THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 8, Issue of March 15, pp. 5018-5024, 1991
Printed in U.S.A.

Anomalous Stimulation of *Escherichia coli* Alkaline Phosphatase Activity in Guanidinium Chloride

MODULATION OF THE RATE-LIMITING STEP AND NEGATIVE COOPERATIVITY*

(Received for publication, February 23, 1990)

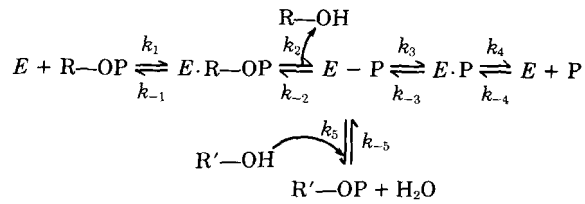
Nalam Madhusudhana Rao‡ and Ramakrishnan Nagaraj

From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Guanidinium chloride stimulates the activity of alkaline phosphatase from *Escherichia coli*, by 3–4-fold. Structural parameters of the enzyme, monitored by fluorescence and circular dichorism, indicate progressive denaturation. This unusual stimulation is shown to be independent of the nature of the substrate and source of the enzyme. Profiles of pH dependence and transphophorylation reaction indicate that the dephosphorylation step of the catalysis is enhanced in the presence of guanidinium chloride. We demonstrate, by fast-flow kinetics and inhibitor titrations, that guanidinium chloride enhances activity by abolishing negative cooperativity and by accelerating the dissociation of rate-limiting enzyme and substrate (E·P) complex.

Protein denaturation by organic solutes, such as guanidinium hydrochloride and urea, and monitoring of subsequent renaturation has been extensively used to gain insight into the mechanism of protein folding (1, 2). Physical properties, such as fluorescence, absorbance, sedimentation, etc. and functional properties, such as activity, ligand binding, etc., of a protein change during denaturation (2, 3), which can be correlated to changes in protein structure (4). Very few studies have been attempted to correlate the structural and functional properties of a protein during unfolding especially when organic denaturants are used, unlike refolding studies, where there is considerable information (2, 5). We have attempted to study the functional and structural aspects of alkaline phosphatase (AP)[1] during GdmCl-mediated denaturation.

AP is a nonspecific monoesterase that hydrolyzes various phosphate monoesters with the same efficiency regardless of the nature of the leaving group (6, 7). The *Escherichia coli* enzyme is a dimer (M_r 94,000) of identical subunits. Apart from this hydrolase activity AP also phosphorylates by transferring a phosphate from donor phosphate to an acceptor alcohol such as Tris, glycerol, etc. (6, 8, 9). AP-mediated phosphohydrolase and transphosphorylation reaction pathways are as follows (6, 10, 11).



Among the enzyme:substrate complexes E·ROP and E·P are noncovalent complexes and E-P is a covalent complex. At acidic pH, the hydrolysis of E-P (*i.e.* k_3) is rate-limiting, whereas at alkaline pH the dissociation of the noncovalent complex E·P (*i.e.* k_4) is the rate-limiting step. The guanidinium group of arginine (Arg-166), located near Ser-102, provides stabilization of the charged intermediates or transient states along the reaction pathway. AP is a classical example of an enzyme demonstrating negative cooperativity or "half-of-sites" activity (12, 13). Subunit interactions keep the dimer in an asymmetric state on substrate or phosphate binding, so that only one unit can accept the substrate or bind to phosphate (14, 15). It has been suggested that subunit interactions bring about asymmetry in the dimer in the sequence of reaction steps in the catalytic mechanism (14).

Recently, Tsou (5), in his detailed studies on denaturation of creatine kinase and other enzymes, suggested that the "active sites are usually situated in a limited region of the enzyme molecule that is more fragile to denaturants than the protein as a whole." Generally, the activity is most susceptible to denaturation in enzymes compared to other structure-related parameters in a protein, which is not unexpected, since the active site is a "niche of distant partners (residues) in a delicate coordination." Any small perturbation is bound to upset the coordination, so the activity suffers first. In our attempts to correlate the changes in structural and functional parameters during denaturation in AP by GdmCl, we have observed an unusual increase in activity, while the structure-related parameters indicate a denaturation of the protein as a whole. We describe in this paper the phenomenon of anomalous increase in activity of AP in GdmCl and also present evidence for the abolishment of negative cooperativity and relaxation of the rate-limiting step, as main contributory factors for this enhancement.

EXPERIMENTAL PROCEDURES

Materials—*p*-Nitrophenyl phosphate (PNPP), Tris, 5-bromo-4-chloro-3-indolyl phosphate, alkaline phosphatase (bovine intestinal mucosa, type VII-S), 3-(*N*-morpholino)propanesulfonic acid (MOPS), and urea were purchased from Sigma. Guanidine HCl (AG) was purchased from Serva, Heidelberg, FRG. All other reagents were of analytical grade.

Guanidine hydrochloride meets the specification of Nozaki (16) and was used without further purification. GdmCl (6 M) solution has an absorbance of < 0.15 at 225 nm and has a melting point of 187 ±

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: AP, alkaline phosphatase; PNPP, *p*-nitrophenyl phosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GdmCl, guanidine hydrochloride; CD, circular dichorism; pNP, *p*-nihophenol; Trp, tryptophan.

1 °C. GdmCl concentrations were calculated from standard refractive index *versus* concentration tables using refractive index values of each solution measured in a Schmidt and Haensch Dur refractometer with water as a standard.

Methods—Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1) was purified from *E. coli* C90, constitutive for alkaline phosphatase, by the method previously described (17) with one additional fractionation on a Sephadex G-100 column. The enzyme was considered to be pure (>97%) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles, G-100 column chromatography, and also by Western blot profile.

AP activity was measured by the method described earlier (18) by following the hydrolysis of 1 mM PNPP in 10 mM Tris-Cl, pH 8.0, spectrophotometrically in a spectrophotometer (Hitachi 150–20) at 410 nm at 25 °C. In all the activity assays, approximately 3 μg of AP was used per assay. To study the GdmCl effects, the enzyme was preincubated with the denaturant for 30 min before the activity was assayed. Molar extinction coefficient of ($\epsilon_{410\,nm}$) is measured to be 14,210 at pH 8.0. BCIP hydrolysis was monitored at 610 nm in the presence of 1 mM substrate (19). Activities were computed from initial velocities and monitored in a recording spectrophotometer or after terminating the reaction by addition of 0.5 M NaOH (2 ml of NaOH to 1 ml of assay volume) at a predetermined time. The activities were linear in the observed periods. Corrections were made for change in extinction value of pNP in 0.5 M NaOH. Background corrections for pNP contamination in PNPP, when used at high concentrations (final concentrations in mM range) were made. Nonenzymatic hydrolysis of PNPP in GdmCl was not observed. In pH experiments, the reactions were stopped by NaOH, since pNP absorbance is strongly dependent on pH below 7.0. Final pH after the addition of 0.5 M NaOH was the same in pH experiments irrespective of the buffer used in the assay.

In competitive inhibition studies, both substrate and inhibitor, *i.e.* inorganic phosphate, were added simultaneously to the enzyme preincubated in GdmCl. Identical results were obtained when enzyme preincubated in GdmCl was added to substrate and inhibitor.

Michaelis-Menton parameters were calculated by Lineweaver-Burk transformation.

Specific activities are defined as μmoles of PNPP hydrolyzed/min/mg of protein. Protein was estimated by the method of Lowry (20) using bovine serum albumin as standard.

Fluorescence studies were performed using a spectrofluorimeter (Hitachi F-4100). Enzyme solutions (15 μg/ml) containing 10 mM Tris-Cl (pH 8.0) and various concentrations of GdmCl or NaCl was excited at 290 nm and the emmision was set at 335 nm. Solute blanks were monitored for correcting the sample fluoresence. Rate of folding of denatured AP or unfolding of native AP was monitored by following the responses of tryptophan (Trp) to polarity changes. For refolding rate measurements, the enzyme in 6 M GdmCl was rapidly added to buffer and the Trp fluorescence was monitored. The digitized data was used to calculate the refolding/unfolding rate, assuming a first order process. These plots were linear confirming the first order nature of the process.

Circular dichroic spectra of AP were obtained using a Jobin-Yvon dicrograph (model V) with a 1-mm path-length cell. The data were collected and analyzed using Apple software. Blank spectra (minus enzyme) with GdmCl were run always to correct the sample spectra. Spectra were recorded in the 210–250-nm range due to high interference of GdmCl signal below 210 nm.

Stopped-flow studies were made on a Hi-Tech spectrophotometer with an SFL-41/592 injection unit supported by an SF-3L support unit and controlled by an SF-40C control unit. The instrument has a dead time of 4 ms and a 10-mm path-length flow cell with a volume of 100 μl. The flow cell was placed in a Dewar flask and the ambient temperature was maintained at 5 ± 0.2 °C. The data were analyzed on an Apple II computer. The sweep times available were from 10 ms to 5 min and were used extensively to follow various stages of the kinetic process. The monochromator was set at 410 nm to monitor the pNP release and corrections were made for the change in the extinction coefficient of pNP at 5 °C. The concentrations expressed in this paper are always the final concentrations *after* mixing.

RESULTS

Enhancement of AP Activity in GdmCl

The activity of AP in various concentrations of GdmCl is shown in Fig. 1*A*. A 3–4-fold increase in activity is clearly discernible in 1.5 M GdmCl, which gradually decreases till the enzyme is totally inactivated in 6 M GdmCl. Nonenzymatic hydrolysis of PNPP in GdmCl was negligible, and substrate hydrolysis with respect to time and enzyme concentration were linear under these conditions (data not shown). In an effort to show that the enhancement in activity was not a function of the substrate used, the activity of AP was determined in varying concentrations of GdmCl with BCIP, another substrate for AP. Stimulation in the activity and parabolic profile observed with BCIP (Fig. 1*B*) indicate that the GdmCl-mediated stimulation is not substrate-dependent. Similar profiles were obtained when AP was assayed in GdmCl using ATP as a substrate (data not shown). In order to further ensure that the stimulation was not due to artifacts of isolation, the activities of commercial samples of AP from *E. coli* as well as calf intestinal AP was determined in varying amounts of GdmCl and the results are depicted in Fig. 1*C*. Stimulation in activity was observed with all the enzyme samples irrespective of the source.

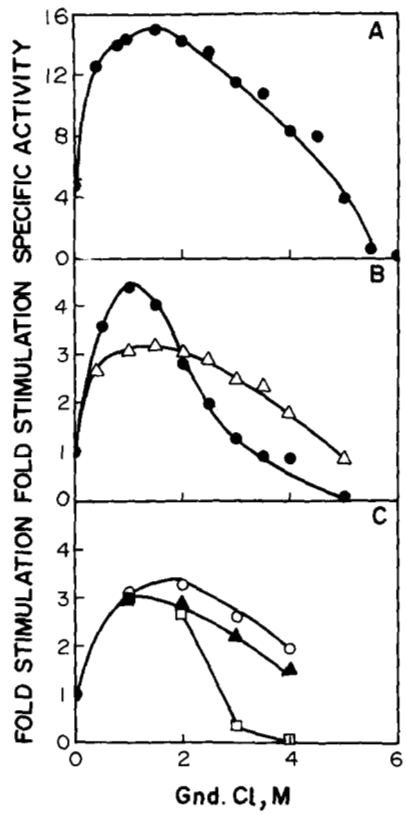

FIG. 1. **Effect of GdmCl on the activity of AP.** *A*, specific activity of AP from *E. coli*, assayed by PNPP hydrolysis in the presence of 10 mM Tris-Cl, pH 8, was plotted against GdmCl concentration. *B*, GdmCl effects on AP activity assayed by different substrates. AP activity was assayed both by PNPP (△) and BCIP (●) and -fold stimulation over control (*i.e.* in the absence of GdmCl) was plotted against GdmCl concentrations. *C*, effects of GdmCl on activity of AP from different sources. Commercial sample of AP from *E. coli* (Amersham Corp.) (▲), calf intestinal mucosal membrane AP (Sigma) (□), and our AP prepatation (○) were assayed in GdmCl using PNPP as the substrate. -Fold stimulation over control (in the absence of GdmCl) was plotted against GdmCl concentration.

Addition of dithiothreitol (10 mM) to the assay medium in the presence and absence of GdmCl reduces the activity to 5% of the control (data not presented). Apparently, intactness of the disulfide bridges is essential for the catalytic function.

pH Dependence of AP Activity in GdmCl—The activity of AP at different pH and GdmCl concentrations is shown in Fig. 2. pH profiles of activity in various GdmCl concentrations

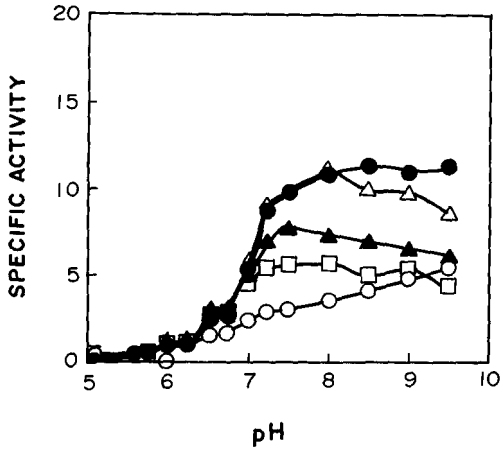

FIG. 2. **Effect of medium pH on GdmCl-mediated stimulation of AP activity.** pH of the assay medium was altered by using 10 mM of Tris-Cl in the pH range of 7.5–9.5 and 10 mM MOPS in the pH range of 5–7.5. Specific activities of AP in control (○), 1.0 M (●), 2 M (△), 3 M (▲) and 4 M (□) GdmCl were plotted against medium pH.

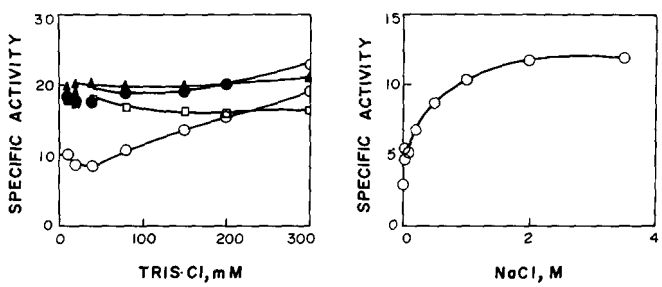

FIG. 3. **Effect of Tris-Cl and sodium chloride on AP activity.** *A*, transphosphorylation catalyzed by AP with Tris, as phosphate acceptor, in the presence of GdmCl. PNPP hydrolysis was monitored in the presence of various Tris-Cl (pH 8) concentrations and in the absence (○), and in the presence of 0.5 M (●), 1.0 M (△), 1.5 M (▲), and 2 M (□) of GdmCl. Specific activity (μmoles of PNPP hydrolyzed/min/mg of protein) was plotted against Tris concentration. *B*, AP activity was plotted against NaCl concentration in the assay medium.

are similar, suggesting that proton dissociation behavior of one or more groups involved in the catalytic mechanism are not altered in GdmCl (21). The stimulation in activity was seen from pH 6.5 and above. Below pH 6.5, *E*-P is the accumulated species in the reaction pathway (22–24), so it is possible that the effect of GdmCl may not be on the enhancement of rate of dephosphorylation, *i.e.* k_3 but a step later, where $E \cdot P$ is the accumulated species, since the stimulation was seen only in alkaline pH.

Effect of Tris on the Specific Activity of AP in GdmCl—Since Tris as well as other alcohols behave as good phosphate acceptors for transphosphorylation reactions catalyzed by AP (8, 9, 25) we investigated whether Tris could stimulate AP activity in the presence of GdmCl. As shown in Fig. 3*A*, stimulation of AP activity by Tris was observed up to 0.3 M Tris, but this stimulatory effect was not seen in the presence of GdmCl.

Salts such as sodium chloride, magnesium sulfate, sodium sulfate, etc., have stimulatory effect on the AP activity (26, 27). It is important to delineate the ionic effects and denaturant effects of GdmCl on AP (28). AP activity was assayed in various NaCl concentrations in the presence of 10 mM Tris-Cl. AP activity profile in NaCl solutions was similar to GdmCl profile up to 2 M NaCl (Fig. 3*B*). Beyond 2 M NaCl the activity plateaus and there is no decrease as in the case of GdmCl.

Effect of Guanidinium Thiocyanate and Urea on the Activity of AP—We studied the effect of the thiocyanate salt of guanidinium on activity to understand the role of counteranion on stimulation. Stimulation of AP activity was also observed with guanidinium thiocyanate, with a maximum stimulation of 0.8 M of the denaturant and complete loss of activity at 2.0 M guanidinium thiocyanate (Fig. 4). Similarly, urea, a non-ionic denaturant, does not stimulate AP activity (Fig. 4).

Conformational Studies on AP

Generally, protein denaturation is complete by 6 M of GdmCl and results in cross-linked random coils, if the disulfide bridges are kept intact (1, 2, 29). It is expected, and also well-documented, that enzymes lose activity progressively in GdmCl (2, 3, 5). It would be of interest to correlate the activity profiles with structural features, as monitored by physical methods, in GdmCl solutions. The loss in enzyme activity with associated loss in the structure with increasing GdmCl concentrations has been well-documented. However, we have observed the stimulation of AP activity rather than the loss of activity with increasing GdmCl (Fig. 1*A*). Hence, the effect of GdmCl on the conformation of AP was next investigated by fluorescence and circular dichorism spectroscopy.

Fig. 5*A* shows the variation in emission of Trp (λ_{max} = 295 nm) fluorescence in AP in various GdmCl concentrations. A sharp fall in the fluorescence intensity is observed from 1 to 2 M GdmCl. Beyond 2 M GdmCl no further change in fluorescence of Trp was observed. These equilibrium fluorescence changes seen in AP were reversible, indicating that the de-

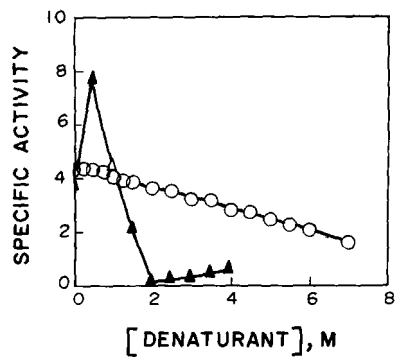

FIG. 4. **Effect of denaturants guanidinium thiocyanate and urea on AP activity.** AP activity was monitored, by PNPP hydrolysis, in the presence of guanidinium thiocyanate (▲) and urea (○). Specific activity was plotted against the denaturant concentration in the assay.

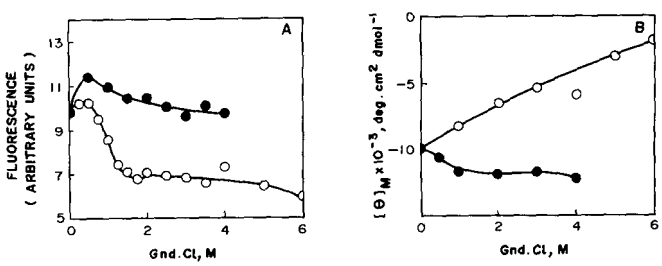

FIG. 5. **Effect of GdmCl on CD and fluorescence profiles of AP.** *A*, equilibrium tryptophan fluorescence (exitation and emission wavelengths set at 295 and 335 nm, respectively) of AP at 25 °C was plotted against GdmCl (○) and NaCl (●) concentration. The data presented was corrected using corresponding blanks. *B*, molar ellipticity values (at 222 nm) were calculated using mean residue weight at 105. θ_{222} values were plotted against the GdmCl (○) and NaCl (●) concentration. Absorbance values were corrected for solute contribution. Enzyme concentration was 0.015 and 0.43 mg/ml in fluorescence and CD studies, respectively.

natured protein completely renatures once GdmCl is removed either by dilution or dialysis. However, the fluorescence profile of AP in NaCl does not show any decrease with increasing NaCl concentrations, but instead shows an increase possibly due to aggregation. The fluorescence profile in GdmCl in different concentrations of the protein or at different time points, was similar.

θ_{222} values obtained from CD spectra of AP in GdmCl (Fig. 5*B*) demonstrate a monotonic decrease up to 6.0 M GdmCl, indicating that the increase in GdmCl concentrations progressively result in denaturation. θ_{222} values of AP do not change with increasing concentrations of NaCl indicating that there is no denaturation in NaCl. It is clear that AP becomes unordered with increasing amounts of GdmCl fluorescence and CD profiles do not correlate with each other. This is probably because they monitor qualitatively different aspects of the protein structure (2). Only 1 Trp residue in AP occurs in a structurally ordered region and it is conceivable that this region is unfolded in 2 M GdmCl resulting in the exposure of the Trp residue to the solvent. The CD profile does not show a sigmoid type of transition, but instead is a smooth monotonic decrease, suggesting that denaturation of AP in GdmCl is not rapid.

Kinetics of Rate Enhancement of AP by GdmCl

It is important to investigate both structural and kinetic constraints on the enzyme catalysis to determine if GdmCl stimulates the activity of AP by removing these constraints. Structural constraints manifest as negative cooperativity between the monomers and reduce the "true" activity to half its value (12, 13). Similarly, kinetic constraints manifest as slow dissociation of the E·P complex which reduces the turnover rate of the enzyme (24).

Michaelis-Menton Parameters of AP in GdmCl—AP behaves like a typical Michaelis-Menton enzyme at a low substrate concentration range, *i.e.* in the micromolar range (6, 26). K_m and V_{max} values were calculated from Lineweaver-Burk plots for AP in various GdmCl concentrations and are shown in Fig. 6. V_{max}, as would be expected, shows a maximum at 2 M GdmCl and decreases beyond 2 M GdmCl. On the contrary, appreciable increase in K_m is discernible only beyond 3 M GdmCl.

Negative Cooperativity of AP in GdmCl—AP is a classic example of an enzyme that demonstrates negative cooperativity *i.e.* only one active site is catalytically active at any moment in the dimeric enzyme (12, 13). It shows a "concave-down" profile in a Lineweaver-Burk plot, when assayed at high substrate concentrations (26). In our attempts to find out the causes for the stimulation of AP in GdmCl, we investigated the phenomenon of negative cooperativity in AP in presence of increasing concentrations of GdmCl. Fig. 7*A* shows the reciprocal plot of initial rates against substrate concentration. At high substrate concentration in the absence of GdmCl, the profiles were concave-down, indicating the negative cooperative effect. However, the plots were linear in the same substrate range, when the assay was performed in the presence of GdmCl (Fig. 7*A*). Thus, negative cooperativity is abolished in the presence of GdmCl and stimulation in activity could conceivably arise because both the active sites in an enzyme molecule are functional at any given instance.

To further substantiate this observation we investigated burst size in the pre-steady state kinetics in the presence of GdmCl by using a stopped-flow spectrophotometer. Using PNPP as substrate for the native enzyme at 8.0 pH, a burst size of one has been demonstrated by many workers (30, 31). Fig. 7*B* shows spectroscopic tracings of hydrolysis of 0.5 mM PNPP in the presence and absence of 0.5 M GdmCl. The extrapolation of the tracing to zero time gives the burst size, which is nearly twice in GdmCl solutions than in the control (Fig. 7*B*). These traces were also obtained with various PNPP concentrations, where the burst size was found to be the same. When experiments were performed in 1.0 M GdmCl, the increase in the amplitude of burst over the control was identical to the value obtained with 0.5 M GdmCl. Burst size was not determined in GdmCl concentrations >1.5 M, due to increase in noise level, possibly, and due to an increase in the viscosity of these solutions.

Dissociation of E·P Complex of AP in GdmCl—Dissociation of E·P complex is the rate-limiting step during catalysis at alkaline pH (24, 32, 33). The dissociation rate of the E·P complex is approximately equal to the steady state k_{cat} of AP at alkaline pH, hence the product dissociation is the rate-limiting step for hydrolysis at alkaline pH, where the enzyme is active (33). Free phosphate is a strong inhibitor of the AP activity (6, 23, 34, 35). The K_m values of various substrates for AP differ little from the K_i for phosphate (34). Inorganic phosphate is not only a competitive inhibitor of the hydrolytic reaction but can be considered as a virtual substrate, since the enzyme catalyzes ^{18}O exchange from water to phosphate (23, 36). The binding of inorganic phosphate to enzyme in the presence of GdmCl was studied by investigating the alteration in K_i of phosphate in GdmCl. Fig. 8 shows the K_i values obtained by replotting K_m/V_{max} (slope) values of individual

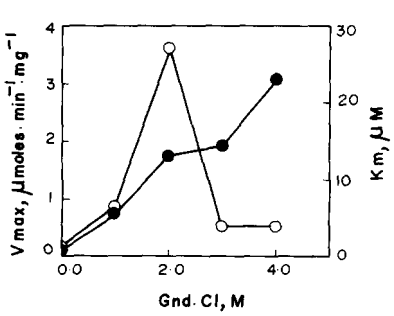

FIG. 6. **Effect of substrate concentration on GdmCl-mediated stimulation of AP activity.** AP was assayed by varying PNPP concentration (2.5–40 μM) in various GdmCl concentration at 25 °C. K_m (μM, ●) and V_{max} (μmoles of PNPP hydrolyzed/min/mg of protein, ○) values calculated from Lineweaver-Burk plots and plotted against GdmCl concentrations. Correaltion coefficient (r) values are above 0.98 in all the plots.

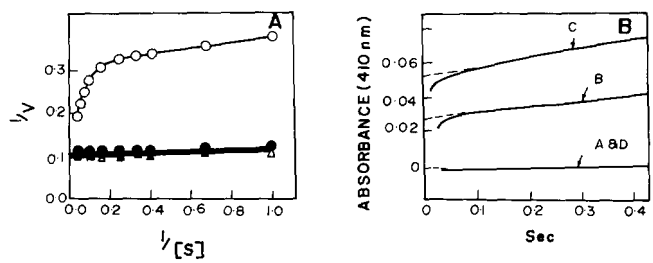

FIG. 7. **Lineweaver-Burk plots of AP activity at high substrate concentration.** AP was assayed using high PNPP concentration (1–40 mM) in the absence (○) and in the presence of 0.5 M (●), 1.0 M (△), and 2 M (▲) GdmCl. Concave-down profile is diagnostic of negative cooperative behavior of the enzyme. *B*, pre-steady state kinetics were performed at pH 8.0 on a stopped-flow spectrometer using PNPP as substrate at 5 °C. Millivolt signals were converted to absorbance units. *Tracing A* represents an enzyme blank run with PNPP and enzyme buffer. *Tracing B* was obtained with PNPP and enzyme (0.3 mg/ml) in 10 mM Tris-Cl. *Tracing C* is a similar run as *tracing B* except that both substrate and enzyme solutions were made in 0.5 M GdmCl. *Tracing D* is a blank run and is similar to *tracing B* except that all the solutions were made in 0.5 M GdmCl.

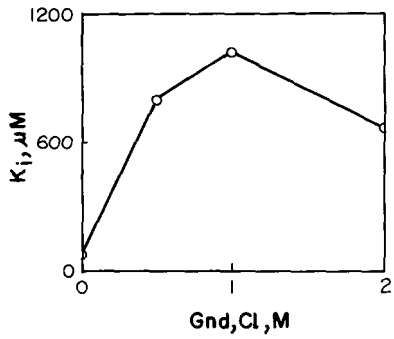

FIG. 8. **Effect of GdmCl on K_i of inorganic phosphate on AP.** Inorganic phosphate was used as a competitive inhibitor in PNPP hydrolysis catalyzed by AP. Individual K_m/V_{max} values obtained in Lineweaver-Burk plots were plotted against phosphate concentration to obtain K_i value. Similarly competitive inhibitions were done in the presence of GdmCl and K_i values, thus obtained, were plotted against GdmCl concentration in the assay.

reciprocal plots of activity *versus* substrate in the presence of various amounts of phosphate, against GdmCl concentrations, suggesting that the affinity for P_i is lowered in the presence of GdmCl.

DISCUSSION

Alkaline phosphatase shows unusual stimulation in activity in the presence of the denaturant GdmCl. This increase in activity, which was 3–4-fold compared to control, was maximal at 1.5 M GdmCl. Enzyme activity was lost completely only at 5 M GdmCl. Stimulation of activity observed in GdmCl was independent of source of the enzyme, nature, and concentration of the substrate. This stimulation was not seen at any concentration of the nonionic denaturant, urea. This increase was shown to be dependent on the intactness of the sulphydryl groups and also on the pH.

Although there is stimulation in the activity of AP, fluorescence and CD studies indicate that the enzyme becomes increasingly unordered in the presence of GdmCl. It thus appears that the structural changes as perceived by fluorescence and CD do not reflect the structural changes in the active site region. Alternatively, the local effects of GdmCl that bring about stimulation dominate at lower GdmCl concentration (*i.e.* < 1.5 M) and denaturing properties of GdmCl dominate at concentrations above 1.5 M. Thus the activity profile of AP in GdmCl is a result of two effects; stimulatory effect and denaturing effect of GdmCl. Miggiano *et al.* (37) explain the "paradoxical" increase in activity of calf intestinal AP by 25% in 0.5 M GdmCl with a possibility of guanidine molecules participating in the reaction. Failure to observe stimulations of AP activity by these authors to the same extent reported in this paper, may be due to the high Tris buffer (100 mM) employed in their assay. We have shown in this paper (Fig. 3*B*) that the stimulation in the presence of GdmCl was absent, if the assay was done in high Tris buffer concentrations. It is a feasible hypothesis that guanidine molecules substitute for the guanidino group of Arg-166, which is an important active site residue, involved in stabilizing the phosphate in catalysis (7). The ascending part of the activity profile could be due to the binding and local effects of GdmCl at low concentrations.

Rate of Trp fluorescence decrease in GdmCl was calculated to be 20 times faster than the rate of the loss in activity. This information along with the activity profile and susceptibility of structural parameters in GdmCl strongly suggest that the active site is unperturbed at low GdmCl concentration and behaves as a relatively stable entity. This is in conflict with the observation of loss of enzyme activity on denaturation in multimeric enzyme such as glyceraldehyde-phosphate dehydrogenase (38), aldolase (39), etc., and in monomeric enzymes with multiple disulfide linkages such as ribonuclease A (M_r 13,500) (40). There is considerable evidence that in renaturation/reactivation processes, gain in enzyme activity is always the slowest step (2). Structural and functional changes in AP in GdmCl do not match similar changes in other enzymes and require a need to include specific interaction terms of denaturant molecules with the macromolecules to account for the denaturation process completely (28). Our investigations suggest that GdmCl brings about subtle changes in the structure of the monomers and also on the monomer-monomer interactions such that the rate of hydrolysis of substrate is enhanced. Enhancement in the activity of AP in GdmCl is primarily due to the release of structural/conformational constraints on the enzyme, which allows a higher catalytic efficiency. AP, functionally, is a dimer with a large subunit interface (16% of the monomer surface). α-Helical regions, which follow directly the metal-binding histidyl residues, from the two subunits, interweave and possibly form a direct pathway for transmission of substrate-induced conformational effects to the second subunit (10). Consequences of structural alterations brought about by GdmCl are, mainly, two: (*a*) on subunit-subunit interaction, *i.e.* negative cooperativity; (*b*) on the dissociation of the $E \cdot P$ complex, the rate-limiting step.

Negative Cooperativity—Negative cooperativity is proposed to explain the observation of anticooperative binding and classical Michaelian kinetics seen with some enzymes (12, 13). The mechanism of negative cooperativity involves, in the first step, conformational change of the active site, which is triggered by the noncovalent binding of 1 mol of substrate per mol of symmetric enzyme (12). The dimeric structure then loses its symmetry (41, 42). Intactness of the dimer is essential for negative cooperativity (12). Molecular weight determinations in the presence of organic and inorganic phosphate support the dimeric nature of the enzyme during catalysis (43). Studies on immobilized AP from *E. coli* on Sephadex columns indicate that the monomers of AP are catalytically inactive (44). Several lines of evidence demonstrate conformational changes in AP on substrate or phosphate binding (41, 43, 45, 46). Our studies on stopped-flow measurements on AP in GdmCl show nearly a two-fold increase in the burst size even at 0.5 M GdmCl. The linear Lineweaver-Burk profiles obtained at high substrate concentrations in GdmCl solutions up to 2 M suggest that negative cooperativity is abolished. Absence of negative cooperativity would result in both subunits being available for activity simultaneously. Since the binding energies are different for substrate (PNPP) to AP in GdmCl solutions, as demonstrated by the changes in the binding parameters, and simultaneous loss of negative cooperativity allows both the subunits to be active, it can be argued that conformational equilibria propagated between subunits on binding of substrate have been compensated in GdmCl (47). Since monomers are not active (44) and induced asymmetry was necessary for negative cooperativity (12, 13) it can be interpreted that the inter subunit "cross-talk" is perturbed to an extent that GdmCl abolishes negative cooperativity but keeps intact those subunit interactions essential for the activity. Hence, we propose that the loss of negative cooperativity is one of the factors that contribute to enhancement in activity.

Dissociation of the $E \cdot P$ Complex—Dissociation of $E \cdot P$ complex is the rate-limiting step in alkaline pH for AP (24, 32, 33). Arguments based on the minimal mechanism for the enzyme suggests that the pK_a of the participating residue(s)

directly controls the equilibrium between E-P and $E \cdot P$ complexes *i.e.*, overall rates of phosphorylation and dephosphorylation of Ser-102 *i.e.*, k_3/k_{-3}. We do not observe stimulation of alkaline phosphatase activity in GdmCl below pH 6.5, suggesting that the GdmCl does not bring about stimulatory effect by increasing the k_3/k_{-3} ratio. Since GdmCl stimulation was seen in alkaline pH, where $E \cdot P$ accumulates, it can be concluded that the GdmCl brings about stimulation in activity by increasing k_4/k_{-4} ratio. Perturbations brought about by GdmCl apparently do not disturb the active site coordination with respect to pK_a values of the active site residues since the pH profiles of activity were similar in GdmCl.

Enhancement of AP activity with Tris was viewed as its better ability, nearly 50-fold better, as a phosphate acceptor than water (8, 9, 48). By doing so Tris eases the rate-limiting step and increases the turnover number of the enzyme. Transphosphorylation by Tris- and GdmCl-mediated changes effect the same step in the reaction scheme, *i.e.* dissociation of the $E \cdot P$ complex. If Tris behaves as an efficient phosphate acceptor for AP by virtue of its binding to AP, that binding is not facilitated in GdmCl denatured protein, since synergistic effects of Tris and GdmCl were not seen.

The activity of AP in media of varying ionic strengths have been studied in detail (23, 26, 27). Our results indicate that there is stimulation of AP activity in the presence of GdmCl but not in urea. Hence it would be pertinent to compare the kinetic parameters in media of different ionic strengths (27) with our results. The dissociation constant of strong binding site of the enzyme-phosphate complex shows a 4-fold decrease in ionic solutions indicating that, unlike GdmCl effect, NaCl increases the binding of phosphate to the enzyme (23, 49). Stopped-flow studies on individual rate constants in 1.0 M NaCl demonstrated that the effect of NaCl is on phosphorylation but not on dephosphorylation (50). Comparison of activity in Cl^- and SO_4^{2-} salts of Mg^{2+}, NH_4^+, Mn^{2+}, and Na^+ at a concentration of 0.5 M indicate that the stimulatory effects are not exclusively dependent on the anions (27). However, studies on phosphate dissociation by ^{31}P NMR in the presence of 1.0 M Cl^-, seems to indicate that an increase in dissociation rate by about 2-fold (33). Detailed study of negative cooperativity of AP by steady state rate measurements revealed that at high NaCl concentrations (only above 1.0 M) binding constants of the two sites become equal (26). These authors demonstrate that the negative cooperativity is progressively abolished with increasing ionic strength, by comparing polynomial quotients using the least-squares fitting method on individual Lineweaver-Burk plots. Role of anions on AP catalysis was evaluated by many workers. Although the detailed mechanism by which salts modulate the activity of AP is not clear, it is evident that salts (ionic strength): (*a*) do not perturb the structure (our present study); (*b*) increase the phosphorylation rate, *i.e.* k_2; (*c*) increases the binding affinity of the phosphate to the enzyme by 4-fold; and (*d*) makes the binding affinity of the substrate to both the sites equal at high salt concentration. However, GdmCl: (*a*) denatures the enzyme; (*b*) enhances the dissociation of the phosphate by 10 times (even at 0.5 M); and (*c*) abolishes negative cooperativity at 0.5 M. Hence, stimulation of AP activity by GdmCl is not similar to general ionic strength effects.

Further substantial evidence for the relaxation of the rate-limiting step comes from the K_i studies. An increase of 10–15-fold in the K_i for inorganic phosphate in GdmCl solutions strongly supports the postulate that the dissociation rate of the $E \cdot P$ complex increases in GdmCl. Kantrowitz (51) altered the phosphate stabilizing residue Arg-166 by site-directed mutagenesis to either serine or alanine. Through the mutant enzymes have similar kinetic properties, the k_{cat} decreased by 30-fold and k_m increased by 2-fold. Also, the k_i for phosphate shows an increase of approximately 50-fold in both mutants. One of the effects of Arg-166 substitution was suggested to be the decrease in rate of hydrolysis of the $E \cdot P$ complex. Our data is similar to Kantrowitz's observations to the extent of increase in K_i, but GdmCl perturbations bring about an increase in k_{cat}/K_m. Based on our data it is possible to speculate that the guanidine group of Arg-166 is replaced by free guanidine, thereby providing the local charge stabilization for the transition state during dephosphorylation but not decreasing K_{cat}/K_m and, since the free guanidine group is not anchored to provide any stability for the $E \cdot P$ complex, thereby increases K_i.

Table I summarizes the influence of metal and amino acid substitutions in AP and also the effect of ionic strength and aritificial substrates, on the dephosphorylation and transphosphorylation steps catalyzed by AP. GdmCl and NaCl effects on the AP kinetic parameters seem to be similar, except for the fact that GdmCl effects are seen at much lower concentrations, thereby ruling out the possibility of exclusive Cl^- effects in GdmCl interactions. Also, NaCl does not bring about any structural perturbations, unlike GdmCl. Furthermore, it seems possible that high specific activities are essential for the observation of negative cooperativity in AP. Exclusive effect of GdmCl can be seen as a combination of all the above effects mentioned, such as altered binding energies, P_i destabilization by easing the rate-limiting step, and also ionic strength effects.

Our results indicate that the unusual stimulation in activity of AP in GdmCl conceivably arises due to the loss of negative cooperativity. We feel that this is a unique evidence for the

TABLE I

Summary of metal and amino acid substitutions in AP and their influence on kinetic parameters

	k_{cat}	$k_{dis(P_i)}$	Negative cooperativity	Transphosphorylation	Influence on enzyme reaction
Native (Zn)	Normal	Normal	Present	Present	
GdmCl (0.5 M)[a]	Increase	Increase	Absent	Absent	
Arg-Ala/Ser[b]	Decrease	Increase	Not observed	Present	P_i destabilization
NaCl (2 M)	Increase	Decrease (?)	Absent	Increase	P_i destabilization
Phosphorothioates[c]	Decrease	No effect	Absent	Not observed	Binding energy
Ser-Cys[d]	Decrease	Not observed	Not observed	Absent	Dephosphorylation impaired
Mn enzyme[e]	Decrease	Not observed	Absent (?)	Not observed	E-P stabilization

[a] Our study.
[b] Chaidaroglou *et al.* (51).
[c] Chlebowski *et al.* (31).
[d] Kendall *et al.* (52).
[e] Chapplet-Tordo.

presence of negative cooperativity in AP, since this experimental approach is free of controversies related to phosphate content of the enzyme (53) and does not involve metal substitutions (30) or use nonphosphorylated substrates such as o-phosphorotioates (31).

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