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THE ROLE OF ARGININE RESIDUES AT ENZYME ACTIVE SITES

THE INTERACTION BETWEEN GUANIDINIUM IONS AND *p*-NITROPHENYL PHOSPHATE AND ITS EFFECT ON THE RATE OF HYDROLYSIS OF THE ESTER

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Summary

In alcoholic solutions a relatively strong complex forms among two guanidinium ions and one *p*-nitrophenylphosphate dianion. The effect of this complex formation on the hydrolysis of the ester is to lower the rate by a factor of 4 in solutions containing 1 M guanidine hydrochloride when compared with solutions of the same total ionic strength containing no guanidinium ion. It is therefore suggested that, for the enzymatically catalyzed hydrolysis of phosphate compounds going via the formation of a metaphosphate intermediate, the role of any arginine residues at the active site is primarily one of binding and positioning the substrate.

Introduction

The recognition that arginine residues are frequently present at the active sites of enzymes and other proteins, particularly those whose substrates, cofactors or ligands contain phosphate, is relatively recent [1—12 **]. Our crystal structure analysis of the enzyme-inhibitor complex formed from Staphylococcal nuclease, thymidine 3′,5′-diphosphate and calcium ion revealed that the 5′-phosphate of the inhibitor forms two good hydrogen bonds to each of the guanidinium ions of Arg-35 and Arg-87 from the enzyme [13—15]. Both of these guanidium ions also from additional hydrogen bonds to other parts of the enzyme structure, thus creating a rather rigid framework around the 5′-

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** Only recent work is cited. Consult the other references especially refs. 1 and 2 for additional citations relevant to arginine-phosphate interactions in proteins and enzymes.

phosphate. Model compounds mimic these observations from the enzyme-inhibitor complex [1,16,22]. Several lines of evidence strongly imply that this particular area is at the point of hydrolytic cleavage in nuclease-substrate complexes [15].

Thus, it seems reasonable to infer that these particular arginine residues in the Staphylococcal nuclease serve to position a substrate rigidly and properly in the enzymatic active site, but we have further speculated that the interaction the two guanidinium ions and the phosphate diester linkage of a substrate makes the diester more susceptible to nucleophilic attack of stabilizes the transition state in the hydrolysis [1,15,16,22]. We are studying model systems to test this hypothesis and here report the results of solution studies of the interaction between guanidinium ions and *p*-nitrophenylphosphate, and the effect of guanidinium ions on the rate of hydrolysis of this monoester.

Methods and Materials

Eastman *p*-nitrophenylphosphate, which was checked for *p*-nitrophenol content [17], and Heico extreme purity grade guanidine hydrochloride were used without further purification. All other chemicals were of reagent grade.

The solubility of *p*-nitrophenylphosphate in 0.01 M guanidine hydrochloride/95% ethanol was determined spectrophotometrically at ambient temperature at a pH sufficiently high (approx. 9) so that the only species present was the phosphate ester dianion.

The proton NMR spectra of 10^{-3} M *p*-nitrophenylphosphate dianion in 95% methanol-d_4/5% D_2O was determined at ambient temperature with a Varian HA-100 spectrometer both in the presence and absence of guanidinium ions. All ultraviolet—visible spectra were determined with a Unicam SP1800 spectrophotometer.

Measurement of the rates of hydrolysis of *p*-nitrophenylphosphate dianion were carried out at 68°C in a solution of 85% ethanol buffered at pH 9.0 with 0.01 M Tris. Ionic strength was maintained at 1.0 with tetramethylammonium chloride, and the guanidine hydrochloride concentration was varied over the range 0—1.0 M in several steps. Samples of the hydrolysis mixtures were taken at several time intervals, appropriately diluted with buffered 85% ethanol and the absorbance read at 400 nm. The substrate concentration was approximately 10^{-3} M for all determinations, but the initial concentration of *p*-nitrophenyl phosphate was directly determined for each sample after complete hydrolysis by reflux in concentrated hydrochloric acid. For the particular guanidine hydrochloride-tetramethylammonium chloride ratios studied, rate constants were estimated, assuming first-order kinetics, by linear least squares fitting of the data from triplicate samples which had been followed for ten or more hours.

Results and Discussion

The solubility measurements of the dianion of *p*-nitrophenylphosphate in 0.01 guanidine hydrochloride/95% ethanol, shown as a Yoe-Jones [18,19] plot in Fig. 1, indicate the formation of a complex containing 2 mol of guanidinium ion per mol of *p*-nitrophenyl phosphate dianion. The ester dianion forms an

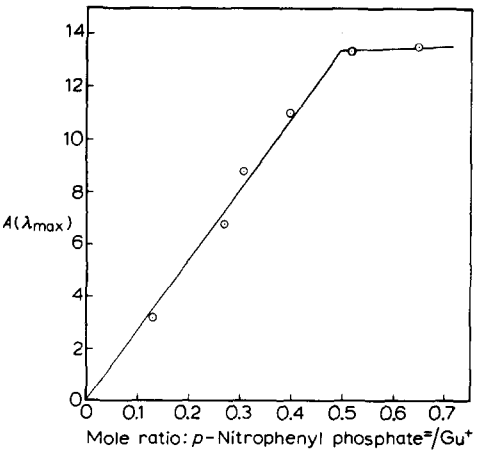

Fig. 1. Yoe-Jones plot of the solubility of *p*-nitrophenylphosphate in 0.01 M guanidine hydrochloride/ 95% ethanol ay a pH of 9. A_{max} is the absorbance absorbance at 307 nm.

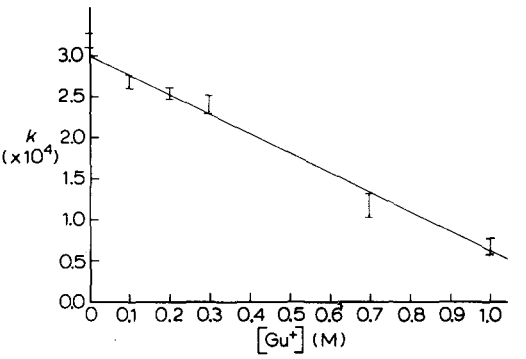

Fig. 2. Plot of the first-order rate contants for the hydrolysis of *p*-nitrophenylphosphate as a function of the concentration of guanidine hydrochloride. The ionic strength was maintained at 1.0 with tetramethyl ammonium chloride and the temperature was 68°C. The solution was 85% (v/v) in ethanol and was buffered at pH 9.0 with 0.01 M Tris. Gu^+ refers to the guanidinium ion. The vertical bars show the range for the replicative measurements.

approx. 10^{-3} M saturated solution in 95% ethanol, but an approx. $5 \cdot 10^{-3}$ M saturated solution when 0.01 M guanidinium ion is added. Thus, guanidine hydrochloride increases the solubility of the ester some 4- to 5-fold. From this data the association constant for the formation of the complex can be estimated to be approximately 10^5—10^6 M^{-2}. Several geometrical arrangements are possible for such a 2 : 1 complex among the guanidinium ions and the ester dianion; it is not possible from the data reported here to tell whether or not the complex formed is a single species or a mixture of several species with different geometries.

The near ultraviolet absorbance maximum of the *p*-nitrophenylphosphate dianion at 315 nm in 95% ethanol shifts to 306 nm upon the addition of guanidinium ions. The absorbance maximum of the monoprotonated ester monoanion is 295 nm in 95% ethanol. Additionally, in NMR studies the resonances of the protons on the phenyl ring ortho to the ester bond show a 3 ppm upfield shift upon the addition of guanidinium ions to the ester dianion dissolved in 95% deuterated methanol 5% D_2O at room temperature. The resonances of the meta protons are not shifted by the addition of the guanidinium ions. Both of these spectral measurements reinforce the concept of complex formation among the guanidinium ions and the phosphate dianion of the ester and further suggest that the interaction is via hydrogen bond formation.

Fig. 2 shows the effect of increasing the concentration of guanidinium ion from 0 to 1 M on the kinetic rate constants for the hydrolysis of the ester dianion. The rate of hydrolysis is four times slower at 1 M guanidinium ion concentration than in solutions of equal ionic strength containing no guanidinium ion. No complex formation or effect on the reaction rate was found for

addition of guanidine hydrochloride to buffered but otherwise wholly aqueous solutions of the ester.

Since comparative rates of reaction depend critically on the relative energies of transition states whose structures are speculative, any attempt to explain an effect as small as that observed here will very probably be at least partly incorrect. However, it is possible to make some sensible and pertinent comments. The generally accepted mechanism for the hydrolysis of phosphate monoesters, except at the extremes of pH, is a monomolecular dissociative one yielding a highly reactive metaphosphate intermediate [20]. Metaphosphate (PO_3^-) is a trigonal planar molecule with considerable multiple character in its P-O bonds. We have presented evidence for a (guanidinium ion)$_2$ · *p*-nitrophenylphosphate complex. If we then make the reasonable assumption that each of these guanidinium ions makes a pair of hydrogen bonds to the phosphate oxygens of the ester, the pattern found in the crystal structures of the Staphylococcal nuclease-thymidine diphosphate complex [13—15] and model compounds [1,16,22], then the complex observed here should have four good P-O . . . H-N hydrogen bonds. Protonation or esterification of phosphate oxygens is well known to lengthen corresponding P-O bonds, an effect that, of course, implies a decrease in P-O bond order. Thus, in a complex between *p*-nitrophenylphosphate and two guanidinium ions having four strong hydrogen bonds, there should be some reduction in P-O bond order of the monoester. It can then be argued that the activation energy for the transition state leading to the metaphosphate intermediate during hydrolysis would be increased by formation of the complex, and the rate of reaction consequently decreased. This picture is consistent with our observations *. Because of the relatively small observed effect of the complex formation among the guanidinium ions and a phosphate monoester on the hydrolytic rate constants for the latter, we conclude that arginine residues, interacting with phosphate-containing substrates at the active sites of enzymes, have only a relatively negligible electronic effect on the rates of such reactions going via metaphosphate formation. The role of such arginine residues would then appear to be solely that of binding and properly positioning the substrate. On the other hand, in enzymatic reactions where the reaction mechanism involves nucleophilic attack on the P (and perhaps the C) of phosphate esters and anhydrides, the formation of guanidinium ion-phosphate complexes can be predicted [1,16,22] to lower the relative energy of the transition state and electronically enhance the reaction rate [21]. However, the demonstration of such an effect and the determination of its magnitude remain to be accomplished **.

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** In unpublished work, however, B. Springs and P. Haake have shown that, in the presence of guanidinium ions, the rate of ester displacement by fluoride ion on bis-(*p*-nitrophenyl)phosphate is increased some 20 times. This observation confirms our prediction [1,15,16] that the guanidinium ions of Arg 35 and Arg 87 play a catalytic as well as a binding and positioning role at the active site of the enzyme.

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