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TEMPERATURE EFFECTS IN AFFINITY CHROMATOGRAPHY OF ALANINE AMINOTRANSFERASE

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SUMMARY

The effects of temperature on the elution parameters of alanine aminotransferase and albumin were studied on substituted agaroses designated for the affinity chromatography of the enzyme.

The elution volume of alanine aminotransferase depended logarithmically and and the elution volume of albumin linearly on temperature. Both decreased when the temperature increased. It was concluded that the observed elution volume of alanine aminotransferase was due to two types of retardation mechanisms: specific (the logarithmic mode) and non-specific (the linear mode), both of which were additive. Thermodynamic parameters were estimated for the specific mode and the calculation resulted in ΔH^0 and ΔS^0 values of *ca.* −40 kJ/mole and −140 J/mole·°K, respectively.

INTRODUCTION

Despite the significant temperature effects in affinity chromatography, very few papers have considered the topic[1–5]. In adsorption chromatography temperature will usually exert a strong influence on the equilibrium between the mobile and stationary phases[6]. At present there are no profound differences between biospecific and non-biospecific adsorptions, and therefore some aspects of adsorption chromatography are worth utilizing in affinity systems. Usually heat is given off during adsorption, and a temperature rise will therefore usually lead to a faster migration of the solutes[6]. In contrast to the ionic forces, the hydrophobic interaction increases with increase in temperature[7]. Thermodynamic parameters describe the sorption process both qualitatively and quantitatively but, unfortunately, much effort is generally required to obtain them.

We have previously examined the affinity purification of alanine aminotransferase (AlaAT*) at 8°C on various agaroses substituted with substrates or inhibitors of

* Abbreviations: AlaAT = alanine aminotransferase; AE-agarose = ethylenediamine coupled to cyanogen bromide-activated agarose; 2-oxoglutaric-AE-agarose = 2-oxoglutarate coupled to AE-agarose; CS-agarose = cycloserine coupled to cyanogen bromide activated agarose.

the enzyme[8,9]. It was suggested that the separation was due to a combination of specific and non-specific affinities[8]. Gradient elution, commonly used in affinity chromatography, greatly increases the complications of any theoretical treatment of the elution process[10]. The enzyme AlaAT was elutable from the column using normal (isocratic) elution when the buffer composition was properly selected. Therefore, and because of the heat stability of AlaAT, the affinity system of AlaAT was suitable for studying temperature effects. As the present study contained much routine work, the analyses were automated.

The results were consistent with the high negative enthalpy of sorption. A new approach for calculating thermodynamic parameters from elution volumes produced results that were parallel to those previously obtained from binding constants[1–3].

EXPERIMENTAL

Materials

Alanine aminotransferase (EC 2.6.1.2) in 1.8 *M* ammonium sulphate solution (93 U/mg protein) from pig heart, lactate dehydrogenase (LDH), salt-free powder (880 U/mg protein), L-alanine, 2-oxoglutaric acid, reduced β-nicotinamide-adenine dinucleotide (NADH), grade III, bovine albumin, fraction V and Sepharose 4B 200 were purchased from Sigma (St. Louis, Mo., U.S.A.) Ultrogel AcA 44 was obtained from LKB (Stockholm, Sweden).

The substrate solution contained 37 m*M* L-alanine, 25 m*M* disodium 2-oxoglutarate, 40 μ*M* pyridoxal 5′-phosphate, 0.3 m*M* NADH and 4400 units of LDH per litre in 0.1 *M* sodium phosphate solution (pH 7.6).

The sample to be chromatographed (190 μl) usually contained 1 μl of the AlaAT solution and 5 mg of albumin dissolved in 1 ml of elution buffer (25 m*M* sodium phosphate, pH 6.0, supplemented with 0.1 *M* sodium chloride).

The preparation of the affinity gels and the methods used for their analyses have been reported earlier[8].

Apparatus

An appartus for automatically recording enzyme activity and protein (absorbance at 280 nm) was constructed. Fig. 1 shows the shceme of the device. Elution buffer of constant composition was introduced at a flow-rate of about 30 ml/h with an Ismatec mp-ge peristaltic pump via a sample applicator[11] into a jacketed glass column of 12 mm I.D., made in our laboratory workshop. The gel volumes were usually 50 ml in the packed state, except that of Ultrogel AcA 44, which was used in a volume of about 90 ml. The temperature in the jacket was maintained by circulating water–ethylene glycol (2:1) (8 l/min) from a Lauda K2 thermostat. The thermostat was provided with a suitable circulation of ethanol at −20°C through its cooling coil. The temperature of the solution entering the column was pre-adjusted so that about 40 cm of the feeding tube was coiled on the jacket.

The eluate was divided after the column into two parts, one moving through an Isco UA-2 UV analyser (280 nm) and the other being taken for enzyme analysis by the pump at a flow-rate of 8 ml/h. An equal volume of the substrate solution was mixed with the flow of enzymic sample and the solution was then

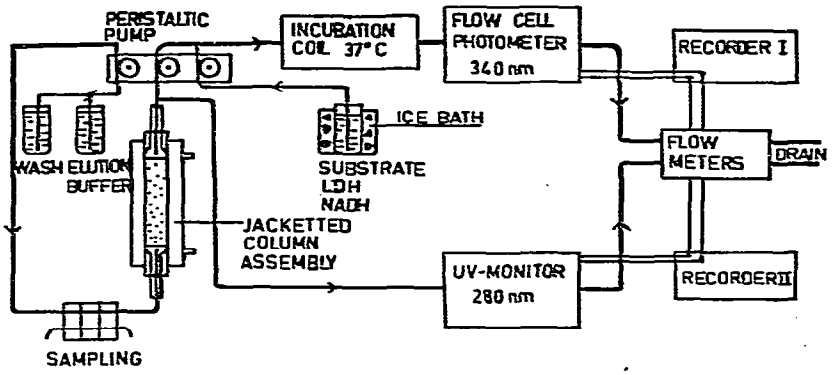

Fig. 1. Apparatus used for studying the effects of temperature on the chromatography of alanine aminotransferase.

introduced through an incubation coil (PTFE, 1 mm I.D., internal volume 12 ml) into a flow cell (volume 0.5 ml) which was continuously monitored by a Beckman DB spectrophotometer. The linear transmission output of the Beckman DB was changed to linear absorbance with an Optilab Multianalog 201 converter. The flow-rates of the enzyme and protein channels were separately monitored semi-continuously by two 10-ml siphon-operated flow meters[12]. The photometers were synchronized to the same time scale by following the absorbance of albumin at 280 nm in both channels. In such runs NADH and pyridoxal phosphate were omitted from the substrate solution. The elution volumes were calculated according to the time delay and according to the check points obtained by the flow meters.

The chromatographic runs were carried out from lower to higher temperatures. The gel was not washed between runs because of the virtually 100% recoveries of the proteins. When the temperature of the column was changed, it was allowed to equilibrate for 1 h before applying a new sample.

The elution volumes of 0.5% Blue Dextran 2000 and tritium oxide were separately measured at temperatures of 3, 25 and 37°C, collecting fractions which were analysed by measuring the absorbance at 265 nm and radioactivity.

RESULTS

The temperature effects were studied on three substituted agaroses, all of which separated AlaAT from albumin at 8°C[8]. The elution buffer was the same as described earlier[8], except that in one experiment on AE-agarose the pH of the buffer was changed from 6.0 to 7.4.

Fig. 2A–C shows that on all three gels an increase in temperature only slightly decreased the elution volume of albumin, but that the decrease was large with AlaAT. The elution volume of albumin was a linear function of the temperature (about −1 ml per 10°C), while that of AlaAT was logarithmic. As estimated from Fig. 2A–C, the change in the elution volume of AlaAT between 5 and 15°C was about 15 ml. At pH 7.4 the curve was of the same form, but the change was only 7 ml.

Stabilities of the gels and AlaAT

Curves similar in form to those in Fig. 2 were reproducible when the runs

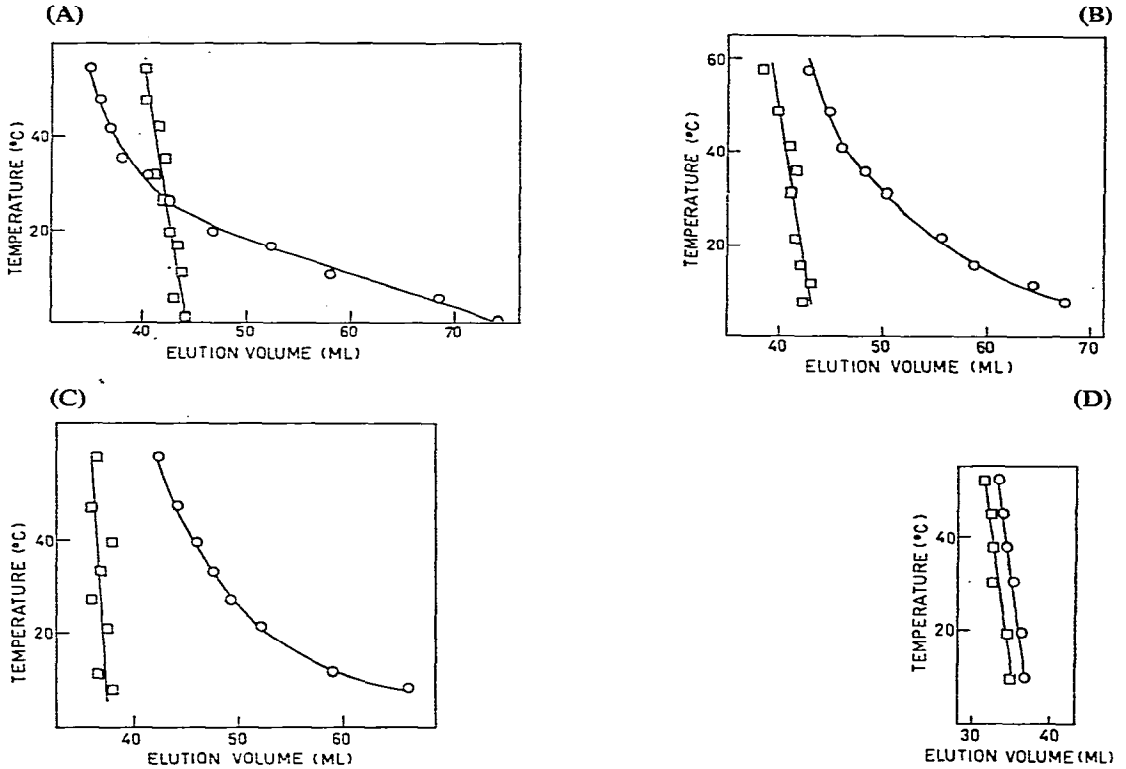

Fig. 2. Effect of temperature on elution volumes of alanine aminotransferase (○) and albumin (□). A = on AE-agarose; B = on 2-oxoglutaric-AE-agarose; C = on CS-agarose; D = on unsubstituted agarose. The gel volume was 50 ml. The elution was carried out with 25 m*M* sodium phosphate supplemented with 0.1 *M* sodium chloride.

were repeated. However, a small decrease in the gel volume (about 1–2%) was observed after exposure to the highest temperatures, which were higher than the manufacturer's recommendations (up to about 40°C). According to amino acid analyses before and after the run cycle with CS-agarose, the ligand concentration (per settled volume of gel) did not change during the cycle. Especially with CS-agarose, leakage of the ligand should have led to significant enzyme inhibition, but this was not detectable. The results thus suggest that the whole matrix either leaked or irreversibly melted at the highest temperatures.

As can be estimated from Fig. 2A–C, the enzyme remained in the column at the highest temperatures for about 1.5 h. The area of the enzyme activity peak was constant to 57–60°C. When a separate enzymic sample was kept in a water-bath of 60°C for 1.5 h it lost 40% of its activity. Hence the protein–gel interaction shielded the enzyme from denaturation. With affinity gels capable of hydrophobic interactions, the shielding may be even larger because the adsorption is stronger at higher temperatures owing to the endothermic nature of the binding[7].

Isothermal runs

No systematic study was made of the effects of the proteins on their elution

volumes because the automatic system was inconvenient for this purpose. On AE-agarose a three-fold increase in the concentrations did not alter the elution volumes at 5 or 35°C.

Basis for dividing the retardation of AlaAT into specific and non-specific modes

Fig. 2D shows that the elution volumes of albumin and AlaAT changed linearly and in a parallel manner on unsubstituted agarose. On Ultrogel AcA 44 the changes were also linear, but not exactly parallel (Fig. 3). There are arguments based on the results presented in Figs. 2 and 3 that the elution volume of AlaAT approaches asymptotically a line parallel to that of albumin. If this assumption is correct, the retardation of AlaAT can be divided into two additive components, one logarithmically dependent on temperature, which probably originates from adsorption, and the other, linearly dependent, originating from a gel chromatographic separation. We shall hereafter call these specific and non-specific contributions, respectively. Nishikawa *et al.*[13] came to similar conclusions when deriving their equation for affinity separation:

$$V_e = V_0 + K_{av}V_i + K_pV_g \quad (1)$$

where V_e = observed elution volume, V_0 = void volume, V_i = internal volume, V_g = volume taken by the gel itself and K_p and K_{av} are the distribution coefficients for adsorption and gel chromatography, respectively.

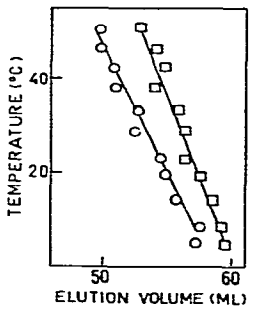

Fig. 3. Effect of temperature on elution volumes of alanine aminotransferase (○) and albumin (□) on Ultrogel AcA 44. The gel volume was 90 ml. Experimental conditions as in Fig. 2.

Calculation of the thermodynamic parameters from elution volumes

Assuming that the difference between the specific (V_e^s) and non-specific (V_e^n) elution volumes of AlaAT at 60°C was 1 ml and that V_e^n is parallel to the V_e of albumin in Fig. 2A–C, log (V_e—V_e^n) plotted against temperature produced a straight line for all runs, which obeyed, at pH 6.0, the equation log (V_e—V_e^n) = $-0.27\,t + 1.6$ where t (°C) is temperature. From this line one can calculate that an increase of 10°C in temperature caused a 1.9-fold decrease in V_e^s.

On the basis of eqn. 1 and with the assumptions made above, $V_e^n = V_0 + K_{av}V_i$ for AlaAT and thus $K_p = (V_e - V_e^n)/V_g$. For the thermodynamic values V_g must be known. Because the elution volume of tritium oxide shows the sum of

TABLE I

THERMODYNAMIC PARAMETERS FOR THE CHROMATOGRAPHY OF ALANINE AMINOTRANSFERASE ON DERIVATIZED AGAROSES

The parameters were calculated from elution volumes for the "specific binding mode" of the enzyme with the assumptions described in the text. The column headed V_g gives the volume taken by the gel at 25°C when the total volume was 50 ml.

Gel	$\Delta H°$ *(kJ/mole)*	$\Delta S°$ *(J/mole·°K)*	$\Delta G_{298°K}$ *(kJ/mole)*	$K^{298°K}$	*Correlation coefficient (r)*	V_g *(ml)*
AE-agarose, pH 6.0	−48.3 ± 1.4	−157 ± 5	−1.40 ± 0.08	1.76	0.995	4.4
AE-agarose, pH 7.4	−43.5 ± 1.8	−147 ± 6	0.36 ± 0.09	0.86	0.995	4.3
2-Oxoglutaric-AE-agarose, pH 6.0	−46.7 ± 2.8	−151 ± 9	−1.67 ± 0.15	1.96	0.980	4.2
CS-agarose, pH 6.0	−35.7 ± 0.9	−115 ± 3	−1.47 ± 0.05	1.81	0.997	5.3

$V_0 + V_i$ (ref. 14), $V_g = V_t - (V_0 + V_i)$, where V_t = total gel volume. Table I shows V_g values (at 25°C), which were used as constants in calculations of the thermodynamic values. The void volume appeared to be essentially independent of the temperature on 2-oxoglutaric-AE- and AE-agaroses (about 16 ml), while the V_e value of tritium oxide increased linearly (about 0.5 ml per 10°C) on increasing the temperature, thus indicating that the water–gel interaction increases with increase in temperature. This agrees with the fact that the protein–gel interaction decreased (about −1 ml per 10°C) with temperature. We could not measure the V_0 of CS-agarose because of the strong adsorption of Blue Dextran.

A plot of ln K_p against $1/T$ gives $\Delta H°$ and $\Delta S°$ values[15]. Fig. 4 shows three such plots. Both lines obtained with AE-agarose (pH 6.0 and 7.4) indicate points of discontinuity at temperatures of about 20°C and 30–35°C. The line with 2-oxoglutaric-

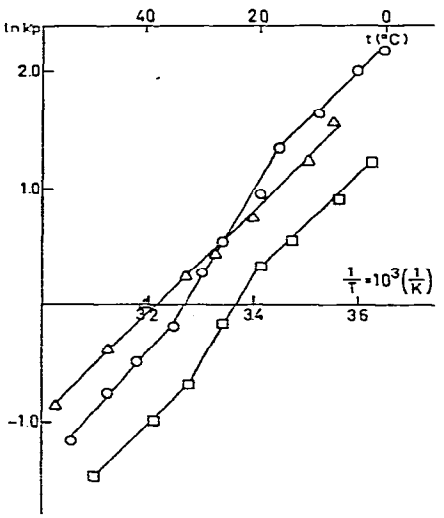

Fig. 4. Van 't Hoff plots obtained on AE-agarose, at pH 6.0 (○), on the same gel at pH 7.4 (□) and on CS-agarose at pH 6.0 (△). The distribution coefficients (K_p) were calculated for the "specific binding mode" with the assumptions described in the text.

AE-agarose (omitted from Fig. 4 for clarity) showed one point of discontinuity at 20°C. Fig. 4 shows that the experimental points on CS-agarose fit the line well. Owing to the assumptions made in the calculations, the points of discontinuity may be artefacts, and therefore only one set of values for $\Delta H°$ and $\Delta S°$ are given in Table I. It is interesting, however, that in two reports[2,3] points of discontinuity near 20°C have been described, which might suggest a common property of the agarose.

Table I also shows $\Delta G°$ and extrapolated K_p values at 25°C obtained from the correlation $\Delta G° = RT\ln K_p = \Delta H° - T\Delta S°$. In spite of the assumptions made above, the thermodynamic values correspond with those found in earlier studies on binding constants[1,2]. This suggests that the assumptions were correct. Because the true effective volume of the stationary phase is only approximated by V_g, the values of the standard entropies, and hence $\Delta G°$ and K_p, are also approximate. They may, however, be useful for comparative purposes.

DISCUSSION

The temperature effects give valuable information about the forces acting between solutes and the matrix. In the context of affinity chromatography, such studies are particularly important for a deeper understanding of the mechanism. An estimate of the temperature effects is also important for practical chromatography.

Even now in many affinity systems the only rational indication of biospecificity is an "exceptional" power to purify a protein. It is indeed very difficult to trace the precise source of separation in affinity chromatography, if for example, an unknown regulatory or "social site"[16] or another unknown effector site takes part in the separation. Only in very succesful affinity systems has biospecificity been proved fairly clearly. In our opinion, there is no absolute method by which biospecificity can be verified. It is not, for example, sufficient to prove that an enzyme is elutable from the column with a substrate, because it is now clear that proteins can be eluted even from ion exchangers using specific elution[17]. The elutability with substrate may be one indication, but the co-occurrence of a few indications of specificity may be considered convincing. When a suitable amount of information has been accumulated concerning the effects of temperature on affinity chromatography, they may be a useful way of describing the degree of biospecificity.

On the gels in Fig. 2A–C the elution volumes of AlaAT relative to albumin at 8°C were 1.4, 1.5 and 1.8, respectively[8]. Based on the present study it seems that AlaAT was best separated on CS-agarose because the non-specific contribution was most favourable on it. Gel chromatography on AcA 44 did not support the suggestion that there would have been great alterations in the structure of AlaAT at different temperatures (*e.g.*, subunit equilibria) which could explain the similar behaviour of AlaAT on different gels. Each ligand of the gels tested contained specificity elements of the substrates of AlaAT. It is known in enzymology that activation energy is more characteristic of the enzyme than of the substrate[18]. This could explain the similarity. Webb[19] has discussed the effects of temperature on enzyme inhibition.

In the course of studying the affinity chromatography of AlaAT, it has been shown that the elution volume of AlaAT is very sensitive to salt concentration, pH and temperature, in contrast to the elution volume of albumin, which is an "inert" reference protein. According to our results, albumin does not show

special chromatographic properties on substituted agaroses and therefore it is probably a suitable reference protein[20].

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