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Interaction of catechol-2,3-dioxygenase of *Pseudomonas putida* with immobilized histidine and histamine

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ABSTRACT

Catechol-2,3-dioxygenase (EC 1.13.11.2) from *Pseudomonas putida* as a model enzyme was purified from a bacterial crude extract in one step using affinity chromatography on immobilized histamine. In order to understand better the nature of the interaction, the adsorption of the enzyme on different gels having histidine and histamine coupled to Sepharose 4B was studied. The dissociation constants determined in the temperature range 4–37°C by an equilibrium binding analysis are between $0.95 \cdot 10^{-7}$ and $2.8 \cdot 10^{-7}$ *M* and between $2.6 \cdot 10^{-7}$ and $4.0 \cdot 10^{-7}$ *M* for histamine-carboxyhexyl Sepharose and histidyl-carboxyhexyl Sepharose, respectively. The standard enthalpy and entropy changes are different for these two gels, reflecting the different natures of the forces involved in the interaction.

INTRODUCTION

Amino acids have been used as pseudo-biospecific ligands in affinity chromatography for several years [1]. For example, arginine, tryptophan and lysine have been coupled to Sepharose for the purification of fibronectin, cellulase and plasminogen [2–4]. Histidine has also been used as a ligand to adsorb proteins [1,5–7]. Owing to its numerous properties such as weak hydrophobicity and charge-transfer ability and a wide range of pK_a values [8,9], which make it unique among the amino acids, it can be used as a general ligand in pseudo-biospecific chromatography [5]. Several workers have observed that the fixation of the ligand to the matrix via a spacer arm can greatly improve the binding properties of the gel towards the molecule to be separated. Thus El-Kak and Vijayalakshmi [7] increased the binding capacity, yield and purification factor during the purification of mouse IgG on immobilized histidine by using aminohexyl as a spacer arm. These parameters were also improved by using histamine instead of histidine. However, there is not much information about the adsorption mechanism and the nature of the interaction forces on which the histidine-ligand affinity chromatography is based.

In this work, the interaction of catechol-2,3-dioxygenase (C-2,3-D) (EC 1.13.11.2) from *Pseudomonas putida* BL 3 as a model enzyme with different supports with immobilized histidine and histamine was studied. This enzyme is an extradiol-type dioxygenase and catalyses the conversion of catechol to 2-hydroxymuconic 6-semialdehyde with the insertion of two atoms of molecular oxygen [10]. The enzyme consists of four identical subunits with a molecular mass of 35 155 [11,12] and contains 4 g-atom of iron(II) per mole of enzyme [11,13]. It is unstable in air owing to the oxidation of iron(II) to iron(III) [14]. The enzyme, which has already been purified by other methods [13], showed a high affinity for histamine-carboxyhexyl Sepharose (hista-CH Sepharose) and histidyl-carboxyhexyl

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Sepharose (his-CH Sepharose) gels. This paper describes the purification of the enzyme with these gels and the determination of the thermodynamic constants of the protein–ligand interaction by a non-chromatographic method using equilibrium binding analysis in order to understand better the nature of the interaction.

EXPERIMENTAL

Materials

Sepharose 4B and carboxyhexyl- and aminohexyl-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, L-histidine and histamine were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical-reagent grade.

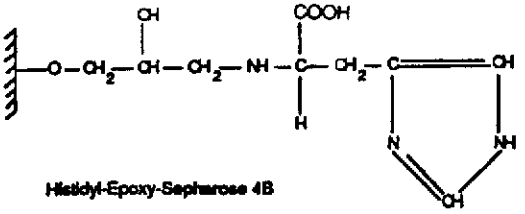
The *Pseudomonas putida* BL3 biomass was supplied by Dr. Schöpp (Department of Biochemistry, University of Leipzig, Leipzig, Germany). This strain, which is a derivative of the strains BL1 and ATCC 33015 [15], obtained after plasmid transfer by Ackermann [16], was fermented on benzyl alcohol as carbon source.

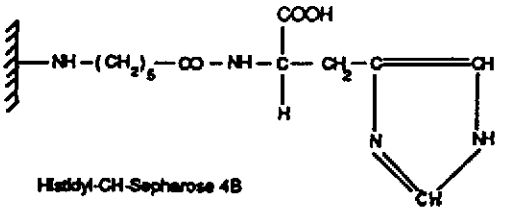
Preparation of the gels coupling histidine and histamine

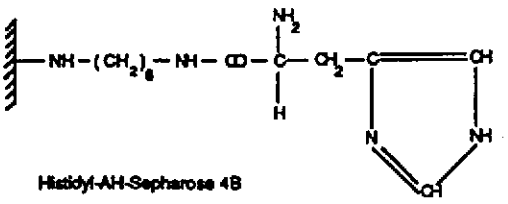
His-CH Sepharose and histidyl-aminohexyl Sepharose were prepared by coupling histidine to carboxyhexyl Sepharose 4B and aminohexyl Sepharose 4B, respectively, by carbodiimide at pH 4.5–6 with constant stirring for 16 h at room temperature as described by the manufacturer. Hista-CH Sepharose was prepared in the same manner by coupling histamine to carboxyhexyl Sepharose 4B. Histidine Sepharose was prepared as described previously [3] by coupling histidine to Sepharose 4B activated with epichlorohydrin. The proposed structures of the gels are shown in Fig. 1.

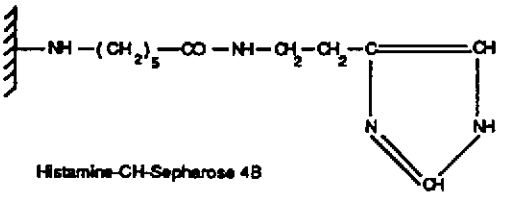
Preparation of the enzyme crude extract

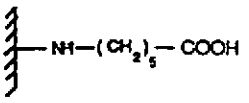
After suspension of the bacterial cells in 25 m*M* Tris–HCl buffer (pH 7.5) to an absorbance of $A_1^{600} = 12$, the cells were disrupted in an Aminco French pressure cell at 5000 p.s.i. (1 p.s.i. = 6894.76 Pa). The resulting suspension was centrifuged for 2 h at 10 000 *g* and the supernatant (crude extract), which had a protein concentration of about 4 mg/ml, was directly applied for chromatography.











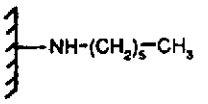


Fig. 1. Proposed structures of the various gels.

Chromatographic procedures

Purification was performed at 20°C using a jacketed column (4 cm × 1.2 cm I.D.) containing hista-CH Sepharose which was thermostated using a water-bath connected to a refrigeration unit (Bioblock). The column was connected to an automated Econo liquid chromatographic system (Bio-Rad Labs.). Chromatography was carried out at a flow-rate of 35 ml/h. A 3–10-ml volume of the crude extract was injected into the column after equilibration with 25 m*M* Tris–HCl buffer (pH 7.5). Elution was performed at the same flow-rate with 25 m*M* Tris–HCl buffer (pH 7.5) containing increasing amounts of sodium chloride (0.1–1 *M*). The absorbance of the eluate was measured at 280 nm; fractions of 2 ml were collected. After each experiment, the column was washed with three column volumes of a 0.05 *M* sodium hydroxide solution followed by water and finally by the equilibration buffer.

Enzyme assay

The activity of catechol-2,3-dioxygenase was assayed spectrophotometrically by measuring the increase in absorbance at 375 nm as described by Nozaki [14]. One unit of enzyme activity is defined as the amount that catalyses the formation of 1 μmol of the product per minute at 20°C.

Protein determination

The protein concentration was determined by the method of Bradford [17]. Crystalline bovine serum albumin was used as a reference protein. In the crude extract, protein was determined according to Kalb and Bernlohr [18]. This method uses the absorbances at 230 and 260 nm and eliminates the interference of nucleic acids.

Native polyacrylamide gel electrophoresis

The purity of the separated enzyme was analysed by native polyacrylamide gel electrophoresis as described by Davis [19] using a 7.5% (w/v) gel. The gel was stained with Coomassie Brilliant Blue. The method described by Müller *et al.* [20] was used to stain for enzyme activity. The gel was incubated for 10 min in a 1 m*M* solution of ammonium iron(II) sulphate in 25 m*M* Tris–HCl buffer (pH 7.5), followed by a 1 m*M* solution of catechol in the same buffer. A yellow band appeared after a few minutes.

Equilibrium binding analysis

The equilibrium binding experiments were carried out according to the method described by Hutchens *et al.* [21]. The gel was equilibrated with 25 m*M* 3-(N-morpholino)propane sulphonic acid (MOPS) buffer (pH 7.5) (hista-CH Sepharose) or 25 m*M* 2-(N-morpholino)ethane sulphonic acid (MES) buffer (pH 6) (his-CH Sepharose) and then allowed to settle. A homogeneous suspension was then prepared with an equal volume of the equilibration buffer, 40-μl aliquots of which were added to a series of Eppendorf incubation tubes (1.5 ml) in duplicate containing 100 μl of the protein solution at various concentrations in the equilibration buffer (between 80 and 1000 μg/ml). For the equilibrium binding experiments, a twice rechromatographed and additionally gel filtration-purified preparation of C-2,3-D was used (during preparation, buffer solutions containing 15% of ethanol were used to prevent oxidative inactivation of the enzyme). The tubes were incubated for 30 min in a thermostated water-bath with intermittent gentle rotation to give adequate mixing. The period of 30 min was chosen from preliminary incubation of a series of control tubes at different temperatures, which did not show any change in the supernatant protein concentration after 20 min. The suspensions were then centrifuged at 180 *g* for 30 s using a thermostated centrifuge and 100 μl of the clear supernatant were taken from each tube to determine the concentration of the unbound protein.

The data were analysed according to Hutchens *et al.* [21] using linear Scatchard plots obtained from the equation $[PL]/[P] = -1/K_D \cdot [PL] + L_t/K_D$, which was obtained from the Langmuir adsorption isotherm after derivation and linearization. The slope gives the dissociation constant K_D of the protein–ligand complex and the intercept gives the maximum binding capacity L_t; [P] is the concentration of unbound protein and [PL] the concentration of the protein–ligand complex at equilibrium. [PL] can be calculated from

$[PL] = [P_0] - [P]$, where $[P_0]$ is the total protein concentration applied to the gel.

The thermodynamic parameters for the adsorption can be obtained by determining K_D at different temperatures. From the Van't Hoff reaction isotherm $\Delta G = \Delta G^0 - RT \ln K_D$, at equilibrium when $\Delta G = 0$, the equation $\Delta G^0 = RT \ln K_D$ is obtained. ΔG^0 can be calculated at a given temperature from the dissociation constant. The temperature dependence of K_D is given by the Van't Hoff reaction isobar. In its integrated form, $\ln K_D = \Delta H^0/RT + l$ (l is an integration constant), when plotting $\ln K_D$ *versus* $1/T$, ΔH^0 is given by the slope if a straight line is obtained (in this case, ΔH^0 does not depend on temperature [22]). From the Gibbs–Helmholtz relationship, $\Delta G^0 = \Delta H^0 - T\,\Delta S^0$, the standard entropy change ΔS^0 can be obtained.

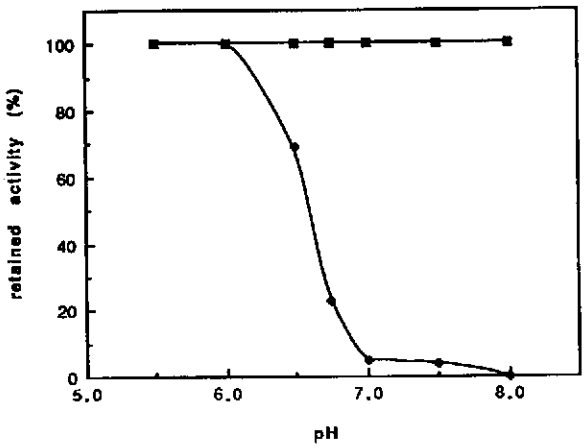


Fig. 2. pH dependence of the adsorption of catechol-2,3-dioxygenase on (◆) his-CH Sepharose and (■) hista-CH Sepharose.

RESULTS

The adsorption of catechol-2,3-dioxygenase on a series of Sepharose gels was studied. The best results concerning the adsorption and elution conditions and the recovery of enzyme activity were obtained with his-CH Sepharose at pH 6 and with hista-CH Sepharose at pH 6–8 (Table I). On histidyl Sepharose and histidyl-aminohexyl Sepharose the adsorption was very low. Carboxyhexyl Sepharose as a reference gel did not adsorb the enzyme, whereas on hexyl Sepharose no elution could be achieved under mild conditions using 1 *M* NaCl in the elution buffer.

The effect of the pH of the adsorption buffer on the retention of catechol-2,3-dioxygenase on hista-CH Sepharose and his-CH Sepharose was investigated. The first gel showed an adsorption of 100% of the enzyme which did not depend on the pH, whereas for his-CH Sepharose the retention was nearly 100% up to pH 6 but decreased to 5% at pH 7 (Fig. 2).

TABLE I

RETENTION OF CATECHOL-2,3-DIOXYGENASE ON DIFFERENT SUPPORTS DEPENDING ON THE pH OF ADSORPTION

RA is retained activity and ECA is eluted cumulating activity at 0.2–1 *M* NaCl in the elution buffer

Support	pH 6		pH 7		pH 8	
	RA (%)	ECA (%)	RA (%)	ECA (%)	RA (%)	ECA (%)
Histidyl Sepharose	20	12	0	0	0	0
Histidyl-AH Sepharose	23	10	0	0	0	0
His-CH Sepharose	100	70	5	5	0	0
Hista-CH Sepharose	100	65	100	100	100	100
Carboxyhexyl Sepharose	0	0	0	0	0	0
Hexyl Sepharose	100	0	100	0	100	0

Chromatography on hista-CH Sepharose

Chromatography was carried out at pH 7.5 on hista-CH Sepharose because the enzyme is unstable at pH values lower than 6.5.

The result of the chromatography on hista-CH Sepharose is shown in Fig. 3. The first large peak contains the unbound protein. Peak 2 contains the protein eluted with 0.1 *M* NaCl in the buffer. In these peaks no C-2,3-D activity could be detected. The enzyme was eluted with 0.15 *M* NaCl (peak 3), whereas peak 4 (0.3 *M* NaCl) and peak 5 (1 *M* NaCl) did not contain any enzyme activity. The specific activity of C-2,3-D increased from 4.9 U/mg in the crude extract to 53 U/mg (purification factor 11); the affinity yield was 42%.

In a native polyacrylamide gel electrophoresis, peak 3 showed an intense protein band with only traces of contaminating proteins (Fig. 4, lane 2). Staining for enzyme activity proved that the main band corresponded to C-2,3-D.

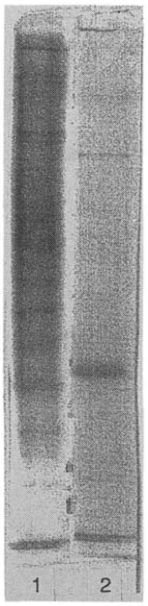


Fig. 4. Native polyacrylamide gel electrophoresis of the active fractions of the chromatography on hista-CH Sepharose. 1 = Crude extract; 2 = peak 3.

Equilibrium binding experiments

The equilibrium binding experiments were carried out at different temperatures (see Table II). In the Scatchard plots, straight lines were obtained (Fig. 5). The K_D and L_t values calculated from the Scatchard plots are given in Table II. The dissociation constant of the protein–ligand complex decreases with increasing temperature, ranging from $2.8 \cdot 10^{-7}$ to $9.5 \cdot 10^{-8}$ *M* for hista-CH Sepharose, and increases with increasing temperature from $2.6 \cdot 10^{-7}$ to $4.0 \cdot 10^{-7}$ *M* for his-CH Sepharose. The apparent maximum binding capacity was found to be between 4 and 6 mg protein/ml gel and between 1.8 and 2.2 mg/ml gel, respectively. In the plot of ln K_D *versus* $1/T$, straight lines were obtained (Fig. 6). The ΔH^0 values calculated from this plot were 5.6 kcal/mol for hista-CH Sepharose and −2.2 kcal/mol for his-CH Sepharose (Table II). The ΔG^0 values calculated for the different temperatures ranged from −8.3 kcal/mol at 4°C to −9.9 kcal/mol at 37°C and from −8.3 kcal/mol at 4°C to −9.1 kcal/mol at 37°C, respectively (Table II). The ΔS^0 values are 49.8 cal/mol·K for hista-CH Sepharose and 22.2 cal/mol·K for his-CH Sepharose (Table II).

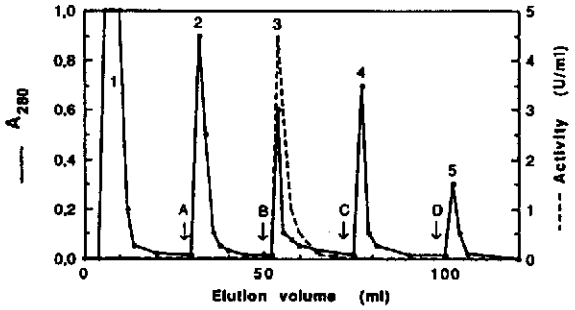


Fig. 3. Elution pattern of the crude extract on hista-CH Sepharose at 20°C. Elution with (A) 0.1 *M* NaCl, (B) 0.15 *M* NaCl, (C) 0.3 *M* NaCl and (D) 1.0 *M* NaCl in Tris–HCl buffer (pH 7.5). The numbers 1–5 indicate the protein peaks obtained.

The K_D values of the protein–ligand complex with hista-CH Sepharose were also determined in the presence of different concentrations of NaCl as neutral salt (relative to solvent structuring ability [23]) and a cosmotropic salt [$(NH_4)_2SO_4$]. The results are shown in Fig. 7. K_D increases with increasing NaCl concentration up to 0.2 *M* and then remains approximately

TABLE II

DISSOCIATION CONSTANTS (K_D) AND MAXIMUM BINDING CAPACITIES (L_t) DETERMINED AT DIFFERENT TEMPERATURES FOR HISTA-CH SEPHAROSE AND HIS-CH SEPHAROSE

r is the correlation coefficient in the linear regression, ΔH^0 is the standard enthalpy change, ΔS^0 the standard entropy change and ΔG^0 the standard free energy change calculated from the dissociation constants.

Parameter	Hista-CH Sepharose				His-CH Sepharose		
	4°C	15°C	26°C	37°C	4°C	20°C	37°C
K_D (10^{-7} M)	2.8	2.3	1.6	0.95	2.6	3.2	4.0
L_t (mg/ml)	4.5	4.3	6.0	5.6	1.9	2.0	2.2
r	0.98	0.98	0.98	0.98	0.99	0.99	0.99
ΔH^0 (kcal/mol)[a]		5.6				−2.2	
ΔS^0 (cal/mol · K)[a]		49.8				22.2	
ΔG^0 (kcal/mol)[a]	−8.2	−8.7	−9.3	−9.8	−8.3	−8.7	−9.2

[a] 1 cal = 4.184 J.

constant up to a concentration of 2 M. In presence of $(NH_4)_2SO_4$, K_D reaches a maximum at a concentration of 0.3 M whereas at higher concentrations better adsorption is obtained. In $(NH_4)_2SO_4$ at a concentration of more than 1.3 M the enzyme protein is not fully soluble.

DISCUSSION

The difference in retention of the enzyme on the different gels shows that adsorption depends on several factors such as hydrophobic and electrostatic interactions and hydrogen bonding, which can change with the surrounding pH depending on the pK values of the different chemical groups involved; interactions are thus modified, as can be seen for his-CH Sepharose (Fig. 2). The increase in adsorption of the enzyme on his-CH Sepharose as compared with histidyl Sepharose might be due to the added carboxyhexyl arm, as histidine is probably not accessible by large molecules such as C-2,3-D. Comparing the adsorption of the enzyme to his-CH Sepharose and to carboxyhexyl Sepharose as reference gel, we can conclude that the imidazole ring is necessary for adsorption. The poor retention on histidyl-aminohexyl Sepharose might be due to an electrostatic repulsion by the positively charged amino group. Apparently, coupling of a hexyl arm alone to Sepharose renders the gel very hydrophobic and elution cannot be achieved under mild conditions.

Chromatography on hista-CH Sepharose shows that this gel has a good specificity for C-2,3-D, which is expressed by the high affinity ($K_D = 0.23$ μM at 15°C; see Table II), and good resolution. Starting from a bacterial crude extract containing a large number of proteins (Fig. 4, lane 1), we observed in the purest fractions eluted with 0.15 M NaCl in native polyacrylamide gel electrophoresis an intense protein band (Fig. 4, lane 2) which corresponds to C-2,3-D, as revealed by an active coloration according to ref. 20. In addition, elution was achieved under very mild conditions. The specific activity for the purified enzyme of 53 U/mg is less than indicated in ref. 13, which is probably due to the oxidative inactivation of the C-2,3-D during the chromatographic procedures. This effect can be overcome by the addition of 15% of ethanol to all the eluting buffers, which also increased the yield from 42% to about 80%. In this instance, the elution pattern was not changed except that the elution volumes decreased (data not shown).

We chose to work with hista-CH Sepharose for the purification of the enzyme owing to the good capacity and resolution of the gel and the possibility of working at pH values above 7 where the enzyme is more stable. Chromatographic results on his-CH Sepharose at pH 6 showed that

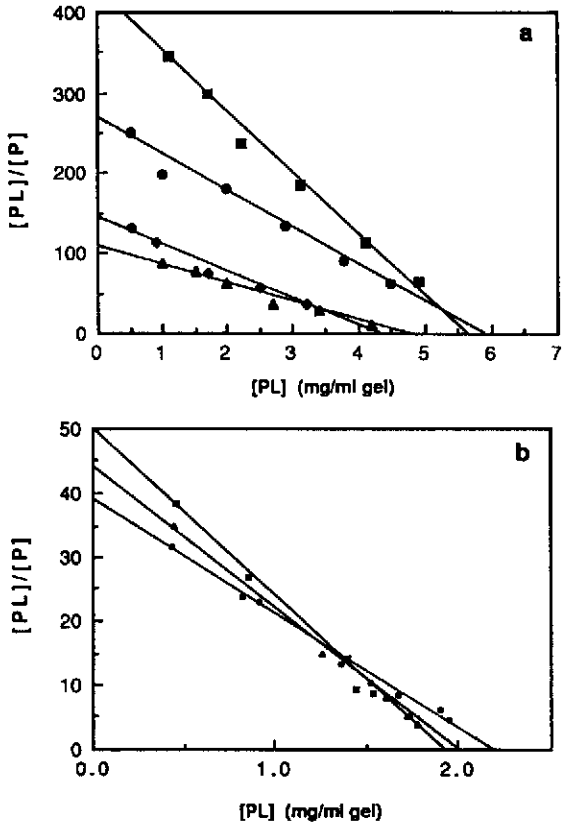


Fig. 5. (a) Scatchard plots of the equilibrium binding analysis of the adsorption of catechol-2,3-dioxygenase on hista-CH Sepharose at different temperatures: ■ = 37; ● = 26; ◆ = 15; ▲ = 4°C. (b) Scatchard plots of the equilibrium binding analysis of the adsorption of catechol-2,3-dioxygenase on his-CH Sepharose at different temperatures: ● = 37; ▲ = 20; ■ = 4°C.

the activity yield is low but the resolution is equal to that obtained with hista-CH Sepharose.

The interaction of C-2,3-D with his-CH Sepharose and hista-CH Sepharose was studied more thoroughly by using an equilibrium binding analysis. This method was put forward by Hutchens and co-workers [21,24], who studied the interactions of model proteins with immobilized transition metals. With a Scatchard plot, the dissociation constant of the protein–ligand complex and the maximum binding capacity can be determined. The latter may be higher when determined in a dynamic mode owing to the formation of a Nernst layer on the surface of the gel beads. As shown by Hutchens *et al.* [21], this method gives the same results as those obtained

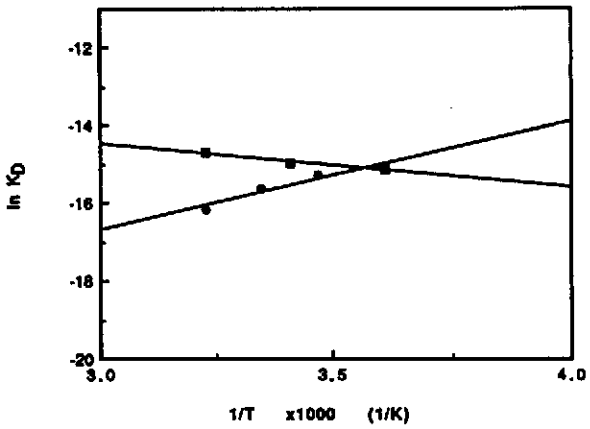


Fig. 6. Dependence of the dissociation constant of the enzyme–immobilized ligand complex on temperature. ■ = His-CH Sepharose; ● = hista-CH Sepharose.

by chromatographic methods (frontal analysis, zonal elution). In addition, the requirement in protein is low and the time of manipulation is short.

We determined the dissociation constants for the adsorption of C-2,3-D on the immobilized ligand at different temperatures (Table II). The increase in adsorption with temperature (K_D decreases) as determined for hista-CH Sepharose (Table II) implies that hydrophobic forces are important for the interaction. Similar results were reported by Cacace and Sada [25] for hydrophobic adsorptions. With his-CH Sepha-

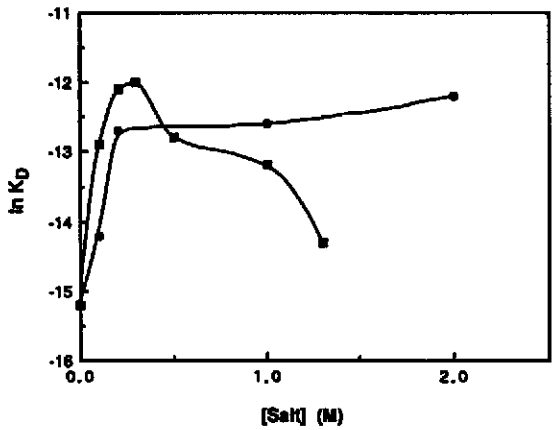


Fig. 7. Dissociation constant K_D of the catechol-2,3-dioxygenase–hista-CH Sepharose complex at different concentrations of (●) NaCl and (■) $(NH_4)_2SO_4$.

rose the adsorption decreased with increasing temperature, which is probably due to the supplementary interaction possibility given by the charged carboxyl group. For this gel, electrostatic forces and hydrogen bonding play a more important role in the adsorption process than with hista-CH Sepharose. As is already well known, hydrophobic interactions are the only ones of the interactions discussed above that increase with temperature [26]. This is probably due to a conformational change of the protein molecule when adsorbed on a hydrophobic matrix [27], which is facilitated at higher temperatures. For his-CH Sepharose, the temperature dependence of the dissociation constants is smaller than for hista-CH Sepharose, indicating that the contribution of hydrophobic forces and the others discussed above to the interaction is well balanced.

The standard free energy value calculated from the dissociation constants is not different for the two gels, but the additional carboxyl group causes a change in the standard enthalpy which is positive for hista-CH Sepharose and negative for his-CH Sepharose, and the positive standard entropy also decreases. This is not surprising because hydrophobic interactions cause a net disordering of the system and the entropy increases. These results indicate that the adsorption might be entropy driven with hista-CH Sepharose and more enthalpy driven for his-CH Sepharose. In the presence of an increasing concentration of ammonium sulphate as cosmotropic salt, hydrophobic interactions are favoured, which is shown by the decrease in K_D values for hista-CH Sepharose when using salt concentrations higher than 0.5 *M*. In the presence of a neutral salt (NaCl) this effect was not observed (Fig. 7).

In conclusion, we can say that histidine and histamine can be successfully used as ligands in affinity chromatography and proteins can be specifically purified even from a mixture of a large number of proteins such as a bacterial crude extract. The contribution of the different molecular forces to the adsorption process depends thereby on different factors such as the coupling mode of the ligand to the polymer support, the presence or not of a spacer arm, the structure of the adsorption site of the molecule to be purified and the adsorption conditions. It seems also that not histidine or histamine alone but the ensemble of amino acid, spacer arm and polymer support is the real ligand.

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