Characterization of a Monomeric *Escherichia coli* Alkaline Phosphatase Formed upon a Single Amino Acid Substitution*

Received for publication, January 31, 2003, and in revised form, April 14, 2003
Published, JBC Papers in Press, April 21, 2003, DOI 10.1074/jbc.M301105200

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Alkaline phosphatase (AP) from *Escherichia coli* as well as APs from many other organisms exist in a dimeric quaternary structure. Each monomer contains an active site located 32 Å away from the active site in the second subunit. Indirect evidence has previously suggested that the monomeric form of AP is inactive. Molecular modeling studies indicated that destabilization of the dimeric interface should occur if Thr-59, located near the 2-fold axis of symmetry, were replaced by a sterically large and charged residue such as arginine. The T59R enzyme was constructed and characterized by size-exclusion chromatography, and circular dichroism (CD) and compared with the previously constructed T59A enzyme. The T59A enzyme was found to exist as a dimer, whereas the T59R enzyme was found to exist as a monomer. The T59A, T59R, and wild-type APs exhibited almost identical secondary structures as judged by CD. The T59R monomeric AP has a melting temperature (Tm) of 43 °C, whereas the wild-type AP dimer has a Tm of 97 °C. The catalytic activity of the T59R enzyme was reduced by 104-fold, whereas the T59A enzyme exhibited an activity similar to that of the wild-type enzyme. The T59A and wild-type enzymes contained similar levels of zinc and magnesium, whereas the T59R enzyme has almost undetectable amounts of tightly bound metals. These results suggest that a significant conformational change occurs upon dimerization, which enhances thermal stability, metal binding, and catalysis.

In some oligomeric proteins, the isolated monomeric subunits must be assembled before the protein becomes functional. Such behavior is exemplified by studies on homodimeric chorismate mutase (1) and the trimeric catalytic subunit of aspartate transcarbamoylase (2). In both of these enzymes, the active site contains residues donated from neighboring chains, and each active site is located at the interface between subunits. The reasons for obligatory subunit association are less obvious for enzymes that do not have a shared active site or metal binding, and catalysis.

Experimental Procedures

Materials—Agar, agarose, ampicillin, *p*-nitrophenyl phosphate, magnesium chloride, and zinc sulfate were purchased from Sigma. Tris, sucrose, and enzyme-grade ammonium sulfate were supplied by ICN Biomedicals (Costa Mesa, CA). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI). DNA sequencing was carried out by Beth Israel Deaconess Medical Center, Molecular Medicine Unit. The oligonucleotides required for site-specific mutagenesis and sequencing were obtained from Operon Technologies (Alameda, CA).

This paper is available on line at http://www.jbc.org

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* This work was supported by Grant GM42833 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: AP, alkaline phosphatase; CD, circular dichroism.
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CA). Plasmids were isolated and purified using the QIAprep Spin Mini-prep kit purchased from Qiagen (Valencia, CA).

Strains and Plasmids—E. coli K12 strain MV1190 (Δlac-proAB), supE, thi, Δ(sri-recA) 306·Tn10(tet')F3 traD36, proAB, lacIq, lacZAM15 was obtained from J. Messing. E. coli K12 strain SM547 (ΔphoA-phoC), phoR, tex::Tn5, Δlac, galK, galU, leu, str') was a gift from H. Inouye. The mutations were constructed in plasmid pEK154, which contains the wild-type phoA gene and its natural promoter in pUC119. This plasmid was derived from plasmid pEK48 (14) by removal of an 882-base pair BstEI-Xhol fragment followed by treatment with T4 DNA ligase after the sticky ends had been filled out with the Klenow fragment of DNA polymerase. Plasmid pEK381 has the T59A mutation in the phoA gene introduced into pEK154 (15).

Construction of a Plasmid for the Expression of the T59R Alkaline Phosphatase—The T59R mutation was induced in the phoA gene contained in the plasmid pEK154 using the procedures outlined by Stratagene in the QuickChange mutagenesis kit protocol. The entire gene was sequenced to ensure that the correct mutation was present and to confirm that no other mutations had been introduced. The final plasmid containing the T59R mutation in the phoA gene was designated pEK161.

Modeling Studies—All molecular modeling studies were carried out using the program QUANTA, and energy minimization was accomplished using X-PLOR (16). The 1.75-A wild-type AP Protein Data Bank structure 1ED8 (7) was used as the base structure.

Protein Expression—SM547, an E. coli strain with the wild-type phoA gene deleted from the chromosome and a mutation in the phoR regulatory gene, was used as the host strain for expression of the phoA gene and its natural promoter in the plasmid present in the host strain.

Protein Purification—The wild-type, T59R, and T59A enzymes were isolated from the periplasmic space by osmotic shock and ammonium sulfate precipitation by the methods previously described (14). Final purification was carried out on a Bio-Rad BioLogic HR system using a Q-Sepharose Fast Flow column (1.6 × 25 cm). A 200-ml gradient from 0 to 0.1 M NaCl in TMZP buffer (0.01 M Tris-HCl, 0.001 M MgCl₂, 10⁻⁵ M ZnSO₄, 10⁻⁴ M NaH₂PO₄, 0.31 × 10⁻⁴ M NaN₃, pH 7.4) was used to elute the protein. The purity of the enzyme was determined by SDS-PAGE (17).

Determination of the Protein Concentration—The concentration of the wild-type enzyme was determined by absorbance measurements at 278 nm with an extinction coefficient of 0.71 cm²/mg (18). The concentration of the mutant enzymes was determined by the Bio-Rad version of Bradford's dye binding assay (19) using wild-type alkaline phosphatase as the standard.

Determination of the Enzymatic Activity—Alkaline phosphatase activity was measured spectrophotometrically utilizing p-nitrophenol phosphate as the substrate at 25 °C by monitoring the release of p-nitrophenolate at 410 nm (20) in 1 M Tris buffer, pH 8.0.

Sucrose-density Gradient Centrifugation—A 4.6-ml gradient of 6–25% sucrose in 50 mM NaH₂PO₄, pH 8.0, was used. After preparation, 200 μl of ~5 mg/ml protein solution was carefully layered on top of the gradient. The tubes were spun at 170,000 × g for 18 h using a Beckman SW 55Ti rotor in a Beckman L-70 centrifuge. The labeled arrows at the top of the plot indicate the migration of carbonic anhydrase (CA) and bovine serum albumin (BSA). The increase in absorbance at 5.0 ml, because of the presence of BSA in the 50% sucrose solution used to push the gradients through the Brandel BR-9620 fractionator, indicates the end of the gradient. WTAP, wild-type AP.

RESULTS

Design of a Monomeric Alkaline Phosphatase—The most appropriate mutation for destabilization of dimeric AP was determined through molecular modeling studies. When a Thr to Arg mutation was introduced at position 59 in the AP structure (Protein Data Bank number 1ED8) using the program QUANTA and subsequently minimized using X-PLOR, large movements of the protein backbone around the location of the amino acid substitution were required to accommodate the mutation, suggesting that the physical mutation may interfere with dimer formation.

Quaternary Structure of the T59A and T59R Alkaline Phosphatases—The quaternary structure of the T59A and T59R enzymes was determined by both sucrose-density gradient sedimentation and size-exclusion chromatography. The sedimentation patterns of the wild-type and T59A enzymes are virtually identical (Fig. 1) indicating that they have the same dimeric quaternary structure. The sedimentation pattern of the T59R enzyme exhibited no species migrating as a dimer; rather a slower migrating species was observed. The sedimentation coefficients of the T59A and T59R enzymes were 6.1 and 3.6 S, respectively. Based upon these sedimentation coefficients, it can be inferred that the T59A enzyme migrates as a dimer and the T59R enzyme migrates as a monomer.

Size-exclusion chromatography was used to determine the Stokes radius of the T59R, T59A, and wild-type enzymes. The elution profiles for the T59A and wild-type enzymes were very similar with nearly identical retention times. However, the
T59R enzyme eluted with a longer retention time. These results further support the proposal of a dimeric structure for the T59A enzyme and a monomeric structure for the T59R enzyme. Based upon these experiments, the Stokes radii of the wild-type, the T59A, and the T59R were determined to be 3.41, 3.35, and 2.93 nm, respectively.

An estimate of molecular mass can be determined from the sedimentation coefficient and Stokes radius (21). The calculated molecular mass for both the wild-type and T59A enzymes was 87,000 daltons, whereas the T59R had a molecular mass of 44,000 daltons, again indicating that the T59R enzyme is monomeric.

**Kinetic Characterization of the T59R Alkaline Phosphatase**—The T59R AP had substantially reduced activity compared with the wild-type enzyme. Although the activity of the T59R AP was 10^4-fold lower than that of the wild-type enzyme, it was still considerably higher than that observed for the noncatalyzed reaction. To rule out the possibility that the observed activity was due to the formation of trace amounts of the more active dimeric form, the effects of protein concentration on the activity was determined. The wild-type and T59A enzymes are virtually identical. Deconvolution of the reaction. To rule out the possibility that the observed activity of the untreated T59R AP.

**Influence of Metals on the Activity of T59R Alkaline Phosphatase**—Two different sets of measurements were performed to determine whether increased amounts of Zn^{2+} and/or Mg^{2+} altered the kinetic properties of the T59R AP. First, the activity of the T59R AP was determined in the presence of Zn^{2+} and/or Mg^{2+} at 100-fold higher concentrations than normally used for the wild-type enzyme. In the second set of experiments, the T59R AP was incubated with 10 mM Zn^{2+} (1000-fold higher concentration than normally used) at 25 °C for 4 h before the activity measurements were performed. The T59R AP in TMZP and in metal-free 0.01 M Tris, pH 8.0, buffer was incubated with Zn^{2+} followed by activity measurements in 1 M Tris, pH 8.0, with 1000 μM p-nitrophenyl phosphate as the substrate. The activity of the T59R AP carried out in the presence of Zn^{2+} and Mg^{2+} and after incubation with 10 mM Zn^{2+} showed very little change in activity when compared with the activity of the untreated T59R AP.

**Analysis of the Mutant and Wild-type APs by Circular Dichroism**—As seen in Fig. 2, the CD spectra of the T59R, T59A, and wild-type enzymes are virtually identical. Deconvolution of these CD spectra (22) indicates that the three enzymes have almost identical secondary structures. Each enzyme contained almost identical percentages of α-helix, β-sheet, β-turn, and random coil.

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**Table I**

| Enzyme  | $k_{cat}$ | $K_m$ | $v_{cat}/K_m$ |
|---------|------------|-------|---------------|
| Wild-type | 7.47 ± 1.2 | 21.8 ± 1.8 | 3.4 × 10^4 |
| T59A | 56.3 ± 3.8 | 24.9 ± 2.4 | 2.3 × 10^4 |
| T59R | 2.12 (± 0.05) × 10^{-4} | 39.2 ± 4.4 | 5.4 |

a The $k_{cat}$ values are calculated from the $V_{max}$ using a dimer molecular weight of 94,000 (35) for wild-type and T59A and a monomer molecular weight of 47,000 for T59R.

b The values for the T59A enzyme were reported previously (15).

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**FIG. 2.** CD spectra for wild-type, T59A, and T59R enzymes. All spectra were obtained using 2 ml of −0.04 mg/ml-enzyme in 10 mM KH₂PO₄ buffer, pH 8.0, at 25 °C. The three spectra have been staggered for clarity. WTAP, wild-type AP.

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**DISCUSSION**

The two active sites in the dimeric *E. coli* alkaline phosphatase are ~32 Å apart. Because no active site residues are contributed from the adjacent subunit, no obvious requirement exists for obligatory dimerization. Yet mutants of the *E. coli* enzyme have been isolated that exhibit intergenic complementation. A heterodimeric form of the enzyme, containing different mutations in the two subunits, exhibits significantly higher activity than would be expected based upon the activity of the homodimeric species used to create the hybrid enzymes (12, 23–26). This observed intergenic complementation suggests that the formation of the dimer interface may result in structural alterations that influence activity.

Although most APs have quaternary structures that are dimeric or higher order aggregates of dimers, there are a few APs reported to be monomeric, such as ones from *Pyrococcus abyssi* (27), *Bombyx mori* (28), *Vibrio sp.* (29), *Bacillus inter-
Unlike the T59R enzyme, the T59A AP exists as a dimer, indicating that the absence of hydrogen bonding when methyl replaced a hydroxy group was not enough to impair dimerization. In contrast, the bulky charged side chain of Arg at position 59 is sufficient to destabilize the interface and prevent dimer formation. To determine whether the T59R mutation caused monomer formation by significantly altering the secondary structure of the enzyme, circular dichroism was used to compare the T59A, T59R, and wild-type enzymes. The monomer form of the T59R enzyme retains the same overall secondary structural fold as the wild-type enzyme. The CD spectra of these three APs were almost identical, indicating that the mutations at position 59 do not significantly alter the secondary structure of the enzyme. Therefore, the loss of the ability of the T59R enzyme to dimerize is not the result of alterations in the secondary structure of the enzyme due to the mutation.

**Altered Properties of the Monomeric Alkaline Phosphatase**—The monomer form of alkaline phosphatase generated in this work has significantly different catalytic activity and structural stability compared with the wild-type enzyme. The thermal melting profiles for the monomer, as determined by CD, indicate an enormous change in thermal stability. Considering that *E. coli* is not a thermophilic organism, *E. coli* AP is an extremely thermally stable protein for its size with a $T_m$ of 97 °C. The more than 50 °C reduction in $T_m$ of the monomeric T59R AP ($T_m = 43 ^\circ C$) indicates that the presence of the dimer interface adds tremendous structural stabilization to the native enzyme. Thus, one of the roles of the interface in *E. coli* AP is to provide thermal stability for the enzyme. It is interesting to note that one of the alkaline phosphatases reported to exist as a monomer was isolated from *Vibrio* sp. (29), a psychrophile. The formation of higher order quaternary structures may well provide a general mechanism for thermal stability for AP in particular and multimeric enzymes in general.

The monomeric T59R AP has severely impaired catalytic ability. In fact, the $k_{cat}$ of the T59R AP is 4-fold lower than that shown by mutant versions of AP in which the critical nucleophilic Ser-102 residue has been replaced by Ala or Gly (32). Even at Zn$^{2+}$ concentrations 1000-fold higher than required for the wild-type enzyme, no significant increase in activity was detected. These results indicate that the low activity of the T59R AP is not simply the result of weakened binding of the metals to the active site of the enzyme. Because the T59A enzyme has almost the same $k_{cat}$ as the wild-type enzyme, the reduction in activity induced by the T59R mutation must be a direct result of the different quaternary structures of the T59A and T59R enzymes. The CD data, which showed that the secondary structures of the T59A, T59R, and wild-type APs were indistinguishable, support this conclusion.

Two points have been made evident by these experiments. First, an intact subunit interface is absolutely required for normal catalytic function of *E. coli* AP. Second, stability of the subunit is greatly enhanced upon association and formation of the dimer interface. As found in earlier studies, maintenance of the structural integrity of the interface has an apparent role in dimer stabilization. In fact, the first 40 residues of the amino terminus of AP have more intersubunit contacts than intra-subunit contacts. This structural feature seems to indicate that the amino-terminal segment of the protein is important for maintaining the dimeric structure. For example, truncation of either the first 10 or 35 residues from the amino terminus greatly destabilizes the dimeric form of the *E. coli* enzyme and reduces the activity by 20% (8, 33).

Both a monomeric and dimeric form of AP exists in *Bacillus subtilis* (31, 34). The amino acid identities found between the *B. subtilis* AP III (dimeric) and AP IV (monomeric) and the

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* S. Zappa, personal communication.
E. coli enzyme are 33% and 34% respectively. The core and active site residues of the B. subtilis enzymes are well conserved when compared with E. coli AP. However, in contrast, the interface residues of the B. subtilis APs are far less conserved when compared with E. coli AP. Another notable difference in the sequence alignment between the two B. subtilis and E. coli APs is residue 59 (E. coli numbering) (34). In the E. coli enzyme, residue 59 is a Thr; and in the dimeric B. subtilis AP, the corresponding residue is a Ser; whereas in the monomeric B. subtilis AP, the residue is Arg. The monomeric T59R E. coli enzyme, therefore, can be compared with that of the monomeric E. coli AP. However, in contrast, B. subtilis AP and of E. coli AP. Another notable difference in the sequence alignment between the two B. subtilis and E. coli AP. However, in contrast, the monomeric T59R AP may not occur because the Arg side chain sterically hinders intersubunit interactions.

This work has elucidated aspects of the functional and structural role of the AP interface, such as the requirement for interfacial interactions, which are critical for the formation of a catalytically functional active site pocket. The monomeric T59R AP bound insignificant amounts of magnesium or zinc, which are required in the proposed catalytic mechanism of wild-type AP (7). Because interfacial interactions are absent in the T59R monomer, the active site pocket remains impaired, and this effect is reflected in the exceedingly low enzymatic activity. However, the loss of the interface interactions and incomplete formation of an active site pocket do not affect the secondary structure, yet they influence the overall folding stability. These findings suggest that the interfacial interactions of AP not only strengthen the protein fold of AP but also align the residues in the active sites that are required for optimum metal-binding and phosphatase activity.

Acknowledgment—We thank Dr. Xu Xu for constructing plasmid pEK154.

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