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Method for the characterization of stationary phases for the separation of proteins by high-performance liquid chromatography

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ABSTRACT

A conformational change of lysozyme and myoglobin can be visualized with transition curves in a high-performance liquid chromatographic (HPLC) system using a diode-array detector and UV difference spectrometry. Transition temperatures calculated from the curves are affected by the mobile phase composition and the column packing material. Flow-rates as used in HPLC do not have a measurable influence.

INTRODUCTION

New and improved stationary phases for protein separations by high-performance liquid chromatography (HPLC) are constantly being announced. Accompanying such product improvements are analytical techniques developed to characterize these materials, such as solid-state NMR, diffuse reflectance infrared Fourier transform spectrometry (DRIFT) and thermogravimetric analysis (TGA).

In certain instances, protein separation by HPLC requires conditions that maintain the tertiary structure of a protein, *e.g.*, in the final stage of a preparative purification. Stationary phases used for this non-denaturing chromatography are often described as "hydrophilic" and should have a very mild interaction with the particular protein. Retention time or retention volume is often taken as a measure of the amount of interaction with the stationary phase. Strongly retained proteins will probably suffer more from denaturation than unretained proteins. Inertness is then regarded as being synonymous with unretained elution.

To synthesize and compare "mild" stationary phases, however, a technique is required that measures a conformational change of a protein in a standardized and reproducible way. There have been a number of papers on the measurement of protein conformational changes in HPLC columns [1–6]. Surface intrinsic fluorescence in a spectroscopic flow cell enabled the study of the surface dynamics of a protein in contact with an adsorbent [3]. Rapid conformational interconversions on a chromatographic detection time scale could be demonstrated with diode-array and low-angle

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laser light scattering detection [2]. Here a method is presented that makes use of a transition curve of an eluted protein as measured by UV difference spectrometry. Related approaches have appeared recently [7–9].

With a stepwise increase in temperature, the fraction of denatured protein will at one point increase rapidly. This fraction, when plotted against temperature, produces a transition curve from which a transition temperature or transition point can be determined at 50% denaturation. Transition temperatures are a measure of the stability of proteins and depend on the nature of the protein and the solvent composition. A worsening of the solvent composition shifts the transition curve and lowers the transition point (Fig. 1).

Transition curves can be recorded with several techniques that measure a protein conformational change, *e.g.*, enzymatic activity [10], circular dichromism [1], viscosity [11], light scattering [12] and UV difference spectrometry [13–16]. In the last method, spectra of a protein under different conditions are subtracted, *e.g.*, at two different temperatures or two concentrations of a solvent modifier.

Spectral differences or shifts are generated by chromophoric moieties, amino acid side-chains, that turn outwards and form complexes with solvent molecules [17–18]. By recording transition curves with different techniques, it can be established that this UV difference indeed represents a conformational change. With lysozyme, comparisons were made with viscosity, optical rotation and light scattering measurements [11,12].

The lysozyme transition is clearly defined with no intermediate states. It is reversible and probably not a complete denaturation to a random coil but a partial opening of the tertiary structure [14,19]. With myoglobin intermediate states are sometimes observed, illustrating that with a transition curve one can "see" a conformation change more directly than, for instance, with a single enzymatic activity assay.

The curves have been used in the past to elucidate protein structures by study-

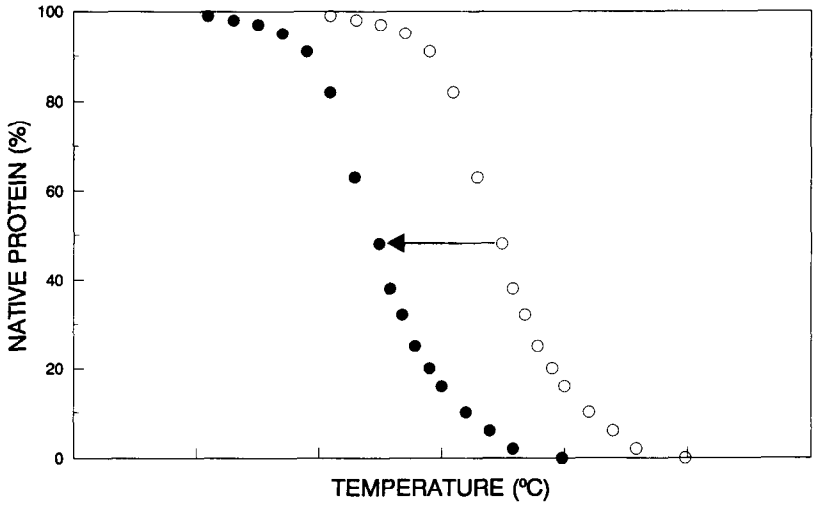

Fig. 1. Shift of transition curve with a change of solvent composition.

ing the influence of intramolecular ionic and hydrophobic bonding [15]. Here they are used to study the interaction of standard proteins with stationary phases in HPLC columns and to develop a method to determine the quality of silica coatings.

EXPERIMENTAL

A silica support (Hypersil WP 300, 10 μm) was obtained from Shandon (Runcorn, U.K.). Lysozyme and myoglobin were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Equipment

HPLC was performed with a constant-flow pump (Spectroflow 400; Kratos, Ramsey, NJ, U.S.A.) and an injection valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop and a stainless-steel column (150 × 2 mm I.D.). The column was thermostated with a Haake thermostat (Berlin, F.R.G.) (Type F4391), plus a water-jacket. Temperatures were measured with a electronic thermometer (Amadigit ad 15th; Amarell Electronic, Kreuzwertheim, F.R.G.) with a probe in the water-jacket. The detector was a Hewlett-Packard (Waldbronn, F.R.G.) diode-array detector (Model 1040A) operated with a Hewlett-Packard (Corvallis, OR, U.S.A.) computer (Model HP-85B) and Hewlett-Packard (Guadalajara, Mexico) disk drives (Model HP-9121).

Procedure

To monitor the conformational change of lysozyme by difference spectrometry a wavelength has to be selected. A value often mentioned in the literature is 292 nm [14,16]. In order to be able to check the total concentration of eluted lysozyme in the peak maximum, the absorbance at 297 nm, an isosbestic point in the difference spectrum, was also recorded. Moreover, an additional measurement was done at 302 nm as a check on the internal consistency of the result. The conformational change in myoglobin is relatively easily measured at 410 nm, because a distinct maximum at that wavelength disappears on denaturation [13]. A check on total concentration was done at 280 nm, where a maximum occurs that is not affected by denaturation.

The mobile phase composition used during the procedure should not interfere with the difference spectrometry and must completely elute the native protein at room temperature.

The procedure starts with a column thermostated at 20°C. The protein sample is injected and the signals are recorded. After elution of the sample the temperature is raised in about 30 steps to 80–90°C. At each predetermined temperature a protein sample is injected and the signals are recorded.

After these measurements the data have to be retrieved and a transition plot calculated. The EVALUATE program from the DAD 1040 supplies signal values in the peak maximum. To determine the UV difference, the 20°C absorbance value, where all the protein is in its native state, has to be subtracted from the values at other temperatures, *e.g.*, $A^{292}(T°C) - A^{292}(20°C)$. However, in this flow system the observed changes in the absorbance values are also due to small variations in the total concentration of the protein in the peak maximum. The absorbance values can be corrected for these variations by normalizing them with the absorbance of the refer-

ence wavelength. This reference wavelength is not affected by the denaturation of the protein and is thus a measure of the amount of protein. With the value of the reference wavelength at 20°C, A_R (20°C), all other temperature values, $A(T°C)$, are recalculated, using

$$[A_R(20°C)/A_R(T°C)]A(T°C)$$

The UV difference is then determined by subtracting the 20°C value. The figures obtained in this way are equivalent to the Δe values used by others [14,20]. They can be plotted against the corresponding temperatures (Figs. 2 and 3). A transition is usually apparent. By identifying the observed asymptotes, with native protein (Δe_N) and complete denaturation (Δe_D) respectively, the vertical axis can be converted into a percentage denaturation scale. The fraction of protein in the denatured form is then calculated using

$$f_d = (\Delta e - \Delta e_N)/(\Delta e_D - \Delta e_N)$$

where Δe is the UV difference at a particular temperature [11].

With lysozyme, the observed asymptotes have a slope, probably due to a temperature dependence of the spectra. The percentage denaturation can be determined by performing an axis transformation. One axis is drawn parallel to the slope of the asymptotes and the other perpendicular to it. The distance between the two asymptotes in this new axis system is then used to determine the fraction of denatured protein (see Fig. 3), and related to the corresponding temperature. More often two values, Δe_N and Δe_D, are chosen visually, symmetrically around the transition (Figs. 4 and 5).

It should be noted that the procedure described so far gives information on the

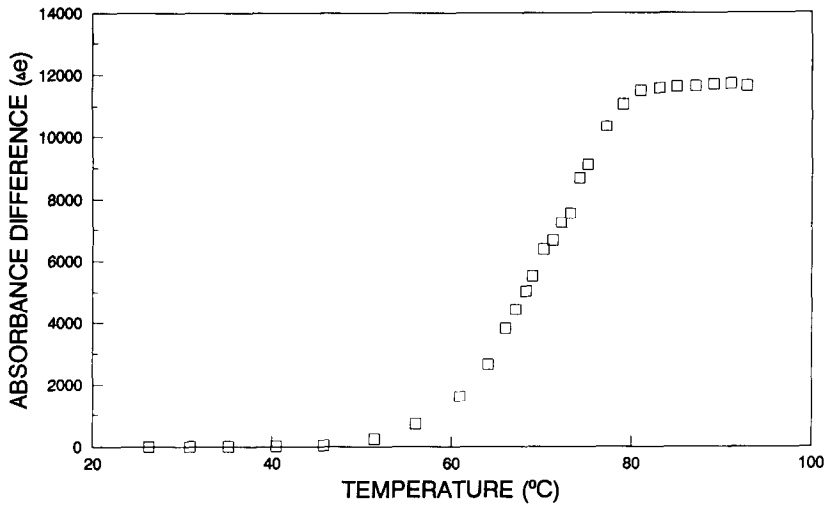

Fig. 2. UV difference of myoglobin plotted against temperature. Calculation as described in the text.

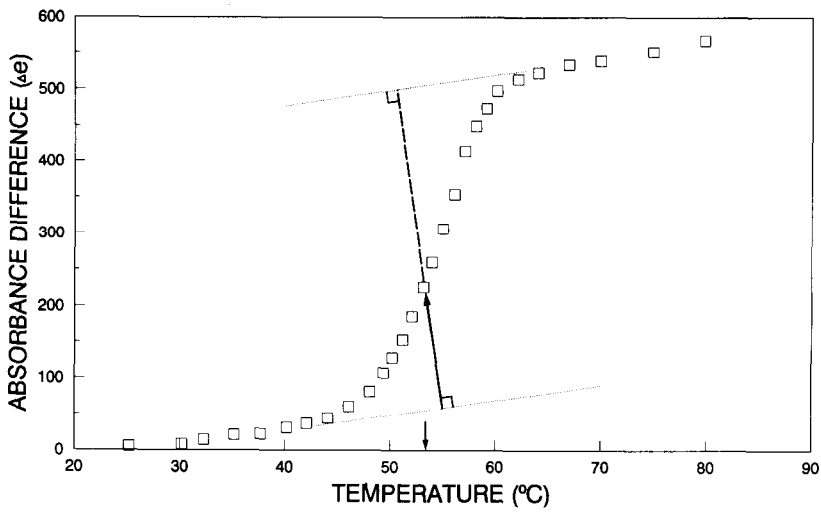

Fig. 3. UV difference of lysozyme plotted against temperature. Calculation as described in the text.

extent of denaturation of the eluted protein. In some instances, however, the protein recovery is drastically reduced at higher temperatures of the transition curve. The mobile phase allows complete elution of the native protein at room temperature. If the temperature is raised and part of the protein denatures, elution need no longer be complete as moieties from inside the protein become exposed. A particular mobile phase may still be desirable in order to study the interaction with a given hydrophilic support. Therefore, a second correction is sometimes necessary with peak-height or peak-area data. The fraction of eluted native protein as calculated from the Δe plot is multiplied by the fraction of protein recovery to give a more accurate figure of f_n, the

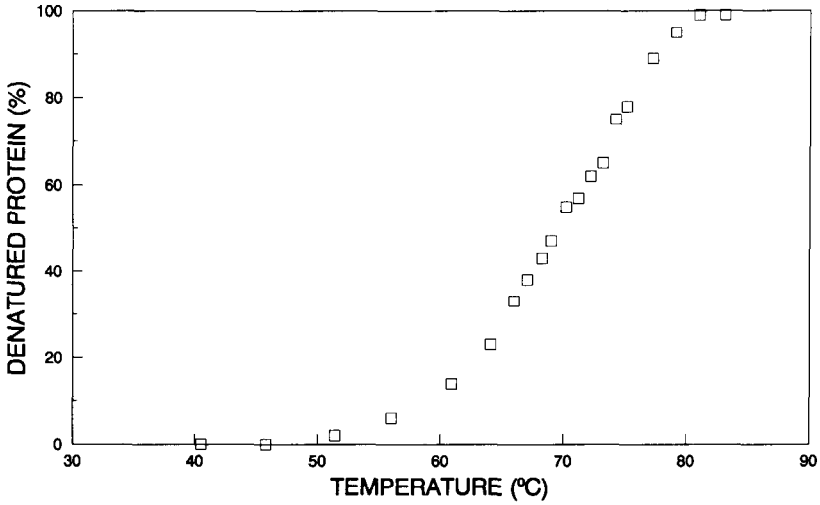

Fig. 4. Fraction denaturation of myoglobin. Calculation as described in the text.

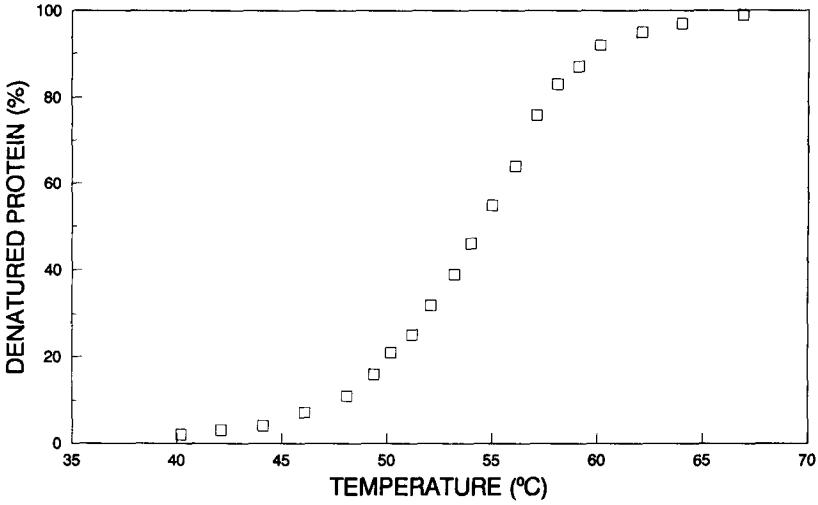

Fig. 5. Fraction denaturation of lysozyme. Calculation with an axis transformation from Fig. 2.

fraction of native protein; f_d is then $1 - f_n$. For most transition temperature determinations, applying this extra correction resulted in insignificant shifts that lay within the experimental error of 1–2°C. Only in the construction of Fig. 11 was a meaningful correction found to be necessary, because a drastic reduction in protein recovery occurred. With further optimization of the procedure and an experimental error below 1°C, peak-area data will have to be used as a standard procedure.

The determination of the transition point is done with a linear regression plot of log K against $1/T$. Here K is the equilibrium constant, assuming an equilibrium between the native and the denatured form of the protein. K is calculated with $f_d/(1 - f_d)$ and log $K = 0$ corresponds to the transition point. A transition slope or steepness is determined from the same plot using four or five values around the transition point.

RESULTS AND DISCUSSION

Mobile phase

First the influence of the mobile phase on the transition temperature of lysozyme and myoglobin is considered. Similar batchwise measurements have been done in the past; here they demonstrate the feasibility in an HPLC situation. The protein is injected in an empty stainless-steel column, using standard equipment.

Transition points for lysozyme are determined in several percentages of methanol and *n*-propanol (Fig. 6). Too high concentrations of organic modifier cause irregular transition curves, so for propanol transition points could only be measured up to 30% organic solvent. A clear relationship is apparent. The transition temperatures decrease with increasing amount of organic modifier. This decrease is greater with *n*-propanol and points at an effect caused by the apolar chain of the molecule. This apolar chain probably interferes with hydrophobic bonds within the protein molecule [10,16]. The transition points also decrease with decreasing pH (Fig. 7), which is probably due to protonation of the protein and electrostatic repulsion between parts of the macromolecule [10,11].

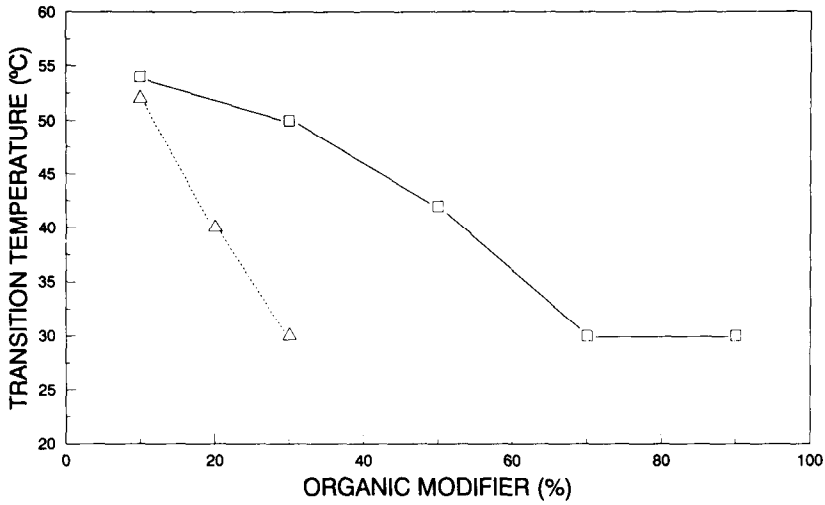

Fig. 6. Transition points of lysozyme in mobile phases containing different amounts of organic modifier, (□) methanol and (△) *n*-propanol. Mobile phase, organic modifier–0.005 *M* HCl + 0.02 *M* NaCl. Flow-rate, 1 ml/min.

It is interesting to note that the transition points of lysozyme do not decrease below 20–25°C. Attempts to decrease the transition temperature at 70% methanol by increasing the acidity were unsuccesful (Table I). An explanation must probably be sought in a stabilizing effect of the lower temperature, making the protein less vulnerable to other environmental influences. Thus it appears that lysozyme is an extremely stable protein that has to be used under very harsh conditions to create an

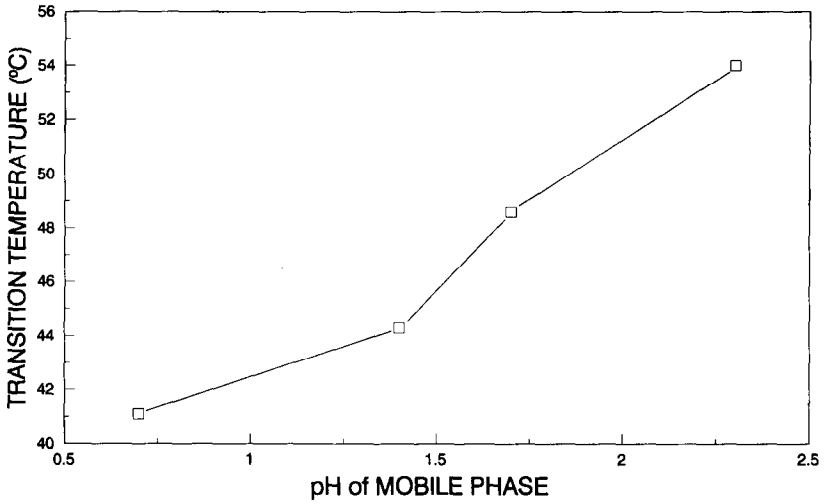

Fig. 7. Transition points of lysozyme in mobile phases with several pH values. pH as measured in the inorganic part before addition of 10% methanol. Mobile phase, methanol–HCl (10:90) + 0.02 *M* NaCl. Flow-rate, 1 ml/min.

TABLE I

EFFECT OF ACIDITY ON THE TRANSITION POINT OF LYSOZYME IN 70% METHANOL AS DETERMINED IN AN EMPTY COLUMN

Mobile phase, 70% methanol–30% HCl + 0.02 *M* NaCl. Flow-rate, 1 ml/min. Lysozyme concentration, 1 mg/ml.

HCl concentration (*M*)	Transition point (°C)
0.005	30
0.05	27
0.5	29

artificial test situation. The consequences of this phenomenon for the reliability of the transition temperature for the characterization of packing materials should be a matter of concern when developing a quantitative technique. As the transition temperatures will be part of a stationary phase classification, insight into the range of applicability is important. A method to compare stationary phases with respect to denaturing effects can probably not be achieved with a single protein as a probe.

For myoglobin similar relationships with pH and organic modifier can be found (Figs. 8–10). A considerable transition point depression is seen to occur under relatively mild mobile phase conditions, *i.e.*, a pH value not differing much from the physiological range.

The preceding measurements demonstrate the applicability of the technique in a standard HPLC flow system.

Residence time

Retention times in a packed column will be influenced by the mobile phase

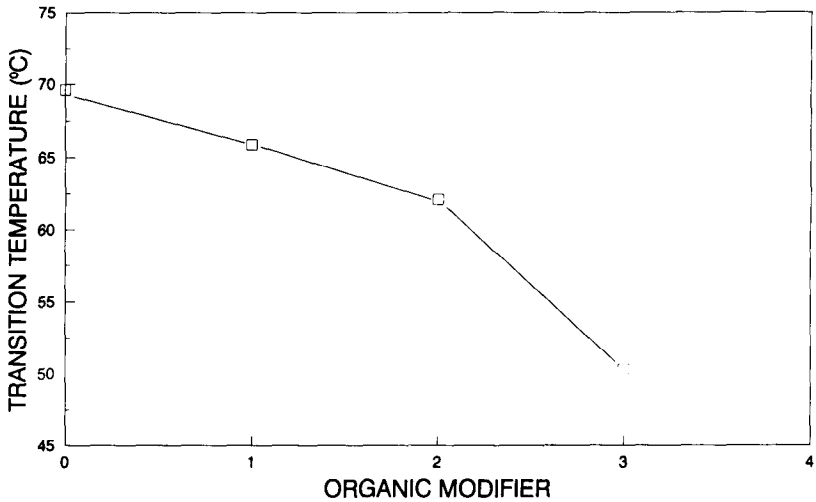

Fig. 8. Transition points of myoglobin in mobile phases containing different organic modifiers. Mobile phase, organic modifier–0.1 *M* phosphate–citrate buffer (pH 4.0) (10:90). 0, No organic modifier; 1 = methanol; 2 = ethanol; 3 = *n*-propanol. Flow-rate, 1 ml/min.

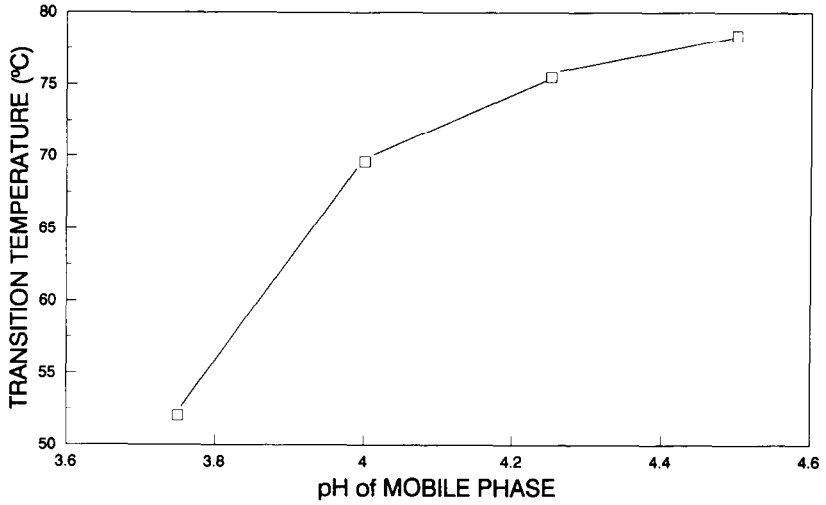

Fig. 9. Transition points of myoglobin in mobile phases with different pH values. Mobile phase, 0.01 *M* phosphate–citrate buffer. Flow-rate, 1 ml/min.

composition. It is therefore necessary to know the effect of residence in the flow system on transition points (Table II). For both lysozyme and myoglobin there appears to be an interval in which the residence time has little effect on the transition temperature, compared with the experimental error of 1–2°C. At a very short residence time in the column the transition point of lysozyme increases, probably because there is not enough time to heat the mobile phase. For myoglobin a residence from 0.5 to almost 7 min has hardly any effect on the transition point.

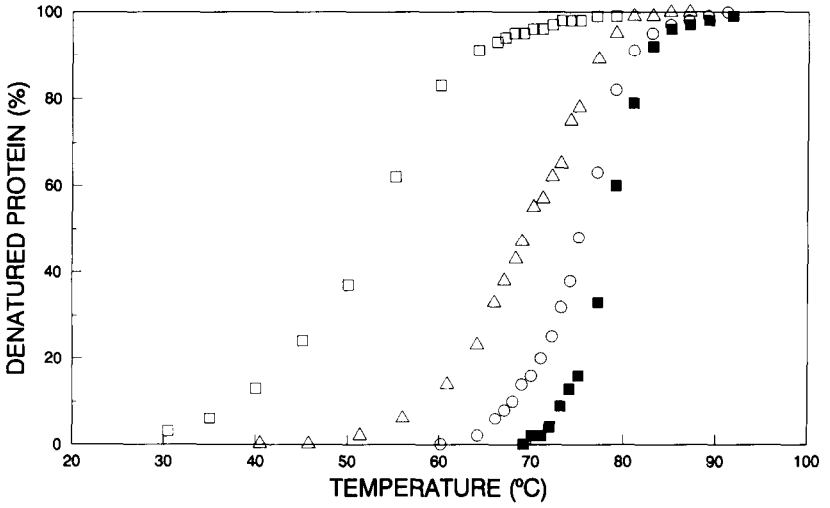

Fig. 10. Multi-transition plot of myoglobin at several pH values: □ = 3.75; △ = 4.00; ○ = 4.25; ■ = 4.50. Conditions as in Fig. 9.

TABLE II

EFFECT OF RESIDENCE TIME IN AN EMPTY COLUMN ON TRANSITION TEMPERATURE

Residence time varied with flow-rate. Mobile phase: LYS, methanol–0.005 *M* HCl + NaCl as specified in the second column; MYO, 0.01 *M* phosphate–citrate buffer (pH 4.0). Lysozyme concentration, 1–10 mg/ml; myoglobin concentration, 2 mg/ml.

Protein	Mobile phase	t_R (min)	T_{tr} (°C)
LYS	10:90 + 0.25 *M* NaCl	0.2	54.8
		0.3	54.0
		0.7	53.7
		1.4	53.1
LYS	30:70 + 0.02 *M* NaCl	0.7	49.9
		0.9	49.6
		1.2	48.5
LYS	50:50 + 0.02 *M* NaCl	0.6	42.0
		0.8	42.2
		1.0	41.8
		1.2	41.1
MYO	See above	0.6	69.6
		1.2	71.4
		2.1	67.8
		3.2	66.9
		6.8	67.6

Silica

Next the influence of a silica packing material was considered. At several methanol concentrations there is no clear effect of the silica on the transition temperature of lysozyme (Table III). It should be noted that under the conditions employed there is hardly any retention on the silica surface. We were not able to record transition curves for lysozyme with a mobile phase that caused a retention on bare silica. To obtain retention of lysozyme on bare silica, the pH would have to be increased. This causes extensive aggregation of the denatured protein [14] and subsequently results in poor transition curves. Changes in salt concentration and flow-rate do not alter the

TABLE III

EFFECT OF BARE SILICA ON TRANSITION TEMPERATURE OF LYSOZYME

Mobile phase, methanol–0.005 *M* HCl + NaCl as specified in the first column. Flow-rate, 1 ml/min. Lysozyme concentration, 1–10 mg/ml. Silica: Hypersil WP 300, 10 μm.

Mobile phase	Bare silica			Empty column		
	[LYS] (mg/ml)	T_{tr} (°C)	t_R (min)	[LYS] (mg/ml)	T_{tr} (°C)	t_R (min)
10:90 + 0.25 *M* NaCl	10	53.6	0.8	10	53.7	0.7
30:70 + 0.02 *M* NaCl	5	51.0	0.7	5	50.0	0.7
50:50 + 0.02 *M* NaCl	1	40.4	0.8	5	42.0	0.6

TABLE IV

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON TRANSITION TEMPERATURE OF LYSOZYME IN A COLUMN PACKED WITH BARE SILICA

Mobile phase, methanol–0.005 *M* HCl (10:90) + NaCl. Flow-rate, 1 ml/min; t_{RO} = 0.7–0.8 min; lysozyme concentration = 1–10 mg/ml.

NaCl concentration (*M*)	[LYS] (mg/ml)	T_{tr} (°C)	t_R (min)
0.02	1	53.5	0.8
0.1	1	52.6	0.8
0.25	10	53.6	0.8
0.5	10	53.0	0.8

picture (Tables IV and V). The results indicate that in the absence of retention, lysozyme can pass through a microparticulate packed bed without measurable denaturation.

A casual finding is a relationship with protein concentration (Table VI). The protein is slightly more stable when a more concentrated solution is injected. The stability of lysozyme makes this protein well suited for handling and subsequent measurement. An error of measurement of 1–2°C could not be avoided, however.

The denaturation of myoglobin by interaction with bare silica cannot be studied easily because in many instances the protein does not elute. Only at pH 10 does a clear, nearly unretained peak come off the column. Then, however, the recording of a transition curve became a problem because high temperatures combined with a high pH resulted in steep pressure rises. The transition clearly lies at lower temperatures than the situation without packing (Fig. 11). Thus, with myoglobin contact with bare

TABLE V

EFFECT OF FLOW-RATE ON TRANSITION TEMPERATURE OF LYSOZYME IN A COLUMN PACKED WITH BARE SILICA

Mobile phase, methanol–0.005 *M* HCl + NaCl as specified in the first column. Lysozyme concentration, 1–10 mg/ml.

Mobile phase	Flow-rate (ml/min)	[LYS] (mg/ml)	T_{tr} (°C)	t_R (min)
50:50 + 0.02 *M* NaCl	1.0	1	40.4	0.8
	0.5	5	40.6	1.5
30:70 + 0.02 *M* NaCl	3.0	1	48.7	0.3
	1.0	1	47.8	0.8
10:90 + 0.25 *M* NaCl	2.0	10	55.7	0.4
	1.0	10	53.6	0.8
	0.5	10	55.9	1.6
10:90 + 0.10 *M* NaCl	2.0	10	54.8	0.4
	1.0	10	55.5	0.8
	0.5	10	53.5	1.5
10:90 + 0.10 *M* NaCl	3.0	1	53.8	0.3
	1.0	1	52.6	0.8

TABLE VI

EFFECT OF LYSOZYME CONCENTRATION ON TRANSITION POINT OF LYSOZYME IN A COLUMN PACKED WITH SILICA

Flow-rate, 1 ml/min. Mobile phase, methanol–0.005 *M* HCl (30:70) +0.02 *M* NaCl.

Protein concentration (mg/ml)	T_{tr} (°C)
5	51.0
2.5	49.1
1	47.8

silica is accompanied by a significant shift in transition. The retention could not be influenced by lowering the salt concentration. It would have been interesting to see if an increase in interaction resulted in a further decrease in transition point. The protein was slightly more stable, however, in 0.01 *M* than in 0.1 *M* sodium chloride solution both in an empty column and in the column packed with silica.

Silica coating

After coating of the silica with epoxy glucose [21], the retention time of lysozyme is dependent on the sodium chloride concentration (Table VII; Fig. 12). With no salt added there is an unretained elution and the transition is about the same as in an empty column. At higher sodium chloride concentration the retention increases while the transition point decreases. In view of previous experiments, where the retention time and the sodium chloride concentration were varied, this decrease is consid-

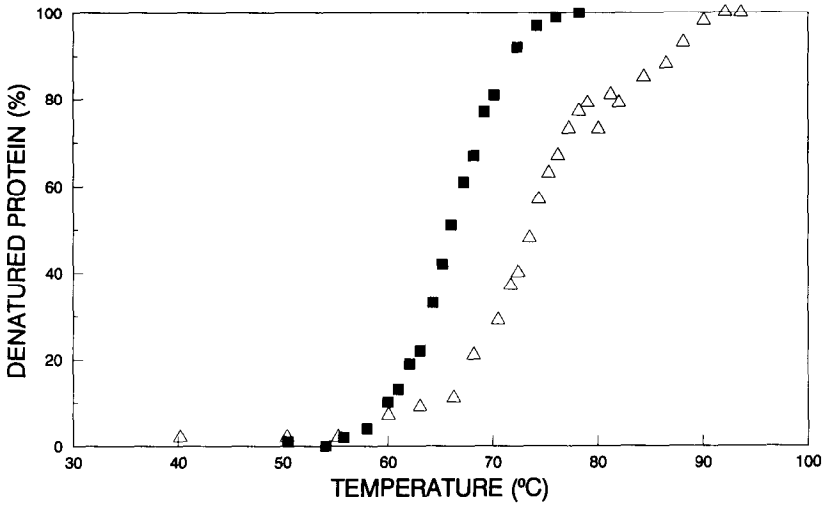

Fig. 11. Transition of myoglobin in column packed with bare silica (■, Hypersil WP) compared with transition in an empty column (△). Mobile phase, 0.01 *M* phosphate buffer (pH 10.0) + 0.1 *M* NaCl. Flow-rate, 1 ml/min. Silica column calculation with correction for protein loss using peak height as a measure of protein recovery.

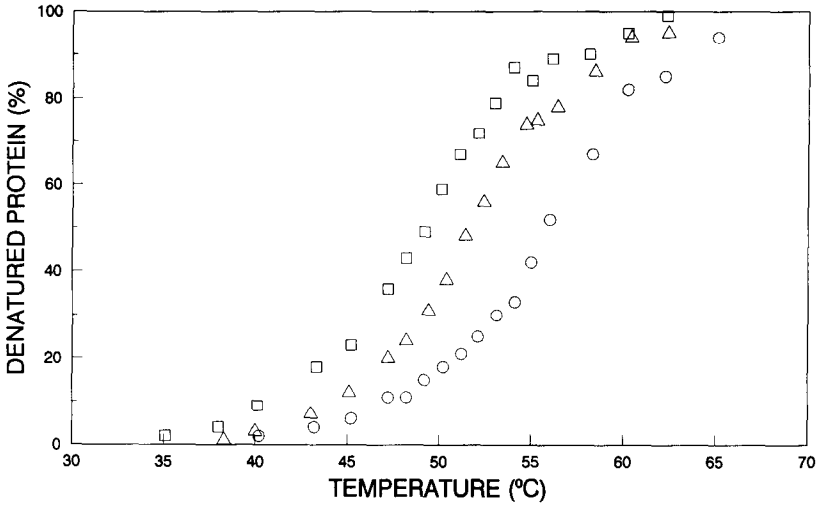

Fig. 12. Transition of lysozyme in a column packed with epoxy glucose-coated silica at three different sodium chloride concentrations: □ = 1.0; △ = 0.1; ○ = 0.01 *M*. Conditions as in Table VII.

ered to be due to the interaction with the stationary phase. This behaviour has been decribed in the past as hydrophobic interaction. Hence with lysozyme the generally accepted relationship between retention and denaturation is seen to apply. This detrimental interaction is not influenced by flow-rate (Table VIII). With the epoxy glucose-coated material, myoglobin transition measurements at pH 4.0 are possible. The epoxy glucose coating causes a clear depression of the transition point with unretained elution of the protein (Fig. 13) (Tables II and VIII).

Relative contribution

After studying a number of transition curves, the relative contribution of the stationary phase compared with the eluent contribution can be established. This should be regarded as an important advantage of transition point determinations. The effect of a packing material on the conformation change of a protein can be expressed in the same terms as the effect of a certain solvent condition. With the two

TABLE VII

EFFECT OF SURFACE INTERACTION ON TRANSITION POINT OF LYSOZYME IN A COLUMN PACKED WITH EPOXY GLUCOSE-COATED SILICA

Mobile phase, methanol–0.005 *M* HCl (10:90) + NaCl. Flow-rate, 1 ml/min. Lysozyme concentration, 2.5 ml/ml.

NaCl concentration (*M*)	t_R (min)	T_{tr}(°C)
0.01	0.68	56.0
0.1	0.74	51.7
1.0	0.81	49.0

TABLE VIII

EFFECT OF FLOW-RATE ON TRANSITION TEMPERATURE IN A COLUMN PACKED WITH EPOXY GLUCOSE-COATED SILICA

Mobile phase: LYS, methanol–0.005 *M* HCl (10:90) + 0.5 *M* NaCl; MYO, 0.01 *M* phosphate–citrate (pH 4.0). Lysozyme concentration, 2.5 mg/ml. Myoglobin concentration, 2 mg/ml.

Protein	Flow-rate (ml/min)	t_R (min)	T_{tr} (°C)
LYS	1.0	0.7	48.8
	0.5	1.3	48.4
MYO	1.0	0.6	58.2
	0.5	1.1	58.1
	0.2	2.7	58.0
	0.1	5.7	57.0

proteins studied in this paper and the mobile and stationary phases that allow good elution, the effect of the stationary phase is of the same order of magnitude as the effect of a change in pH or the addition of some modifier. This can be convenient in more theoretical considerations, as the representation of denaturation as used for solvent modifiers might be applicable to the stationary phase effect.

Transition steepness

From the transition curves, a transition steepness or slope can be determined around the transition point. The large slope is ascribed to the phenomenon of "cooperativity" [22]. A certain small change in the conformation of the protein triggers the unwinding of the complete molecule. There seems to be a favoured route in the transition from native to denatured state and *vice versa*, with no intermediate states

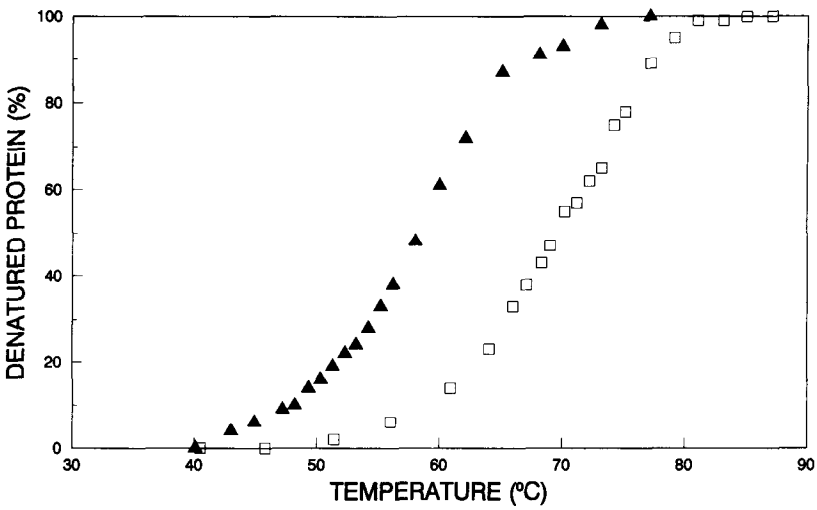

Fig. 13. Transition of myoglobin in (▲) a column packed with epoxy glucose-coated silica compared with (□) an empty column. Mobile phase, 0.01 *M* phosphate buffer (pH 4.0). Flow-rate, 1 ml/min.

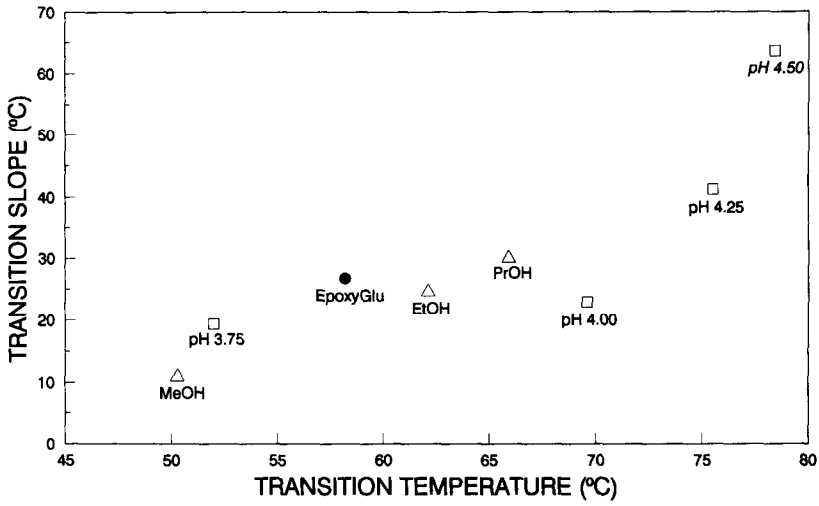

Fig. 14. Steepness of transition of myoglobin plotted against transition temperature. Calculation from log K *vs.* $1/T$ plot. Conditions as in Figs. 8, 9 and 13. □ = pH 3.75, 4.00, 4.25, 4.50; △ = methanol (MeOH), ethanol (EtOH), *n*-propanol (PrOH); ● = epoxy glucose (EpoxyGlu).

that are stable enough to be detected. A relationship with temperature seems likely (Fig. 14). Transitions in the neighbourhood of 80°C are steeper than those at 50–60°C, but the relationship is more complicated. With an increase in pH from 3.75 to 4.0 the transition temperature rises from 50 to 70°C without a significant increase in steepness. Steepness is probably also a function of factors that promote the transition such as protonation of the molecule and addition of organic modifier.

The steepness or slope of the transition has been used in the past to calculate a reaction enthalpy. The figures calculated from our results seem to correspond with values from the literature [11], given the differences in the conditions used. The precise meaning in this flow system is not completely clear, however.

Attention was concentrated on differences between packed and empty columns. An intermediate state was found repeatedly for the transition of myoglobin at pH 10, as can be seen in Fig. 11. In the transition there is a small interval where the protein has not reached in completely denatured state, but is relatively insensitive to temperature changes. On a further increase in temperature the UV difference again changes sharply to reach a final state. More information on the residual structure of denatured proteins, in particular lysozyme, can be found in the work of Aune *et al.* [19] and Kurono and Hamaguchi [14]. In a packed column, however, this transition is slightly steeper with no discernible unevenness that could suggest a stable intermediate state. This could indicate that the silica cooperates in some way in the denaturation of the protein. With the calculation of this plot as correction was made for the protein loss on the column. It is not known how intermediates elute compared with the native and the denatured molecule.

At pH 4.0 in an epoxy glucose column, where the transition of myoglobin shifts drastically, the steepness changes very little. More elaborate measurements are necessary to demonstrate a clear effect and to relate the steepness to *e.g.*, the flow-rate.

CONCLUSION

From both lysozyme and myoglobin a transition curve can be obtained in an HPLC system using a diode-array detector and the phenomenon of UV difference spectrometry. The position of the curves can be influenced by the mobile phase composition. Shifts of the transition curves also occur in the presence of packing material. The depression of the transition point is not a function of flow-rates as used in ordinary HPLC. The procedure needs further optimization to demonstrate effects in the 0.1°C range.

The relative contribution to denaturation of the stationary phase compared with those due to mobile phase changes is easily measured with transition curves. The procedure and the findings should lead to a method for comparing stationary phases as regards denaturing effects on proteins, knowing that a conformation change is only one aspect of the broader concept of denaturation. Measurements were done with only two proteins and a limited number of mobile phase compositions. Comparisons of stationary phases will involve proteins that elute under widely different conditions. A useful study of denaturing effects should be done under normal chromatographic conditions. It is unlikely that this will be possible with a limited number of test proteins and difference spectrometry. An alternative approach would be to use another detector, *e.g.*, a flow-through light-scattering detector or a post-column enzyme reaction detector. With the latter, unpurified proteins could also be tested.

To direct synthetic approaches, however, a small number of proteins and mobile phase compositions are probably sufficient. Used in an artificial environment, these proteins become part of the product characterization. They allow the probing of surfaces and may be an important tool in the fine tuning of coating chemistry. Further efforts in this direction are being made.

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