Improving *Escherichia coli* Alkaline Phosphatase Efficacy by Additional Mutations inside and outside the Catalytic Pocket

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*We describe a strategy that allowed us to confer on a bacterial (*E. coli*) alkaline phosphatase (AP) the high catalytic activity of the mammalian enzyme while maintaining its high thermostability. First, we identified mutations, at positions other than those occupied by essential catalytic residues, which inactivate the bacterial enzyme without destroying its overall conformation. We transferred concomitantly into the bacterial enzyme four residues of the mammalian enzyme, two being in the catalytic pocket and two being outside. Second, the gene encoding the inactive mutant was submitted to random mutagenesis. Enzyme activity was restored upon the single mutation D330N, at a position that is 12 Å away from the center of the catalytic pocket. Third, this mutation was combined with other mutations previously reported to increase AP activity slightly in the presence of magnesium. As a result, at pH 10.0 the phosphatase activity of both mutants D330N/D153H and D330N/D153G was 17-fold higher than that of the wild-type AP. Strikingly, although the two individual mutations D153H and D153G destabilize the enzyme, the double mutant D330N/D153G remained highly stable ($T_m = 87\,^{\circ}C$). Moreover, when combining the phosphatase and transferase activities, the catalytic activity of the mutant D330N/D153G increased 40-fold ($k_{cat} = 3200\ s^{-1}$) relative to that of the wild-type enzyme ($k_{cat} = 80\ s^{-1}$). Due to the simultaneous increase in K_m, the resulting k_{cat}/K_m value was only increased by a factor of two. Therefore, a single mutation occurring outside a catalytic pocket can dramatically control not only the activity of an enzyme, but also its thermostability. Preliminary crystallographic data of a covalent D330N/D153G enzyme – phosphate complex show that the phosphate group has significantly moved away from the catalytic pocket, relative to its position in the structure of another mutant previously reported.*

KEYWORDS:

alkaline phosphatases · enzymes · hydrolases · mutagenesis · protein structures

Introduction

Providing enzymes with higher catalytic efficiencies often remains a challenge. This is no surprise in view of the complexity of a catalytic reaction which involves several elements (a specific binding pocket, an appropriately coupled catalytic machinery, etc.) that need to be adequately adjusted for an efficient transition-state stabilization to occur.[1] To improve the catalytic efficiency of an enzyme, one or more of these elements need to be modified in an appropriate, and most probably delicate manner. Additional difficulties are presented by the fact that the introduced mutations must not interfere with the folding and secretion processes. Despite these potential drawbacks we have searched for a strategy that would allow us to concomitantly improve the catalytic efficiency of an enzyme and provide it with the desired thermostability. The enzyme that was used in this study is the alkaline phosphatase (AP) from *Escherichia coli*.

E. coli AP is a well-known dimeric metalloenzyme which catalyzes the hydrolysis of a variety of phosphomonoesters.[2] Each monomer contains 449 amino acids and two zinc ions located at the metal-binding sites M1 and M2 and one magnesium ion located at site M3. Elucidation of its three-dimensional structure by X-ray crystallography[3] and other mechanistic studies[4–6] suggested a four-step catalytic mechanism (Scheme 1). In short, nucleophilic catalysis associated with phosphorylation of the hydroxy group of Ser 102 is followed by hydrolysis of the serine phosphate ester and dissociation of inorganic phosphate. Above pH 8.0 the rate-limiting step is the release of phosphate from the noncovalent complex of enzyme bound to inorganic phosphate,[2, 7] whereas below pH 6.0 hydrolysis of the phosphoenzyme is rate-limiting. In the presence of a high concentration of a phosphate acceptor, such

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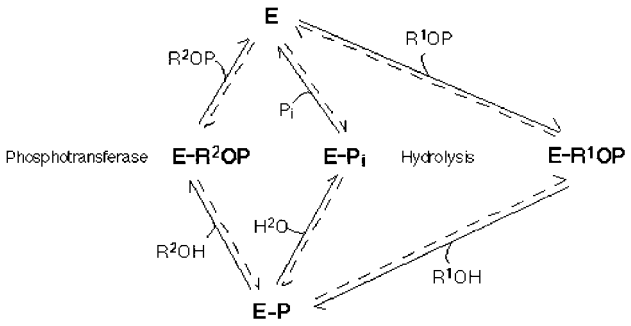

Scheme 1. *Schematic representation of the reaction mechanism of alkaline phosphatase.*

as Tris or ethanolamine, the enzyme also transfers the phosphate from the substrate to the alcohol with a higher efficiency at pH 8.0.[2]

Mammalian APs share a number of physicochemical features with the bacterial enzymes, including 25 – 30% amino acid sequence identity and an active site that may be similarly organized.[8, 9] Though much more active, the mammalian enzymes harbor an esterase activity characterized by a k_{cat} value of about 2000 s^{-1},[10] and once combined with the transferase activity,[2] a k_{cat} value of 3000 – 4000 s^{-1};[11] they are much less thermostable than bacterial enzymes, the respective T_m values being about 65 °C and 95 °C.[8, 12]

The bacterial enzyme has the potential of becoming an industrial tool for applications in diagnostics. However, its turnover rate is too low ($k_{cat} = 65 – 80\ s^{-1}$) and needs to be increased to a level comparable to that of mammalian enzymes. A number of mutations introduced within the catalytic site provided an *E. coli* enzyme with optimal k_{cat} values of 400 – 500 s^{-1}[8] and at most of 1070 s^{-1} when Asp 101 was mutated to a serine residue.[13, 14] Moreover, the mutations occurring at position 153 were shown to provide the bacterial enzyme with some of the properties of the mammalian enzyme, such as the activity-versus-pH profile.[7, 8, 15, 16] The beneficial mutations always caused a substantial decrease in thermostability. Overall, these studies could have suggested that an increase in catalytic efficacy, even a small one, might correlate with a decrease in stability. In this work, however, we show that the bacterial enzyme can reach the catalytic (esterase plus transferase) efficiency of the mammalian enzyme while also largely retaining its original high stability.

Our strategy was based on several considerations. First, it was previously shown that the activity of an enzyme can be abolished by mutating a residue critical for the catalytic process and then partially restored by subsequent compensating mutations.[17] Second, it is becoming clear that residues other than those that are essential in a catalytic reaction, including some that may be outside the catalytic site, may act distantly on the catalytic activity. We decided, therefore, to search for residues not essential for the catalytic reaction of the enzyme whose mutations would inactivate it. Our goal being to reactivate the enzyme by a random mutational analysis, it was important that these mutations should not destroy the overall conformation of the enzyme. Since the mammalian and bacterial enzymes adopt the same overall conformation,[3] we decided to transfer progressively four mutations from the mammalian enzyme to the bacterial one. Thus, we not only successfully inactivated the bacterial enzyme but we also fully restored its activity by a random mutational experiment. Strikingly, a single mutation (D330N), located 12 Å from the center of the catalytic pocket, sufficed to reactivate the enzyme entirely. Third, we combined this mutation with those previously known to slightly increase the catalytic activity of the enzyme.[8, 15, 16, 18] Thus, we found that the double mutant D153G/D330N has a combined phosphatase and transferase activity characterized by a k_{cat} value of 3200 s^{-1}, a value that is highly similar to those of mammalian enzymes, but also a high thermostability, comparable to that of the bacterial enzyme. Finally, a preliminary crystallographic study of the mutant shed light on how the mutation D330N may contribute to the increase in the enzyme's catalytic efficacy.

[*] *Members of the Editorial Advisory Board will be introduced to the readers with their first manuscript.*

Results and Discussion

Preparation of an inactive AP mutant

At first, our attention was drawn by the previous observation that in the absence of magnesium, introduction of the mammalian mutation K328H slightly decreases the activity of the bacterial enzyme.[8, 19] The crystal structure of the bacterial enzyme revealed that Lys 328 is located in the small helix 325 – 331, with Asp 327 and His 331 that are both conserved in mammalian and bacterial APs and essential for stabilizing the critical Zn ion at site M1.[3] However, the sequences which, on the small helical stretch, are framed by these two critical residues are different in the bacterial and mammalian enzymes, being Lys-Gln-Asp and His-Gly-His, respectively. We therefore decided to provide the whole helical region 327 – 331 of the bacterial enzyme with the corresponding mammalian sequence. In addition, it was reported that, still in the absence of magnesium, the mutation D153H also causes a slight activity decrease.[8] Therefore, the bacterial enzyme concomitantly received the four mutations K328H/Q329G/D330H and D153H.

The different forms of the enzyme were expressed from three vectors. Thus, the plasmids pJC2431[20] and pLIP4.0.B, respectively, encode the wild-type bacterial (AP) and a wild-type-like form (APl) which allows insertion of foreign proteins between its codons +6 and +7 (Figure 1).[21] In the present work we used

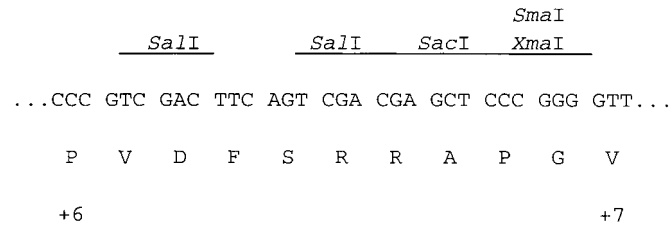

Figure 1. *Nucleotide and deduced amino acid sequences of the in-frame insertion between codons +6 and +7 of the alkaline phosphatase gene. The included restriction sites are also shown.*

this form rather than the real wild-type enzyme. A third vector encodes APl that received the mutations D153H/K328H/Q329G/D330H; we named this protein APbm1. After chromatofocusing of the periplasmic extracts from bacteria transformed with each vector, SDS-PAGE analysis revealed that the three resulting proteins were all 95% pure (data not shown). As expected, the apparent molecular weight of the AP was close to 43 kDa, its electrophoretic mobility being higher than those of APl and APbm1 which both possess nine amino acids previously added to the N-terminal region of the enzyme.[21] The production level of the three proteins was similar, indicating that the expression of the different genes as well as the exportation process were not significantly altered by the four mutations. The fact that the recombinant proteins were not degraded by the proteolytic system of the bacteria suggests that they are correctly folded.[22] The apparent molecular mass of the enzymes as determined under nondenaturing conditions by size exclusion chromatography was 84–87 kDa for both AP and APl, which is in agreement with the expected mass of a dimer of alkaline phosphatase (86 kDa), as determined by sedimentation equilibrium analysis.[23] APbm1 had a molecular mass of 90–95 kDa (data not shown). This result is not incompatible with the view that this protein is a dimer of alkaline phosphatase; however, since APbm1 is uniquely characterized by approximately an additional 10 kDa in molecular mass, we assumed that one or more of the mutations D153H/K328H/Q329G/D330H might have altered the hydrodynamic properties of the enzyme. Presumably APbm1 had undergone some structural modification.

The kinetic constants (k_{cat} and K_m) of purified AP, APl, and APbm1 were determined by using *p*-nitrophenyl phosphate (pNPP) as substrate. The experiments were performed in the presence of magnesium with 1 M Tris-HCl which acts as phosphate acceptor and hence enhances the rate of release of *p*-nitrophenolate.[2] The pH was 8.0 which corresponds to the optimal pH for the transferase activity of the wild-type enzyme.[2] The results of these experiments are shown in Table 1. AP and APl displayed similar kinetic characteristics that agreed with those previously reported for the wild-type enzyme.[7, 8] The k_{cat} values of AP and APl were 65 and 78 s^{-1}, respectively, whereas the K_m values were 23 and 30 μM. These results indicate that the presence of nine additional residues in the N-terminal part of the enzyme had no detectable effect on its catalytic characteristics, in agreement with our previous report.[24] In contrast, APbm1 had no detectable catalytic activity (Table 1). Clearly, the slight decrease in activity caused by the two mutations D153H/K328H[8] in the absence of magnesium has been dramatically enhanced by either or both of the two additional mutations Q329G and D330H, although these residues do not belong per se to the catalytic pocket.

Table 1. *Kinetic parameters of the wild-type (AP and APl), inactive (APbm1), and revertant (APbm2) enzymes in 1.0 M Tris at pH 8.0.[a]*

Enzyme	k_{cat} [s^{-1}][b]	K_m [μM]	k_{cat}/K_m ($\times 10^{-6}$) [$M^{-1} s^{-1}$]
AP (pJC2431)	65 ± 1	23 ± 1	2.8
APl (pLIP4.0.B)	78 ± 4	30 ± 5	2.6
APbm1 (D153H/K328H/Q329G/D330H)	< 0.7	n.d.	n.d.
APbm2 (D153H/K328H/Q329G/D330N)	82 ± 2	49 ± 6	1.7

[a] Assays were performed at 25 °C in 1 M Tris-HCl (pH 8.0) with *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} values by assuming a dimer molecular mass of 96000 Da. – n.d. = not determined.

Restoring catalytic activity

By using the polymerase chain reaction (PCR) we imposed an error-prone mutagenesis[25] through almost the whole coding region of the *phoA* gene coding for the inactive APbm1 protein. Search for a revertant was based on the expression of catalytic activity, which yielded colored colonies when an appropriate phosphatase substrate was added (see Experimental Section). Approximately 50000 clones were obtained, and two of them reacted positively to substrate addition. Both were sequenced, revealing the presence in their *phoA* gene of a single mutation at codon 330. In both cases, the original His330 mutation was reverted to an asparagine. In both AP and APl, this position is occupied by an aspartic acid residue; however, introduction of this residue into APbm1 does not restore its activity (data not shown). As the mutant D153H/K328H/Q329G has no catalytic activity, the mutation Q329G and not necessarily D330H may be responsible for the lack of activity of APbm1. Therefore, the mutation H330N is a real intragenic suppressor mutation.[17] We named APbm2 the phenotypic revertant containing the four mutations D153H/K328H/Q329G/H330N. At pH 8.0 in the presence of magnesium and in 1 M Tris-HCl buffer, APbm2 had a catalytic constant (k_{cat} = 82 s^{-1}) similar to that of APl (k_{cat} = 78 s^{-1}) with a slightly higher K_m value (49 instead of 30 μM; Table 1)

Effect of an asparagine at position 330 on catalytic activity

We studied the catalytic parameters of the enzymes at pH 10.0, in the presence of magnesium and in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, which are the optimal esterase conditions for both mammalian enzymes and D153H-modified bacterial enzymes. At first, we redetermined the

catalytic constants and the K_m values of the two single mutants D153H and K328H and found them to be highly similar to those previously determined by other authors.[7, 8] Under the same conditions, the catalytic parameters of APbm2 are, respectively, about threefold ($k_{cat} = 227\ s^{-1}$) and sixfold ($K_m = 562\ \mu M$) higher than the corresponding values of the wild-type-like API.

We then studied, under similar conditions, the effect of the mutation D330N, alone or associated with other mammalian mutations (D153H and K328H) previously shown to increase the efficacy of the enzyme.[8] Clearly, all the tested mutants possessing the mutation D330N have a higher k_{cat} value than API (Table 2). Thus, the k_{cat} values of both the single mutant D330N and the double mutant K328H/D330N are about two- to threefold higher. The mutant with the triple mutation D153H/K328H/D330N displays a nearly eightfold higher k_{cat}, an increase that is slightly greater than the highest rate enhancement (approximately sixfold) previously observed with the double mutant D153H/K328H.[8] However, the most interesting result was obtained with the mutant having the two mutations D153H/D330N whose k_{cat} value was approximately 17-fold higher than that of the wild-type-like mutant. Interestingly, the K_m value of this mutant was 350 μM, which is close to the K_m values of mammalian enzymes. To our knowledge, this is the first time that a mutant of bacterial alkaline phosphatase has such a high k_{cat} value. Clearly, the presence of an asparagine residue at position 330 has dramatically enhanced the slight activity increase observed upon the single mutation D153H.

Table 2. Kinetic parameters of the wild-type and mutant enzymes in 0.1 M CAPS at pH 10.0.[a]

Enzyme[b]	k_{cat} [s^{-1}][c]	K_m [μM]	k_{cat}/K_m ($\times 10^{-6}$) [$M^{-1}s^{-1}$]
API (pLIP4.0.B)	80 ± 3	90 ± 8	0.9
APbm2 (D153H/K328H/Q329G/H330N)	227 ± 4	562 ± 45	0.4
D153H	240 ± 12	320 ± 30	0.8
K328H	214 ± 11	105 ± 11	2.0
D330N	201 ± 10	47 ± 4	4.3
K328H/D330N	254 ± 20	112 ± 9	2.3
D153H/D330N	1389 ± 110	350 ± 30	4.0
D153G/D330N	1400 ± 60	566 ± 20	2.5
D153H/K328H/D330N	650 ± 43	170 ± 16	3.8

[a] Assays were performed at 25 °C in 0.1 M CAPS (pH 10.0), 10 mM Mg^{2+} (ionic strength adjusted to 0.5 M with NaCl), with *p*-nitrophenyl phosphate as substrate. [b] The enzymes were preincubated for 2 h at 25 °C in 0.1 M CAPS buffer (pH 10.0), 10 mM Mg^{2+}, before the assays were carried out. [c] The k_{cat} values are calculated from the V_{max} values by assuming a dimer molecular mass of 96 000 Da.

Position 153 of the bacterial enzyme has been previously explored by various groups who introduced three other mutations, D153G,[18] D153A, and D153N.[15] Like D153H, the mutations D153G and D153A caused an increase in esterase activity, the mutation D153A being apparently the most efficient mutation. Since the presence of an asparagine residue at position 330 improved the positive effect of the mutation D153H, it was of interest to examine the effect of the asparagine on the three other mutations. D153G alone had previously been shown to provide AP with a five- to sixfold higher k_{cat} value.[18] When D153G was combined with D330N, the enzyme displayed a k_{cat} of 1400 $s^{-1} \pm 60$ and a K_m of 566 ± 20 μM (Table 2), which are virtually identical to the values observed with the double mutant D153H/D330N. Surprisingly, the double mutant D153A/D330N was less potent than the single mutant D153A.

Thermostability of the two mutants D153H/D330N and D153G/D330N

We tested the thermostability of the two double mutants displaying the highest activity, D153H/D330N and D153G/D330N. It was previously reported that a mutant of bacterial AP carrying the mutation D153H is less thermostable than the wild-type enzyme.[8] We found that the additional presence of the mutation D330N did not improve this situation. In fact, the T_m of the double mutant D153H/D330N was close to 70 °C (Figure 2), which is only 5 °C higher than that of the phosphatase from calf intestine, but almost 25 °C lower than that of the *E. coli* enzyme. Moreover, its activity disappeared after a few days of storage at 4 °C. This low thermostability was solely due to the mutation D153H and not to the mutation D330N, since the sole presence of D330N had virtually no effect on the stability of the bacterial enzyme (Figure 2). In sharp contrast to that, the double mutant D153G/D330N was almost as stable as the original bacterial enzyme, with a T_m of 87 °C, as compared to the T_m of 95 °C for bacterial enzymes (Figure 2). Moreover, the double mutant D153G/D330N remained fully stable for at least one year in a storage buffer containing antibiotics and protease inhibitors. This result is all the more striking as the AP mutant with the single mutation D153G was previously reported to be poorly stable.[18] Therefore, the presence of the mutation D330N largely contributed to the stability of the double mutant D153G/D330N.

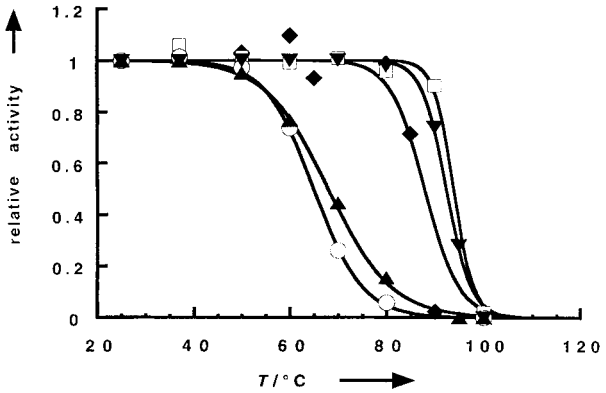

Figure 2. Heat stability of API, the wild-type-like E. coli *enzyme (□), and of its mutants D330N (▼), D153H/D330N (▲), and D153G/D330N (◆). The heat stability of calf intestinal alkaline phosphatase is also shown (○).*

The double mutant D153G/D330N is as active as mammalian APs

Intestinal mammalian APs are more active than the *Escherichia coli* enzyme,[5] the respective k_{cat} values being around 3000 –

4000 s^{-1} and 65 – 80 s^{-1} under the respective optimal conditions. These values are reached at pH 10.0 and pH 8.0 for mammalian and bacterial enzymes, respectively, with a concomitant expression of esterase and transferase activities for both types of enzymes.[26] The transferase activity occurs only if the buffer contains a high concentration of a phosphate acceptor like diethanolamine (DEA) or Tris.[2] Therefore, to obtain a better estimate of the activity of the double mutant D153G/D330N and to compare it with those of mammalian enzymes,[26] we tested it in the presence of 1 M DEA at 37 °C. As shown in Figure 3, the

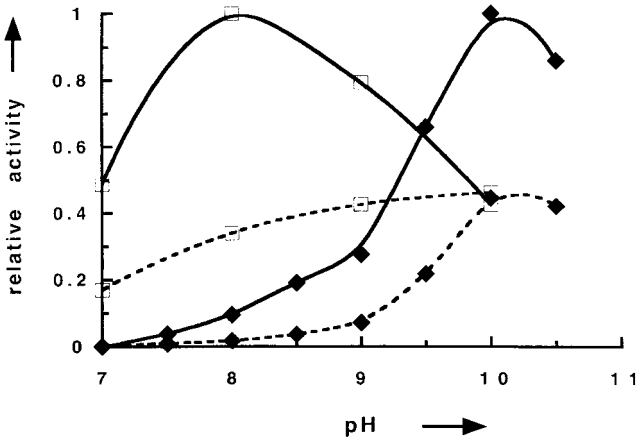

Figure 3. *Effect of the pH on the activity of API (□) and the mutant D153G/D330N (◆). The esterase activity (dotted lines) was measured at 25 °C in the following buffers: 50 mM Tris-HCl, pH 7.0 – 9.0; and 0.1 M CAPS, pH 9.5 – 10.5, both in the presence of 10 mM $MgCl_2$, 0.5 M NaCl, and 5 mM* p*-nitrophenyl phosphate. The sum of the hydrolytic and transferase activities (continuous lines) was measured at 25 °C in the following buffers: 1 M Tris-HCl, pH 7.0 – 9.0; and 1 M DEA, pH 9.5 – 10.5, both in the presence of 10 mM $MgCl_2$ and 5 mM* p*-nitrophenyl phosphate. Phosphatase activity was measured by monitoring the release of* p*-nitrophenolate at 410 nm after 10 min.*

activity of the mutant is pH-dependent with an optimal activity around pH 10, which is the same as for the mammalian enzymes. Under these conditions, the double mutant D153G/D330N displayed virtually the same k_{cat} and K_m values as those of bovine intestinal phosphatase (Table 3). Thus, the activity of the double mutant ($k_{cat} = 3200\ s^{-1}$) was 40- to 50-fold higher than that of the wild-type bacterial enzyme ($k_{cat} = 65 – 80\ s^{-1}$).

Table 3. *Biochemical comparison of different alkaline phosphatases.*[a]

Enzyme	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m ($\times 10^{-6}$) [$M^{-1}s^{-1}$]
E. coli wild-type	65 ± 1	23 ± 1	2.80
E. coli D153G/D330N	3202 ± 75	567 ± 7	5.65
bovine intestinal[b]	3435 ± 84	660 ± 23	5.20

[a] Assays were performed under optimal conditions defined for each enzyme: 1 M Tris (pH 8.0) and 25 °C for the wild-type bacterial enzyme; 1 M diethanolamine (pH 10.0) and 37 °C for the bacterial double mutant. [b] The catalytic parameters for bovine alkaline phosphatase are taken from ref. [11].

Preliminary structural data on the double mutant

In order to understand the molecular basis associated with the enhancement of the catalytic activity observed with the double mutant D153G/D330N, we crystallized it under conditions similar to those previously described.[3] These conditions included soaking the crystal in a solution containing 100 mM phosphate (NaH_2PO_4) and 10 mM magnesium ($MgCl_2$). After refinement of the model to 3.1 Å resolution with the XPLOR program,[27] the working R factor was 16.6% and R_{free} was 24.2%. As expected, the structure of the double mutant and that of the wild-type enzyme are highly similar with an rms deviation of 0.4 Å for the 852 target Cα atoms used for the comparison. To illustrate further that the substitution D330N caused no structural deviation, we noted that the side chain of Asn 330 was fully superimposable on that of Asp 330.

Previous studies have shown that the wild-type enzyme crystallizes with a phosphate group held in the active site by noncovalent interactions.[3] Close inspection of the active site of the double mutant revealed the presence of a phosphate group, but the distance between phosphate and the hydroxy group of Ser 102 is in agreement with a covalent bond (Figure 4a). This result is particularly surprising because the only other model containing a phosphoseryl intermediate was obtained through

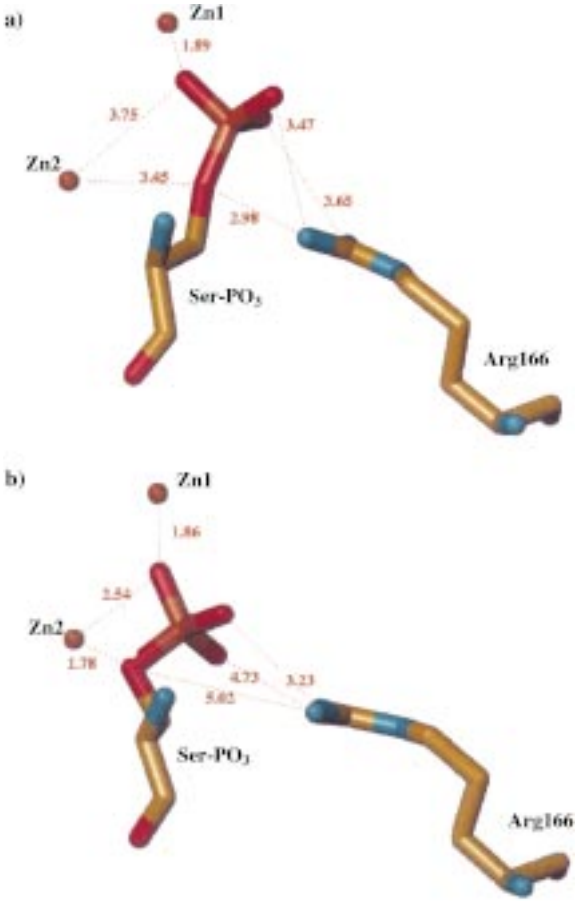

Figure 4. *Schematic representation of interactions in phosphoserine intermediates. a) Mutant D153G/D330N; b) mutant H331Q (Protein Data Bank entry code 1HJK).*

the inhibition of the phosphoseryl hydrolysis step. This was performed either by substituting the zinc ion in binding site M1 by a cadmium ion[3] or by the mutation H331Q, which affected the stability of zinc binding at M1.[28] The authors considered mutation H331Q to mimic the phosphoseryl intermediate of the wild-type enzyme, and we compared it to the phosphoseryl intermediate of mutant D153G/D330N (Figures 4b and 4a, respectively). First, one should notice that the mutations are too distant to interfere directly with the zinc ion at M1. Second, the phosphate group in D153G/D330N is shifted by 1.6 Å relative to that in H331Q, toward the entrance of the active-site pocket. This shift affects the distance between the zinc ion at M2 and the phosphate group, which becomes too long for a direct interaction. Third, the distance between the phosphate group and the zinc ion at M1 is not affected, suggesting that the latter can still act as an activator in the phosphoseryl hydrolysis step.

The observation of the phosphoseryl intermediate implies that the covalent enzyme–phosphate complex is more stable than the noncovalent one. This may be due either to a slowing down of the phosphoseryl hydrolysis step or to a speeding up of the phosphate release from the noncovalent complex. The catalytic data presented above are consistent with an acceleration process. It is therefore tempting to assume that the phosphate release has been accelerated and that the limiting step may now be the phosphoseryl hydrolysis. However, the catalytic rate constants of the different steps of the reaction must now be investigated.

Conclusions

Previous reports have shown that improvement of the catalytic activity of bacterial alkaline phosphatase is possible.[8, 13] In these studies, however, the highest activities[13] remained substantially below those of the mammalian enzymes.[14] Moreover, improved catalytic activities were accompanied by substantial decreases in thermostability.[8, 18] We have shown here that introduction of only two mutations sufficed not only to provide a bacterial enzyme with the high catalytic activity of mammalian enzymes, but also to almost maintain its high original thermostability. This result is clearly associated with the mutation D330N outside the catalytic pocket, 12 Å away from the substrate-binding site.

The major question which now remains to be solved is how the mutation D330N causes the observed beneficial effects. Preliminary crystallographic data suggest that the mutation D330N may be uniquely responsible for the acceleration of the release of the product in the medium. It remains to be elucidated how this phenomenon occurs.

Experimental Section

Bacterial strains and plasmids: *E. coli* K12 strains MV1190 and M13 phages M13mp18 and M13mp19 were from Biorad. The Δ*phoA* *E. coli* K12 strain CC118[29] was used for plasmid transformation according to the procedure previously described[30] and for plasmid electroporation according to the procedure described by Biorad. The pLIP4.0.B plasmid was a derivative of pLIP4.0 described by Gillet et al.,[21] in which a *Bam*HI restriction site was introduced downstream from the *phoA* promoter in order to create an exchangeable promoter cassette. The vector, containing the in-frame *phoA* gene, included several restriction sites between codons +6 and +7 of the mature PhoA protein corresponding to an insertion of nine amino acids (Figure 1). These sites were used to insert foreign genes, resulting in the production of recombinant immunoenzymatic conjugates.[21, 31, 32]

Mutagenesis procedures: The method described by Kunkel[33] was used to introduce simultaneously a nucleotide sequence encoding the mammalian enzyme residues histidine, glycine, and histidine at positions 328, 329, and 330 of the bacterial alkaline phosphatase, respectively. Independently, the previously described D153H mutation[8] was similarly constructed and recombined with the modified triplet to generate a chimera containing four mammalian-type residues. Oligonucleotides for site-directed mutagenesis were obtained from Bioprobe Systems. Site-directed mutagenesis was carried out on a *Sac*I/*Sph*I fragment of the structural gene encoding *E. coli* alkaline phosphatase (*phoA*). The presence of the expected mutations was checked by DNA sequencing.[34] Random mutagenesis was performed as previously described[25] on a *Sac*I/*Sph*I fragment of the fourfold mutated vector pLIP4.0.B/D153H-K328H-Q329G-D330H. For amplification, the 5′ primer (GACTTCAGTCGACGAGCTCCCGGG) and the 3′ primer (AACAACATTGGCGGCATGCGGGCC) were used. The Taq polymerase (Gibco BRL) used for the reaction committed approximately 1 error every 30000 incorporated nucleotides. The PCR product (1250 bp) was then subcloned into the plasmid pLIP4.0.B/D153H-K328H-Q329G-D330H between the *Sac*I and *Sph*I restriction sites, and the clones expressing an active alkaline phosphatase were selected on plates containing Xp substrate as previously described.[35] 50000 white clones and two blue clones were obtained. DNA sequencing of the selected *phoA* genes revealed the presence of the same nucleotide mutation in the two clones.

Purification of AP proteins: Transformed cells were grown overnight at 37 °C in LB medium supplemented with 200 μg mL^{-1} ampicillin and then seeded to a 1/50 dilution in 200 mL of low-phosphate medium.[35] After 5 h incubation at 37 °C, periplasmic proteins were extracted from the bacterial culture by cold osmotic shock as previously described.[36] The resulting periplasmic extracts were concentrated from 20 mL to 1 mL and dialyzed against 0.02 M Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$, at 4 °C by using a Centricon-30 microconcentrator (Amicon). Chromatofocusing of AP proteins was performed on a fast protein liquid chromatography system using a Mono P HR 5/5 column and Polybuffer 74 and 96 as described.[14] The active fractions were submitted to SDS-PAGE. The fractions migrating with an apparent molecular mass of 48 kDa were pooled and concentrated to 100 μL. The polybuffers were removed by chromatography on Sephadex G-75, equilibrated in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. SDS-PAGE revealed a single major band which represented more than 95% of the total stained protein. The enzymes were stored at −20 °C in 20 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 0.02% (w/v) NaN_3, 50% glycerol. The concentrations of the purified wild-type and mutant enzymes were determined by absorbance measurements at 278 nm with an extinction coefficient of 0.71 cm^2 mg^{-1}.[37]

Determination of enzyme activity: Measurements were performed at 25 °C when Tris or CAPS buffers were used and at 37 °C when DEA buffer was used. The enzymatic activity was measured spectrophotometrically by using *p*-nitrophenyl phosphate (pNPP) as substrate, by monitoring the release of *p*-nitrophenolate (pNP) at 410 nm (molar extinction coefficient 1.62×10^4 M^{-1} cm^{-1}). The buffers used were either 1 M Tris-HCl (pH 8.0), 0.1 M CAPS (pH 10.0, 0.4 M NaCl,

10 mM $MgCl_2$), or 1 M DEA (pH 10.0, 1 mM $MgCl_2$, 20 μM $ZnCl_2$). The k_{cat} values were computed from the V_{max} values by assuming a dimer molecular mass of 96 000 Da. Heat stabilities of the wild-type and modified enzymes were determined according to the procedure described by Janeway et al.[8]

Determination of the X-ray crystal structure: The D153G/D330N enzyme was crystallized by vapor diffusion using the sitting-drop method. The crystallization conditions previously reported[3, 38] had to be adjusted. The optimal conditions were 1.1 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 20 mM $ZnSO_4$, 100 mM Tris-HCl (pH 8.0), with a protein concentration of 30 – 40 mg mL^{-1}. The crystals were soaked sequentially in a solution containing 2.2 M $(NH_4)_2SO_4$, 0.05 M Tris-HCl at pH 8.0, 10 mM $MgCl_2$ for two weeks, then in a solution containing 2.4 M $(NH_4)_2SO_4$, 0.05 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaH_2PO_4 for five weeks. The data were collected to 3.1 Å resolution on a 300-mm diameter MAR research imaging plate system mounted on a Rigaku H2R rotating anode, and processed with the HKL package.[39] The structure was refined with XPLOR version 3.1[27] to an R_{work} of 16.6% and an R_{free} of 24.2%. The final model includes 888 residues including 2 phosphoserines, 6 metal ions, and 146 water molecules. The coordinates have been deposited in the Protein Data Bank under entry code 1i23.

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