Unfolding Pathway in Red Kidney Bean Acid Phosphatase Is Dependent on Ligand Binding*

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Anil G. Cashikar and Nalam Madhusudhana Rao‡
From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

The unfolding pathway for KBPAP; however, in the presence of phosphate, KBPAP unfolds with a single intermediate. Based on the crystal structure, we propose that the Arg238 may have an important role to play in stabilization mediated by phosphate.

Increase in the free energy upon ligand binding to a protein could translate into specific conformational changes that may have stabilizing or destabilizing effects on the protein (1, 2). Native proteins are only marginally more stable than the denatured proteins (3). Since unfolded proteins are better substrates for proteolytic degradation, ligand-mediated stabilization was considered to have influence on the protein turnover (3, 4). Physiological ligands such as substrate analogues, inhibitors, etc., were shown to enhance enzyme stability (5–7). Conformational rearrangements in a protein on ligand binding was documented, and in several instances ligand-protein complexes were crystallized (8, 9). Ligand-induced enzyme stabilization was quantitated by the protective role ligand binding plays against denaturation, e.g. heat or denaturant, and the reported values of the energy of stabilization were in the range of 1–4 kcal/mole (10, 11). However, the causal relation between the ligand-induced structural changes and the ensuing stability was not addressed. It would be of interest to know whether the ligand merely shifts the unfolding pathway to a higher value on a temperature/denaturant scale or if it alters the way the protein responds to temperature/denaturant, i.e. alters the unfolding pathway. Such information would be of interest in our attempt to design enzymes with improved stability (6).

Acid phosphatase from red kidney beans (KBPAP) is a homodimeric glycoprotein with a subunit molecular mass of 55 kDa (12–15). The binuclear metal center of the protein with a tyrosine to Fe(II) charge transfer transition was responsible for its characteristic purple color (15). Based on amino acid sequence alignment, it was shown that the protein has ββαββ motif and has same amino acids ligating the metal ions as in uteroferrin, its mammalian counterpart (16). The N-terminal 120-amino acid stretch was absent in uteroferrin and does not have any active site or metal ion-ligating amino acid residues (16). The enzyme has limited subunit interactions between the α5 helix and a loop formed by amino acids from 253 to 260 beside an intersubunit disulfide bond at Cys345 (17). KBPAP shows a rather narrow substrate specificity toward ATP, unlike other plant acid phosphatases, and its exact physiological function has not been ascertained until recently (18). Plant acid phosphatases have been observed to be intimately involved in plant phosphate metabolism (19). We observed significant enhancement in the stability of KBPAP toward solvent and heat denaturation in the presence of phosphate. In this communication, we report data on the influence of phosphate binding on the denaturation pathway of KBPAP and demonstrate that phosphate not only stabilizes KBPAP to denaturation but also alters the unfolding pathway.

EXPERIMENTAL PROCEDURES

Materials

Guanidinium chloride (GdmCl) was from Serva Feinbiochemica GmbH & Co. (Germany); 8-anilino-1-naphthalene 1-sulfonic acid (ANS) was from Aldrich Chemical Co.; p-nitrophenyl phosphate was from Sisco Research Laboratory (India); Superose 12 gel filtration column and the FPLC system were from Pharmacia LKB Biotechnology (Sweden); water used for the experiments was purified over a Milli-Q water purification system from Millipore.

Methods

Protein Purification—Purification of the red kidney bean purple acid phosphatase was according to the procedures described earlier (12, 13). The KBPAP purified from a local variety of beans shows small differences in amino acid composition but matches with the reported enzyme in substrate specificity, number, and location of disulfide bond and molecular weight (14–17).

Denaturation of Protein—GdmCl stock solutions were made in Milli-Q water, and the concentration was determined by measuring the refractive index on a Schmidt-Haensch-DUR refractometer thermostated at 25 °C. The denaturation mix contained 10 mM Tris buffer (pH 7.4), 500 mM NaCl, and necessary concentrations of GdmCl with or without 10 mM NaH₂PO₄. The protein was incubated in the denaturing conditions for 10 min at 25 °C. SDS-PAGE of the denatured protein was used to estimate the extent of denaturation.

The abbreviations used are: KBPAP, red kidney bean purple acid phosphatase; ATP·S, adenosine 5′-O-(thiotriphosphate); ANS, 8-anilino-1-naphthalene 1-sulfonic acid; GdmCl, guanidinium chloride; FPLC, fast protein liquid chromatography; SEC, size exclusion chromatography.

Structural basis for ligand-induced protein stabilization was investigated in the case of an acid phosphatase (red kidney bean purple acid phosphatase, KBPAP) from red kidney bean. Phosphate, a physiological ligand, increases the stability against solvent denaturation by 3.5 kcal/mol. Generality of phosphate stabilization was shown by similar effects with other KBPAP ligands viz. adenosine 5′-O-(thiotriphosphate), a nonhydrolyzable ligand, and arsenate, an inhibitor. The dissociation constant of phosphate obtained from denaturation curves matches with the dissociation constant estimated by conventional methods. The guanidinium chloride-mediated denaturation of KBPAP was monitored by several structural and functional parameters viz. activity, tryptophan fluorescence, 8-anilino-1-naphthalene 1-sulfonic acid binding, circular dichroism, and size exclusion chromatography, in the presence and absence of 10 mM phosphate. In the presence of phosphate, profiles of all the parameters shift to a higher guanidinium chloride concentration. Noncoincidence of these profiles in the absence of phosphate indicates multistate unfolding pathway for KBPAP; however, in the presence of phosphate, KBPAP unfolds with a single intermediate. Based on the crystal structure, we propose that the Arg238 may have an important role to play in stabilization mediated by phosphate.

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† To whom correspondence should be addressed. Tel.: 91-40-672241; Fax: 91-40-671195; E-mail: madhu@ccmb.uunet.in.
mix for 24 h at 25 °C except in case of tryptophan fluorescence quenching by potassium iodide, wherein the enzyme was incubated for 6 h.

Enzymatic Activity—Enzyme activity was measured by diluting an aliquot of the enzyme at the appropriate concentration of GdmCl into reaction mixture containing 50 mM sodium acetate buffer (pH 5), 500 mM NaCl, and 10 mM p-nitrophenyl phosphate. The reaction was stopped by addition of 2 volumes of 0.5 M NaOH. Absorbance measured at 410 nm was corrected with respective blanks. Binding constant of phosphate to KBPAP was calculated from the denaturation curves by using nonparametric methods (20).

Circular Dichroism (CD)—CD spectra were recorded on a J710 from Autodichrograph Mark V spectropolarimeter. A protein concentration of 0.1 mg/ml in the denaturing mix as indicated in the earlier section and pathlength of 1 mm was used. Base-line corrections were done by subtracting the buffer spectra from the sample spectra.

Fluorimetry—Fluorescence spectra were recorded on Hitachi F-4010 spectrophotometer. Intrinsic tryptophan fluorescence spectra were recorded by exciting the sample at 295 nm with excitation and emission slit widths set at 5 nm each. Protein concentration was 0.1 mg/ml. ΔG values were calculated using the tryptophan emission wavelength maximum (λmax) obtained at various GdmCl concentrations, according to the equation ΔG = RT(lnKd)l, where Kd = (λmax, λmax) and λmax and λD are λmax of the native and the denatured protein, respectively.

Potassium iodide quenching of tryptophan fluorescence was done on the protein incubated at various concentrations of GdmCl for 6 h at 25 °C. A stock of potassium iodide was prepared in buffer containing the respective concentration of GdmCl. Small aliquots of this stock were added to the denatured protein up to a final potassium iodide concentration of 0.25 M. Fluorescence spectra were recorded after each addition of 2 volumes of 0.5 M NaOH. Absorbance measured at 410 nm was corrected with respective blanks. Binding constant of phosphate to KBPAP was calculated from the denaturation curves by using nonparametric methods (20).

ANs fluorescence spectra were recorded after adding twice-crystallized ANs to the protein sample to a final concentration of 112 μM. Excitation wavelength was set at 350 nm with a slit width of 10 nm. Emission spectra were recorded between 450 and 570 nm with a slit width of 5 nm. Base-line corrections were done with buffer without protein in all cases.

Size Exclusion Chromatography (SEC)—SEC was performed on the Superose 12 column on a Pharmacia FPLC system (22). The column was routinely calibrated using the manufacturer’s instructions and found that the reproducibility of elution volumes of standards was accurate to 99%, even after extensive use. The column was equilibrated with solutions identical to the solutions in which protein was incubated. Protein was denatured by preincubating in various GdmCl concentration for 24 h at 25 °C before loading onto the column. Protein elution was monitored at 280 nm. The elution profiles were deconvoluted using a commercial software (Jandel Corp.), and molar ratios of different fractions were computed.

RESULTS

Activity—Stabilization of KBPAP on phosphate binding was most significant in case of its activity (Table I). The D1/2, of the activity profile in the presence of phosphate increased by 2.3 M GdmCl, in comparison to that in the absence of phosphate (Fig. 1A). Denaturation transition was steeper in the presence of phosphate. Similar shifts in D1/2 were also observed with ATPγS, a nonhydrolyzable substrate analogue, and arsenate, an inhibitor of KBPAP (Fig. 1B and Table I). With ATPγS, we consistently observed a 2.5-fold enhancement in activity in GdmCl.

KBPAP is a homodimer with an intersubunit disulfide bond at Cys345, and an intact disulfide is necessary for native conformation and activity (13). To study the accessibility of the intersubunit disulfide after denaturation for 24 h at 25 °C, we incubated the protein in the denaturing mix for further 3 h after addition of β-mercaptoethanol to a final concentration of 50 mM. In the presence of phosphate, the D1/2 decreased to 1.6 M GdmCl, while in the presence of phosphate the D1/2 remained unchanged (Fig. 1C and Table I). Even in the samples without GdmCl, a 32% loss in the specific activity was caused by β-mercaptoethanol in the absence of phosphate and only a 7% loss in the presence of phosphate (data not shown).

Based on theoretical calculations, it was predicted that the binding constants of ligands could be calculated from the denaturation curves (23). We calculated the binding constant of phosphate to KBPAP by plotting the D1/2 values against phosphate concentration (Fig. 1D) and obtained a binding constant

| Ligand/effector | Activity | Tryptophan fluorescence λmax | Circular dichroism (θ) 220 | SEC area under native peak |
|-----------------|----------|-----------------------------|---------------------------|---------------------------|
| No ligand       | 2.2      | 3.4                         | 3.7                       | 2.3                       |
| 2 10 mM phosphate | 4.5      | 4.2                         | 4.5                       | 4.5                       |
| 3 10 mM arsenate  | 3.3      |                             |                           |                           |
| 4 2 mM ATPγS     | 3.9      |                             |                           |                           |
| 5 50 mM β-mercaptoethanol | 1.6 |                             |                           |                           |
| 6 10 mM phosphate + 50 mM β-mercaptoethanol | 4.4 |                             |                           |                           |

*The assay was done as in the case of 1 and 2, and β-mercaptoethanol was added to a final concentration of 50 mM and incubated further for 3 h at 25 °C.
of 1.2 mM, which was similar to the inhibition constant ($K_i = 1.8$ mM) obtained by activity inhibition studies. The equilibrium binding constants were calculated by the non-parametric method described by Cornish-Bowden (20). Protection by phosphate was also observed against thermal denaturation, wherein the midpoint of transition was shifted by 5°C (data not shown).

**Tryptophan Fluorescence**—Tryptophan fluorescence emission intensity and wavelength are very sensitive to the polarity of its surroundings, hence Trp fluorescence was very effectively used to monitor the protein denaturation (24). An 11-nm shift in the emission maximum and a 30% decrease in the relative fluorescence intensity was observed upon denaturation of KBPAP in 6 M GdmCl. The $D_{1/2}$ of the change in $\lambda_{max}$ increases by 0.6 M in the presence of phosphate, and the transition is typical of a two-state transition (Fig. 2). The final state of unfolding seems to be independent of phosphate in the medium. Arsenate also brings about similar effects on Trp fluorescence as phosphate (data not reported). A red shift in the Trp fluorescence indicates increased accessibility of Trp to the bulk solvent. $D_{G}$ of denaturation were calculated for the protein in the presence and absence of phosphate, using the tryptophan emission wavelength maxima, by the procedure described under "Experimental Procedures" (25). Tryptophan emission data were selected for energy calculations since the size of the data set in the transition zone was significant. We obtained values of 2.5 and 6 kcal/mol in the absence and in the presence of phosphate, respectively. This results in a ligand stabilization energy of 3.5 kcal/mol (see inset in Fig. 2). Iodide was used as a polar quencher of Trp fluorescence. Detailed quenching experiments were performed on KBPAP in various concentrations of GdmCl in the presence and absence of phosphate to obtain the Stern-Volmer constants ($K_{SV}$) at each concentration of GdmCl (21). $K_{SV}$ plotted against GdmCl shows a sharp increase in exposure of Trp at 3.5 M GdmCl in the absence of phosphate and at 4.5 M in the presence of phosphate (Fig. 3).

**ANS Fluorescence**—Solvent denaturation of protein results in exposure of occluded hydrophobic pockets, which could be quantitated by binding of ANS (26). Equilibrium binding of ANS to KBPAP enhances by more than 12-fold in 1.5 M GdmCl. ANS binding profiles reveal two peaks at 1.5 and 4 M, which were independent of phosphate (Fig. 4). In the presence of phosphate, ANS binding to KBPAP was approximately 50% of the value obtained in the absence of phosphate.

**Circular Dichroism**—CD of peptide backbone is an excellent parameter to study loss in protein secondary structure on solvent denaturation. Ellipticity value at 220 nm is a good indicator of the helical content in the protein. The $D_{1/2}$ of protein ellipticity profile shifts to the right by 1.2 M in the presence of phosphate (Fig. 5). An initial decrease in the ellipticity value was observed at low GdmCl concentrations in the absence of phosphate at 1.5 M GdmCl, wherein ANS shows enhanced binding. Even at 6 M GdmCl the ellipticity value in the presence of phosphate does not reach the value obtained in the absence of phosphate, indicating presence of residual structure in the protein.

**Size Exclusion Chromatography**—SEC of protein, where exchange times between various forms are longer than the separation time, is a powerful technique to study the equilibrium distribution of various intermediates during protein denaturation (22), particular advantages of SEC being that the information on the size of the intermediates could be obtained and also intermediates could be obtained in pure form for subsequent characterization. Elution profiles of KBPAP in various concentrations of GdmCl in the absence (Fig. 6A) and presence...
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Fig. 5. Change in mean residue ellipticity ([θ]220) at 220 nm as a function of GdmCl. Open circles indicate absence and solid circles indicate presence of 10 mM phosphate in the denaturation mix. CD spectra were obtained at a concentration of 0.1 mg/ml using a path-length of 1 mm.

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DISCUSSION

To understand the structural basis of ligand-induced stabilization in KBPAP, equilibrium solvent denaturation was studied by various structural and functional parameters in the presence of its physiological ligand, phosphate. In the presence of 10 mM phosphate, D1/2, i.e., the denaturant concentration at 50% denaturation, shifted to higher GdmCl concentration by more than 1 M, indicating substantial stabilization of KBPAP toward solvent denaturation (Table I). A ∆(ΔG) of 3.5 kcal/mol was obtained for phosphate protection, according to ∆G calculations based on fluorescence data (see inset in Fig. 2). Reported ligand binding energies were in the order of 1-4 kcal/mol (2, 4, 27, 28). Observed stabilization with ATP>γS, a nonhydrolyzable substrate analogue, and arsenate, an inhibitor, indicated that the stabilization were general and not specific for phosphate alone. In all these assays, enzyme was preincubated in the presence or absence of phosphate/ligand and GdmCl and assayed in the absence of phosphate/ligand and GdmCl. Phosphate was used at about 10 times its inhibitory constant in the stability experiments. We also observed that the inhibitory constant of phosphate increases with GdmCl (data not shown).

Similar information was not available with ATP>γS as ligand, and the enhanced specific activities in 0-3 M GdmCl region may be due to decreased binding of ATP>γS. Phosphate inhibition was competitive in nature when studied with ATP and p-nitrophenyl phosphate as substrates, indicating that primary effect of phosphate was by binding to the active site. Ligand binding energy could, for reasons of energy conservation, have structural consequences that might stabilize or destabilize a protein (2, 4). Such substrate effects were implicated in the protein turnover in vivo (3). In this case, dissociation and denaturation of KBPAP mediated by GdmCl was delayed by phosphate ligation. The crystal structure of KBPAP has been reported at 2.9 Å resolution and was shown to be a homodimeric glycoprotein with an intersubunit disulfide bond (13, 17). KBPAP has a limited subunit interface at α5 helix and a loop (amino acids 253-260) participating in the intersubunit interaction beside the intersubunit disulfide bond (17). Reduction of the disulfide bond results in inactivation and aggregation of the protein.2 Here, we observe that in the presence of phosphate, accessibility of the intersubunit disulfide bond was reduced, indicating that the active site bound ligand can stabilize subunit interactions.

By overlapping various parameters on a common GdmCl scale, one could identify and approximately characterize the intermediates in the unfolding pathway (29). Unfolding pathway of KBPAP was multistate in nature, with all the parameter profiles showing clear dependence on the presence of phosphate. At low concentrations of GdmCl, surface hydrophobicity of the KBPAP increased significantly resulting in a 12-fold increase in ANS fluorescence. Substantial suppression of ANS binding in the presence of phosphate suggests prevention of underlying structural changes by phosphate. In this concentration range of GdmCl, i.e., up to 1.5 M, except ANS binding, other parameters including activity, the most susceptible parameter to denaturation, were invariant. KBPAP has an N-terminal 120-amino acid domain, which does not have any active site amino acids and was shown to be absent in the mammalian counterpart of KBPAP, uteroferrin (16). The structural changes occurring in 0-1.5 M range of GdmCl may be limited to the N-terminal domain.

On further denaturation in the absence of phosphate, i.e. in 1.5-3 M GdmCl, we observed a 90% decrease in enzyme activity, the mole ratio of the native protein, ANS binding, and only a marginal change in fluorescence and ellipticity. In this region, KBPAP may be an inactive, swollen (probably aggregated to a limited extent) intermediate with reduced surface hydrophobicity and little change in the secondary structure but substantial loss in tertiary and quaternary structure. Phosphate at 10 mM concentration prevents all the changes induced in the range of 1.5-3 M GdmCl and apparently shifts the equilibrium of N = I(1) more to the left. Thus, on phosphate ligation, KBPAP in 3 M GdmCl has all the properties as a native enzyme, whereas nonligated enzyme loses most of its tertiary and quaternary structure. In the range of 3-5 M GdmCl, both in the absence and presence of phosphate, significant loss in the secondary structure and “inside out” opening of the protein indicated by Trp fluorescence and quenchability by a polar quencher occurs. These structural rearrangements resulted in an intermediate with a 5-fold enhancement in ANS binding and a significant portion of the protein appearing as an aggre-

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gate. Both of these events were substantially reduced by the presence of phosphate (see Figs. 4, 6, and 7). In presence of phosphate, even in 6 M GdmCl, KBPAP has residual structure, indicated by higher negative $\beta$ value compared to the value in the absence of phosphate.

In the absence of phosphate, the nonoverlapping profiles of ANS binding, activity, SEC, fluorescence, and CD demonstrate that unfolding pathway of KBPAP was multistate with at least three intermediates populating the pathway. In the presence of phosphate, the $D_{1/2}$ of all of these profiles was in the narrow range of 4–4.5 M. However, SEC analysis of KBPAP denaturation was rewarding. In the range of 2–4 M GdmCl, even in the presence of phosphate, a small fraction (<20%) of the protein was in aggregated form. SEC demonstrates that the phosphate ligation to KBPAP alters the unfolding pathway with three intermediates into a pathway with a single intermediate. Additionally, in the absence of GdmCl, phosphate-induced structural changes reduce the accessibility of the intersubunit disulfide bond to the reducing agents.

To stabilize the pentacordinate phosphorous transition state in KBPAP, His$^{202}$ and His$^{206}$ from one subunit and Arg$^{258}$ from other subunit participate (17). It appears that the stabilizing effect of phosphate on binding to active site of KBPAP was steric. By restricting the movement of the loop containing Arg$^{258}$, opening up of the subunit interface could be prevented. In the phosphate-stabilized protein, the restriction on movement of various segments restricts the number of intermediates that could form, and consequently it unfolds with a single intermediate. Similar ligand stabilizations were observed with the catalytic trimer of Escherichia coli aspartate transcarbamylase in the presence of carbamyl phosphate (2) and in E. coli dehydroquinase with covalently bound substrate (10, 28). In the case of 20$\beta$-hydroxysteroid dehydrogenase, NADH was shown to alter the unfolding pathway (30). The effects reported in this paper were observed at a phosphate concentration in the range of its inhibition constant (i.e. 1.8 mM). The dissociation constant of phosphate calculated based on the denaturation curves was similar to the $K_d$ value obtained based on activity inhibition. Further, these effects were duplicated with other active site binding ligands, indicating that phosphate-induced
stabilization was strictly ligand-induced effects and not specific to phosphate. Also, the protective effect of phosphate toward KBPAP denaturation was observed even during thermal denaturation. Stability of a protein was known to be intimately related to its in vivo life times, since unfolded proteins are considered to be substrates for cytosolic proteases (3). Near doubling in stabilization energies indicate phosphate may have a physiological role of stabilizing KBPAP in vivo.

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