STABILITY AND PARTITION OF ALCOHOL DEHYDROGENASE IN POLYETHYLENE GLYCOL/PHOSPHATE AFFINITY AQUEOUS TWO-PHASE SYSTEMS USING A TRIAZINE DYE

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The conformational changes in baker's yeast alcohol dehydrogenase (ADH) caused by the interaction of the affinity ligand Procion Blue MX-R, is investigated at various pH values in polyethylene glycol/potassium phosphate aqueous two-phase systems (ATPS). The affinity partitioning of ADH is effectively carried out at neutral pH without any damage to the structural conservation. However, the protein becomes unstable at alkaline pH and is further denatured in the presence of Procion Blue MX-R. The oligomeric ADH is completely dissociated to the monomer, and the tertiary structure of the subunits is affected by the interaction of the Blue ligand. Hydrophobic residues such as tryptophan initially buried inside may become accessible to a solvent and make denatured ADH more hydrophobic than in the native state. This affinity ligand promotes partition of the denatured fraction to the top phase, and of the active fraction to the bottom phase, at alkaline pH. The analysis of partitioning behavior in ATPS could be utilized for sensitive detection of the conformational changes in protein structure.

Introduction

Affinity partition is a technique based on the biospecific interaction between proteins and affinity ligands. A strong interaction gives rise to stable ligand-protein complexes and thus improves the partition of the proteins to the ligand-containing phase. In work previously reported,^{1,4)} triazine dyes such as Procion Blue MX-R and Procion Olive MX-G were found to be good affinity ligands for baker's yeast alcohol dehydrogenase (ADH) and some other dehydrogenases in polyethylene glycol (PEG)/potassium phosphate systems as well as in PEG/dextran systems. Addition of the dyes to PEG/phosphate systems at pH 7.5 increases the partition coefficients of the dehydrogenases, defined as the ratio of the enzyme activities in the top phase to that in the bottom phase. Yeast ADH is, however, labile and its thermal stability changes with pH.⁷⁾ In the present work, the stability of ADH in PEG/phosphate aqueous two-phase systems and the conformational changes of the enzyme resulting from the binding of the affinity ligand Procion Blue MX-R have been investigated under the conditions of neutral and alkaline pH. The influence of the protein structure on partitioning behavior has also been studied.

1. Experimental

1.1 Reagents

Yeast alcohol dehydrogenase (EC 1.1.1.1, MW = 140 kDa, pI = 5.4), papain (EC 3.4.22.2, MW = 23 kDa, pI = 8.7) and chymopapain (EC 3.4.22.6, MW = 35 kDa, pI = 10.1–10.4) from papaya were supplied by Sigma Chemical Co. (U.S.A.). Procion Blue MX-R (MW = 681.4) was obtained from Serva Feinbiochemica Heidelberg (New York). The coenzyme and substrate, β -nicotinamide-adenine dinucleotide (β -NAD⁺) and ethanol, used for the measurement of ADH activity, were supplied by Oriental Yeast Co. (Japan) and Wako Pure Chemical Industries, Ltd. (Japan), respectively. Polyethylene glycol 1540 (PEG, MW = 1500 Da), PEG4000 (MW = 3000 Da), potassium phosphate (phosphate) and the PEG4000 derivative of Procion Blue MX-R (Blue-PEG) were the same as reported previously.^{1,4)} Other chemicals were of analytical grade.

1.2 Procedures

Aqueous two-phase systems used were composed of PEG1540 (12 w/w%) and phosphate (12 w/w%), or PEG4000 (11, 12 w/w%) and phosphate (11, 12 w/w%) (total 8 g). PEG-bound Blue or non-bound (free) Blue was used as an affinity ligand. The concentration of protein partitioned is 0.13–0.16 mg/g-system. It was measured by a Shimadzu spectrophotometer UV-265FW. The activity of ADH was determined by measuring the changes in β -NADH absorbance at 340 nm in the following enzymatic

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reaction:²⁾

One unit of ADH activity was defined as the amount of enzyme that oxidized 1.0×10^{-6} mol of ethanol to acetaldehyde per minute in 0.01 M pyrophosphate buffer at pH 8.8 and 25°C. The partition coefficient of active ADH, K_{ADH} , and that of total protein, K_{pr} , were defined as the ratio between ADH activity (U/ml) and total protein concentration (mg/ml), respectively. The binding of PEG-bound Blue or free Blue to ADH was evaluated by the inhibition constant, K_i , which is the dissociation constant of the inactive ADH-Blue complex. The value was determined by Dixon and Lineweaver-Burk plots as described in the previous paper.¹⁴⁾ The hydrophobic factor of a phase system (HF) and that of a solute (HFS) were analyzed to express their hydrophobicities as described before.^{8,9)}

The molecular weights of ADH and its derivatives were analyzed by size exclusion HPLC equipped with a Shimadzu Shim-pack Diol-150 column, using UV-265FW as a detector and followed at 280 nm. The mobile phase was 0.01 M phosphate buffer (pH 7) with 0.2 M Na_2SO_4 . Absorption spectra of protein solutions in the region of 250 to 310 nm contain contributions from phenylalanine, tyrosine and tryptophan.¹⁵⁾ Interaction of the residues in the protein molecules with solvents appears in the difference spectra.^{4,5,11)} Therefore, changes in the spectra can account for general configurational changes in proteins due to denaturation. In this work, the difference extinction coefficient ($\Delta\epsilon$) of tryptophyl residues in ADH was measured at 290 nm with a Shimadzu UV-265FW spectrophotometer. Dimethyl sulfoxide was used in the range less than 40 v/v%, since higher solvent concentration may result in denaturation of ADH. An ADH aqueous solution with the same pH and the same concentration of dye was used as a blank.

Circular dichroism (CD) measurement was carried out with a Jasco spectropolarimeter, Model J-500A. The results were expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{\text{obsd}}/(lc)$, where θ_{obsd} is the observed ellipticity in degrees, c the concentration of residue in mols per liter, and l the length of the light path in centimeters. The residue number of ADH monomer is about 347.⁶⁾ The pH value of systems for partition was adjusted with phosphate buffer and that of protein solutions for ultraviolet absorption and CD measurement with NaOH or HCl.

2. Results and Discussion

2.1 Partition of total protein and ADH

The effect of the affinity ligand, Blue or Blue-PEG,

Fig. 1. Effect of Blue concentration in the top phase of the systems on K_{ADH} and K_{pr} at neutral pH

Fig. 2. Effect of Blue concentration in the top phase of the system on K_{ADH} and K_{pr} at alkaline pH

on the partition of ADH at pH 7.0–7.5 is shown in Fig. 1. The PEG1540 (12 w/w%)/phosphate (12 w/w%) system with free (non-bound) Blue and the PEG4000 system of the same concentration with Blue-PEG were used. The partition coefficients of ADH and total protein, K_{ADH} and K_{pr} , at neutral pH increase with addition of Blue or Blue-PEG. This is due to the affinity and hydrophobic interaction between ADH and Blue, as described previously.¹⁴⁾ The results obtained for the PEG4000 (11 w/w%)/phosphate (11 w/w%) system at pH 9.4 are shown in Fig. 2. The partition coefficient of ADH at this pH is much lower than that of total protein, and decreases slightly with increase in the concentration of Blue-PEG. The total activity yield in the system at neutral pH without the Blue ligand was around 80%, whilst that at pH 9.4 was 26% and further decreased to 2% when the concentration of Blue-PEG in the top phase increased to 8.3 mM. The yield of

total protein at pH 9.4 still showed the same tendency as at neutral pH. The inhibition constants of ADH by the Blue ligand, K_i , in the PEG1540/phosphate system and those by Blue-PEG in the PEG4000/phosphate system are shown in Table 1. As found in the previous study,¹⁴⁾ phosphate is a non-inhibitor for ADH and the inhibition constants in the presence of the two phases are similar to those in the absence of the system. Additionally, both Blue and Blue-PEG show one-sided partition in the top phase of PEG/phosphate systems. The chemical modification of Blue to PEG and the molecular weights of phase-forming PEG are unlikely to influence the affinity between Blue and ADH. Therefore, Blue and Blue-PEG are expected to be effective for ADH to the same extent in systems with various PEG molecular weights and compositions of PEG and phosphate.

2.2 pH effect on ADH activity

The effect of pH on ADH activity is shown in Fig. 3. When ADH solutions with the same neutral pH were analyzed in enzymatic assay buffers of various pH values, an apparent maximum of ADH activity was observed at pH 8.1. This value is close to the traditional condition, pH 8.8, commonly used for the enzymatic assay.²⁾ However, when the pH of ADH solutions had been changed with NaOH or HCl before use, the activity was greatest at neutral pH and decreased significantly at alkaline pH, as reported by Sund and Theorell.¹³⁾ In this case, all the samples were analyzed at a pH of 8.8, and thus the decrease in ADH activity at alkaline pH was likely to be caused by destabilizing changes in ADH structure. This corresponds to the pH effect on ADH thermal stability obtained by Kennedy *et al.*⁷⁾

2.3 Changes in ADH quaternary structure

Alcohol dehydrogenase solutions (8.1×10^{-7} M) in the absence and presence of the Blue ligand were analyzed by chromatography. The results at pH 6.8 and pH 9.2 are compared in Fig. 4. The active ADH at pH 6.8 containing no Blue gives a single peak at a retention time of 13 min (the left side in Fig. 4a). This corresponds to tetrameric ADH with a molecular weight of 140 kDal. The influence of contaminating proteins is negligible. The same result is obtained for samples standing at 4°C for two hours and also for those containing Blue in the range of $5.3\text{--}53 \times 10^{-6}$ M (the left side in Fig. 4b–d). However, when the pH of the ADH solution is adjusted to 9.2, the peak of tetrameric ADH splits and an additional peak is detected at longer retention time (the right side in Fig. 4a). The peak at longer retention time corresponds to a new component with a lower molecular weight, which is a dissociation product of ADH denatured at alkaline pH. The intensity of the new fraction relative to that of tetrameric ADH increases after two

Table 1. Inhibition constants of ADH by Blue and Blue-PEG4000 in PEG1540/phosphate and PEG4000/phosphate systems

$K_i \times 10^6$ [M]	In the simple buffer	In the presence of the top phase	In the presence of the bottom phase
Blue (PEG1540/KPi)	128–246	4–95	81–270
Blue-PEG (PEG4000/KPi)	48–268	128	134–176

Compositions of the systems: PEG 12%, Phosphate (KPi) 12%; pH: 7.0–7.5.

Fig. 3. Effect of pH on the relative activities of alcohol dehydrogenase

(a) solutions with different pH analyzed at pH 8.8; (b) identical solutions with pH 6.8 analyzed at different pH

hours standing (the right side in Fig. 4b), indicating that the dissociation in ADH proceeds during the preparation. It also shows that a small amount of Blue results in almost complete and immediate peak-shift from the short retention time to the long retention time. The latter peak has a similar retention time. The latter peak has a similar retention time to that of chymopapain, whose molecular weight is 35 kDal, indicating that the dissociated component may be monomeric ADH. Consequently, the quaternary structure of ADH is disrupted by binding of the Blue ligand at alkaline pH, whilst it is conserved well at neutral pH.

2.4 Changes in ADH tertiary structure and secondary structure

The effect of the Blue ligand on the difference extinction coefficients ($\Delta\epsilon$, at 290 nm) of tryptophyl residues in ADH is shown in Fig. 5. The difference extinction coefficients are linearly correlated with

Fig. 4. Chromatogram of alcohol dehydrogenase (a) [Blue]=0 M, 10 min standing after solubilization; (b) [Blue]=0 M, 2 hr standing at 4°C; (c) [Blue]= 5.3×10^{-6} M, 10 min standing after solubilization; (d) [Blue]= 5.3×10^{-5} M, 10 min standing after solubilization

Fig. 5. Influence of coexistent Blue on the difference extinction coefficient at pH 9.4
●, pH 6.8, [Blue]=0 M; ▲, pH 9.4, [Blue]=0 M; ■, pH 9.4, [Blue]= 1.3×10^{-6} M; ▼, pH 9.4, [Blue]= 2.7×10^{-6} M

dimethyl sulfoxide concentration up to 40 v/v%. The slope of the line corresponds to the interaction between exposed tryptophan and the solvent. The slope is the lowest for active ADH at pH 6.8. It increases with the change of pH to 9.4 and further with increase in the concentration of coexistent Blue at pH 9.4. Therefore, tryptophyl residues in ADH are exposed to the solvent to a higher extent at alkaline pH. The same tendency was observed at a Blue concentration of 5.3 – 53×10^{-6} M. The tertiary structure of ADH subunits may be disturbed to some extent when the

Fig. 6. CD spectra of alcohol dehydrogenase

Fig. 7. Comparison of hydrophobicities of alcohol dehydrogenase with those of papain and chymopapain

oligomeric ADH is dissociated to the monomer.

Figure 6 shows the CD spectra of ADH under conditions similar to those obtained for changes in tertiary structure. The ellipticities in the region of 190–250 nm are nearly identical at neutral and alkaline pH, and show little change with addition of the Blue ligand at alkaline pH. The result at 222 nm becomes a little more negative when ADH is denatured by increasing both pH and the concentration of the Blue ligand at alkaline pH. This indicates that the secondary structure of dissociated ADH is stable even in the presence of Blue at alkaline pH.

2.5 Changes in the hydrophobicity of ADH with denaturation

The surface hydrophobicity of active ADH is compared with those of papain and chymopapain in **Fig. 7**. The hydrophobicities of the PEG/dextran systems, HF , were controlled by changing the molecular weights and compositions of PEG and dextran in the systems. The logarithmic partition coefficients of the proteins at pI are plotted as linear

Table 2. The proportion of hydrophobic amino residues in ADH, papain and chymopapain

	Hydrophobic amino residues								Total amino residue number	Proportion (%)
	Ala	Val	Leu	Ile	Met	Pro	Phe	Trp		
ADH*	35	36	24	20	6	13	8	5	347	42
Papain	14	18	11	12	0	10	4	5	212	35
Chymopapain	19	21	15	9	1	14	7	4	284	32

* Monomer of yeast alcohol dehydrogenase.

function of *HF*. The larger the slope of the line, the more hydrophobic the protein becomes.^{8,9)} Values of the surface hydrophobic factor of solutes (*HFS*) of the proteins are determined as -243 kJ/mol, -46 kJ/mol and 25 kJ/mol for ADH, chymopapain and papain respectively. ADH is the least hydrophobic in the aqueous two-phase systems.

The proportions of hydrophobic amino acid residues in these three proteins^{1,3,6)} are shown in **Table 2**. Proteins containing relatively more hydrophobic residues are likely to have more hydrophobic surfaces, as the relationship between papain and chymopapain. The proportion of hydrophobic amino residues in ADH is the highest among these three proteins, but it is found to be the least hydrophobic. This indicates that hydrophobic residues in ADH are comparably concentrated at the interior of the molecules. According to Jornvall,⁶⁾ many residues are unevenly distributed in the primary structure of ADH, and all the tryptophan and most of the proline are distributed in the N-terminal half. Hydrophobic regions are common and the following three long segments are found to involve no charged residues, *i.e.* (1) from His (171) to Ala (190), (2) from Ala (262) to Ala (274) and (3) from Ser (287) to Asn (297), which are poorly soluble in aqueous solution. These structural characteristics are particularly important to the folding of ADH and to the stabilization of such a large tetrameric molecule in aqueous environment. Once the protein is denatured and the conformational changes occur, those hydrophobic residues initially buried inside may become accessible to a solvent and thus make the surface of the protein more hydrophobic than in the native state.

The hydrophobic interaction of Blue with the whole ADH molecule becomes more important than its group-specific interaction with the active site of ADH. This results in an increase in partition coefficient of total protein concentration. Denatured ADH is expected to partition in the top phase prior to active ADH, and the denaturation of ADH may be enhanced in the top phase. Since partitioning behaviour is very susceptible to the conformational characteristics of proteins, aqueous two-phase partition can also be applied to the detection of structural changes in

proteins.

Conclusion

The effect of pH conditions on the effective partition of baker's yeast ADH in the PEG/phosphate aqueous two-phase systems was studied, using a triazine dye, Procion Blue MX-R, as an affinity ligand. Structural changes in the enzyme caused by the interaction of the affinity ligand were investigated, with the following results.

(1) Alcohol dehydrogenase is stable at neutral pH and shows no structural changes in the presence of the Blue ligand. The dye can successfully be used at neutral pH to improve the extraction of active ADH to the top phase of PEG/phosphate systems.

(2) The quaternary structure of the enzyme is completely disrupted and the tertiary structure is affected at alkaline pH resulting from the interaction of the Blue ligand, whilst the secondary structure remains well conserved.

(3) Alcohol dehydrogenase is hydrophilic in the native state, but becomes much more hydrophobic when denatured by the Blue ligand at alkaline pH. Addition of the Blue ligand to the PEG/phosphate systems at alkaline pH increases the partition coefficient of total protein concentration, whilst decreasing that of ADH activity.

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Nomenclature

<i>HF</i>	= hydrophobic factor of two-phase system	[mol/kJ]
<i>HFS</i>	= hydrophobic factor of solutes (protein)	[kJ/mol]
K_{ADH}, K_{pr}	= partition coefficient of ADH activity and that of total protein concentration	[—]
K_i	= inhibition constant (dissociation constant) of enzyme-inhibitor complex	[M]
PB_u	= Blue concentration in the top phase	[M]
<i>RA</i>	= relative activity of ADH	[%]

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