity was small enough to make acceptable the two-state approximation. The transitions showed a slight asymmetry when compared to theoretical fits. The higher-temperature side of the endothermic peak was generally steeper than expected. It has been reported to be associated with exothermic irreversible processes such as protein aggregation, deamidation, proline isomerization, and sulfhydryl oxidation (17).

The calorimetric data are presented in Table I, with ANOVA and regression results listed in Table II. The multiple R^2 value, more than 0.99 for T_m and $T_{o,e}$, reflected the good fit between the regression equations and the data for these two variables. For T_o, the regression equation did not explain the data as well as for the two other responses since the multiple R^2 was only 0.81, which was related to the larger scatter of T_o data as compared to T_m and $T_{o,e}$ data. With F probability always less than 0.001, the regressions for the three responses were highly significant. The p values for the t-tests indicated that all four factors had significant effects on T_m and $T_{o,e}$, while only cyclodextrin concentration and pH significantly influenced T_o. For the three responses, both first-order and higher-order terms were found to be significant. Standard errors were 0.04°C for the overall T_m mean (75.7°C), 0.34°C for the overall T_o mean (59.4°C), and 0. 05°C for the overall $T_{o,e}$ mean (69.7°C). The residual analysis showed that the sum of the residuals was zero and, plotting the residuals versus the predicted responses, no pattern was observed, which validated the assumption that the observations were independently distributed random variables.

Table I. Calorimetric Data for the T_m, T_o, and $T_{o,e}$ of Lysozyme

Sample	T_m (°C)	T_o (°C)	$T_{o,e}$ (°C)
1	75.56	58.55	69.41
2	75.61	58.84	69.41
3	75.57	59.92	69.38
4	74.00	55.82	67.73
5	75.55	59.29	69.38
6	77.19	59.71	71.15
7	74.22	57.09	67.93
8	68.76	53.64	62.06
9	75.34	57.21	69.14
11	71.89	57.42	65.55
12	75.59	60.80	69.55
13	73.76	57.22	67.49
14	77.60	60.38	71.50
15	75.25	59.61	69.17
16	74.05	57.62	67.90
17	76.83	60.81	70.69
18	71.78	56.65	65.49
19	75.66	60.00	69.62
20	75.53	61.34	69.46
21	71.88	54.98	65.68
22	73.67	59.75	67.90
23	73.40	56.47	67.38
24	75.63	58.58	69.73
25	75.63	59.73	69.62
26	77.10	61.84	70.91
27	75.61	57.50	69.50
28	75.23	57.80	69.03
29	73.59	57.73	67.37
30	75.23	59.43	69.13

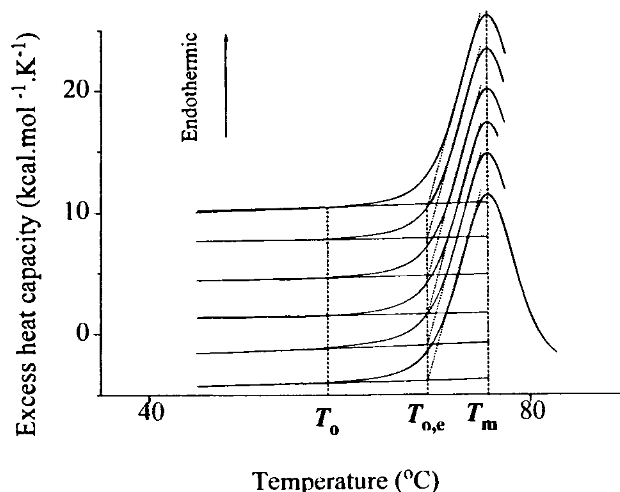

Fig. 1. Six independent replicates of a calorimetric experiment with lysozyme. Conditions: 0.77 mM protein, 10% w/w hydroxypropyl-β-cyclodextrin, 10% w/w sucrose, pH 5.2, citrate-phosphate buffer.

Searches were made for maxima for T_m, T_o, and $T_{o,e}$. The optimum factor levels found are given in Table III, and maximum T_m, T_o, and $T_{o,e}$ were 79.6°C, 61.4°C, and 73.4°C respectively.

Some response surfaces are shown in Fig. 2. Each surface corresponds to one response as a function of two factors, the other two factors being fixed at their optimum values.

The pH Effect

Analysis of the data revealed that the pH, having large coded β coefficients, was the main factor that explained the variation of the responses about their means, even though for T_o, the coefficient for the cyclodextrin concentration term was similar to that for the pH quadratic term. This was ascribed to the wide pH range covered in the study and the known strong influence of pH on protein thermal stability (21). The electrostatic environment thus contributed to lysozyme stability. The overall pH effect was positive for T_m and $T_{o,e}$ (~ +0.8°C per

Table II. Statistical Analysis of the Calorimetric Data

(a) Analysis of variance (ANOVA)

T_m analysis:

	Degrees of freedom	Sums of squares	Mean squares	F-ratio	F probability
Regression	8	95.58	11.95	1000.4	$p < 0.001$
Residual	16	0.19	0.01		
Total	24	95.77			

T_o analysis:

	Degrees of freedom	Sums of squares	Mean squares	F-ratio	F probability
Regression	2	62.93	31.46	21.0	$p < 0.001$
Residual	22	32.92	1.50		
Total	24	95.85			

$T_{o,c}$ analysis:

	Degrees of freedom	Sums of squares	Mean squares	F-ratio	F probability
Regression	7	105.53	15.08	449.0	$p < 0.001$
Residual	17	0.57	0.03		
Total	24	106.10			

(b) Linear regression carried out with the coded factors[a]

T_m multiple $R^2 > 0.99$		T_o multiple $R^2 > 0.81$		$T_{o,c}$ multiple $R^2 > 0.99$	
Parameters	Estimates	Parameters	Estimates	Parameters	Estimates
β_0	75.673	β_0	59.386	β_0	69.693
β_2	−0.828	β_2	−1.129	β_2	−0.875
β_3	0.795	β_{44}	−1.138	β_3	0.819
β_4	−1.513			β_4	−1.540
β_{22}	0.085[b]			β_{44}	−1.420
β_{44}	−1.312			β_{14}	0.112[c]
β_{14}	0.089[b]			β_{24}	−0.101[c]
β_{24}	−0.073[c]			β_{444}	0.611
β_{444}	0.573				

[a] P value < 0.001 unless otherwise indicated.
[b] P value < 0.01.
[c] P value < 0.1.

pH unit increase when other factors were optimal) and was zero for T_o. However, the higher-order terms had such coefficients that it was not appropriate to consider the overall effect. This can be visualized on the response surfaces, which have a marked curvature along the pH axis (Figures 2B and 2C). As seen on these surfaces, the negative coefficient for the pH quadratic term created a hilly shape. The T_m, T_o, and $T_{o,c}$ responses increased as the pH was raised in the range 3.2–4.8, then reached maxima, and decreased. For T_m and $T_{o,c}$, a positive third-order effect was superimposed on this quadratic behaviour. Therefore, the change over the 1.6 pH-unit-wide 3.2–4.8 range induced larger changes in the responses than those observed due to change in the 2.4 pH-unit-wide 4.8–7.2 range (e.g. $\Delta T_{o,c}$ ~ +8.5°C as compared to −3.2°C respectively). The pH effect showed small but significant [HP-β-CD] and [HEL] dependences for T_m and $T_{o,c}$. The existence of pH*[HEL] terms was in agreement with the known interdependence of the unfolding and association-dissociation equilibrium processes (28) since protein association state is expected to have an impact on the susceptibility of the monomer towards unfolding and reciprocally. The positive sign of the pH*[HEL] coefficients (β_{14} ~ +0.09 for T_m and β_{14} ~ +0.11 for $T_{o,c}$) indicated that, as [HEL] was increased, the pH was more effective at increasing T_m. A likely event that was favoured by increases in both pH and [HEL] and that resulted in an increase in T_m and $T_{o,c}$ is self association of the lysozyme monomer. However, it is known that lysozyme association is minor when pH and [HEL] are less than 6.5 and 10 $mg.ml^{-1}$ respectively (9), which is reflected in the small magnitude of the β_{14} coefficients observed for T_m and $T_{o,c}$. This means that the main contribution to the observed increase in T_m was due to charge effects on the monomer, independently from association with other monomers. Moreover, when association was favoured, T_m only decreased. Therefore, the observed overall pH dependence of T_m was mainly due to protonation changes during thermal unfolding of the monomer. Further, although maximum conformational stability

Table III. Optimized Conditions Within the Ranges Investigated

	x_1 [HEL] ($mg.ml^{-1}$)	x_2 [HP-β-CD] (% w/w)	x_3 [Sucrose] (% w/w)	x_4 pH
T_m	2	0	20	4.75
T_o	[2; 20]	0	[2; 20]	4.70
$T_{o,c}$	2	0	20	4.77

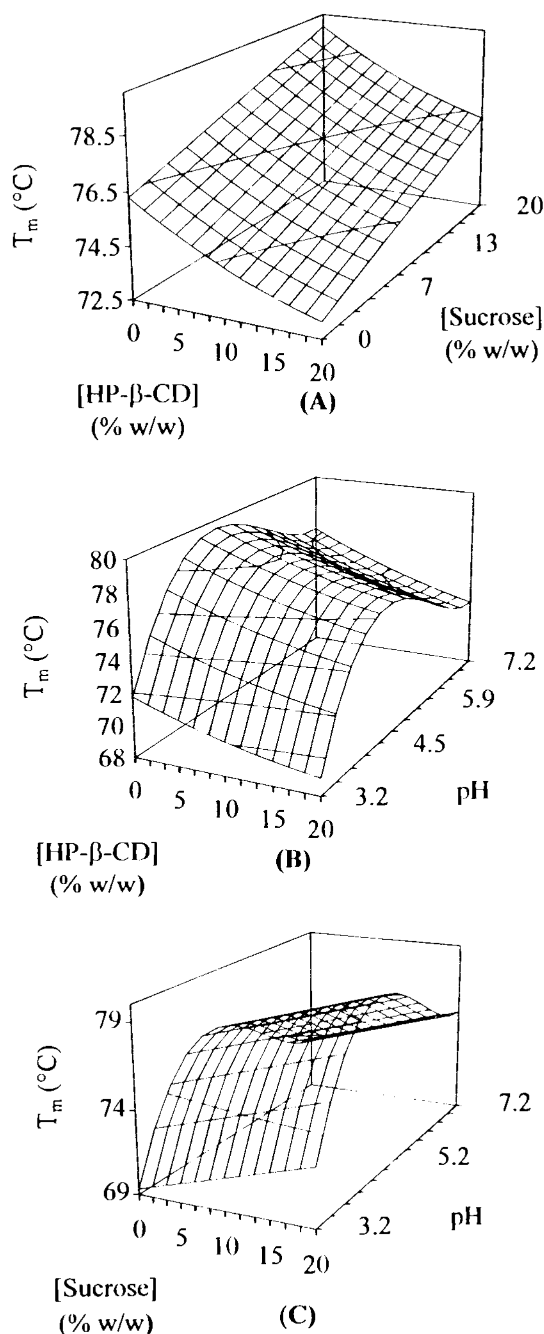

Fig. 2. Response surfaces for the denaturation temperature (T_m) of 0.14 mM lysozyme as a function of: **A.** Sucrose and cyclodextrin concentrations (at pH 4.75). **B.** pH and cyclodextrin concentration (at 20% w/w sucrose). **C.** pH and sucrose concentration (at 0% w/w cyclodextrin).

of proteins should occur at their isoelectric point (pI) (29), i.e. around pH 10 for lysozyme, the burial of nontitratable groups often leads to maximum stabilities at pH values other than the pI. Counterions, i.e. citrate and phosphate ions in this work, may also shift the pH of the maximum stability due to intrachain charge repulsion shielding effects (30). Regarding the pH*[HP-β-CD] term, the negative sign of its coefficient ($\beta_{24} \sim -0.07$ for T_m and $\beta_{24} \sim -0.10$ for $T_{o,e}$) supported the observation that cyclodextrins reduce protein aggregation (5). Nonetheless, no [HEL]*[HP-β-CD] term was detected, probably due to the small magnitude of the [HEL] effect.

The Protein Concentration Effect

Unlike T_o, both T_m and $T_{o,e}$ displayed a significant protein concentration dependence, the protein concentration effect being itself a function of pH. However, this effect ($\sim +0.01$°C per mg.ml^{-1} increase in [HEL]) was weak in comparison to the potential dramatic changes in the association-dissociation behaviour of the monomer due to the wide [HEL] and pH ranges spanned. The low magnitude of the pH*[HEL] coefficient ($\beta_{14} \sim +0.10$) indicated that, while monomer-monomer interactions were greatly enhanced above certain conditions tested in this study, they had little impact on the thermal stability of the monomer. The dependence of the [HEL] effect on the pH pointed out the partially hydrophilic nature of protein molecule surfaces, since purely hydrophobic protein-protein interactions would not in principle depend on environmental electrostatic conditions. This observation was consistent with the finding that hydrophilic bridges contribute to protein association (28).

The Cyclodextrin Concentration Effect

Cyclodextrin concentration had primarily a main (i.e. first-order) negative effect on T_m, T_o, and $T_{o,e}$. The overall [HP-β-CD] influence was negative, about -0.2°C per percentage increase in cyclodextrin concentration (w/w) when other factors were optimal. The [HP-β-CD] effect on T_o was larger than that on T_m and $T_{o,e}$ ($\beta_2 \sim -1.13$ as compared to $\beta_2 \sim -0.83$ and $\beta_2 \sim -0.88$ respectively), which corresponds to the expected broadening of the peaks with decreasing temperature. Higher-order terms for cyclodextrin concentration had minor coefficients. The nature of cyclodextrin effects on protein thermal stability in solution does not seem to vary with the nature of proteins (31) or CDs (32). The usual observation is that CDs favour protein thermal denaturation. Protein-CD interactions stabilize non native states, and reduce, as well as T_m, the changes in enthalpy (ΔH_{cal}) and heat capacity (ΔC_p) upon denaturation (31). Since a relationship has been found between the magnitude of the protein stabilizing effect (ΔT_m) by sugars and their water structure-making or structure-breaking effect (2), the effects of cyclodextrins on protein stability might also be related to their impact on water properties. This was particularly likely in this study because, unlike natural cyclodextrins, HP-β-CD is surface active (33). For the cyclodextrin concentration total effect, the predominence of the main effect ($\beta_2 \sim -0.83$) over that of the quadratic and crossproduct terms ($\beta_{22} \sim +0.09$ and $\beta_{24} \sim -0.07$ respectively) was consistent with a simple equilibrium model describing protein-CD interactions (31). This model assumes n identical and independent CD binding sites on the unfolded macromolecule and predicts a linear CD concentration dependence of protein transition temperature at low CD concentrations. This model has been used to provide estimates of $K_b = 1.6$ M^{-1} and $n = 12$ for the binding constant per α-CD binding site (at T_m) and the number of sites on lysozyme, consistent with the aromatic profile of the protein (6 Trp, 3 Tyr, 3 Phe) (31). Although the magnitude of the higher-order coefficients were small, they were significant and brought additional information. The nonzero positive quadratic term for T_m

indicated a flattening of the main negative effect of [HP-β-CD]. This was consistent with a decrease in the number of available CD binding sites on the lysozyme molecule as an increasing number of these sites interact with other CD molecules. The pH*[HP-β-CD] interactive term revealed an interrelationship that was unexpected if protein-CD interactions were assumed to be purely hydrophobic. This term suggested a small electrostatic dependence of protein-CD interactions, which may be explained by the occurrence of polar interactions between CD outer surface and polar protein groups. This crossproduct effect may also result from local pH-induced changes at hydrophilic residues on the protein surface, which e.g. alter the accessibility of nonpolar protein groups to CD molecules. Under conditions which favour the protein monomer, cyclodextrins may only decrease T_m. But in conditions favourable to association, cyclodextrins could increase the T_m of proteins : insulin has been observed to undergo an increase in T_m of about +10°C due to the presence of sulfate-β-cyclodextrin which favoured self association (34). However, we did not observe any such effect with the hydroxyalkylated cyclodextrin, which is uncharged unlike the anionic sulfate-β-cyclodextrin.

The Sucrose Concentration Effect

Sucrose concentration did not influence T_o but did affect T_m and $T_{o,e}$. This is interesting because, while both sucrose and cyclodextrin influenced T_m and $T_{o,e}$, only cyclodextrin affected T_o. The overall effect of sucrose was purely a positive, first-order effect, as expected for many sugars and polyols. Each percentage increase in sucrose concentration (w/w) gave rise to an increase in T_m or $T_{o,e}$ of approximately +0.2°C. Hence, the magnitudes of the [sucrose] and [HP-β-CD] effects were approximately the same, and only the signs were different. Interestingly, the [sucrose] factor did not interact with any other factor. It was not altered by wide changes in pH, demonstrating the absence of electrostatic nature of the sugar effect. This is in agreement with the report that the influence of 50% (w/w) sucrose on the T_m of lysozyme (about +15°C) is the same at pHs 3 and 7 (2). The pH independent behaviour of the sucrose concentration effect was consistent with the mechanism whereby sugars and polyols stabilize proteins through their influence on water properties, which in turn determine the strength of hydrophobic interactions. The [sucrose] effect was also independent of the concentration and subsequent association behaviour of lysozyme, in contradiction with the idea that an extensive dimerization, by affecting the interface of the protein, might influence any interfacial process such as protein hydration and thus protein-sucrose interactions. The absence of a [sucrose]*[HEL] term also indicated that the protein concentration effect was the same regardless of the sucrose concentration. This was not expected since sucrose increases the water surface tension (35), and as a consequence the protein association behaviour. The explanation may lie both in the small magnitude of the [HEL] effect observed and in the fact that unfolding transitions occur at constant surface tension since this property of water decreases with an increase in temperature (36). Finally, since sugars change water properties, which affect hydrophobic interactions, protein-CD interactions might be expected to change upon addition of sucrose. This hypothesis was not supported by our data.

The Buffer Effect

Although the nature and the concentration of buffer components were not varied in this work, they did not have a neutral behaviour towards protein stability. Phosphate ions probably interacted extensively with the positively charged lysozyme since these ions usually bind positively charged and polar residues in proteins (37). Additionally, while sodium ions are not active in this respect, citrate and phosphate ions disrupt the structure of water, markedly enhance its surface tension, and decrease the solubility of non polar solutes (38).

CONCLUSIONS

Response surface methodology (RSM), an economical approach allowing maximum information to be collected with a minimum of experiments, was effective in the construction of a highly significant statistical model describing the thermal stability of lysozyme. The high precision of the data (e.g. $\sigma < 0.04$°C for T_m) resulting from the combined use of a central composite design and high-resolution calorimetry facilitated the identification of minor effects important in understanding of mechanisms but often not detected.

From the factors examined, the pH of the solution had the largest effect on lysozyme thermal stability. The observed pH dependences of the transition temperature (T_m), the onset temperature (T_o), and the extrapolated onset temperature ($T_{o,e}$) were primarily ascribed to ionization changes during thermal unfolding of the monomer, virtually independent from association with other monomers. A significant pH dependence of the lysozyme concentration effect was found which was in agreement with hydrophilic bridges contributing to protein association (28). As expected, hydroxypropyl-β-cyclodextrin concentration had mainly a first-order negative effect, consistent with a simple equilibrium model describing protein-cyclodextrin interactions (31), while sucrose concentration had a purely first-order positive effect. In contrast to the sucrose effect, the cyclodextrin effect exhibited a significant pH dependence, indicating the partially hydrophilic nature of protein-cyclodextrin interactions.

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