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DENATURATION AND THE EFFECTS OF TEMPERATURE ON HYDROPHOBIC-INTERACTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

BIO-GEL TSK-PHENYL-5-PW COLUMN

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SUMMARY

Cytochrome *c*, myoglobin and lysozyme, as well as two synthetic peptides, TM-22 and TM-36, were used to examine denaturation of protein structure on a hydrophobic-interaction column, the Bio-Gel TSK-Phenyl -5-PW high-performance liquid chromatography column. The first three proteins were chosen because they have a monomeric structure while both synthetic peptides, which have the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide where $n = 3$ for TM-22 and $n = 5$ for TM-36, are dimeric under solvent conditions used for hydrophobic-interaction chromatography. Only TM-36 is dimeric under reversed-phase conditions. Thus, denaturation of both tertiary and quaternary structure can be examined. The column was operated in both reversed-phase and hydrophobic-interaction modes. This, in combination with temperature variation between approximately 0–50°C, provided conditions where denaturing effects of the support could be examined. In reversed-phase mode, cytochrome *c*, myoglobin, and TM-22 were eluted in a denatured form throughout the temperature range. In contrast, lysozyme and TM-36 eluted primarily in their native conformation at low temperatures, but experienced partial or total denaturation at higher temperatures. Significantly, all of the polypeptides studied were denatured at room temperature by a conventional reversed-phase column, the Altex Ultrapore RPSC C-3 indicating that the Bio-Gel TSK-Phenyl-5-PW column is less denaturing. In the hydrophobic-interaction mode, the dimeric structure of TM-22 was totally disrupted at all temperatures, while TM-36, myoglobin, and cytochrome *c* underwent various degrees of partial denaturation as the temperature increased. Comparison of the temperatures at which the various polypeptides underwent denaturation on the column with their normal melting temperatures (where half the molecules are unfolded) demonstrated that the hydrophobic column itself, rather than the temperature, was primarily responsible for denaturation. Hence, even relatively "gentle" hydrophobic columns can promote denaturation of protein structure. Since the tertiary and quaternary structures of most proteins are stabilized by hydrophobic interactions, the possibility of denaturation must always be taken into consideration when a hydrophobic column is used.

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INTRODUCTION

The tendency of proteins to become denatured during reversed-phase high-performance liquid chromatography (RP-HPLC) has spurred the development of a related technique: hydrophobic-interaction chromatography (HIC). While both techniques rely on the strength of hydrophobic interactions between the protein and a non-polar support they differ in the solvent conditions utilized and the relative hydrophobicity and density of the bonded ligand. For HIC, ligands of lower hydrophobicity, lower ligand densities and non-denaturing aqueous solvents are used. These conditions have been shown to be significantly less harsh on protein tertiary structure than those used in reversed-phase chromatography (RPC)[1–3].

Recently, BioRad has introduced the Bio-Gel TSK-Phenyl-5-PW column for HIC. Goheen[3] has demonstrated the utility of this column in purifying a number of monomeric enzymes without loss of catalytic activity. Nevertheless, it is quite conceivable that some of these enzymes underwent reversible denaturation while bound to the column. This raises the possibility that multi-subunit proteins may not always be recovered in their native state following purification, due to partial or complete dissociation into the component polypeptide chains.

In this study, we have investigated the effects of HIC on the tertiary and quaternary structures of selected proteins. This was accomplished by examining the effects of temperature on the retention of two groups of proteins on the Bio-Gel TSK-Phenyl-5-PW column while operating the column in either the reversed-phase or hydrophobic-interaction mode*. The first set of proteins consists of myoglobin, lysozyme, and cytochrome *c*, while the second consists of synthetic analogues of tropomyosin (TM-22 and TM-36). The study of the former monomeric proteins provides a criterion for evaluating changes in tertiary structure resulting from temperature change. In contrast, TM-22 and TM-36 can form dimers, providing an excellent model for evaluating chromatographic effects on quaternary structure[4].

Comparison of the results obtained in the reversed-phase and hydrophobic-interaction modes yields information about the conformational states of the various polypeptide chains under the differing solvent conditions. In addition, the differing solvent conditions utilized in the two modes alters the structural stability of the various polypeptides so that the denaturing effects of the hydrophobic column can be more readily observed.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were reagent grade: acetonitrile (HPLC grade, Fisher Scientific, Fairlawn, NJ, U.S.A.); trifluoroacetic acid (TFA) was distilled following purchase from Halocarbon Products (Hackensack, NJ, U.S.A.); and ammonium sulfate (HPLC grade, BioRad Lab., Richmond, CA, U.S.A.). Doubly-distilled water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Proteins used were obtained as follows: protein

* Reversed-phase mode implies a gradient of increasing concentration of organic solvent. Hydrophobic-interaction mode implies a gradient of decreasing salt concentration. The solvent composition and gradients are described in the Materials and Methods section.

standards for HIC (BioRad Lab., Richmond, CA, U.S.A.); lysozyme, cytochrome *c*, and myoglobin (Sigma, St. Louis, MO, U.S.A.).

Peptide synthesis and purification

The two TM peptide analogues (TM-22 and TM-36) were synthesized and purified as described by Lau *et al.*[4].

Hydrophobic-interaction and reversed-phase chromatography

The HPLC instrumentation consisted of a Spectra-Physics SP8700 solvent delivery system and SP8750 organizer module in conjunction with a Hewlett-Packard HP1040A detection system, HP3390A integrator, HP85 computer, HP9121 disc drive and HP7470A plotter.

Proteins and peptide analogues were separated on a Bio-Gel TSK-Phenyl-5-PW column (BioRad Lab., Richmond, CA, U.S.A.; 75 × 7.5 mm I.D.). Additional experiments were performed on an Altex Ultrapore RPSC C-3 reversed-phase column (Beckman, CA, U.S.A.; 75 × 4.6 mm I.D.) and a TSK G2000SW size-exclusion column (Toyo Soda, Tokyo, Japan; 600 × 7.5 mm I.D.). For RPC, samples were dissolved in 0.1% aq. TFA at pH 2.1, and the column was eluted with a gradient constructed from 0.1% aq. TFA (solvent A) and 0.05% TFA in acetonitrile (solvent B). The gradient program was as follows: linear gradient from 100% A to 49% A, 51% B at 17 min (3% B/min), which was then maintained at this percentage of B for an additional 5 min. For HIC, samples were dissolved in solvent A containing 1.7 *M* ammonium sulfate and 0.1 *M* sodium phosphate buffer (38 m*M* NaH_2PO_4 and 62 m*M* Na_2HPO_4) at pH 7.0; column elution was effected with a linear gradient from 100% A to 100% B containing 0.1 *M* sodium phosphate buffer (38 m*M* NaH_2PO_4 and 62 m*M* Na_2HPO_4) at pH 7.0 at 15 min, followed by an additional 5 min at 100% B. Size-exclusion experiments with myoglobin were performed with either 0.1% aq. TFA or 0.1% TFA in water–acetonitrile (75:25).

A circulating Haake water bath with variable temperature control was used to adjust the temperature of the column, which was enclosed by a 500-ml jacket, constructed from a plastic graduated cylinder and rubber stoppers. A 50% aq. ethanol solution was used as the circulating solvent.

Circular dichroism experiments

Circular dichroism spectra were recorded on a JASCO-J5006 spectropolarimeter, attached to a JASCO DP-500N data processor. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-camphorsulfonic acid. Constant nitrogen flushing was employed. Ellipticity data were converted into conformation parameters by the procedures and equations described by Chen *et al.*[5]. The reproducibility of all spectra was within ± 3% for wavelengths greater than 205 nm.

RESULTS AND DISCUSSION

Temperature effects on chromatography of myoglobin, lysozyme and cytochrome c on an HIC column operated in reversed-phase mode

The effects of temperature on the retention of myoglobin, lysozyme and cytochrome *c* on the Bio-Gel TSK-Phenyl-5-PW column during operation in the re-

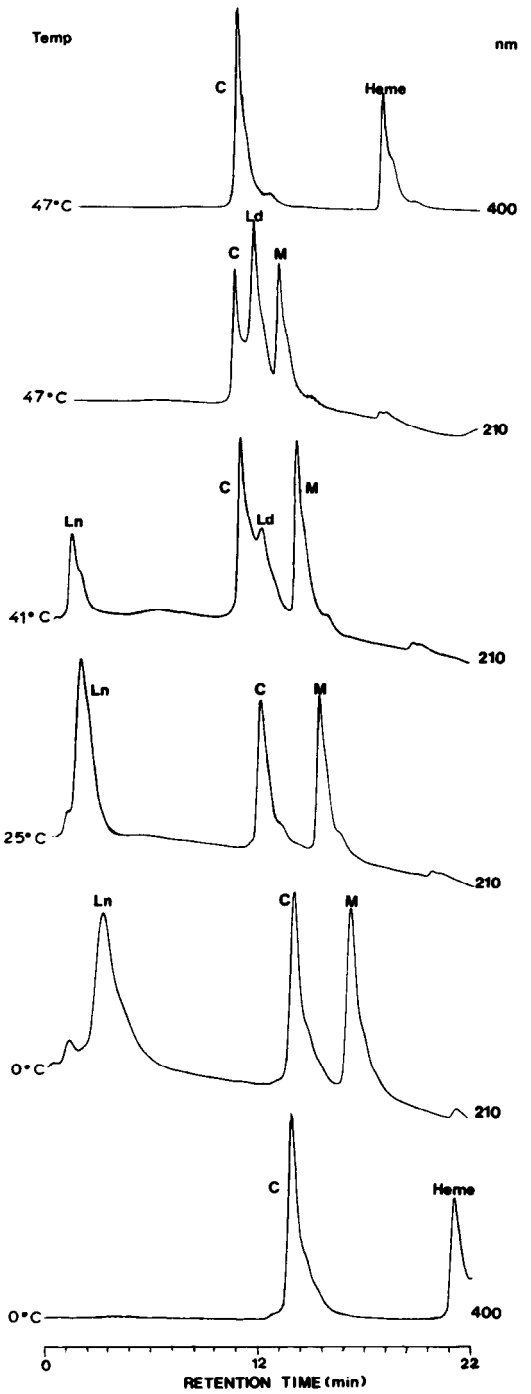

Fig. 1. Effects of temperature on the RPC elution profile of a mixture of myoglobin, cytochrome *c* and lysozyme on the Bio-Gel TSK-Phenyl-5-PW column. The values to the left of each profile are the temperatures (°C) at which the column was operated; the values to the right are the wavelengths monitored. Peak identification: C, M, Ln and Ld stand for cytochrome *c*, apomyoglobin, native lysozyme and denatured lysozyme, respectively. Conditions: AB gradient, solvent A consisted of 0.1% aq. TFA and solvent B of 0.05% TFA in acetonitrile. See Materials and Methods for gradient details. Flow-rate, 1 ml/min.

versed-phase mode are displayed in Fig. 1. For these experiments a linear AB gradient was used, with A consisting of 0.1% aq. TFA and B containing 0.05% TFA in acetonitrile. The chromatographic experiments performed with these proteins were followed simultaneously at wavelengths of both 210 nm and 400 nm. This was done to enable monitoring of the elution of both the polypeptide chains (210 nm) and the two heme groups (400 nm). In the 210-nm elution profile at 0°C, the first peak to be eluted is lysozyme, with a retention time of 4.7 min, followed by cytochrome *c* (13.6 min) and apomyoglobin (16.2 min). The 400-nm elution profile indicates the presence of the covalently bound heme group of cytochrome *c* and the subsequent elution of the heme group (21.2 min), released from myoglobin as a result of the solvent conditions utilized, and possibly the hydrophobicity of the column. We have shown by size-exclusion HPLC that the heme is not dissociated from myoglobin in the starting solvent (0.1% aq. TFA). However, the heme is dissociated from myoglobin when size-exclusion chromatography is carried out with 25% acetonitrile in starting solvent. This effect of an acidified organic solvent on myoglobin is consistent with the observation of Theorell and Akeson[6] that apomyoglobin can be prepared by extraction of the heme in acidified acetone. Two effects of temperature are demonstrated with these proteins. First, there is a decrease in the retention times of individual components with increasing temperature (Fig. 1). This inverse relationship between retention time and temperature during RPC has been observed previously by other investigators[7,8]. Its practical usefulness lies in the fact that the shift in retention time which occurs with temperature change is dependent upon the specific protein involved. The retention time shifts between 0°C and 47°C for apomyoglobin and cytochrome *c*, for example, are 3.7 and 3.1 min respectively. Such differences could be used to improve the resolution of proteins with similar retention times[7,8]. This effect is solvophobic and does not involve changes in protein conformation, since it occurs with the heme group as well. The second temperature effect is denaturation. In standard RPC the primary cause of denaturation is the hydrophobicity of the matrix which disrupts the hydrophobic interactions stabilizing the native conformation[9]. However, in matrices used for HIC the hydrophobic ligands are sparsely distributed. This reduction in hydrophobic density can result in proteins being separated in their native conformation, even when the column is operated in the reversed-phase mode. This is clearly demonstrated for lysozyme in Fig. 1. Lysozyme at 0°C exists in its native conformation, and only with increasing temperature does thermally induced denaturation occur. The shift to the denatured state is accompanied by a large increase in the retention time of the molecule. At 41°C, the molecule exists in an equilibrium in which both the native and denatured conformations are present in significant amounts. Under these conditions, the native molecule has a retention time of 3.0 min, while the denatured protein is eluted following cytochrome *c* at 11.8 min. Utilizing differential scanning calorimetry as a probe, Privalov and Khechinashvili[10] have determined the unfolding temperature of lysozyme as a function of pH. They observed a steady decrease in the thermal stability of the enzyme with lowering pH. The melting temperature, that is, the temperature at which 50% of the molecules are unfolded, was found to be approximately 56°C at pH 2.0[10]. Since it appears in Fig. 1 that nearly 50% of the lysozyme is denatured at 40°C on the column, this indicates that denaturing aspects of the column itself, in addition to elevated temperatures, contribute to lysozyme unfolding. A similar statement can be made with regard to

cytochrome *c*, which appears to be denatured throughout the temperature range, even though it has a melting temperature of approximately 52°C at pH 3.0[10]. In order to examine this further, we investigated the behaviour at 22°C of this protein mixture (lysozyme, cytochrome *c* and myoglobin) on a standard type of reversed-phase column, the Altex Ultrapore RPSC C-3 column. Significantly, all of the lysozyme was eluted in the denatured form between the cytochrome *c* and apomyoglobin peaks. Based on this comparison it is clear that although the Bio-Gel TSK-Phenyl-5-PW column has a denaturing effect, it is still less harsh in its effects on protein tertiary structure than is a conventional type of reversed-phase column.

The increase in retention time which occurs upon denaturation is presumably a result of the exposure of numerous hydrophobic residues previously buried in the interior of the molecule. We interpret this observation as indicating that the majority of the lysozyme molecules bind to the column in either the native or denatured state and are subsequently eluted in the same state without undergoing either refolding or denaturation. This suggests that the kinetics for conversion between the two conformational states are on the same timescale as passage through the column. If this were not the case, that is, if all of the molecules were in rapid equilibrium between the two conformational states while in the column, we would expect that the lysozyme peak would move as a single peak toward longer retention times as the amount of time spent in the denatured state grows with increasing temperature. The fact that this does not occur is good evidence that significant numbers of lysozyme molecules do not undergo large-scale conformational change as they move through the column during the experiment.

In contrast to the situation with lysozyme, our data indicate that apomyoglobin and cytochrome *c* are eluted in their denatured forms throughout the temperature range studied. Several pieces of evidence point to this conclusion. The first concerns the relative order of elution of the three polypeptide chains. As mentioned previously, on a column which is more strongly denaturing than the Bio-Gel TSK-Phenyl-5-PW, namely the Altex Ultrapore RPSC C-3 column, the relative elution order at 22°C is cytochrome *c*, lysozyme and apomyoglobin. This elution order is seen only at 47°C on the Bio-Gel TSK-Phenyl-5-PW column. In the denatured state the relative hydrophobicities of the three polypeptides would be based on their content of nonpolar residues. Significantly, the elution order which would be predicted on the basis of this criterion was identical to that observed on the HIC column at 47°C or the conventional RPC column at room temperature, that is, cytochrome *c*, lysozyme and apomyoglobin, respectively. In addition to these observations, the dissociation of myoglobin into apomyoglobin and heme discussed previously is certainly suggestive of disruption of the tertiary structure of this protein. Further evidence that cytochrome *c* and apomyoglobin are eluted in their denatured state under reversed-phase conditions will be discussed in the following section.

Temperature effects on the chromatography of myoglobin, lysozyme and cytochrome c on an HIC column operated in the hydrophobic-interaction mode

For this portion of the study, the chromatographic experiments were carried out on the individual proteins, rather than a mixture of myoglobin, cytochrome *c*, and lysozyme. Fig. 2 displays the elution profiles of cytochrome *c* and myoglobin as a function of temperature. In each case, an AB gradient was used with A containing

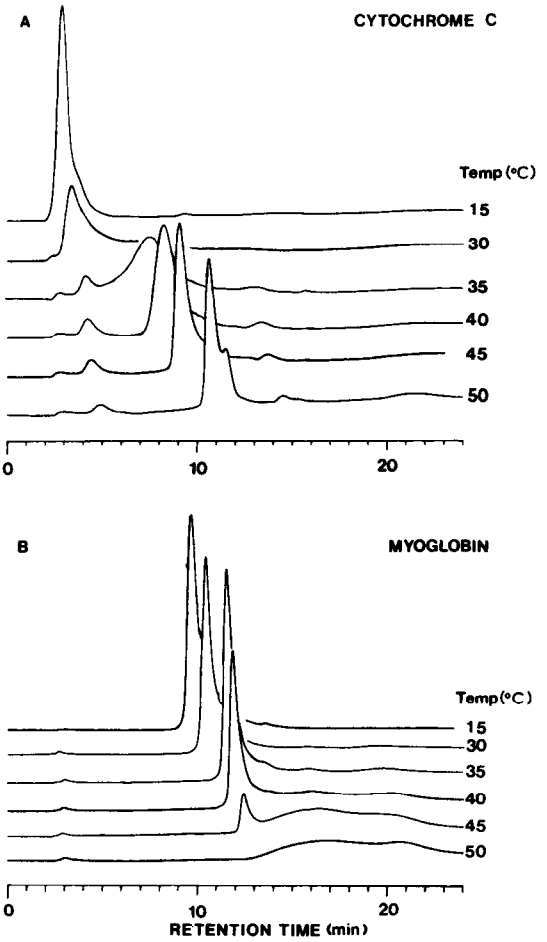

Fig. 2. Effects of temperature on the HIC elution profile of cytochrome *c* and myoglobin on the Bio-Gel TSK-Phenyl-5-PW column. The profiles were monitored at both 210 nm and 400 nm. The normalized absorbances are identical for the two wavelengths. Conditions: AB gradient, solvent A contains 0.1 *M* sodium phosphate and 1.7 *M* ammonium sulfate at pH 7.0, and solvent B contains 0.1 *M* sodium phosphate at pH 7.0. See Materials and methods for gradient details. Flow-rate, 1 ml/min.

1.7 *M* ammonium sulfate and 0.1 *M* sodium phosphate at pH 7.0 while B contained only the 0.1 *M* sodium phosphate at pH 7.0. In HIC, the presence of a high concentration of a salt such as ammonium sulfate, increases the strength of the hydrophobic interaction between the phenyl groups of the column material and the hydrophobic residues present either on the surface of the protein if it is in its native conformation, or throughout its sequence, if it is unfolded. In contrast to RPC, increasing the temperature at which HIC is performed generally enhances protein binding to the matrix without change in conformation[11]. Our data demonstrate this tendency. Panel B of Fig. 2 displays the elution profiles of myoglobin as a function of temperature. It is clear from this figure that elevated temperatures enhance the binding of this molecule to the hydrophobic matrix. At 15°C, the retention time of the peak is 9.3 min, whereas at 45°C it is 12.4 min. However, as the temperature increases above

40°C thermal unfolding of myoglobin is observed. The original myoglobin peak at 12.0 min disappears between 40 and 50°C with the appearance of a very broad envelope between 12 and 24 min. We interpret this result as indicating that myoglobin undergoes unfolding at these elevated temperatures. Unlike the situation with myoglobin under reversed-phase conditions, total denaturation is not observed. When the elution profile obtained at 400 nm (where heme absorbs) was compared to that observed at 210 nm, it was found to be identical. This indicates that the heme group is not released from the polypeptide chain. Hence, denaturation of the molecule at these temperatures cannot be total. Instead, it appears that myoglobin can exist in various partially unfolded states under these conditions. The exposure of differing amounts of buried hydrophobic residues in these states would substantially increase retention time, and a rapid equilibrium between the various states would allow individual molecules to bind in several different conformations during the course of a run, with resultant peak broadening.

Panel A of Fig. 2 shows that at 15°C cytochrome *c* is eluted as a sharp peak following the column dead volume. Hence, at 15°C, this protein is not retained by the column. Doubling the temperature to 30°C shifts the retention time from 2.8 min to 3.6 min, and results in a broadening of the peak. Increasing the temperature to 35°C results in additional broadening accompanied by a retention time shift to 7.7 min. Experiments performed at 40, 45 and 50°C show a reversal of the previous broadening trend with peak sharpening and successively longer retention times. The observed progression in peak shape, from sharp to broad to sharp, suggests that cytochrome *c* undergoes a localized conformational change between 15 and 40°C, which increases its hydrophobicity. Such an occurrence would be expected to contribute to the increase in retention time and also to produce peak broadening at intermediate temperatures, where the portion of the molecule undergoing conformational change would be in equilibrium between differing nearly isoenergetic states. According to this interpretation, by 40°C, the equilibrium has shifted heavily toward one particular conformation, which results in peak sharpening. The peak observed at 50°C is definitely not the totally denatured form of cytochrome *c*. Exposure of the buried hydrophobic groups would result in such strong binding to the column that organic solvents would have been necessary to elute cytochrome *c* from the column, as was observed in the reversed-phase mode. In addition, at pH 7.0 cytochrome *c* has an unfolding temperature of approximately 82°C[10]. Thus, it seems most likely that cytochrome *c* has undergone a very minor localized conformational change, resulting in a stable intermediate. To a certain extent, the changes in peak shape observed with cytochrome *c* and the explanation for them are similar to those found with myoglobin. In addition, this intermediate may well undergo thermally induced denaturation, as observed with myoglobin if the temperature were to be increased beyond 50°C. Although not displayed in Fig. 2, we have also measured the effect of temperature on the retention of lysozyme during HIC. Surprisingly, there does not appear to be much of a temperature effect for this protein. Retention times of 14.7, 15.0, 15.1, 15.6 and 15.5 min were recorded for 15, 30, 35, 40 and 45°C, respectively. The apparent near-independence of lysozyme retention time on temperature during HIC has also been observed by Goheen[3]. At present, we do not have an explanation for this phenomenon. However, the fact that it occurs with lysozyme suggests that the degree to which temperature affects retention time may vary significantly between

proteins. Hence, utilizing temperature variation during HIC may be used to promote better resolution of protein peaks as well as to sharpen individual peaks.

Comparison of the data for RPC and HIC for these proteins provides additional evidence that cytochrome *c* and apomyoglobin are eluted in their denatured form when the Bio-Gel TSK-Phenyl-5-PW column is operated in the reversed-phase mode. The relative elution order for the native proteins in HIC is cytochrome *c*, myoglobin and lysozyme. This indicates that of the three, lysozyme has the most hydrophobic surface in its native conformation, followed by myoglobin and cytochrome *c*, respectively. Denatured lysozyme is eluted before apomyoglobin in RPC. Given their respective surface hydrophobicities, this is an unlikely occurrence, unless the buried nonpolar residues of apomyoglobin have been exposed by denaturation. Similarly, cytochrome *c* has a much longer retention time than native lysozyme in the reversed-phase mode, but a considerably shorter one in the hydrophobic-interaction mode. This also is quite improbable, unless cytochrome *c* is denatured in the former mode.

Temperature effects on chromatography of TM-22 and TM-36 on an HIC column operated in reversed-phase mode

In our laboratory, we have constructed a series of peptide analogues of the two-stranded α-helical coiled-coil protein, tropomyosin[4]. Two of these analogues, TM-22 and TM-36, are of particular interest for this study. The sequence of these peptides is Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide where $n = 3$ for TM-22 and $n = 5$ for TM-36. We have previously shown that the former peptide is monomeric and contains relatively little α-helical structure in aqueous medium (0.1% aq. TFA)[4,9]. In contrast, the latter peptide, TM-36, forms an extremely stable dimer in 0.1% aq. TFA, which exhibits a circular dichroic spectrum typical of a 100% α-helical coiled-coil molecule[4,9]. These features make these peptides excellent candidates for examining the effects of the Bio-Gel TSK-Phenyl-5-PW hydrophobic column on protein quaternary structure.

The retention of these peptides on the column was examined with a standard AB gradient. The monomeric TM-22 peptide had elution times of 4.8, 5.5, 5.5, 5.2 and 4.9 min at 0, 15, 29, 35, 40 and 49°C respectively. The change in retention time *versus* temperature is only 0.6 min with TM-22 between 0–49°C. The fact that this change is significantly smaller than the comparable values obtained with cytochrome *c* and apomyoglobin demonstrates again the potential utility of temperature variation in selectively altering the retention times of different polypeptides.

The elution profiles for TM-36 as a function of temperature are displayed in Fig. 3. From this figure it is clear that temperature has a dramatic denaturing effect on the quaternary structure of TM-36. The occurence of such an effect at these temperatures is surprising in view of the fact that at pH 2.5, this peptide retains nearly 90% of its coiled-coil structure, even at 50°C, and well over that at lower temperatures[4]. Despite this high thermal stability, the peptide is eluted in multiple peaks throughout the temperature range examined. The first peak eluted is only slightly retained by the column. Its retention time varies from 3.0 min at 0°C to 2.5 min at 49°C. The second to be eluted is not discernible below 29°C. The retention time for this broad intermediate peak is 10.0 min at 29°C, and increases to 12.0 min at 49°C. The final peak has a retention time of 18.3 min at 0°C, which decreases to

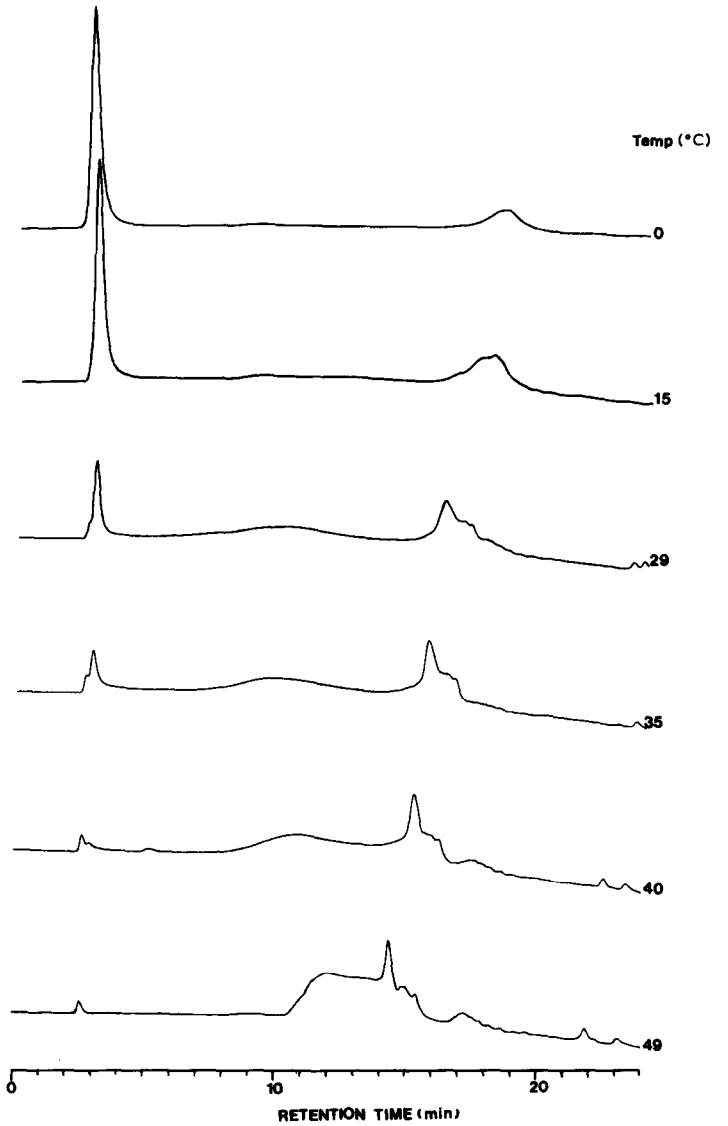

Fig. 3. Effects of temperature on the RP-HPLC elution profile of TM-36 on the Bio-Gel TSK-Phenyl-5-PW column. The TM-36 peptide has the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where $n = 5$. The values to the right of each profile are the temperatures (°C) at which the column was operated. Conditions: AB gradient, solvent A consisted of 0.1% aq. TFA and solvent B of 0.05% TFA in acetonitrile. See Materials and methods for gradient details. The effluent was monitored at 210 nm. Flow-rate, 1 ml/min.

14.3 min by 49°C. Comparison of the retention times for the first peak to those obtained with TM-22 shows that TM-22 is retained longer. In a previous study, where standard hydrophobic columns were used, Lau *et al.*[9] demonstrated the denaturing effect of these columns. TM-36 binds much more tightly to reversed-phase columns than does TM-22 due to the increased hydrophobicity of the polymer in its

monomeric form[9]. The fact that the first peak is only weakly retained on this column indicates that it is in the dimeric native form of the peptide. By analogy with the denaturation of lysozyme discussed previously, we attribute the final peak, which increases in height as the first decreases, to the totally denatured form of the peptide. However, the phenomenon observed with TM-36 differs from that with lysozyme in two significant ways. First, denaturation of TM-36 involves disruption of both tertiary and quaternary structure, while that of lysozyme involves only the former. Second, lysozyme did not have a significant intermediate peak between the native and denatured forms. Two explanations for the existence of the TM-36 intermediate peak present themselves. The first is that the native dimeric and denatured monomeric molecules are in fairly rapid equilibrium with each other as they move through the column. As a result, a significant fraction of the individual molecules bind sometimes as the weakly retained dimer and sometimes as strongly bound monomer. The intermediate peak retention time is an average of these binding events. The alternative explanation is that TM-36 undergoes partial denaturation on the column. These partially denatured molecules would still be dimeric, but would bind more tightly to the column as some portion of the coiled-coil quaternary structure is unravelled, thereby exposing hydrophobic residues. Our data cannot distinguish between these two explanations. Regardless of which explanation is correct, it is apparent from this example that the use of temperature variation to alter retention times can have relatively complex effects on the elution of multi-subunit proteins. In addition, the fact that some of the TM-36 undergoes denaturation at temperatures as low as 0°C indicates that the hydrophobicity of the column material can destabilize protein quaternary structure. However, as was observed with lysozyme, utilization of lower temperatures appears to favour significantly elution of the polypeptide in its native form.

Temperature effects on chromatography of TM-22 and TM-36 on a HIC column operated in hydrophobic-interaction mode

The profiles for elution of TM-22 and TM-36 from the Bio-Gel TSK-Phenyl-5-PW column, when it is operated in the hydrophobic-interaction mode are shown as a function of temperature in Fig. 4. At 0°C, both TM-36 and TM-22 are eluted as single peaks with retention times of 5.2 and 9.6 min, respectively. Significantly, the relative elution order for the two peptides is identical to what we observed when the column was operated in the reversed-phase mode at 0°C. On the basis of the same reasoning as before, this observation indicates that TM-36 is eluted as a dimer under these conditions while TM-22 is eluted in its monomeric form. As the temperature is increased, both peaks shift to longer retention times and are resolved into doublets. The retention time shift is presumably due to the temperature dependence of the hydrophobic effect[11]. More surprising is the resolution of the two peaks into doublets. The possibility that the doublets were due to contamination of the peptides with related deletion peptides was ruled out by careful purification and analysis by use of both standard RPC, as discussed previously[4,9], and the Bio-Gel TSK-Phenyl-5-PW column in the reversed-phase mode. Consequently these observations suggest that the presence of multiple conformational states of the peptides is responsible for doublet formation at elevated temperatures. To gain insight into this possibility, we examined the structure of these molecules in the starting buffer (1.7 *M* ammonium sulfate and 0.1 *M* sodium phosphate, pH 7.0) by using circular dichroism as a probe. Fig.

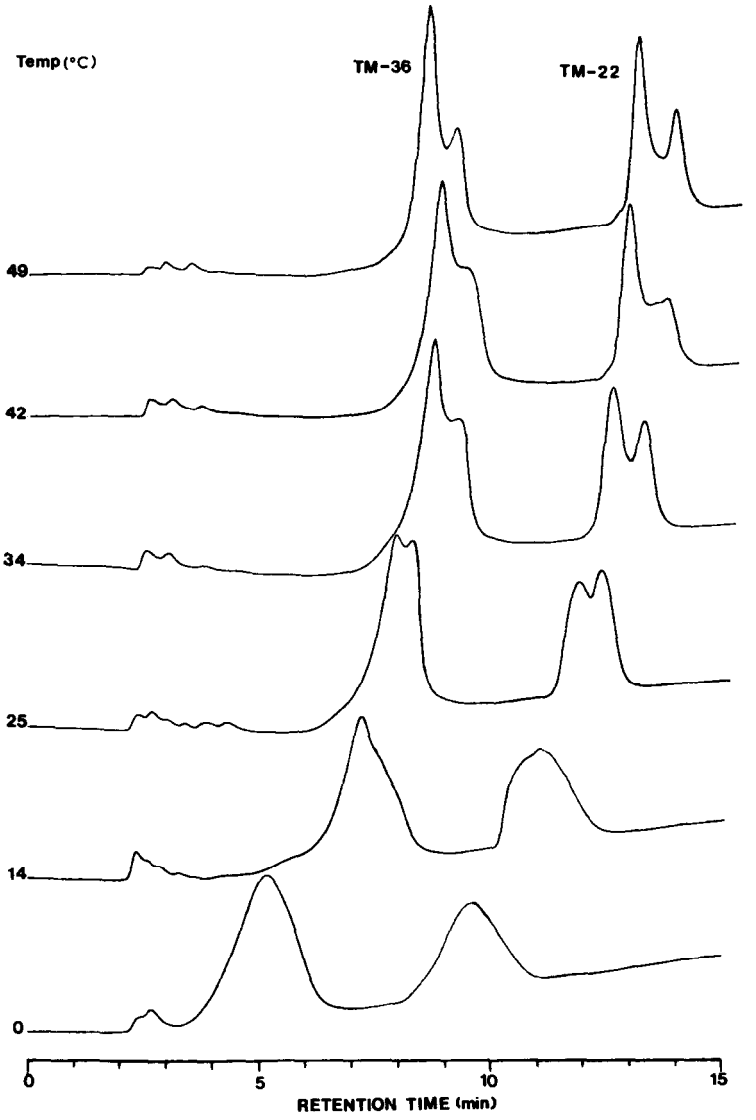

Fig. 4. Effects of temperature on the HIC elution profile of a mixture of TM-22 and TM-36 on the Bio-Gel TSK-Phenyl-5-PW column. These peptides have the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where n = 3 for TM-22 and 5 for TM-36. The values to the left of each profile are the temperatures (°C) at which the column was operated. Conditions: AB gradient, solvent A contained 0.1 *M* sodium phosphate and 1.7 *M* ammonium sulfate at pH 7.0 and solvent B contained 0.1 *M* sodium phosphate at pH 7.0. See Materials and Methods for gradient details. The effluent was monitored at 210 nm. Flow-rate, 1 ml/min.

5 displays the circular dichroism spectra for the two peptides at a temperature of 7.8°C. The molar ellipticities (θ) at 222 nm are $-32\,960°$ cm^2 dmole^{-1} and $-33\,250°$ cm^2 dmole^{-1} for TM-22 and TM-36, respectively. Using an equation derived by Chen *et al.*[5], Lau *et al.*[4] calculated the predicted molar ellipticities of these peptides at 220

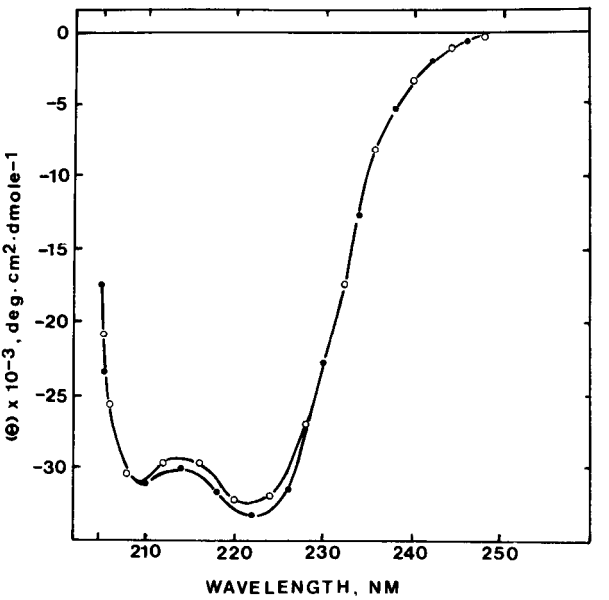

Fig. 5. Circular dichroism spectra of TM-22 and TM-36 in HIC starting buffer (0.1 *M* sodium phosphate, 1.7 *M* ammonium sulfate, pH 7.0) at 7.8°C. Peptide sequences are Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where n = 3 for TM-22 and 5 for TM-36. The open and closed circles are for TM-22 and TM-36, respectively.

nm assuming 100% α-helical structure. Values of $-32\,000$ and $-33\,700°$ cm^2 dmole^{-1} were obtained for TM-22 and TM-36, respectively. Comparison of the predicted and actual values demonstrates clearly that both molecules are 100% α-helical. This result was expected for TM-36, which has been shown previously to exist as a two-stranded α-helical coiled-coil in benign buffer systems[4]. In contrast, when the circular dichroism spectrum of TM-22 was previously studied in a buffer system containing 1.1 *M* potassium chloride and 0.05 *M* sodium phosphate at pH 7.0, the molar ellipticity at 220 nm was found to be only $-12\,500°$ cm^2 dmole^{-1} and the peptide was monomeric[4]. It is apparent from comparison of the values in the two buffer systems that the presence of ammonium sulfate induces α-helix formation in this peptide. More importantly, it also promotes formation of TM-22 dimers. In a previous study, our laboratory demonstrated that dimeric α-helical coiled-coils exhibit circular dichroism spectra which differ significantly from those obtained with monomeric α-helices[4]. In a typical monomeric α-helix, the ratio of the molar ellipticities at 222 and 209 nm is approximately equal to 0.8, while a value of approximately 1.0 would be typical for a coiled-coil structure. In starting buffer, this ratio is 1.05 for TM-22 and 1.08 for TM-36, which is strong evidence that both peptides enter the Bio-Gel TSK-Phenyl-5-PW column as dimers. The fact that only TM-36 is eluted as a dimer may be explained on the basis of their relative stabilities. Fig. 6 is a thermal melting profile of TM-22 and TM-36 in the ammonium sulfate starting buffer. It is quite clear from these data that TM-36 is a considerably more stable molecule than TM-22. Nevertheless, TM-22 is 100% α-helical at 0°C and retains approximately 80% of its α-helical content even at 50°C. This suggests that the dimeric quaternary structure of this peptide is disrupted by binding to the hydrophobic matrix of the column. Thus,

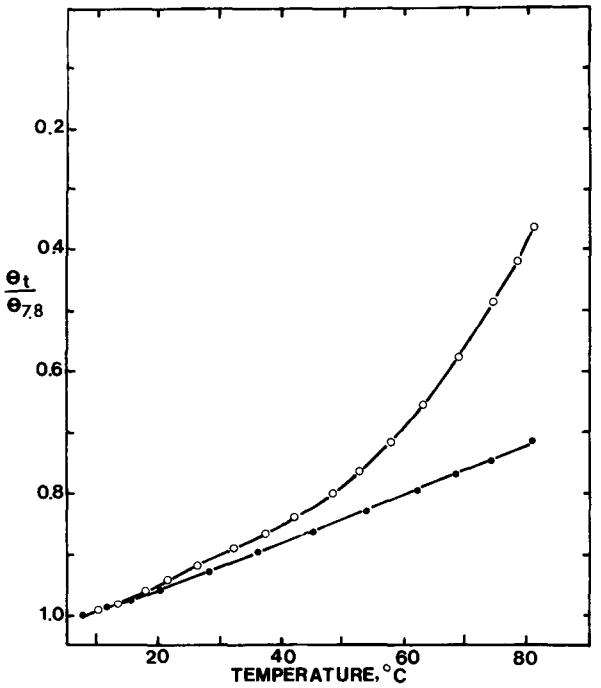

Fig. 6. Thermal melting of TM-22 and TM-36 in HIC starting buffer (0.1 *M* sodium phosphate, 1.7 *M* ammonium sulfate, pH 7.0) determined by circular dichroism. The open and closed circles are for TM-22 and TM-36, respectively. $\Theta_t/\Theta_{7.8}$ Represents the ratio of the ellipticity at 222 nm at the indicated temperature to the ellipticity at 7.8°C.

the destabilizing effect of the matrix on TM-22 under hydrophobic interaction conditions is reminiscent of the same effect on TM-36 under reversed-phase conditions. Presumably, the reason TM-36 does not lose its dimeric structure under these conditions as it did in the reversed-phase mode is due to stabilization brought about by the presence of ammonium sulfate.

In light of the preceding observations, we interpret the existence of the doublet TM-22 and TM-36 peaks in the following manner. On the column at 0°C, there is only one predominant conformational state for both TM-36 and TM-22. In the case of the former, this is a coiled-coil structure, while for the latter it is a monomer. Destabilization of both peptides occurs as the temperature is raised. For TM-36, this results in partial unravelling of the coiled-coil structure of some of the molecules present. These partially denatured dimeric molecules are retained slightly longer by the column. This, in conjunction with slow kinetics for conversion between differing conformational states would result in the appearance of a second peak. In contrast to the situation with TM-36 involving partial disruption of quaternary interactions, it appears that increased temperatures disrupt only interactions in secondary structure for TM-22. Despite column-induced dissociation of the TM-22 dimer, this peptide would still be expected to retain some of its α-helical structure. Differences in the extent to which the α-helical structure is retained would affect the surface hydrophobicity of the peptide and could result in the two peaks observed.

CONCLUSIONS

These results demonstrate the difficulty of preparing a hydrophobic column material for isolating proteins in their native conformation. The monomeric proteins, cytochrome *c* and lysozyme, and the dimeric peptides, TM-22 and TM-36, all underwent partial or total denaturation under temperature and solvent conditions where the molecules are native in the absence of the support. This indicates that, even though the Bio-Gel TSK-Phenyl-5-PW column is less harsh in its effects on protein structure than reversed-phase columns, the phenyl ligand density is still high enough to cause some denaturation of tertiary and quaternary structure. However, there are practical limits to decreasing ligand density or hydrophobicity to prevent denaturation since the effectiveness and capacity of the column would decrease simultaneously.

Since the stability of protein tertiary and quaternary structures are heavily dependent on hydrophobic interactions, there will always be competition between hydrophobic groups, either buried within the protein core or at a subunit interface and the hydrophobic support of the column. This competition will necessarily weaken the structural stability of the protein. However, as one would expect, the utilization of lower temperatures can significantly shift the native and denatured equilibrium toward the native side.

An interesting contrast to the situation with HIC or RPC is provided by ion-exchange chromatography. The surfaces of most native proteins have a significant number of accessible charged residues. Thus, the interaction between the column and the charged surface would be expected to minimize destabilization of the molecule. Significantly, Parente and Wetlaufer[12,13] have recently shown this to be true for lysozyme and chymotrypsinogen using cation-exchange HPLC. This suggests that HIC is most likely to succeed without denaturation when the desired protein has accessible hydrophobic residues on its surface.

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