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Enzyme conformational alterations detected by partition column chromatography

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Abstract

In this paper, we demonstrate the ability of liquid–liquid partition chromatography (LLPC) to detect conformational alterations occurring in well-characterized enzymes. The conformational changes induced in dehydrogenases such as alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenases (LDH) and malate dehydrogenase (MDH) upon binding of ligand(s) were detectable by LLPC. The ligand-dependent equilibrium between two forms of citrate synthase (CS), glutamate-oxaloacetate transaminase (GOT), hexokinase (HK) and 3-phosphoglycerate kinase (PGK) could also be demonstrated. Furthermore, different conformational forms of some of the apoenzymes could also be detected and separated by LLPC. The results obtained here are discussed in relation to those obtained by other methods.

Keywords: Partition column chromatography; Conformational changes; Enzymes

1. Introduction

Structural flexibility plays an important role in the biological function of many proteins. For instance, several enzymes undergo conformational changes in response to changes in their state of ligation and these changes in conformation are essential to the catalytic activity of many enzymes [1]. In several cases, as for the immunoglobulins [2], the events upon complexation are still not entirely understood and there is a need for methods that allow a simple, rapid and sensitive comparison of the overall surface properties of biomolecules in solution before and after binding of ligand.

Conformational changes can be examined by direct methods such as NMR and X-ray crystallography [3]. However, these methods are not only complicated and time-consuming, but NMR is also restricted to analysis of small proteins (<30 kDa) [3], while X-ray crystallography requires the molecules to be in a crystalline state [3]. Several indirect methods can be used to obtain information about structural flexibility for large biomolecules in solution. However, each of these methods usually detects a single property such as shape, charge or hydrophobicity. This may also be the case for partitioning in aqueous two-phase systems, although the unique feature of this method is that it can also be used to detect differences in the overall surface properties, i.e. three-dimensional structures and general surface properties [4–8]. Some experiments have indeed indicated that partitioning in aqueous two-phase systems may be used to study the binding of ligand to protein [5,9–13].

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A column chromatographic approach, liquid–liquid partition chromatography (LLPC), which increases the selectivity and sensitivity of the two-phase technique, has been developed [14–18]. LLPC has been demonstrated to be a rapid and sensitive method that can be used to separate and fractionate proteins [16,19], to study surface properties of proteins which are related to their biological specificity [16,20,21] and to examine possible conformational changes occurring upon binding of ligand by specific biomolecules [17,21,22]. We have recently shown that LLPC may be used to detect changes in the surface properties of monoclonal and polyclonal IgG antibodies when they bind their antigen [21,22] as well as to demonstrate that enzymes (lactate dehydrogenase and hexokinase) may exist in a ligand-dependent equilibrium between two (conformational) forms [22].

The purpose of this study was to further explore the general applicability of LLPC to detect conformational changes that may occur upon binding of ligand by biomolecules like enzymes. Eight well-characterized enzymes from three classes (dehydrogenases, lyases and transferases) were used. Each of the chosen enzymes are known to undergo one of two major types of conformational changes upon binding of ligand, determined by several methods including X-ray crystallography. In the first group, including isoforms of malate dehydrogenase [23,24], the enzyme exists in an open or closed form depending on the conformation of a flexible loop at the active site. In the second group, the active site of the enzyme is situated in a cleft between two domains and the structural rearrangement involves a rotation of the domains to close the active site over the bound ligand(s). This group includes yeast and liver alcohol dehydrogenase [25,26], citrate synthase [27,28], glutamate-oxaloacetate transaminase [29,30], glyceraldehyde-3-phosphate dehydrogenase [31,32] and 3-phosphoglycerate kinase [33,34].

In this paper, we show that LLPC can be used to detect conformational changes occurring upon binding of ligand(s) by enzymes. The changes in surface properties detected by LLPC are discussed and compared with results obtained by several indirect or direct methods used to study the conformation of biomolecules.

2. Experimental

2.1. Materials

LiParGel 650 was obtained from Merck AG (Darmstadt, Germany). Poly(ethylene glycol) 8000 (PEG) (M_r 6000 – 7500) was purchased from Union Carbide (New York, USA) while dextran T 40 (M_r 40 000) and dextran T 500 (M_r 500 000) were supplied by Pharmacia Biotech Norden AB (Uppsala, Sweden). The sodium salt of NADH as well as the free acid, NAD^+, were purchased from Boehringer Mannheim (Stockholm, Sweden). Pyridoxal 5-phosphate (PDP), oxaloacetic acid, α-methyl-aspartic acid, glucose, pyrazole, dimethyl sulfoxide (DMSO) and the lithium potassium salt of acetyl phosphate were supplied by Sigma (St. Louis, MO, USA), as were the sodium salts of 3-phosphoglycerate (3-PGA), oxamate, citrate and GDP. Horseradish peroxidase was obtained from Merck and whale skeletal myoglobin from Sigma. Yeast and horse liver alcohol dehydrogenase (YADH and LADH), pig heart citrate synthase (CS), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pig heart glutamate-oxaloacetate transaminase (GOT) and yeast 3-phosphoglycerate kinase (PGK) were purchased as purified preparations from Boehringer Mannheim. Purified pig heart mitochondrial and cytoplasmic isoforms of malate dehydrogenase (mMDH and sMDH), pig heart and muscle isoforms of lactate dehydrogenase (LDH-H_4 and LDH-M_4), and two forms of bakers yeast hexokinase (fraction I, HK (I) and II, HK (II), of Kaji et al. [35]) were supplied by Sigma.

2.2. Control of the homogeneity of the enzymes

All enzymes, except for mMDH, were homogeneous as determined by agarose gel electrophoresis (0.8% agarose in 75 m*M* barbital buffer, 2 m*M* calcium lactate, pH 8.6), HPLC–SE on an Ultropac TSK G3000 SW column (600×7.5 mm I.D., Pharmacia) in 10 m*M* sodium phosphate, 0.1 *M* NaCl, pH 7.4, and SDS–PAGE on a 5% polyacrylamide gel [36]. mMDH contained equal amounts of monomer and dimer.

2.3. Preparation of enzyme–ligand complexes

In order to remove possible traces of ligand(s), the enzyme preparations were extensively dialysed against solutions with a pH 0.5–1 units below their optimum pH prior to LLPC analysis, i.e. 50 m*M* sodium phosphate, 1 m*M* Zn^{2+}, pH 7.0 (LADH and YADH), 20 m*M* triethanolamine, 0.5 m*M* EDTA, pH 7.0 (GAPDH), 10 m*M* Tris–acetate, 0.2 m*M* PDP, pH 6.9 (GOT), 50 m*M* sodium phosphate, pH 7.0 [HK(I), HK(II), LDH-H_4, LDH-M_4, mMDH and sMDH], or 20 m*M* sodium phosphate, 0.5 m*M* EDTA, 1 m*M* Mg^{2+}, pH 6.8 (PGK), prior to LLPC analysis. The enzymatic activity (ΔA/min) of each dialysed preparation was determined prior to the LLPC analyses [37]. The activities of the enzymes were not, except in the case of CS (lowered activity), affected by the dialysis. CS was therefore not dialysed prior to the LLPC analyses.

Enzyme–ligand complexes were prepared by mixing 30–200 μg enzyme and 0.1–30 μg ligand in a suitable buffer at the optimum pH, i.e. 0.1 *M* sodium phosphate, 1 m*M* Zn^{2+}, pH 8.0 (LADH and YADH), 0.1 *M* Tris–HCl, pH 8.0 (CS), 0.1 *M* triethanolamine, 0.5 m*M* EDTA, pH 7.6 (GAPDH), 0.1 *M* potassium phosphate, 0.2 m*M* PDP, pH 7.4 (GOT), 50 m*M* sodium phosphate, pH 7.0 [HK (I), HK(II), LDH-H_4 and LDH-M_4], 0.1 m*M* sodium phosphate, pH 7.4 (mMDH and sMDH) or 0.1 *M* triethanolamine, 0.5 m*M* EDTA, 1 m*M* Mg^{2+}, pH 7.6 (PGK), making a final volume of 100 μl.

Enzyme–ligand complexes were prepared over a wide range of molar ratios but results are presented only for mixtures prepared at the lowest molar ratio of ligand to enzyme at which no further changes in the LLPC chromatograms could be observed (Table 1). The enzyme–ligand mixtures were first incubated for 15 min at 20.0°C. The concentration of PEG in the mixtures was then adjusted to that of the top phase by adding appropriate volumes of a 40% (w/w) PEG 8000 stock solution, whereafter top phase of the phase system was added, making a final volume of 150 μl. A 100-μl volume of this mixture was immediately applied to the LLPC column. All LLPC analyses were performed in such a way that free ligand did not interfere with the chromatogram of the complexes, i.e. either the free ligand could not be detected at 280 nm in the concentrations used here or it was well separated from the complexes. The elution profiles obtained for complexes made at identical molar ratios were reproducible both with respect to the proportions between the components and to the K_C values (see below).

2.4. Preparation of the two-phase systems

The experiments were, unless otherwise stated, performed in a 4.4% (w/w) PEG 8000/6.2% (w/w)

Table 1
Molar ratios of the enzyme–ligand complexes used in this investigation

Enzyme–ligand complexes		Molar ratio	Enzyme–ligand complexes		Molar ratio
LADH	+NAD^+ or NADH	1:2	HK (II)	+glucose	1:4
	+NAD^++pyrazole	1:2:2	LDH-H_4	+NAD^+ or NADH	1:10
	+NADH+DMSO	1:2:2		+NADH+oxamate	1:10:10
YADH	+NAD^+ or NADH	1:5	LDH-M_4	+NAD^+ or NADH	1:10
	+NAD^++pyrazole	1:5:5		+NADH+oxamate	1:10:10
	+NADH+DMSO	1:5:5	mMDH	+NAD^+ or NADH	1:4
CS	+oxaloacetate	1:2		+NAD^++citrate	1:4:10
GAPDH	+NAD^+ or NADH	1:4	sMDH	+NAD^+ or NADH	1:4
	+NAD^++acetyl phosphate	1:4:4		+NAD^++citrate	1:4:10
GOT	+α-methyl aspartate	1:2	PGK	+3-PGA or GDP	1:2
HK (I)	+glucose	1:4		+3-PGA+GDP	1:2:2

The complexes were prepared at the lowest molar ratio of ligand to enzyme where no further changes were observed in the LLPC chromatogram and the experiments were performed in such a way that free ligand did not interfere with the chromatogram of the complexes (see Experimental).

dextran T 500 two-phase system containing 50 m*M* sodium phosphate, 0.1 *M* NaCl and 0.1 *M* glycine at pH 7.0. The two-phase system was prepared by thoroughly mixing 110 g 40% (w/w in distilled water) PEG and 248 g 25% (w/w in distilled water) dextran with some of the additional water. Dry sodium chloride, glycine and sodium phosphate were added and pH was adjusted with 4 *M* HCl before the rest of the water was added until a 1000 g two-phase system was obtained. The system was equilibrated in a separation funnel at 20.0°C for 72 h and the clear phases were separated. In some experiments, a 5.2% (w/w) PEG 8000/10% (w/w) dextran T 40 two-phase system containing 50 m*M* sodium phosphate, 0.1 *M* NaCl and 0.1 *M* glycine at pH 7.0, prepared in the same way, was used.

2.5. Preparation of LLPC columns

The matrix, LiParGel 650, was equilibrated with the dextran-rich bottom phase (stationary phase) overnight at 20.0°C. The coated matrix was rinsed with the PEG-rich top phase (mobile phase) in order to remove excess stationary phase, suspended in mobile phase and poured into a thermostated (20.0°C) steel column (300×8.0 mm I.D.) with a filling reservoir. The column was first packed under gravitational sedimentation (≤30 h) and then at flow-rates ≤ 1.0 ml/min (≤25 MPa) using an HPLC pump (2248, Pharmacia) [18]. A 100-μl sample (see above) was applied to the column at a flow-rate of 0.12 ml/min. Eluates were continuously monitored at 280 nm.

2.6. Calculations

The performance and capacity of the LLPC columns were determined by daily application of two reference proteins, peroxidase and myoglobin (20–40 μg), to the columns. The partition coefficients of the reference proteins, K_{batch}, were determined in batch experiments [16]. $1/K_{batch}$ was used as K_C, the ratio of the concentration of a molecule in the two phases:

$$K_C = C_{\text{stationary phase}}/C_{\text{mobile phase}} \quad (1)$$

The volumes of the stationary and mobile phases, V_S and V_M, were calculated from the retention volumes for the references, V_R, using the relationship

$$V_R = V_M + K_C V_S \quad (2)$$

The plate number, N, was calculated from the peak width at half height (w_h) of the myoglobin peak according to

$$N = 5.54(V_R/w_h)^2 \quad (3)$$

The resolution of the peroxidase and myoglobin peaks, R_S, was calculated as

$$R_S = (\sqrt{N}/4)(k/(1+k))(\alpha - 1) \quad (4)$$

where k is the capacity factor ($k = (V_S/V_M)K_C$) and α is the ratio of the partition coefficients of the references ($\alpha = K_{\text{batch,peroxidase}}/K_{\text{batch,myoglobin}}$).

The parameters of the LLPC columns used were $V_S/V_M = 1.5\pm0.2$, $V_S/V_C = 0.40\pm0.04$, $V_M/V_C = 0.27\pm0.04$, $N = 650\pm75$ and $R_S = 3.4\pm0.3$ in the dextran 500 system, and $V_S/V_M = 0.8\pm0.1$, $V_S/V_C = 0.29\pm0.02$, $V_M/V_C = 0.36\pm0.02$, $N = 500\pm50$ and $R_S = 3.5\pm0.3$ in the dextran 40 system, where V_C is the column volume. The variation in column parameters with repeated use/storage is exemplified by the ratio of V_S/V_M in Fig. 1. V_S/V_M was found to be constant and the same column could be used for at least one year. In order to facilitate the comparison of chromatograms obtained from columns with different parameters, retention volumes were expressed as K_C, according to Eq. 2. The relative standard deviation [100(standard deviation/mean)] of K_C was

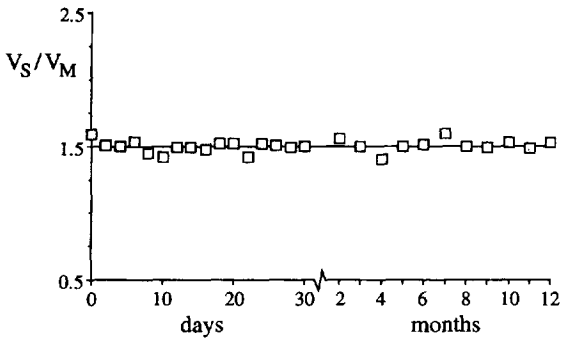

Fig. 1. Variations in the column parameters, exemplified by the volume ratio of the stationary phase (V_S) to the mobile phase (V_M), for a LLPC column in the dextran 500/PEG 8000 system run 8 h/day, 0–5 days per week, during a period of 12 months.

≤ 2% and the 95% confidence limit of K_C was ≤ ±0.05. All LLPC analyses were run three to eight times.

2.7. Statistical analysis of data

A two-tailed Mann–Whitney U-test was used to determine whether the difference between two K_C values was statistically significantly at the 95% significance level ($P = 0.05$).

3. Results

The ability of liquid–liquid partition chromatography (LLPC) in aqueous PEG/dextran two-phase systems to detect conformational changes was examined using a set of well-characterized enzymes. The LLPC analyses of HK and LDH have been published but are included here in order to facilitate a direct comparison of the results.

The partition properties of some dehydrogenases, determined before and after binding of ligand(s), are shown in Table 2. Except for GAPDH, the apoenzymes were fractionated into two components by LLPC, in spite of the fact that they were homogeneous both with respect to net charge and size distribution (estimated by agarose gel electrophoresis, HPLC–SE and SDS-PAGE). For YADH, LDH-H_4, LDH-M_4 and sMDH (the minor component constituted ≥20%), the two components were collected and reinjected separately. Two-peak chromatograms similar to those of the original preparations were obtained for each of the two the components of YADH, LDH-H_4 and LDH-M_4 (data not shown). For the two sMDH components, a single component with a K_C value identical to that of the applied one was obtained (see Fig. 2A–C). Thus, the results demonstrated an equilibrium between two components with different surface properties for YADH, LDH-H_4 and LDH-M_4 but not for sMDH.

The binding of NAD^+ or NADH by the dehydrogenases is shown in Table 2. It should be noted that the LLPC analyses of complexes were performed in

Table 2
LLPC of some dehydrogenases before and after binding of ligand(s)

Enzyme	K_C				
	Apoenzyme	Binary complexes		Ternary complexes	
		E–NAD^+	E–NADH	E–NAD^+–S_1	E–NADH–S_2
LADH	1.56 (10%)[a]	1.56	1.56	1.56	1.56
	1.64 (90%)				
YADH	1.41 (75%)	1.53	1.48	1.53	1.48
	1.54 (25%)				
GAPDH	1.37	1.46	1.46	1.46	1.46
LDH-H_4 [b]	1.7 (80%)	1.7 (65%)	1.7 (80%)	–	1.7 (80%)
	2.0 (20%)	2.0 (35%)	2.0 (20%)		2.0 (20%)
LDH-M_4 [b]	1.7 (20%)	1.7 (20%)	1.7 (40%)	–	2.2
	2.2 (80%)	2.2 (80%)	2.2 (60%)		
mMDH	1.57 (10%)	1.57 (10%)	1.57 (10%)	1.66	–
	1.75 (90%)	1.75 (90%)	1.75 (90%)		
sMDH	1.31 (45%)	1.31 (45%)	1.31 (45%)	See Fig. 1	–
	2.11 (55%)	2.11 (55%)	2.11 (55%)		

The complexes were prepared at the molar ratios of ligand to enzyme given in Table 1; 50–200 μg enzyme and 0.3–30 μg ligand were applied to the LLPC column (300 × 8 mm I.D.; $V_S/V_M = 1.5 \pm 0.2$; $N = 650 \pm 75$; $R_S = 3.4 \pm 0.3$) in 100 μl mobile phase and the flow-rate was 0.12 ml/min. The retention volumes are expressed as partition coefficients (K_C) according to Eq. 2. The relative standard deviation of K_C was ≤ 2%. The 95% confidence limits of the K_C values were ±0.01 (LADH and YADH), ±0.02 (GAPDH, mMDH and sMDH) and ±0.05 (LDH-H_4 and LDH-M_4).

S_1: substrate analogue 1; pyrazole (LADH and YADH); acetyl phosphate (GAPDH); citrate (mMDH and sMDH).

S_2: substrate analogue 2; DMSO (LADH and YADH); acetyl phosphate (GAPDH); oxamate (LDH-H_4 and LDH-M_4).

[a] Proportion of each component.

[b] Data adopted from Ref. [22].

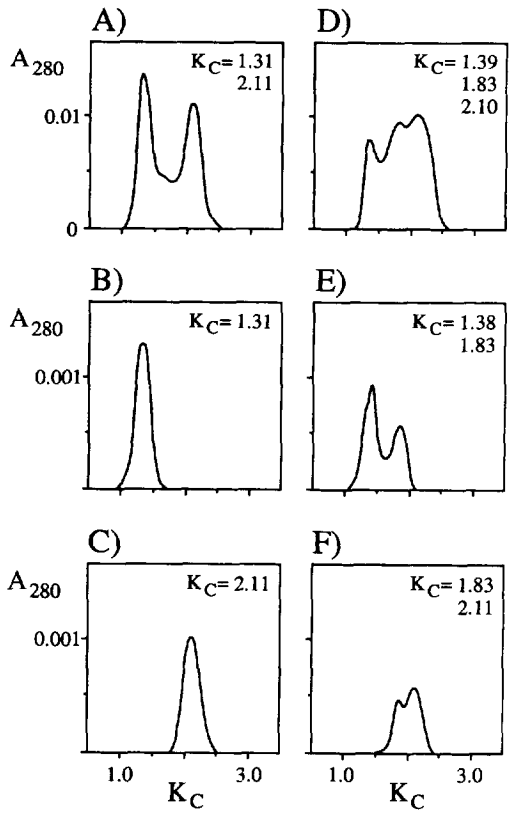

Fig. 2. LLPC of sMDH and sMDH–NAD^+–citrate complexes prepared at the molar ratio given in Table 1. (A) sMDH. (B) Reinjection of the first component (peak I) of sMDH. (C) Reinjection of the second component (peak II) of sMDH. (D) sMDH–NAD^+–citrate complexes. (E) sMDH(peak I)–NAD^+–citrate complexes. (F) sMDH(peak II)–NAD^+–citrate complexes. 55 μg enzyme and 0.3–3 μg ligand were applied to the LLPC column (300 × 8 mm I.D.; $V_S/V_M = 1.5 \pm 0.2$; $N = 650 \pm 75$; $R_S = 3.4 \pm 0.3$) in 100 μl mobile phase and the flow-rate was 0.12 ml/min. The retention volumes are expressed as partition coefficients (K_C) according to Eq. 2. The relative standard deviation of K_C was $\leq$ 2%. The 95% confidence limit of K_C was $\pm$ 0.02.

such a way that free ligand did not interfere with the chromatogram of the complexes (see Experimental). The K_C values of mMDH and sMDH were not affected by the binding of either NADH or NAD^+ (Table 2). In the case of the LDHs, changes in the chromatograms were only observed upon binding of NAD^+ by LDH-H_4 and NADH by LDH-M_4 (Table 2). However, the partition properties of LADH and GAPDH were affected upon binding of both NADH and NAD^+ (Table 2). Both LADH and GAPDH bound cofactor in such a way that the binary complexes, E–NADH and E–NAD^+, had identical K_C values. The K_C of YADH was also affected upon binding of NAD^+ or NADH (Table 2), but the YADH–NADH and YADH–NAD^+ complexes had different K_C values, i.e. the cofactors seemed to induce different conformational changes in the enzyme.

The conformations induced by cofactor-binding to LADH, YADH and GAPDH were retained in the ternary complexes with substrate analogues (Table 2). This was also the case for LDH-H_4–cofactor complexes, while the LLPC chromatogram for LDH-M_4–NADH complexes was affected by the addition of oxamate (Table 2).

The formation of ternary MDH–NAD^+–citrate complexes also caused a shift in the elution profiles (Table 2 and Fig. 2). Only one homogeneous peak with a K_C value between those of the two components of the free enzyme was observed for the ternary complexes of mMDH (Table 2). In contrast, the sMDH–NAD^+–citrate complexes were eluted as three components (Fig. 2D). It should be noted that ternary complexes formed by either of the two components in free sMDH, peaks I and II (Fig. 2A), were eluted as two peaks (Fig. 2E,F). It is interesting to note that peaks I and II, in spite of having quite different surface properties (K_C values) also formed complexes which had identical K_C values (1.83) (see Fig. 2E,F).

In an attempt to improve the sensitivity and resolution of the method even further, a dextran 40/PEG 8000 two-phase system was used instead of the dextran 500/PEG 8000 system. The elution volumes and, thereby, the numerical differences between the K_C values increased. The relative differences between the K_C values were not affected, however. For instance, the two components in mMDH were eluted with K_C values of 4.16 and 4.67 in the dextran 40 system compared with 1.57 and 1.75 in the dextran 500 system. Employing the dextran 40 system did not provide any new information and the time required for analysis increased (from 2 to more than 4 h). Therefore, the dextran 500/PEG 8000 system was used throughout the rest of this study.

The binding of ligand(s) by some transferases and one lyase was then examined (Table 3). The

Table 3
LLPC of four transferases and one lyase before and after binding of ligand(s)

Enzyme	K_C	
	Apoenzyme	Binary complexes E–S
HK(I)[a]	1.69 (45%)[b]	1.69 (15%)
	1.85 (55%)	1.85 (85%)
HK(II)	1.69 (45%)	1.69 (15%)
	1.85 (55%)	1.85 (85%)
PGK	1.83	1.83
GOT	1.39	1.46
CS	1.93	1.77

The complexes were prepared at the molar ratios of ligand to enzyme given in Table 1; 30–120 μg enzyme and 0.1–20 μg ligand were applied to the LLPC column (300 × 8 mm I.D.; $V_S/V_M = 1.5\pm0.2$; $N = 650\pm75$; $R_S = 3.4\pm0.3$) in 100 μl mobile phase and the flow-rate was 0.12 ml/min. The retention volumes are expressed as partition coefficients (K_C) according to Eq. 2. The relative standard deviation of K_C was ≤ 2%. The 95% confidence limits of the K_C values were ±0.01 (PGK, CS and GOT) and ±0.03 [HK(I) and HK(II)].
S: substrate/substrate analogue; glucose (HK(I) and HK(II)); 3-PGA (PGK); oxaloacetate (CS); α-methyl-aspartate (GOT).
[a] Data adopted from Ref. [22].
[b] Proportion of each component.

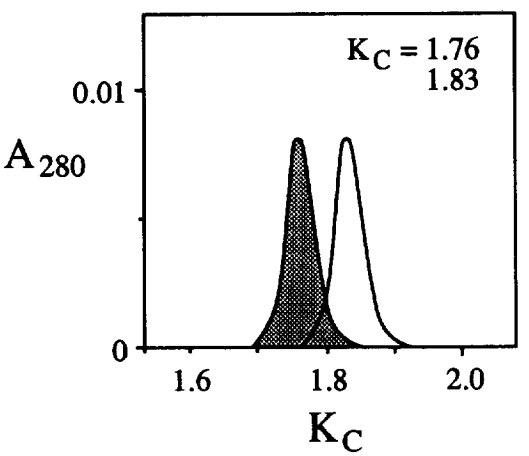

Fig. 3. LLPC of PGK and PGK–ligand complexes prepared at the molar ratios given in Table 1. □, PGK, PGK–GDP binary complexes or PGK–3-PGA binary complexes. Shaded area represents PGK–GDP–3-PGA ternary complexes. 100 μg enzyme and 0.9–2 μg ligand were applied to the LLPC column (300 × 8 mm I.D.; $V_S/V_M = 1.5\pm0.2$; $N = 650\pm75$; $R_S = 3.4\pm0.3$) in 100 μl mobile phase and the flow-rate was 0.12 ml/min. The retention volumes are expressed as partition coefficients (K_C) according to Eq. (2). The relative standard deviation of K_C was ≤ 2%. The 95% confidence limit of K_C was ± 0.01.

apoenzymes were eluted as single homogeneous peaks by LLPC and they were also homogeneous with respect to net charge and size distribution (estimated by agarose gel electrophoresis, HPLC–SE and SDS–PAGE). In contrast, the HKs were fractionated into two components by LLPC (Table 3) and re-chromatographing experiments showed that there was an equilibrium between these two components with different surface properties (data not shown).

The binary complexes formed by these enzymes were also eluted as single homogeneous peaks (Table 3 and Fig. 3). However, the CS complex shifted towards a smaller K_C whereas GOT, as the HKs, shifted towards larger K_C values (Table 3). A shift in the elution profile of PGK was detected by LLPC only upon the formation of ternary complexes (Fig. 3), indicating that both ligands were required to induce detectable conformational changes.

4. Discussion

The partitioning of a protein molecule in aqueous PEG–dextran systems is related to its overall surface properties [4–8,16]. The method is mild so the conformation of the partitioned molecule is not likely to be disturbed [5,8]. In this paper, the LLPC analyses of the enzyme–ligand complexes were performed in such a way that free ligand did not interfere with the chromatogram of the complexes. Since ligands which bind to the cleft/crevice of the active site of the enzymes used here are 'not' solvent exposed [1,38], observed changes in elution profiles for the enzyme–ligand complexes could not be related to the surface properties of the bound ligands. Hence, changes observed in LLPC chromatograms following binding of ligand are likely to reflect changes in the overall surface of the enzyme.

Several techniques, such as X-ray crystallography, NMR, small-angle neutron scattering, small-angle X-ray scattering and fluorescence spectroscopy, have shown that the enzymes used in this study all can exist in an open or closed conformation depending on the absence/presence of suitable ligands [1,23–34,38–41]. These experiments have also shown that each of the enzymes undergo one of two major types of conformational changes upon binding of ligand. In

the first group, including MDH and LDH, the active site is situated within a subunit, and the site is open or closed depending on the conformation of a flexible loop [23,24,38,42]. The flexible loop protrudes from the molecular surface in the open form and is folded down over the active site in the closed form [42]. In the second group, including the remaining enzymes, the enzymes have an intersubunit active site and the access to this site is dependent on the orientation of the two domains [1,25–34]. Upon binding of ligand(s), one of the domains rotate relative to the other to close the active site over the bound ligand(s) [1,25–34]. In order to adopt the correct conformation, additional minor changes occur at the interfaces between the domains and the subunits, within the domains and/or on the surface of the enzyme [1,25–34].

Using LLPC, we have previously demonstrated that there are two forms of unliganded LDH with different surface properties that were in equilibrium with each other [22]. These findings agree with the results of electron spin resonance experiments, showing that unliganded LDH occurs in an equilibrium between the open and closed form in solution [43]. In the case of MDH, there are no data available concerning the conformation of the apoenzyme in solution but X-ray crystallography has shown that it may adopt the open form [23,44]. However, there are reports of two forms of sMDH with different abilities to bind NADH [45] or to be phosphorylated [45]. Indeed, sMDH was fractionated into two distinct components by LLPC which were not in equilibrium with each other (see Fig. 2A–C). Hence, LLPC is a simple and rapid method to detect and even separate different forms of enzymes in a way that may not be achieved by other techniques.

In the second group of enzymes, a conformational equilibrium has been suggested for unliganded LADH, although X-ray crystallography has shown that the open form is favoured [46]. We found that LADH was eluted as one major component and one minor one by LLPC (Table 2). YADH was also fractionated into two components by LLPC (Table 2) which were in equilibrium. These results agree with those previously obtained for HK [22]. Thus, LLPC can be used to detect and even separate different conformational forms of enzymes.

As far as we know, there are no reports of two conformational forms of either unliganded CS, GAPDH, GOT or PGK in solution. The available data indicate that these apoenzymes exclusively adopt the open form [1,27–34]. Accordingly, they were also eluted as single homogeneous peaks by LLPC (Tables 1–3) which then may be suggested to represent the open form of the enzymes.

The binding of ligand by MDH is not as thoroughly studied as that by LDH. However, in both cases, X-ray crystallography has shown that the open form is favoured after binding of cofactor [23,38,42]. Subsequent binding of substrate shifts the conformation to the closed form [24,38,39]. A shift in the elution profiles was indeed observed when MDH–NAD^+ complexes bound citrate and when LDH-H_4–NADH complexes bound oxamate (Table 2 and Fig. 2). Thus, our results showed that LLPC can be used to detect the transition between open and closed conformation induced by ligand binding.

For LADH, YADH, CS, GAPDH, GOT, the HKs and PGK, the closed conformation is induced upon binding of cofactor and/or substrate [25–34,40,41]. Table 2 and Table 3 demonstrate that these changes may also be detected by LLPC. In this context, it may be of interest to note that the surface properties of LADH and YADH, which have similar substrate specificities [47], were similar before binding of cofactor and almost identical for the enzyme–cofactor complexes. Hence, the surface properties responsible for the specificity of the enzymes may dominate over other surfaces of the molecules when the molecules are dissolved in aqueous solution. We have recently published results strongly indicating such a dominance of ligand-binding sites also for antibodies [20].

We have also previously shown that LLPC indicate that antigen-binding induces changes in the surface properties of monoclonal antibodies [22]. Another interesting observation is the fact that hapten–antibody complexes display similar surface properties even if the complexes are prepared at different molar ratios [22], i.e. one unliganded and one liganded form of the antibody is observed. Thus, antibodies as well as enzymes may occur in two different forms which are detected by LLPC.

The observed differences between the K_C of the apoenzyme and that of the enzyme–ligand complex were generally relatively small (<20%). The mag-

nitude of the conformational changes occurring in the first group of enzymes may also be regarded as being small (confined to the movement of a loop). In the second group of enzymes, the conformational changes include a domain rotation and various rearrangements. Still, the changes on the surfaces of the enzymes may, except for the closure of the active site, be regarded as being small. However, a general relationship between the magnitude of a conformational change and the observed difference in K_C cannot be established. All indirect methods used to analyse flexible biomolecules in solution detect only net changes and conformational changes may then occur that partly cancel out each other. Neither is it possible to relate a decrease or increase in K_C to a certain type of conformational change. Such information is still not available even if the PEG/dextran two-phase systems have been successfully used for many years to separate and analyse biomolecules. Rather than being seen merely as a source of frustration, the complexity of the parameters determining K_C may be regarded as the beauty of the method in providing a selectivity which may not be obtained by any other method or combination of methods. LLPC is a simple and sensitive method to obtain rapid information about conformational changes occurring upon binding of ligand(s) by enzymes in solution.

Abbreviations

CS	citrate synthase
DMSO	dimethyl sulfoxide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GOT	glutamate-oxaloacetate transaminase
HK (I)	hexokinase (fraction I of Kaji)
HK (II)	hexokinase (fraction II of Kaji)
LADH	liver alcohol dehydrogenase
LDH-H_4	heart lactate dehydrogenase
LDH-M_4	muscle lactate dehydrogenase
LLPC	liquid–liquid partition chromatography
mMDH	mitochondrial maltate dehydrogenase
sMDH	cytoplasmic malate dehydrogenase
PDP	pyridoxal 5-phosphate
PEG	poly(ethylene glycol)
3-PGA	3-phosphoglycerate
PGK	3-phosphoglycerate kinase
YADH	yeast alcohol dehydrogenase

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