Journal of Chromatography, 367 (1986) 1–8
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 811

DETERMINATION OF THERMODYNAMIC PARAMETERS FOR THE INTERACTION OF A LIPID-BINDING PEPTIDE AND INSULIN WITH A REVERSED-PHASE COLUMN

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(First received July 22nd, 1985; revised manuscript received May 21st, 1986)

SUMMARY

The effect of temperature was investigated on the reversed-phase chromatography of a synthetic lipid associating peptide (LAP), with the following sequence (LESFLKSWLSALEQALKA) and on insulin. The LAP was chromatographed on a μBondapak-alkylphenyl column with 1% aq. triethylammonium phosphate, pH 3.2–2-propanol (80:20) as the isocratic mobile phase. The insulin was separated on a Resolve-C_{18} column with a mobile phase that contained 0.1 *M* sodium phosphate, pH 2.0–acetonitrile (71.5:28.5). With the LAP the Van 't Hoff plot (ln k' *vs.* $1000/T$) was linear and the value of enthalpy for association of the peptide with the reversed-phase column was large and negative. The phase ratio was estimated for the column used in the separation and then derivation of the corresponding entropic term demonstrated that the association was enthalpy and not entropy drive. By contrast the corresponding Van 't Hoff plot derived or the insulin study was non-linear and with a positive slope. Further study indicated that the formation of the insulin–reversed phase complex could be attributed to a favorable entropy change. It is probable that the non-classical thermodynamics observed during the insulin chromatography could be related to conformational changes in the insulin structure during the chromatographic process.

INTRODUCTION

In a previous paper[1] we published evidence that the hydrophobic environment provided by a reversed-phase column could induce in a synthetic lipid-associating peptide (LAP) the formation of an amphipathic helix. The formation of the helical structure promoted hydrophobic interactions between the sample and the stationary phase. The results of this study[1] suggested that reversed-phase high-performance liquid chromatography (HPLC) may be of general utility in investigating the pertur-

0021-9673/86/$03.50

bations in structure that can occur when a polypeptide is located adjacent to a hydrophobic environment. In an attempt to extend this study, we have investigated the effect of temperature on the retention times observed for polypeptide samples in reversed-phase HPLC.

THEORY

Calculation of phase ratio

Since the capacity factor $k' = K\varphi$, where K is the thermodynamic equilibrium constant, and φ is the phase ratio (which reflects the ratio of amounts of stationary and mobile phase) and $\Delta G^0 = -RT \ln K$, we can derive

$$\ln k' = -\Delta G^0/RT + \ln \varphi \tag{1}$$

From eqn. 1 ΔG^0 could be expressed as

$$\Delta G^0 = -RT (\ln k' - \ln \varphi) \tag{2}$$

For the μBondapak-alkylphenyl column (30 cm × 4 mm I.D.) where φ was estimated as 0.084 from the approximation that φ is the ratio of the weight of the stationary phase to the volume of the mobile phase[2]. In this calculation the weight of the packing material in the column was 2.1 g, the fraction of weight of organic ligands present on the packing material was 10% and the volume of the mobile phase was 2.5 ml.

For the Resolve-C_{18} column (10 cm × 8 mm I.D.) the phase ratio was calculated to be 0.108. The weight of the reversed phase was 2.25 g, the fraction of organic ligands was 13% and the volume of the mobile phase was 2.7 ml.

Calculation of entropy

ΔS^0 was calculated from the expression

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{3}$$

which was rearranged to

$$T\Delta S^0 = \Delta H^0 - \Delta G^0 \tag{4}$$

With the LAP the value of ΔH^0 was −12.05 kcal/mol at 40°C and 39% solvent B (see Experimental). The value of $T\Delta S^0$ was −8.67 kcal/mol (for a phase ratio φ = 0.084). It should be noted that if the phase ratio is much smaller or larger than its proposed value, the entropic term does not change sign: *e.g.* if φ = 0.84, then $T\Delta S^0$ = −10.11 kcal/mol, and if φ = 0.0084, $T\Delta S^0$ = −7.24 kcal/mol.

The enthalpy values (ΔH^0) listed in Table I were calculated in the following manner. The values of $\Delta H^0{}_1$ and $\Delta H^0{}_2$ were calculated by measurement of the slope derivative of the Van't Hoff plot either by the use of convolution integers[4] or by the explicit three-point sliding least-squares procedure[5], respectively, after the insertion of additional calculated data points. The value of $\Delta H^0{}_3$ was derived from the non-linear fit of the equation $R \ln k' = a \ln T + b/2T - c/T + d'$; where $d' = d + R \ln \varphi$ and R = 1.987 cal/(mol · deg). This fit gave the following parameters: a = 1012;

TABLE I

THE THERMODYDANAMIC PARAMETERS DETERMINED FOR THE INTERACTION OF BOVINE INSULIN WITH A RESOLVE-C_{18} COLUMN

The chromatographic conditions were the same as described in the legend to Fig. 3.

Temp. (°C)	*Capacity factor* (k')	*Enthalpy**			*Heat capacity***	*K(eq)****	$\Delta G^{0\S}$	$\Delta S^{0\S\S}$
		ΔH_1^0	ΔH_2^0	ΔH_3^0	ΔCp			
21	2.91	13.3	13.2	13.7	−0.178	26.9	−1.92	51.4
40	8.97	8.50	8.51	8.4	−0.288	83.1	−2.75	36.0
45	10.3	6.89	6.91	6.79	−0.315	95.4	−2.88	30.8
50	12.9	5.15	5.18	5.08	−0.343	119.4	−3.07	25.5
55	14.2	3.3	3.32	3.27	−0.370	131.5	−3.18	19.8
60	15.2	1.34	1.36	1.36	−0.398	140.7	−3.27	13.9
65	14.9	− 0.71	− 0.71	− 0.66	−0.425	138.1	−3.31	7.7
70	14.3	− 2.84	− 2.86	− 2.79	−0.453	132.4	−3.33	1.4
75	12.8	− 5.04	− 5.08	− 5.02	−0.480	118.5	−3.30	− 5.1
80	12.3	−7.31	−7.38	−7.35	−0.508	113.9	−3.32	−11.5

* The values of ΔH_1^0,and ΔH_2^0 were calculated by measurement of the slope derivative of the Van't Hoff plot and ΔH_3^0 mathematically (see Theory); units are kcal/mol.

** Values are expressed as kcal/(mol · deg). The calculation method is described in the Theory section.

*** The equilibrium constant (K) for the association of insulin with the reversed-phase column is related to the capacity factor (k') by the expression $k' = \varphi K$, where φ is the phase ratio (0.108 for the Resolve-C_{18} column).

§ The free energy change (ΔG^0) was calculated from the expression $\Delta G^0 = -RT \ln K$; units are kcal/mol.

§§ The entropy (ΔS^0) was calculated from the expression $\Delta G^0 = \Delta H^0 - T\Delta S^0$ and using the values of ΔH_2^0; units are eu or cal/(mol · deg).

$b = -4.218$; $c = -101\,600$; $d' = 5473$. The enthalpy was then calculated[6] from the expression $\Delta H^0 = aT + b/2T^2 + c$; units are kcal/mol.

Calculation of heat capacity

The heat capacity values listed in Table I were calculated[6] from the expression $-\Delta H^0/\Delta T = \Delta Cp^0 = a + bT$ and using the values of $\Delta H^0{}_2$. Values are expressed as kcal/(mol · deg). It should be noted that the heat capacity is represented by the second derivative of the initial data plot and thus is relatively imprecise. Clearly the HPLC procedure can only be used to calculate heat capacity changes that are relatively large.

EXPERIMENTAL

Apparatus and chemicals

The analyses were performed on a Waters high-performance liquid chromatograph equipped with a 660 or 721 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). A Model 450 UV spectrophotometer (Waters Assoc.) and a data module (Waters Assoc.) were used to monitor and record the separation. Sample injections were made using either a WISP (Waters Assoc.) or a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.). The columns were either a μBondapak-alkylphenyl (30 cm × 4 mm I.D.) or a Resolve-C_{18} (10 cm × 8 mm I.D.). Water was purified by dis-

tillation of RO water in an all-glass still. Organic solvents (LC grade) were obtained from BDH (Poole, U.K.). The aqueous mobile phase was prepared with phosphoric acid (AR grade; J. T. Baker, Phillipsburg, NJ, U.S.A.), triethylamine (Aldrich, Milwaukee, WS, U.S.A.) and sodium phosphate (Merck, Darmstadt, F.R.G.).

Separation conditions

The LAP was chromatographed on a μBondapak-alkylphenyl column with solvent A (1% triethylammonium phosphate) and solvent B (2-propanol–solvent A, 80:20). The flow-rate was 1 ml/min.

A Resolve-C_{18} column was used in the chromatography of insulin and the mobile phase was 0.1 *M* sodium phosphate (pH 2.00)–acetonitrile, 71.5:28.5. For comparative purposes we have listed the mobile phases used by other workers on the effect of temperature changes on the chromatography of insulin. Lloyd and Corran[7] used an Ultrasphere-ODS column with a mobile phase that contained 0.1 *M* sodium dihydrogen phosphate (pH 2.00)–acetonitrile, 70:30. Vigh *et al.*[8] used a LiChrosorb-C_{18} column with a mobile phase of 0.05 *M* tetramethylammonium hydroxide and 0.1 *M* phosphoric acid (pH 3.2) with 45.9% methanol.

RESULTS AND DISCUSSION

In general it has been observed for the chromatography of small molecules that the retention time decreases with an increase in temperature[9–11]. This temperature effect is opposite to that observed for many biochemical systems in which an increase in temperature often results in strengthened hydrophobic interactions[12]. Such an increase is usually attributed to an entropy-driven process, with the enthalpic term being negligible or even unfavourable. As shown in Fig. 1A, The temperature dependence of the retention in reversed-phase HPLC of LAP 202 is consistent with the effect observed for small molecules, *i.e.*, an increase in temperature decreases retention. The plot of ln *k'* *vs.* solvent composition is translated to a lower percentage of solvent B by an increase in temperature. The data from Fig. 1A can be plotted in the form of a Van't Hoff plot for evaluation of the enthalpies of association of the

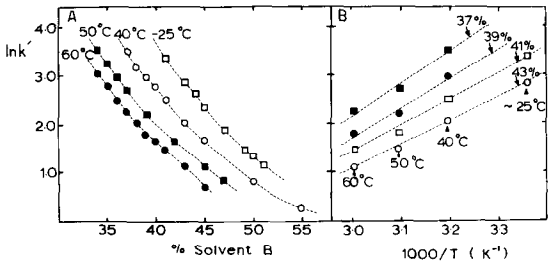

Fig. 1. (A) Plots of ln *k'* (capacity factor) *vs.* percent solvent B for the isocratic elution of LAP 202 at different temperatures. The amino acid sequence of the peptide was LESFLKSWLSALEQALKA. The chromatographic system consisted of a μBondapak-alkylphenyl column with 1% triethylammonium phosphate (pH 3.2) as solvent A and 2-propanol–solvent A (80:20) as solvent B. The flow-rate was 1 ml/min. In the analysis, 1.4 μg of peptide were injected dissolved in 0.02 ml of solvent A, 3 *M* guanidine hydrochloride. (B) The Van't Hoff plot of ln *k'* *vs.* 1000/*T* for the isocratic elution of LAP 202 at different temperatures. The percentage figures refer to the amount of solvent B used in the mobile phase for the isocratic analysis.

solute with the reversed-phase at different solvent compositions (Fig. 1B). The linearity of each plot indicates that when conditions of elution are held constant, apart from temperature, the enthalpy of association is relatively constant at least over the temperature range used here. An estimation of the errors involved in preparation of a given solvent composition indicated that the lines drawn in Fig. 1B are within experimental error for the points.

The enthalpies of association at 40°C and 60°C are shown in Fig. 2, and clearly demonstrate the linear relationship between the enthalpy of association and ln k'. At least over the limited concentration range used in this study, this result indicates that the mechanism of interaction of peptide 202 with the reversed-phase column does not change with the different concentrations of organic solvent[14]. As can be seen from Fig. 2 the enthalpy of association of peptide 202 with the reversed phase is large and negative (−13.6 kcal/mol at 37% solvent B). An estimation of the $T\Delta S^0$ term as −8.67 kcal/mol (see Theory) showed that the association of the LAP with the reversed phase was enthalpy-driven not entropy-driven. This observation is consistent with other studies on the chromatography of small molecules[9-11] and with the effect of temperature on the retention values obtained for nitropentane in our chromatographic system[16]. The results of this study on peptide 202, when contrasted with the variable temperature effects observed for the chromatography of insulin with different mobile phases[7,8], as well as biochemical studies such as protein–protein associations[15], suggest that hydrophobic interactions may appear to be either entropy- or enthalpy-driven.

In a previous publication[1] it was postulated that the LAP adopted a nearly 100% helical structure so as to maximize hydrophobic interactions between the reversed phase and the non-polar face of the amphipathic peptide. The linearity of the plots in Fig. 1A and B and the observation of linear plots of ln k' *vs.* the enthalpy at different temperatures suggest that the helical structure is not significantly altered over the chosen temperature range.

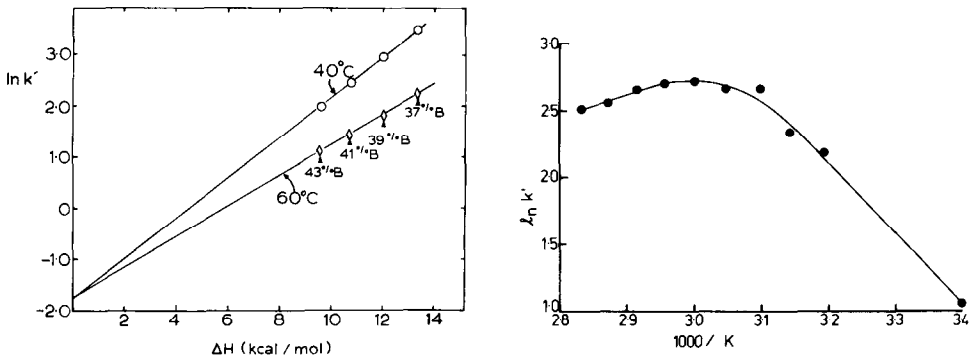

Fig. 2. Plots of ln k' *vs.* enthalpy of association for the isocratic elution of peptide 202 at different temperatures and concentrations of organic solvent. The mobile phase composition (percent solvent B) is shown on the graph at two different temperatures: (○) 40°C and (◇) 60°C.

Fig. 3. The plot of ln k' *vs.* 1000/K for the isocratic elution of insulin from a Resolve-C_{18} column at different temperatures. The flow-rate was 1 ml/min, and the mobile phase contained 0.1 *M* sodium phosphate (pH 2.0)–acetonitrile (71.5:28.5). The data points represent the mean of thirteen determinations with a percentage coefficient of variation of 0.68–2%.

Vigh *et al.*[8] studied the effect of temperature on the retention of nitropentane and bovine and porcine insulins in a reversed-phase system in which methanol was used as the organic modifier. These authors found that the Van't Hoff plot was non-linear but with a positive slope. We have observed a similar temperature dependence for the chromatography of porcine insulin using a different chromatographic system (see Methods). Therefore, the formation of the complex between insulin and the reversed phase is associated with a favourable enthalpy change[16]. However, Lloyd and Corran[7] showed that with acetonitrile as the organic modifier the temperature effect was opposite, as an increase in temperature resulted in an increase in retention time. With a similar chromatographic system (see Methods), we have extended this study over a greater temperature range, and Fig. 3 shows a Van't Hoff plot of the data. As the plot was curvilinear the enthalpy (ΔH^0) was calculated from thermodynamic parameters by graphical methods (see Theory). Similar values of ΔH^0 were obtained using the three different methods (see Table I).

As was originally noted by Edsall[17], the association of hydrophobic groups results in a decrease in the apparent heat capacities of solutes in aqueous solutions. This effect is attributed to the formation of cages of structured water of abnormally high heat capacity when the non-polar groups are exposed to the aqueous medium[3,15]. These studies have noted a dependence on temperature of changes in heat capacity that are similar to the values listed in Table I.

The phase ratio (φ) was calculated as 0.108 for the Resolve-C_{18} column (see Theory). We have assumed, as is common in related thermodynamic studies[18], that either the phase ratio does not change significantly with temperature or that ΔH^0 includes contributions due to changes in that ratio. With this value of the phase ratio the values for the equilibrium constant (K), the free energy (ΔG^0) and the entropy (ΔS^0) could be calculated for each of the temperatures. The values for these parameters are listed in Table I. Although the free energy change for the formation of the complex between insulin and the reversed phase is favourable over the whole temperature range, the values of ΔH^0 and ΔS^0 both decrease continuously and finally become negative with increasing temperatures.

It is possible that conformational changes in the insulin structure cause the significant changes observed in the thermodynamic parameters with the change in temperature. The non-classical thermodynamics cannot be attributed to the effects of temperature on the dimer monomer equilibrium, as it has been shown that the dimer is not present in significant concentrations in the high levels of acetonitrile (28.5%) that are present in the mobile phase[19]. Vigh *et al.*[8] attributed the curvature of the Van't Hoff plot to the changes with temperature of the protonation constants of both inslulin and components of the buffer. However, in the study described here a lower pH value was chosen (pH 2.0) so that minor pH fluctuations would not effect the ionisation of carboxyl groups. In addition, Waelbroeck *et al.*[20] corrected the pH values for temperature effects in a related study and found that the correction had only a moderate effect on the thermodynamic values obtained for the reaction of insulin with its receptor.

Hearn *et al.*[21] made a similar observation of non-classical thermodynamics for the effect of temperature on the association of the S-peptide and protein of ribonuclease, which could be related to changes in protein structure. Unlike LAP 202, which adopted a structure that maximized hydrophobic interactions, insulin could undergo

a reversible structural change that would allow buried hydrophobic groups to interact with the reversed phase and therefore increase the free energy of association. Despite these differences in three-dimensional structure, insulin and the LAP show a similar mode of interaction with the reversed phase, as indicated by the demonstration of an enthalpy–entropy compensation (linear ΔH^0 *vs.* ΔS^0 plots) for both the formation of the insulin–reversed phase complex and the LAP–reversed phase complex. Laufer[12] has noted that the compensation effect is an indication that a hydrophobic interaction is closely associated with a given process. A compensation temperature (β) of 350 K was calculated from the ΔH^0 *vs.* ΔS^0 plot for insulin[13]. Hsu *et al.*[13] have noted that a β value of greater than zero is further evidence of the hydrophobic effect and that the β values for biological reactions range from 265 to 420 K.

The applicability of thermodynamic data derived from reversed-phase HPLC studies to fully aqueous systems is supported by the following observations. There is excellent agreement between partitioning studies based on HPLC and those based on classical partition systems for various amphiphiles, including phospholipids[22], and for polypeptides[1]. Recently we have demonstrated a striking similarity between the thermodynamic parameters from the association of insulin with a reversed-phase column and with the corresponding data of Waelbroeck *et al.* for insulin receptor present on the surface of cultured human lymphocytes[20].

In conclusion, the studies described here support an earlier suggestion[23] that reversed-phase HPLC can be used successfully for studying the structure of proteins in hydrophobic environments, and it can be expected that the technique will become an important probe in protein chemistry.

ACKNOWLEDGEMENTS

This research study was supported by a grant from the National Heart Foundation and Medical Research Council of New Zealand. Part of this study was carried out while one of the authors (W.S.H.) was a visiting scientist at the Food and Drug Administration (Washington, DC, U.S.A.). The non-linear fit of the chromatographic data was performed by Dr. D. Martire and Mr. B. N. Barman (Georgetown University, Washington, DC, U.S.A.). Dr. Martire also developed the thermodynamic expressions for the enthalpy values. The following colleagues are acknowledged for their enthusiastic support and helpful advice: Dr. E. Titus (FDA), Dr. P. Hensley (Georgetown University), Drs. J. Roth, H. Edelhock and I. Chaiken (NIH).

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