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SPECTRAL STUDIES OF THE INTERACTION OF *ESCHERICHIA COLI* ALKALINE PHOSPHATASE WITH 4-(4-AMINOPHENYLAZO)-PHENYLARSONIC ACID

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Summary

Escherichia coli alkaline phosphatase (EC 3.1.3.1) is reversibly inhibited by a variety of phenylarsonic acids, including some *N*-haloacetylated derivatives. The inhibition is of the competitive type, and K_i values are reported.

The action on the enzyme of one of the arsonate inhibitors, the azo dye, 4-(4-aminophenylazo)-phenylarsonic acid was studied in detail, using spectrophotometric and kinetic methods. The azo dye binds more strongly to *E. coli* alkaline phosphatase than do the other arsonates. Spectrophotometric titration indicates the presence of a single, strong dye-binding site on the enzyme dimer molecule in the concentration range covered. In 0.1 M Tris · HCl buffer pH 8.0, 25°C K_{diss} for the dye · enzyme complex is $1.50 \cdot 10^{-5}$ M as determined by spectrophotometric titration. This value is in good agreement with the $K_i = 1.30 \cdot 10^{-5}$ M obtained from kinetic measurements.

The dye can be displaced from alkaline phosphatase by phosphate and competitive inhibitor 2-aminoethyl phosphonate. These results indicate that the dye binds with its arsonic acid group to the anion binding site of the active site of the enzyme.

The binding of the dye to the native enzyme is associated with a red shift in the visible spectrum of the dye. It seems that the aromatic portion of the dye interacts with a hydrophobic region close to the anion binding site.

The spectrum of the dye is not changed in the presence of the apoenzyme. When zinc is added to an apoenzyme-dye solution, the spectral changes of the dye depend on both the ratio of zinc per apoenzyme and the pH. The presence of Mg^{2+} had no effect on the observed phenomenon.

Introduction

Phenylarsonates are known to reversibly inhibit some of the serine esterases, such as α-chymotrypsin, trypsin and the subtilisins, apparently by binding to the active site of these enzymes [1].

The arsonates can be regarded as analogs of the phosphonates, known to inhibit *Escherichia coli* alkaline phosphatase (phosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) [2—6]. This enzyme also is a serine esterase, and at the same time a metalloenzyme consisting of two apparently identical monomers. The apoenzyme, obtained by removing the metal ions from the native enzyme, is inactive and does not bind phosphate. Since the interaction of arsonates with alkaline phosphatase has not been studied earlier, it seemed to be a good reason to investigate the action of arsonates on this enzyme. It was of special interest to use a chromophoric arsonate since changes in spectral properties of the dye, accompanying binding to the enzyme, made it an attractive tool for studying the nature of the inhibitor site.

The present report describes an attempt to use the changes in spectral properties accompanying dye binding to the enzyme to obtain information on the stoichiometry and the nature of this binding.

Materials and Methods

Reagents. All reagents were of analytical grade. 4-Aminophenylarsonic acid and 4-nitrophenylarsonic acid were obtained from K and K Laboratories, Ca., U.S.A., and 4-tolylarsonic acid was from Alfa Inorganics, Beverly, Mass., U.S.A. *N*-Bromoacetylaminophenylarsonic acid and *N*-chloroacetylaminophenylarsonic acid were prepared as follows: 4-aminophenylarsonic acid (3.3 g, 15 mmol) was dissolved in acetic acid and saturated aqueous sodium acetate (25 ml of each). Chloroacetyl chloride (1.5 ml, 20 mmol) or bromoacetyl bromide (1.8 ml, 20 mmol) was added in small portions to the vigorously shaken and ice-cooled solution. The *N*-acylated derivative crystallized from the solution within 10 min, in both cases and was collected and washed with 50% acetic acid and water. The derivatives were dissolved in 15 ml of 1 M NaOH, the solutions filtered and acidified with 15 ml of 1 M HCl. After washing with 1 M HCl and water followed by drying in vacuo over KOH the two compounds were judged pure from analysis of their nitrogen content and thin layer chromatography. Their infrared spectra as KBr pellets were in accord with the structures given in Table I. The two compounds crystallize well from ethanol solutions by adding a mixture of diethyl ether and light petroleum.

The dye 4-(4-aminophenylazo)-phenylarsonic acid was from Aldrich Chemical Co., Milwaukee, Wisc., U.S.A. Stock solutions of $9.00 \cdot 10^{-4}$ M were prepared in 50% aqueous ethanol. These solutions were stored at 4°C in the dark [7]. The ϵ_M of the dye was $1.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 390 nm, in 0.1 M Tris · HCl buffer pH 8.0, containing 2.4% alcohol (by volume). The dye gave the same spectra in the pH range 5.3—10.3.

p-Nitrophenyl phosphate (Sigma 104) was from Sigma Chemical Co., St. Louis, Mo. U.S.A. and Chelex 100 (200—400 mesh) from Bio-Rad Laboratories,

Richmond, Calif. U.S.A. Metal-free solutions were prepared by extraction with dithizone in CCl_4 [8] and glassware was treated as described by Thiers [9].

Enzyme. Preparation of native alkaline phosphatase including heat treatment and preparation of the apoenzyme was carried out as described previously [8,10,11]. The zinc content was 3.3 g · atom/mol of protein. The enzyme did not possess significant amounts of endogenous phosphate [cf. ref. 12]. Determination of protein concentrations, activity measurements and Zn^{2+} analyses were performed as described previously [11]. The molecular weight was assumed to be 80 000 per enzyme dimer [13].

Spectrophotometric measurements. All spectra were obtained at 25° C with a Gilford model 240 recording spectrophotometer. 1 mm quartz cells of 1 cm light path were used. Difference spectra were recorded by comparing the light absorption of the dye in protein containing solutions with a reference solution of the dye where the protein was omitted. Titrations were performed directly in the cell and appropriate corrections were made for the dilution.

Determination of the K_i values. K_i values for all inhibitors were determinated from double reciprocal initial velocity vs. substrate plots. Cleland's computer programs for competitive inhibitions [14] were utilized. The total volume in the reaction mixture was 1 ml and the quantity of enzyme was 1.44 μg.

Results

Inhibition with phenylarsonates

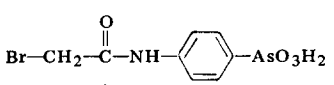
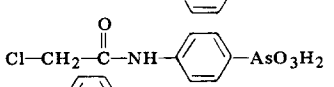
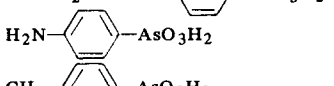
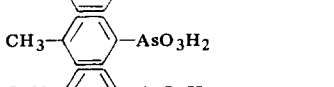
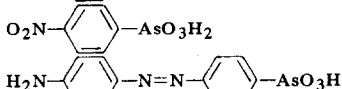
The various K_i values are shown in Table I. All the inhibitors show the competitive type of inhibition. No irreversible inactivation of the enzyme by the action of the *N*-haloacetylated phenylarsonates was observed.

Binding of 4-(4-aminophenylazo)-phenylarsonic acid to the enzyme

Addition of native *E. coli* alkaline phosphatase to the dye produces a shift to

TABLE I

K_i values were determined in 0.1 M Tris · HCl buffer pH 8.0, 25° C.

Inhibitor		K_i ($\times 10^3$ M)
N—Bromoacetylamino phenylarsonic acid		5.5 ± 0.6
N-Chloroacetylamino phenylarsonic acid		6.2 ± 0.4
4-Aminophenylarsonic acid		4.7 ± 0.4
4-Tolylarsonic acid		12.6 ± 2
4-Nitrophenylarsonic acid		17.0 ± 2
4-(4-Aminophenylazo)-phenylarsonic acid		0.013 ± 0.005

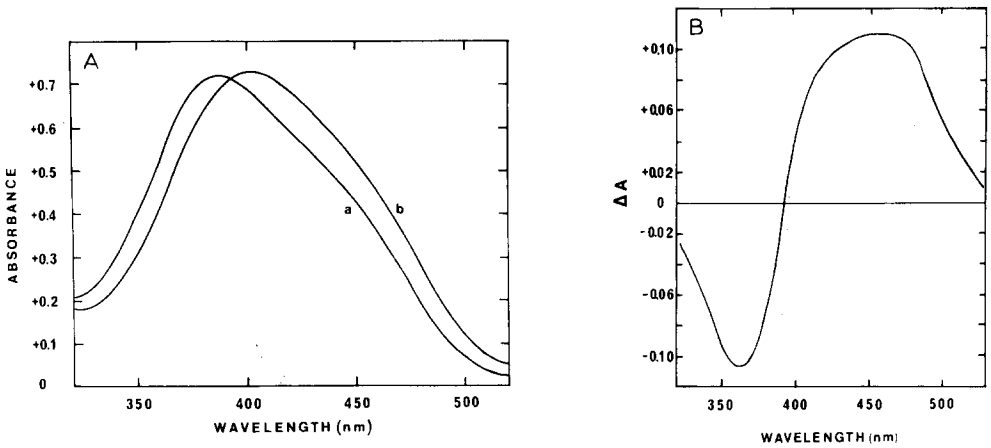


Fig. 1. Effect of *E. coli* alkaline phosphatase on the visible spectrum of the dye, 4-(4-aminophenylazo)-phenylarsonic acid. The spectra were obtained in 0.1 M Tris · HCl buffer, pH 8.0, 25°C containing 2.4% ethanol (by volume). Dye concentration was $4.24 \cdot 10^{-5}$ M and the enzyme concentration $7.96 \cdot 10^{-5}$ M. The light path was 1 cm. A. Curve a, dye vs. buffer; curve b, dye in the presence of enzyme. B. Difference spectrum: dye plus enzyme vs. dye.

a longer wavelength in the visible absorption spectrum of the dye (Fig. 1A). The λ_{max} for the dye in the presence of alkaline phosphatase was 400 nm as compared with 390 nm for the free dye. This spectral shift was associated with a characteristic difference spectrum with a maximum difference at 440—465 nm (Fig. 1B). The shift was the same whether both zinc and magnesium [cf. refs. 15,16] or only zinc had been added to the apoenzyme to produce the active enzyme. Titration of the enzyme solution with the dye gave the curve shown in Fig. 2, and $K_{diss} = 1.50 \cdot 10^{-5}$ M [cf. ref. 17].

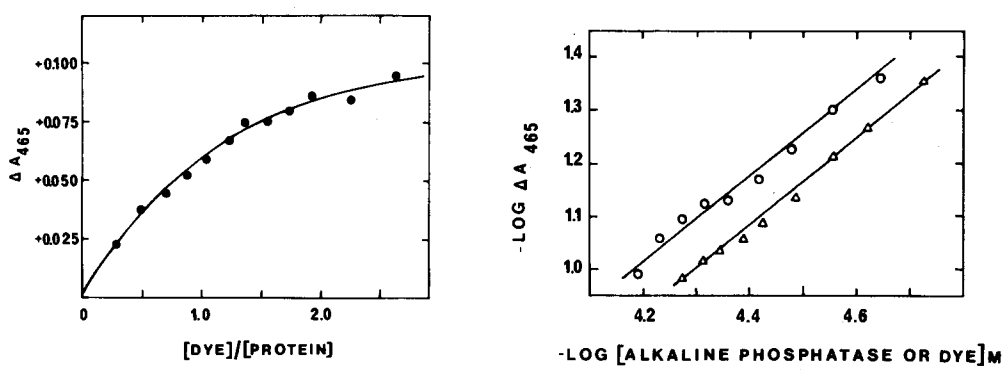


Fig. 2. Spectrophotometric titration of *E. coli* alkaline phosphatase (initial concn. $3.26 \cdot 10^{-5}$ M) with the dye, followed by measuring the increase in absorbance at 465 nm. The solvent was 0.1 M Tris · HCl buffer pH 8.0, 25°C. The line is the theoretical curve for 1 : 1 protein · dye complex having $K_{diss} = 1.50 \cdot 10^{-5}$ M and $\Delta A_{max} = 0.12$.

Fig. 3. Determination of the stoichiometry of the dye · enzyme complex in 0.1 M Tris · HCl buffer pH 8.0, 25°C. Titration of alkaline phosphatase ($3.29 \cdot 10^{-5}$) with dye (○) and of the dye ($5.09 \cdot 10^{-5}$ M) with alkaline phosphatase (△).

Stoichiometry and site of dye binding on the enzyme molecule

Stoichiometry of dye binding was determined by plotting the logarithms of proteins and dye concentrations against log ΔA_{465} (the latter being proportional to enzyme · dye complex concentration). The equality of the slopes of the log plots shown in Fig. 3 indicates that the interaction between the dye and alkaline phosphatase exhibits 1 : 1 stoichiometry under the conditions studied [cf. ref. 18]. The same stoichiometry was obtained with the enzyme reconstituted from the apoenzyme by the addition of slightly more than three equivalents of Zn^{2+}.

Evidence that the dye is bound to the active site of the enzyme was obtained from the fact that phosphate is capable of displacing the dye from the enzyme (Fig. 4) as is the competitive inhibitor 2-aminoethyl phosphonate (K_i = 8.25 · 10^{-4} M in 0.1 M Tris · HCl buffer pH 8.0, 25°C, determined as described in Materials and Methods).

When incremental amounts of phosphate or phosphonate were added to the dye/protein mixture there were corresponding quantitative decreases in the difference spectra. With one mole of phosphate per mole of enzyme the difference spectrum has disappeared completely (Fig. 4). No other changes occur up to a 1000-fold molar excess of phosphate.

The existence of an unique binding site for the dye molecule, which is the same as the binding site for substrate, was shown by another approach, namely by determining the dye-enzyme inhibition constant (K_i). The value of K_i determined by utilizing the dye as an inhibitor of the enzyme, should be identical to the dissociation constant of the enzyme · dye complex as determined above in the absence of substrate. The competitive inhibition constant for the dye, K_i = 1.30 · 10^{-5} M, was determined as illustrated in Fig. 5. This inhibition constant is in good agreement with the enzyme-dye dissociation constant K_{diss} = 1.50 · 10^{-5} M determined in the absence of substrate. (Ethanol in the concentrations

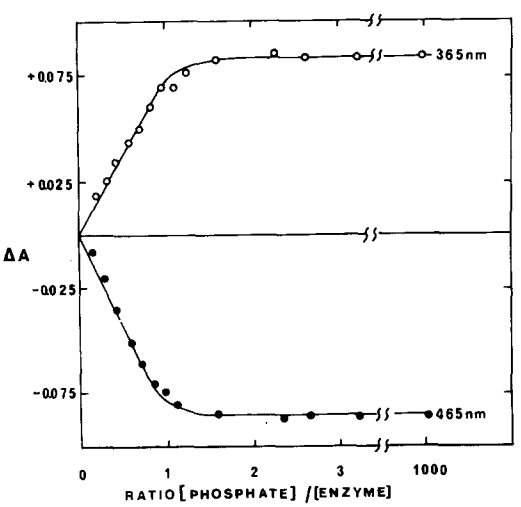


Fig. 4. Displacement of the dye from alkaline phosphatase by phosphate. Plot of ΔA_{365} (○) and ΔA_{465} (●) against mol phosphate added per mol protein. The base-line was the difference spectrum of a dye ($1.05 \cdot 10^{-4}$ M) plus enzyme ($2.90 \cdot 10^{-5}$ M) vs. dye. The solvent was 0.1 M Tris · HCl buffer, pH 8.0, 25°C.

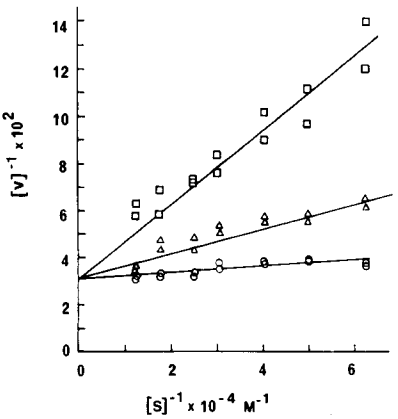


Fig. 5. The inhibition of alkaline phosphatase activity by the dye. The substrate was *p*-nitrophenyl phosphate. The solvent was 0.1 M Tris · HCl buffer, pH 8.0, 25°C. ○, no inhibitor; △, $8.30 \cdot 10^{-5}$ M dye; □, $1.74 \cdot 10^{-4}$ M dye. Velocities are expressed as μmol of substrate hydrolysed per min per mg of enzyme.

used has no effect on the activity of the enzyme). The above observations show that the dye binds at a single site on the enzyme dimer molecule and that this site overlaps with the region responsible for substrate binding.

Zinc dependence of the dye binding

The apoenzyme does not bind the dye in the same way as the native enzyme, since no difference spectrum is obtained. The enzymatic activity of *E. coli* alkaline phosphatase can be restored by the addition of Zn^{2+} to the apoenzyme [10,13]. Titration of a solution containing apoenzyme and dye with Zn^{2+} shows (Figs. 6A and 6B) that the addition of two equivalents of Zn^{2+} per mole of apoenzyme restores the specific dye-enzyme spectrum completely, at pH 6.0 and at pH 8.0. A more complicated picture is seen, when more than two Zn^{2+} ions per apoenzyme molecule are added, and the difference spectra obtained

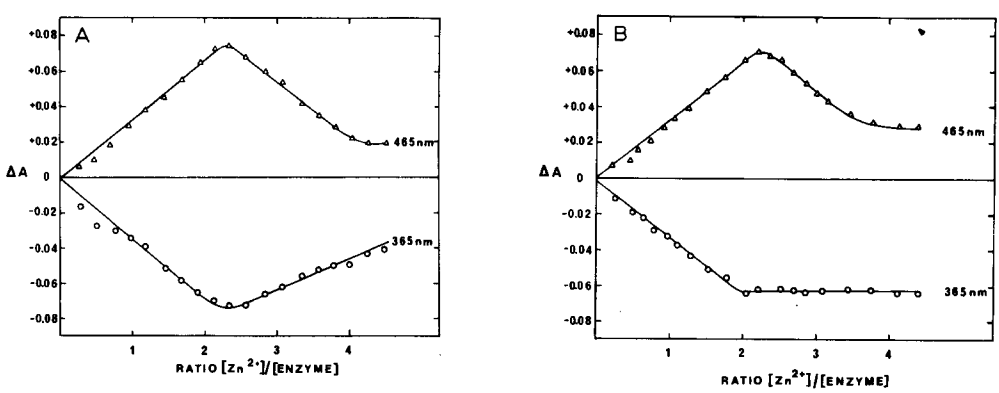


Fig. 6. Spectrophotometric titration of apoalkaline phosphatase with Zn^{2+} in the presence of the dye. A. Apoenzyme concentration was $2.32 \cdot 10^{-5}$ M, dye concentration was $4.70 \cdot 10^{-5}$ M, 0.1 M Mes buffer pH 6.0, 25°C. B. Apoenzyme concentration was $3.76 \cdot 10^{-5}$ M, dye concentration was $9.67 \cdot 10^{-5}$ M, 0.1 M Tris · HCl buffer pH 8.0, 25°C. △, absorbance at 465 nm; ○, absorbance at 365 nm.

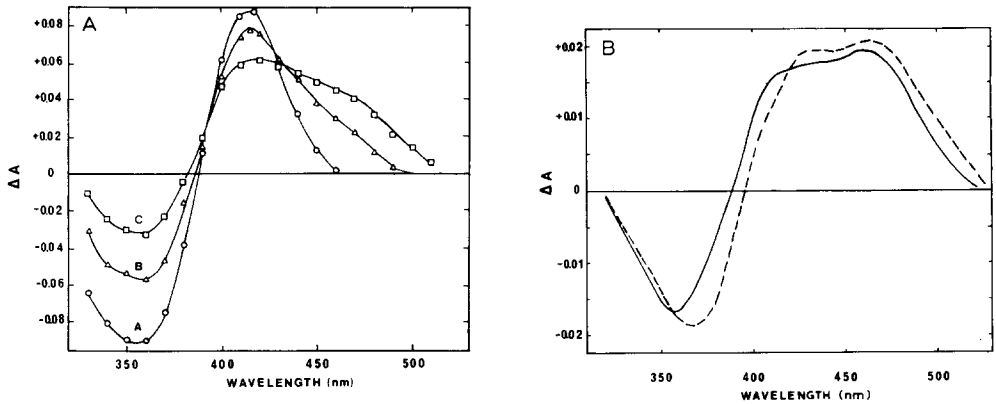


Fig. 7A. Difference absorption spectra of dye in buffer plus dioxane vs. dye in buffer. Dye concentration was $1.95 \cdot 10^{-5}$ M, the buffer was 0.1 M Tris · HCl pH 8.0, 25°C. Dioxane concentrations (v/v) were: curve A (○), 75%; curve B (△), 50%; curve C (□), 25%. B. Difference absorption spectrum of dye in buffer plus 2% phenol vs. dye in buffer. Dye concentration was $5.00 \cdot 10^{-5}$ M, the buffer was 0.1 M Tris · HCl pH 8.0, 25°C (———). Difference absorption spectrum of dye ($1.07 \cdot 10^{-5}$ M) in buffer plus enzyme ($3.02 \cdot 10^{-5}$ M) vs. dye in buffer. The buffer was 0.1 M Tris · HCl pH 8.0, 25°C (- - - - -).

depend on the order in which the components are mixed as well as on the pH. If first zinc is added to the apoenzyme, i.e. restoring the active enzyme before the addition of dye, the ΔA_{465} reaches a maximum value at 2 Zn^{2+} ions per enzyme and remains at this value up to 4 Zn^{2+} ions per dimer. However, when the zinc is added to a solution of apoenzyme and the dye, the ΔA_{465} gradually decreases with increasing zinc concentration above the ratio 2 Zn^{2+} ions per mole of enzyme. Simultaneously, the ΔA_{365} decreases with increasing zinc concentration at pH 6.0 but remains constant at pH 8 (Figs. 6A and 6B). Control experiments have shown that zinc alone does not change the dye spectrum at the pH values studied.

Origin of the difference spectrum

The spectrum of the dye is shifted to longer wavelengths and shows an increase in the absorbance when the polarity of the solvent is decreased by the addition of organic solvents. The resulting dye difference spectra caused by dioxane (Fig. 7A) and especially phenol (Fig. 7B) resemble those caused by the enzyme. Therefore, it appears that the aromatic portion of the dye interacts with a hydrophobic region of the enzyme.

Discussion

The kinetic data presented above show that arsonates similar to arsenates [2,19] are competitive inhibitors of alkaline phosphatase from *E. coli*. Consequently, the use of different arsonates offers possibilities for studying the active site of alkaline phosphatase. We did include two *N*-haloacetylated arsonates in our study to see if there were any amino acid side chains near the anion binding site of the enzyme that were reactive enough to be labeled covalently by alkylation. However, no irreversible inactivation was observed.

The chromophoric enzyme inhibitor, 4-(4-aminophenylazo)-phenylarsonic acid, seemed to be especially useful and therefore we concentrated our investigations on the interaction of this compound with the enzyme. It is known, that this dye is a strong inhibitor of the subtilisins but it does not act on either trypsin or chymotrypsin [7].

Our data show that the enzyme · inhibitor complex formation between the dye and alkaline phosphatase is associated with a red-shift of the dye spectrum. A characteristic absorption difference spectrum is produced, with a maximum difference at 440—465 nm, and this spectral property has been utilized to determine the stoichiometry, the dissociation constant of the complex and the nature of the dye binding.

The results shown in Figs. 2 and 3 establish the presence of one single strong inhibitor-binding site per alkaline phosphatase dimer. The protein · dye complex is characterized by a $K_{diss} = 1.50 \cdot 10^{-5}$ M. This value is in good agreement with the $K_i = 1.30 \cdot 10^{-5}$ M obtained (Table I and Fig. 5) from the kinetic studies of the competition by substrate and the dye for the binding site of the enzyme.

The displacement of the dye by phosphate (Fig. 4) or 2-aminoethyl phosphonate confirm that the binding site for the dye overlaps with the active site region of *E. coli* alkaline phosphatase. Furthermore, the addition of the dye to the metal-free apoenzyme does not produce the difference spectrum which is characteristic for the dye binding to the native enzyme. This observation is consistent with the previous findings that the apoenzyme is incapable of binding phosphate [13] and phosphonates [20]. The spectral changes typical for binding 4-(4-aminophenylazo)-phenylarsonic acid to the native enzyme are also observed when Zn^{2+} ions are added to a solution of the dye and the apoenzyme. However, when the number of metal ions added per apoenzyme molecule exceeds 2, the spectral changes become dependent on the Zn^{2+} concentration as well as on the pH, as mentioned under the Results (cf. Figs. 6A and B). These observations suggest that the protein · dye complex formed by adding zinc to the apoenzyme plus dye in some way differs from the complex formed from native enzyme plus dye. The former complex seems to undergo a pH-dependent conformational change in the dye-binding site when the Zn^{2+}/enzyme ratio exceeds 2.

The spectral red-shift observed on binding of the dye to the enzyme can be simulated by lowering the polarity of an aqueous solution of the dye by adding organic solvents such as dioxane (Fig. 7A) or phenol (Fig. 7B). This can be taken as an indication that the aromatic portion of the dye binds to a hydrophobic region near the active site of *E. coli* alkaline phosphatase. The penetration of the second benzene ring of the dye into such a hydrophobic region may account for the strong binding of the dye to the active site. The other phenylarsonates studied, do not have this second benzene ring and bind about 500—1000 times more weakly to the enzyme. The absence of alkylation by the haloacetylated arsonates might be a further indication for the presence of such a hydrophobic region, devoid of reactive amino acid side chains.

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