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Staphylococcal nuclease: Proposed mechanism of action based on structure of enzyme–thymidine 3′,5′-bisphosphate–calcium ion complex at 1.5-Å resolution

(micrococcal nuclease/arginine residues/x-ray crystallography)

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ABSTRACT **The structure of the staphylococcal nuclease (EC 3.1.4.7)–thymidine 3′,5′-bisphosphate–Ca^{2+} (enzyme–inhibitor) complex has been extended to 1.5-Å resolution by using much additional data and a phase refinement scheme based on an electron-density map modification procedure. By correlating this structure with the known properties of the enzyme, a mechanism of action is proposed that involves nucleophilic attack on phosphorus by a water molecule, which is bound to Glu-43, in line with the 5′-$CH_2O(H)$ leaving group. The carboxylate of Glu-43 promotes this attack by acting as a general base for the abstraction of a proton from the attacking water molecule. Nucleophilic attack is further facilitated by polarization of the phosphodiester by an ionic interaction between a Ca^{2+} ion and a phosphate oxygen atom and by four hydrogen bonds to phosphate oxygen atoms from guanidinium ions of Arg-35 and Arg-87. These interactions may also catalyze the reaction by lowering the energy of a trigonal bipyramidal transition state. The hydrolysis of nucleic acid substrate proceeds by cleavage of the 5′—P—O bond to yield a free 5′-hydroxyl group and a terminal, 3′-phosphate monoester group. In the inhibitor complex the only general acid group found in a position to donate a proton to the leaving 5′-oxygen is the guanidinium ion of Arg-87. Alternative proton donors, presently lacking direct structural support, could be the phenolic hydroxyl group of Tyr-113 or a water molecule. The precision and rigidity of the location of the reactants at the active site and the probable dual binding and catalytic roles of the guanidinium ions of Arg-35 and Arg-87 are especially noteworthy.**

Staphylococcal or micrococcal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) is a Ca^{2+}-dependent, extracellular enzyme produced by certain strains of *Staphylococcus aureus*. As isolated, the enzyme has a single peptide chain of 149 amino acid residues with no intrachain crosslinkages and a molecular weight of about 16,800. The small size, availability in highly purified form, and interesting properties of this enzyme have led to its use as a model in several significant studies in protein chemistry and as a practical tool in nucleic acid research. We have recently prepared a comprehensive, four-part review of the nuclease (1–4), and other reviews dealing with this enzyme have also appeared (5, 6). We restrict ourselves here to a description of the structure of the active site of the enzyme based on our interpretation of the nuclease–thymidine 3′,5′-bisphosphate (pdTp)–Ca^{2+} complex at 1.5-Å resolution and to the proposal of a plausible mechanism of action based on this structure and certain known chemical and enzymological properties of the nuclease. We note that, to the best of our knowledge, this is one of a very few enzyme structures that has been determined at a resolution as high as 1.5 Å.

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MATERIALS AND METHODS

Crystals of the nuclease–pdTp–Ca^{2+} complex were grown as described (7, 8). Data were collected with a Syntex $P\bar{1}$ autodiffractometer by using an abbreviated ω scan. Our earlier high-resolution structure (8) of the enzyme–inhibitor complex was based on some 4600 independent reflections phased by the use of two heavy-atom derivatives, but, between *d*-spacings of 4 Å and 2 Å, the data set included only that third of the reflections having the highest intensities. By using these phased reflections as a base, phases were extended and refined by an iterative, electron-density map modification procedure (9, 10) to a total of some 18,400 (of a possible 25,000) measured reflections within a *d*-spacing of 1.5 Å. The resultant electron-density map was fitted to a model by using an interactive computer graphics system developed in the laboratory of Edgar Meyer at this university (9). A full description of these methods, the detailed results of their application, and the nominal 1.5-Å structure of the nuclease–pdTp–Ca^{2+} complex are as yet unpublished.

RESULTS AND DISCUSSION

Enzymological properties of the nuclease

Staphylococcal nuclease catalyzes the hydrolysis of both DNA and RNA at the 5′ position of the phosphodiester bond yielding a free 5′-hydroxyl group and a 3′-phosphate monoester (1–3, 11). The pH optimum is between 8.6 and 10.3 and varies inversely with Ca^{2+} concentration, but at any pH rather high levels of Ca^{2+}, typically 0.01 M, are required for optimal activity (1–3, 11). With minor exceptions, which will be discussed later, Ca^{2+} is required for activity with all substrates and cannot be replaced by other ions, although Ca^{2+} and a number of other ions do promote the binding of various inhibitors (1–3, 11–13). The 5′-*p*-nitrophenyl esters of pdT and pdTp are the simplest known good substrates for the nuclease. Both these esters are hydrolyzed at essentially the same rate via P—O bond cleavage of the 5′—C—O—P bond, but the pdTp-based ester has a K_m showing almost two orders of magnitude tighter binding to enzyme (1–3, 11–13). Both the 5′-methyl ester of pdT and thymidine 5′-fluorophosphate are poor substrates. With these simple substrates, the products of the enzyme-catalyzed reaction are exclusively *p*-nitrophenyl phosphate and dT or dTp for the first two and methylphosphate or fluorophosphate plus dT for the second two [as Dunn *et al.* (13) pointed out], a striking and significant contrast to the products of a nonenzymatic hydrolysis of these compounds where *p*-nitrophenol, fluoride ion, or at least some methanol along with pdT or pdTp

Abbreviation: pdTp, thymidine 3′,5′-bisphosphate.

would result (1–3, 11–13). Simple diesters of phosphate are not substrates, so this enzyme is indeed a nuclease and not a general phosphodiesterase (11, 12). For a series of dinucleotides, $dN^{\alpha}pdN^{\beta}$, as substrates, there is a distinct order of preference ($dT \leq dA \gg dC \gg dG$) for the base in the β-nucleotide position, but little base specificity in the α-position (14, 15). The presence of a terminal 3′ phosphate results in a better substrate than the plain dinucleotide; a terminal 5′ phosphate results in a poorer substrate (14, 15). In the presence of Ca^{2+}, or a wide variety of other metal ions, pA, pdA, and pdT are good inhibitors ($K_i \simeq 10^{-5}$); pdTp is the best known inhibitor ($K_i \simeq 10^{-7}$) (1–3, 11). The binding of the nucleoside 5′-monophosphates involves a single Ca^{2+} ion; the binding of pdTp and of DNA and RNA appears to involve two (2, 3, 16). Nucleosides themselves and nucleoside 2′- or 3′-monophosphates are not inhibitory (2, 3, 17). Mapping studies of the active site region with oligonucleotides having a terminal 5′ phosphate, $(pdT)_n$, show maximum binding when $n = 3$, suggesting the presence of a third, probably ionic, binding site in addition to those for the 5′- and 3′-phosphates of pdTp (11). Studies of the interactions among the inhibitors, the simple substrates, DNA, and RNA strongly imply a single, common binding and hydrolytic site on the nuclease (11, 17), although, as noted elsewhere (3), there are differences between DNA and RNA as substrates that may indicate different hydrolytic mechanisms.

The point of the preceding brief, but fairly comprehensive, review of the enzymological properties of staphylococcal nuclease is to establish that the crystal structure of the nuclease–pdTp–Ca^{2+} complex should resemble fairly closely that of the actual nuclease–substrate–Ca^{2+} complex. Because a diester substrate will have a single negative charge on the phosphate at the hydrolytic site and pdTp has two negative charges on its 5′ phosphate, some difference is, of course, to be expected.

In our following mechanistic arguments, water molecules are critically involved. Thus, any difference in hydration between an enzyme–inhibitor complex, dianionic at the 5′ phosphate, and an enzyme–substrate complex, monoanionic at this phosphate, could be of particular importance. We have attempted to more closely mimic the structure of an enzyme–substrate complex by preparing nuclease crystals containing suitable dinucleoside *phosphonate* analogues; these studies have not yet yielded any definitive information bearing on the mechanism of action (3).

Phosphate ester hydrolysis is complex; in postulating a mechanism, we shall make the following two assumptions that are reasonable for the pH and the diester substrates concerned. (*i*) Hydrolysis proceeds via direct nucleophilic attack on P with the formation of a five-coordinate, trigonal bipyramidal transition state or meta-stable intermediate followed by breakdown to give a new phosphate species. (*ii*) Attacking groups can enter and leaving groups leave only at the apical positions of the bipyramid (3, 13).*

The structure of the nuclease–pdTp complex

Even in our single-derivative, 4-Å resolution electron-density map of the nuclease–pdTp–Ca^{2+} complex, the location and orientation of the nucleotide was clear (18). In our nominal 2-Å resolution structure of the complex, the specific interactions between the nucleotide and the enzyme were clearly revealed, and the position of the single, clear-cut Ca^{2+} ion was determined (8, 19–21). However, presumably because the Ca^{2+} was replaced by Ba^{2+} to provide one of the heavy-atom derivatives, the Ca^{2+} position had abnormally low electron density and the delineation of the electron density in the area around the Ca^{2+} was not clear. Extension of the data set to include reflections to 1.5 Å and refinement of the phases have significantly improved the appearance of the entire electron density map, but most gratifying and useful has been the improvement at and around the Ca^{2+}-binding site (9). In the new map, the position of the Ca^{2+} is now sharp, and the ion has an electron density consistent with its atomic number; moreover, the area around the Ca^{2+}-binding site is much clearer and more readily interpreted.

Fig. 1 is a stereo view showing both our previous interpretation and our new interpretation of the pdTp/Ca^{2+} binding site. Fig. 2 is a schematized drawing of the active site. The major difference between our previous interpretation of the 2-Å map and our interpretation of the 1.5-Å map is the movement of Asp-19 and Glu-43 away from their positions as direct Ca^{2+} ligands and the insertion of three water molecules, two as Ca^{2+} ligands and one as a bridge between the carboxylate group of Glu-43 and one of the 5′-phosphoryloxygen atoms. Fig. 2 shows the major interactions of the enzyme, the nucleotide, and the Ca^{2+} ion, and only a brief additional description is required.

* Space does not permit a discussion of the mechanistic possibilities for phosphodiester hydrolysis. Ref. 3 has a brief discussion and leading references.

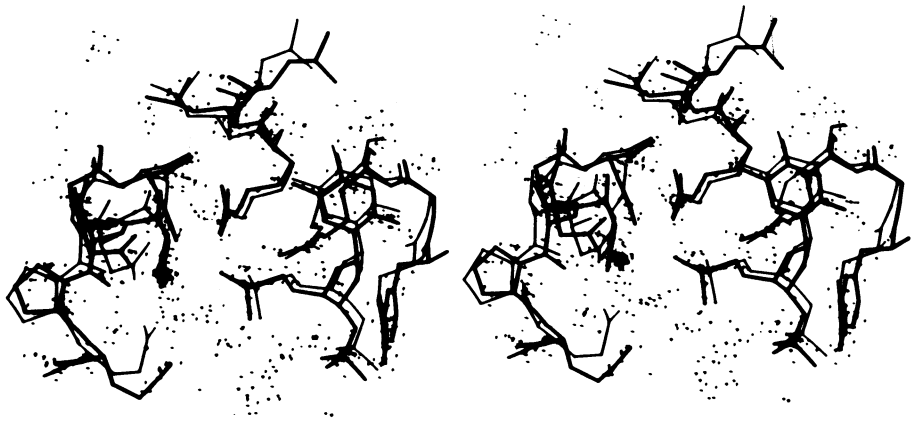

FIG. 1. Stereo view of binding-site region in nuclease–pdTp–Ca^{2+} complex. The stick models having light lines represent the interpretation at 2-Å resolution; the model with heavy lines, that at 1.5-Å resolution. The Ca^{2+} position is shown by a heavy cross (+). The peptide chain in the right background is -NH-Tyr(85)-Gly(86)-Arg(87)-C=O (reading outside to inside). The peptide chain in the upper center is -NH-Arg(35)-Leu(36)-Leu(37)-C=O (reading bottom to top). The peptide chain in the left background is -NH-Asp(19)-Gly(20)-Asp(21)-C=O (reading back to front). The final peptide chain in the left foreground is -NH-Asp(40)-Thr(41)-Pro(42)-Glu 43-C=O (reading top to bottom). Instead of contour lines, each point of electron density ≥ 0.6 e/Å^3, on a particular grid, is represented by a small cross.

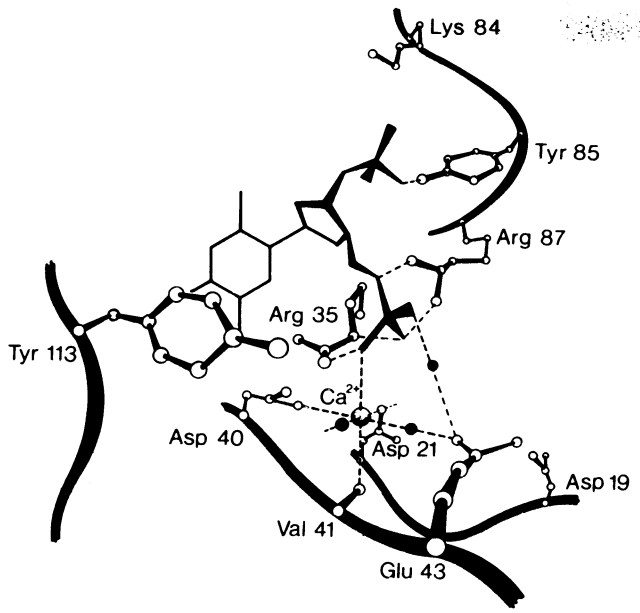

FIG. 2. Schematic view of binding site in nuclease–pdTp–Ca^{2+} complex. The heavy dots represent regions of electron density assigned as water molecules. Hydrogen-bond and ionic interactions are shown as dotted lines. The interaction between the Ca^{2+} and Val-41 is through the carbonyl oxygen of the peptide bond.

The thymine ring fits into a well-defined, hydrophobic pocket with the 5-methyl group down as viewed in Fig. 2 (8, 19–21). Not only do the guanidinium ions of Arg-35 and Arg-87 each form a pair of hydrogen bonds to the 5′ phosphate, but both these groups form additional hydrogen bonds (not shown) back to the enzyme and directed away from the phosphate. Arg-35 binds to the carbonyl oxygen atoms of Leu-36 and Val-39, and Arg-87 binds through the carboxylate group of Asp-83 to the peptide nitrogen atom of either Tyr-85 or Gly-86 (or both). These additional interactions, along with those shown in the figure, give the strong impression that the pdTp, particularly its 5′-phosphate, and the activating Ca^{2+} are precisely and rigidly locked into position at the hydrolytic site.

A proposed mechanism

Based on our 2-Å structure of the nuclease–pdTp–Ca^{2+} complex, we tentatively suggested a mechanism based on the nucleophilic attack at the phosphorus by a Ca^{2+}-bound hydroxide in line with the leaving group (8, 19, 20). This hypothesis, though plausible, suffered from the lack of any electron density corresponding to the postulated hydroxide ion. Now, as can be seen in the figures, we have an excellent candidate for an attacking nucleophile in the water molecule that bridges, through hydrogen bonds, the carboxylate group of Glu-43 and one of the oxygen atoms of the 5′-phosphate. Therefore, as illustrated in Fig. 3, we postulate a nucleophilic attack by the oxygen atom of this water molecule on the 5′-P atom in line with the 5′—C—O—P ester bond, the formation of a trigonal bipyramidal transition state or metastable intermediate with the 5′—C—O—P in an apical position, and finally the formation of the products by the breaking of the 5′—P—O bond. In addition to its function in accurately positioning the reactants, the carboxylate group of Glu-43 can serve as a general base for abstraction of a proton from the attacking water molecule. The ionic bond between the Ca^{2+} ion and a 5′-phosphate oxygen atom plus the total of four hydrogen bonds from Arg-35 and Arg-87 to the phosphate group can serve to neutralize the charge and to polarize the phosphate group so

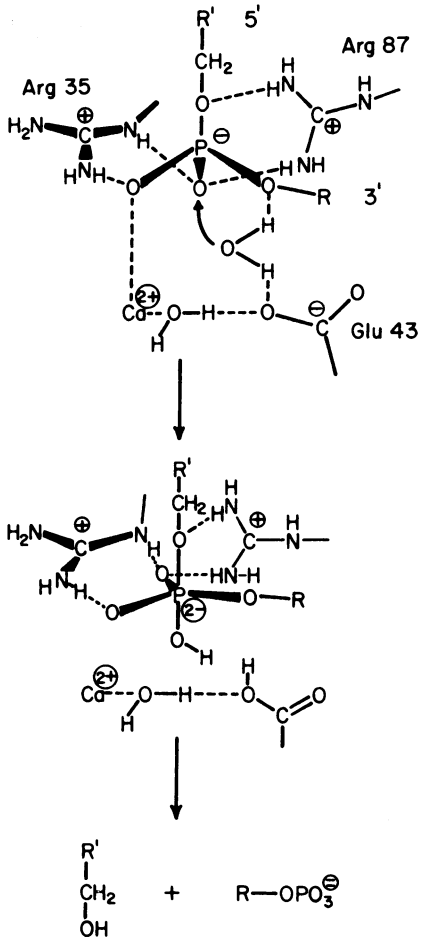

FIG. 3. Diagrammatic representation of proposed mechanism of action of staphylococcal nuclease. Nucleophilic attack by the water molecule bound between Glu-43 and an oxygen atom of the 5′-phosphate is promoted by interactions among the 5′ phosphate, the Ca^{2+} ion, and the guanidinium ions of Arg-35 and Arg-87. See text for further details. The actual stereochemical location of the 3′-R group within the active site is not known.

as to make it more susceptible to nucleophilic attack or to lower the energy of the transition state or intermediate. Efficient cleavage of the 5′—P—O bond should require the simultaneous protonation of the oxygen atom by some group acting as a general acid. Because it is indeed hydrogen-bonded to the leaving oxygen, the guanidinium ion of Arg-87 is an available if improbable candidate. It is perhaps not an entirely impossible candidate, because striking perturbation of the pK_as for weak acid groups in nonaqueous environments has indeed ample precedent in enzymology (22). Other possible general acids for this role are the phenolic hydroxyl group of Tyr-113 or water molecules, but we lack decisive structural evidence on this point. Fig. 1 shows that there is electron density in the area between the 3′- and 5′-phosphate groups of pdTp. Some of this can be accounted for by the presence of an ϵ-amino group of Lys-71 from another nuclease molecule in the crystal structure, but there remains some otherwise unaccounted for electron density in this area. Without evidence, we can only observe that both structurally and catalytically this is a logical place for the binding of the second Ca^{2+} ion noted in solution studies with pdTp and the natural substrates (16). Such a binding site is consistent with the relatively high levels of Ca^{2+} ions needed for optimal activity and for the stimulation of activity by Mg^{2+} only when Ca^{2+} is also present (11, 17). Dunn *et al.* (13) studied the effects of pH variation on the parameters common to the

analysis of enzyme kinetics and have analyzed these "titration" curves in terms of several apparent pK_as for the enzyme or enzyme–substrate complex. In view of the relatively minor, but clear-cut conformational change known to occur in nuclease upon inhibitor binding and known to involve tyrosine residues (2, 3, 11, 23), no easy correlation of these pK_as with specific groups appear to us to be possible at this time.

Comments and supporting data

We noted above the precise positioning of the reactants, and, if it is a near truism that one of the critical roles for groups at the active site of an enzyme must be to align the reactants accurately, it is one worth repeating. It is known that the staphylococcal nuclease is almost wholly intolerant to replacement of its active-site Ca^{2+}. Only Sr^{2+} gives any significant level of activity and then only for DNA, not RNA, hydrolysis, and Sr^{2+} is inhibitory in the presence of Ca^{2+}, as are a wide variety of other metal ions (17). Furthermore, by using a proteolytically modified but active variant of nuclease, Anfinsen and his associates (24, 25) have demonstrated synthetically that variation in the size or charge of the active-site residues Asp-19, Asp-21, Arg-35, Asp-40, and Glu-43 leads to complete inactivation or, where asparagine replaces Asp-40, to marked reduction in activity. Arg-87 was not replaced synthetically, but Tucker *et al.* (3) have shown that phenylglyoxal rapidly abolishes nuclease activity and that activity is retained and two arginines are protected from modification in the presences of pdTp and Ca^{2+}.

Although it might appear surprising that the carboxylate of Glu-43 is not a direct ligand of the Ca^{2+} ion, as are the carboxylates of Asp-21 and Asp-40 (Fig. 2), we note that, were this carboxylate directly bonded to the Ca^{2+}, it could scarcely function as a general base for proton abstraction. The water between the Ca^{2+} and the Glu-43 CO_2^- can serve a positioning role and simultaneously "insulate" the carboxylate from the positive charge on the calcium ion.

The now well-known idea that an important factor in the efficiency of enzyme catalysis could be that enzymes bind the transition state more tightly than the substrate was suggested by Pauling a number of years ago. Based upon the yet structurally unproven postulate that complexes of uridine with VO^{2+} have a trigonal bipyramidal geometry and are thus a transition-state analog, Lienhard and his associates (26, 27) have shown for ribonuclease that the K_i for the VO^{2+}–uridine complex is 1000 times greater than the K_m for the substrate, uridine 2′,3′-cyclic monophosphate, where both constants are very probably true enzyme–substrate and enzyme–inhibitor dissociation constants. By using VO^{2+} and deoxythymidine, we have found (3) for the staphylococcal nuclease a $K_i = 10^{-8}$, where $K_m = 10^{-3}$ for a comparable substrate, *p*-nitrophenyl-pdT. Here again, the K_m is very probably a true enzyme–substrate dissociation constant (11–13), and, as for enzyme activity or strong inhibitor binding, Ca^{2+} is required for the formation of a nuclease–VO^{2+}–thymidine complex, indicating specificity of interaction. Thus, the case for the VO^{2+}–thymidine complex as a trigonal bipyramidal transition-state analog is plausible, though enthusiasm for this notion should be tempered by the fact that the K_i for the nuclease–pdTp–Ca^{2+} complex is approximately 10^{-7} and, in the crystal structure of the complex, the 5′ phosphate is clearly tetrahedral.

Another limiting mechanism for the action of staphylococcal nuclease—namely, the attack on a nucleophile adjacent to, rather than in line with, the leaving group and a subsequent pseudorotation of the trigonal bipyramid to place the leaving group in a favored, apical position (3)—must be evaluated. Possible nucleophiles for this role are the phenolic hydroxyl group of Tyr-113 (see Fig. 2) or yet unlocated water molecules, but we consider this mechanism untenable on the following grounds. Because the exclusive products of the nuclease-catalyzed hydrolysis of the 5′-*p*-nitrophenyl or 5′-methyl esters of pdT nd of thymidine 5′-fluorophosphate are *p*-nitrophenyl-phosphate, methylphosphate and fluorophosphate, in the mechanism of action of this enzyme, two conditions must hold: (*i*) A nucleophile can never attack in line to the *p*-nitrophenyl, methyl, or fluoro groups, for this would place these groups in an apical and favored-for-leaving position. (*ii*) No pseudorotation of a trigonal bipyramidal transition state could take place that would place any of these groups in an apical and favored leaving position. For a mechanism involving adjacent nucleophilic attack, it is perhaps possible to imagine that the enzymatic binding site has sufficient stereochemical discrimination to position the 5′-*p*-nitrophenyl ester of thymidine 5′-phosphate in such a way that the *p*-nitrophenolate moiety could never assume an apical leaving position. However, we very much doubt that this argument can be extended to include the 5′-methyl ester and, especially, the 5′-fluorophosphate ester, and we therefore conclude that a mechanism involving adjacent nucleophilic attack and subsequent pseudorotation is not plausible.

We and others have called attention to the frequent occurrence of arginine residues at the active sites of enzymes and other proteins (28, 29) and to their consequent structural and functional roles (28). We are postulating here that the guanidinium ions of arginine residues can serve both *to bind and to catalytically activate a substrate.* The reality of this latter function has received important support from the recent study of Springs and Haake (30) who have shown that guanidinium ions produce a 27-fold rate enhancement for the cleavage of phosphodiesters by the fluoride ion.

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