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Fluoride and Beryllium Interact with the (Na + K)-Dependent ATPase as Analogs of Phosphate[1]

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Abstract

Fluoride irreversibly inhibits the (Na + K)-ATPase, and this inactivation requires divalent cations (Mg^{2+}, Mn^{2+}, or Ca^{2+}), is augmented by K^+, but is diminished by Na^+ and by ATP. Prior incubation with the aluminum chelator deferoxamine markedly slows inactivation, whereas adding 1 μM $AlCl_3$ speeds it, consistent with AlF_4^- being the active species. Prior incubation of the enzyme with vanadate also blocks inactivation by fluoride added subsequently. Fluoride stimulates ouabain binding to the enzyme, and thus the analogy between AlF_4^- and both orthophosphate and orthovanadate is reflected not only in the similar dependence on specific ligands for their enzyme interactions and their apparent competition for the same sites, but also in their common ability to promote ouabain binding. Beryllium also irreversibly inhibits the enzyme, and this inactivation again requires divalent cations, is augmented by K^+, but is diminished by Na^+ and by ATP. Similarly, prior incubation of the enzyme with vanadate blocks inactivation by beryllium added subsequently. Inactivation by beryllium, however, does not require a halide, and, unlike inactivation by fluoride, increases at basic pHs. These observations suggest that beryllium, as beryllium hydroxide complexes, acts as a phosphate analog, similar to AlF_4^- and vanadate.

Key Words: Fluoride; aluminum; beryllium; vanadate; ouabain; (Na + K)-ATPase.

Introduction

Sternweis and Gilman (1982) demonstrated that aluminum is required for fluoride to activate the regulatory component of adenylate cyclase, and

[1] Abbreviations. EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol-bis(β-aminoethyl-ether)-*N,N'*-tetraacetate; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid)1,4-piperazine diethanesulfonic acid.

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proposed that the effective species is AlF_4^-. Recently, Bigay *et al.* (1985) argued that this activation results from AlF_4^- mimicking phosphate, specifically as an analog of the γ-posphate of GTP when AlF_4^- is bound in conjunction with GDP to the regulatory component. They also suggested that such mimicry could account for the known inhibitory effects of fluoride on phosphatases.

Fluoride can irreversibly inhibit the (Na + K)-ATPase (Yoshida *et al.*, 1968; Robinson, 1975). Inactivation requires a divalent cation such as Mg^{2+} and is enhanced by K^+, but no relationship of this process to Al^{3+} has been reported. However, the possibility that AlF_4^- interacts like orthophosphate with the (Na + K)-ATPase raises the possible parallel with the interaction between orthovanadate and enzyme, which also require a divalent cation, is enhanced by K^+, and appears to bind as a transition-state analog of phosphate (Cantley *et al.*, 1978). We describe here experiments not only indicating that inhibition of the (Na + K)-ATPase proceeds via an aluminum fluoride complex, but also supporting the analogy between that complex and both phosphate and vanadate, represented by fluoride-enhanced binding of ouabain to the enzyme.

Be^{2+} shares with Al^{3+} the ability to form halides with a strong covalent character, and is the only metal that could substitute for Al^{3+} to activate adenylate cyclase with fluoride (Sternweis and Gilman, 1982). Be^{2+} irreversibly inhibits the (Na + K)-APTase also (Toda *et al.*, 1967; Robinson, 1973), and these characteristics, as shown here, closely resemble those found for fluoride: a divalent cation such as Mg^{2+} is required, and K^+ enhances whereas Na^+ and ATP diminish inactivation. Nevertheless, inactivation by Be^{2+} does not require halide anions, and instead seems to reflect the structural similarity between beryllium hydroxides and orthophosphate (or even oligophosphates).

Materials and Methods

The enzyme preparation was obtained from medullae of frozen canine kidneys, following the procedure of Jorgensen (1974). (Na + K)-ATPase activity was measured in terms of phosphate production after incubation for 8 min at 37°C in the assay medium containing, routinely, 20 mM HEPES/triethylamine (pH 7.5), 0.1 mM EGTA, 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl (final concentrations).

Inactivation by fluoride was measured by a 5-fold dilution into the assay medium immediately following a prior incubation, routinely at 37°C for 8 min, in an inactivation medium containing 20 mM HEPES/triethylamine (pH 7.5), 0.5 mM $MgCl_2$, and 1.5 mM LiF, or with alterations as noted.

Protection by deferoxamine was tested by first preincubating the enzyme with that reagent for 8 min at 37°C in the inactivating medium without LiF, which was then added to initiate the inactivation incubation. Inactivation by beryllium was measured analogously by a 5-fold dilution into the assay medium [but, routinely, with $Mg(NO_3)_2$, $NaNO_3$, and KNO_3 instead of the chlorides] immediately following a prior incubation, at 37°C for 8 min, in an inactivation medium containing 20 mM HEPES/triethylamine (pH 7.5), 1.5 mM $Mg(NO_3)_2$, and 3 μM $Be(NO_3)_2$, or with alterations as noted.

Mg-dependent [^{3}H]ouabain binding was measured after an 8 min prior incubation in 20 mM HEPES/triethylamine (pH 7.5) and 0.1 mM EDTA, in the absence or presence of $MgCl_2$, LiF, and other ligands; 1 μM [^{3}H]ouabain was then added and the incubation continued for 10 min more before being stopped by membrane filtration followed by four washes with ice-cold water (Van Winckle *et al.*, 1972). Alternatively, ouabain binding was measured in terms of fluorescence changes in the FITC-labeled enzyme (Steinberg *et al.*, 1983): fluorescence (excitation 490 μm; emission 520 μm) was recorded from a stirred, temperature-controlled cuvette at 25°C; the media contained 50 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 0.5 mM $MgCl_2$, and either 1.5 mM LiCl or LiF, followed by the addition of 30 μM ouabain.

Frozen canine kidneys were obtained from Pel Freeze, [^{3}H]ouabain from New England Nuclear, deferoxamine from CIBA, and ATP, ouabain, EGTA, and EDTA from Sigma.

Results and Discussion

Studies with Fluoride

When the (Na + K)-ATPase is first incubated with LiF in the presence of a divalent cation such as Mg^{2+}, hydrolytic activity measured in a subsequent incubation is lost (Table I). In these experiments the residual ATPase activity is measured after dilution into the assay medium. The extent of inhibition does not change during the assay incubation both because of this dilution and the protective effects of Na^+ and ATP in the assay medium (Table I); moreover, the inhibition persists after dilution, centrifugation, and resuspension of the enzyme (see below), and thus will be referred to as inactivation.

The extent of inactivation during the initial incubation with LiF and $MgCl_2$ followed an apparent first-order time course (Fig. 1). With an 8-min inactivating incubation the loss of (Na + K)-ATPase activity was increased by adding K^+ (Fig. 1; Table I) and by decreasing the pH of that medium (Table II).

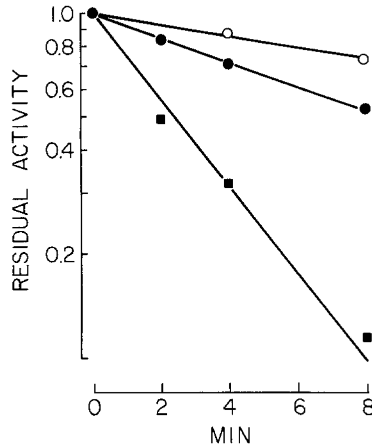

Fig. 1. Time course of inactivation by fluoride. The enzyme was incubated at 37°C in media containing 20 mM HEPES/triethylamine (pH 7.5), 0.5 mM $MgCl_2$, and 1.5 mM LiF (●), or in that medium containing, in addition, 10 mM KCl (■), for the times indicated. Residual activity was then measured by a 5-fold dilution into the assay medium; data are plotted relative to the activity of corresponding controls in the absence of LiF. In addition, the enzyme was first incubated at 37°C for 8 min in media containing 0.5 mM deferoxamine plus buffer and $MgCl_2$, and then 1.5 mM LiF was added for the times indicated before assay of the residual activity (○).

Table I. Factors Influencing Inactivation by Fluoride[a]

Additions	Residual activity		Percent loss of activity
	Without LiF	With 1.5 mM LiF	
None	100	97 ± 2	3
$MgCl_2$, 0.5 mM	88 ± 3	46 ± 2	48
+ Deferoxamine, 0.5 mM	100 ± 4	72 ± 3	28
+ $AlCl_3$, 1 μM	90 ± 3	23 ± 4	74
+ KCl, 10 mM	87 ± 4	11 ± 2	87
+ NaCl, 100 mM	99 ± 5	99 ± 4	0
+ ATP, 3 mM	109 ± 4	103 ± 4	6
$MgCl_2$, 0.1 mM	96 ± 2	72 ± 3	25
$CaCl_2$, 0.1 mM	88 ± 2	71 ± 2	19
$MnCl_2$, 0.02 mM	62 ± 3	35 ± 2	44

[a]The enzyme was first incubated for 8 min in media containing 20 mM HEPES/triethylamine (pH 7.5) in the absence and presence of LiF plus the additions noted, and (Na + K)-ATPase activity was then measured (see Materials and Methods). Residual activity is presented relative to that with no additions, ± S.E.M., and the effects of LiF are also presented relative to the corresponding control without LiF.

Table II. Effect of pH on Inactivation[a]

Inactivating incubation with	Percent loss of activity after incubation		
	At pH 6.5	At pH 7.5	At pH 8.1
LiF, 1.5 mM	82 ± 1	46 ± 3	25 ± 3
$AlCl_3$, 2 mM	8 ± 3	0 ± 2	13 ± 3

[a] Residual (Na + K)-ATPase activity was measured after first incubating the enzyme for 8 min at 37°C in media containing (i) 0.5 mM $MgCl_2$ and 1.5 mM LiF, or (ii) 1.5 mM $MgCl_2$ and 2 mM $AlCl_3$, and either 20 mM PIPES/triethylamine (pH 6.5), 20 mM HEPES/triethylamine (pH 7.5), or 20 mM HEPES/triethylamine (pH 8.1). In all cases activity is compared to the corresponding control without (i) LiF or (ii) $AlCl_3$. The $AlCl_3$ stock solution used was 10 mM $AlCl_3$ adjusted only to pH 6.2 because of the insolubility of $Al(OH)_3$. The buffer for the assay incubation was 20 mM HEPES/triethylamine (pH 7.5) in all cases.

No aluminum was added in these experiments, but it is a ubiquitous contaminant of glassware (Sternweis and Gilman, 1982). Correspondingly, preincubation with 0.5 mM deferoxamine, a chelator of aluminum (Blackmore *et al.*, 1985), diminishes inactivation (Fig. 1; Table I); by contrast, 0.1 mM EGTA, a divalent cation chelator, had little effect (data not shown). On the other hand, adding 1 μM $AlCl_3$ greatly increases inactivation in the presence of fluoride and Mg^{2+} but does not cause inactivation in the absence of fluoride (Table I).

This enhancement of inactivation by aluminum and the protection by deferoxamine thus strongly suggest that aluminum participates with fluoride in the inactivation process. In the light of the predominance of AlF_4^- at this fluoride concentration (Sternweis and Gilman, 1982), AlF_4^- is probably the active species. (Experimentally, the range of Mg^{2+} and F^- concentrations are limited by the notable insolubility of MgF_2.)

Increasing the $AlCl_3$ concentration in the absence of fluoride to 2 mM still did not cause appreciable inactivation over the pH range 6.5–8.1 (Table II). Although the insolubility of $Al(OH)_3$ prevents a precise evaluation of the concentrations available, it would seem that neither Al^{3+}, $AlCl_3$, nor $Al(OH)_3$ is particularly effective as an inactivator of the enzyme.

Divalent cations are required for the inactivation by fluoride, with an order of potency (in terms of concentration) of Mn > Mg > Ca (Table I). This sequence is identical to that for [^{48}V]vanadate binding (Robinson and Mercer, 1981). This similarity with vanadate also extends to the ability of K^+ to augment the interaction with the enzyme and of Na^+ and ATP to diminish it (Table I).

Inactivation by fluoride is thus diminished by reagents favoring the E_1 family of enzyme conformations (Na^+, ATP) but enhanced by those favoring the E_2 family (K^+, decreased pH). These observations further support the similarity between effects of fluoride, as the AlF_4^- complex, mimicking

phosphate since vanadate is a close analog of phosphate and both bind preferentially to E_2 conformations. (The possibility that ATP may also act by steric blockade of the phosphate-binding site cannot, however, be excluded.)

If both vanadate and fluoride act by binding to the same enzyme site, the phosphate site on E_2, then they should compete for that site. This should be readily demonstrable in terms of vanadate protecting against inactivation by fluoride: prior incubation with vanadate should hinder access by fluoride. This experiment is feasible since inactivation by fluoride is nearly irreversible, whereas vanadate binding has a reported half-life on the enzyme of 3.5 min at 25°C (Cantley *et al.*, 1978), although it is much longer at 0°C. Indeed, almost all inhibition by vanadate is removed by dilution, centrifugation, resuspension, and incubation for 8 min at 37°C before assay, whereas inhibition by fluoride persists (Table III). On the other hand, when vanadate is first reacted with the enzyme for 10 min at 37°C, a subsequent incubation for 10 min with fluoride added to that medium results in little inactivation (Table III). Conversely, initial incubation with fluoride followed by vanadate results in about as much inactivation as in the absence of vanadate (the small difference is probably due to vanadate competing with fluoride for further binding during the second 10 min incubation). These experiments thus support the contention that AlF_4^- can react with the enzyme like vanadate, as a phosphate analog.

Both phosphate and vanadate promote binding to the enzyme of the specific inhibitor, ouabain, and ouabain stimulates enzyme phosphorylation by orthophosphate (Lindenmayer *et al.*, 1968; Hansen, 1979). Fluoride similarly promotes ouabain binding, and this stimulation is diminished by

Table III. Protection by Vanadate against Inactivation by Fluoride[a]

Initial inactivating medium	Subsequent addition	Percent loss of activity
LiF, 1.5 mM	None	68 ± 2
LiF, 1.5 mM	Vanadate, 0.01 mM	59 ± 2
Vanadate, 0.01 mM	None	3 ± 3
Vanadate, 0.01 mM	LiF, 1.5 mM	6 ± 2

[a]The enzyme was first incubated at 37°C for 10 min in media containing 20 mM HEPES/triethylamine (pH 7.5), 0.5 mM $MgCl_2$, and either 1.5 mM LiF or 0.01 mM vanadate. Then to half the media containing fluoride was added vanadate to a final concentration of 0.01 mM, and to half the media containing vanadate was added LiF to a final concentration of 1.5 mM; this incubation was continued at 37°C for 10 min. The media were then diluted 10-fold with ice-cold 0.25 M sucrose and centrifuged for 30 min at 35,000 rpm in a Beckman #40 rotor. The pellets were resuspended in 0.25 M sucrose and the enzyme preincubated for 8 min at 37°C in the assay medium without ATP, which was then added to initiate the assay incubation. Residual activity, corrected for the protein content recovered, is expressed relative to that of the corresponding controls treated identically, except for the absence of LiF and vanadate.

Table IV. Factors Influencing Mg-Dependent [^{3}H]Ouabain Binding[a]

Additions	Relative binding of [^{3}H]ouabain
$MgCl_2$, 0.5 mM	100
+ Deferoxamine, 0.5 mM	102 ± 5
+ Phosphate, 5 mM	174 ± 10
+ LiF, 1.5 mM	148 ± 8
+ LiF and Deferoxamine	106 ± 6

[a]Media contained 20 mM HEPES/triethylamine (pH 7.5) and 0.1 mM EDTA, in the absence or presence of the additions listed, plus 1 μM [^{3}H]ouabain. Binding is presented relative to the difference between incubations with and without added $MgCl_2$.

deferoxamine (Table IV). The increased rate of ouabain binding induced by fluoride is also apparent in fluorescence changes in the enzyme labeled with FITC (Fig. 2).

The argument that fluoride acts through the AlF_4^- complex as a phosphate analog thus rests on: (i) potentiation of inactivation by added $AlCl_3$ and antagonism by the aluminum chelator deferoxamine; (ii) similarities to vanadate binding and inhibition in terms of divalent cation requirements, potentiation by K^+, and antagonism by Na^+ and by ATP; (iii) protection against inactivation by fluoride through prior incubation with vanadate; and (iv) the similarity with phosphate and vanadate in promoting ouabain binding.

Finally, the potential usefulness of fluoride (or AlF_4^-) should be emphasized. Although no practical radioisotopes of F or Al are available, AlF_4^- has a major advantage over phosphate and vanadate in forming a more stable complex with the (Na + K)-ATPase. Moreover, for nuclear magnetic resonance studies of the enzyme active site, sensitivity to ^{19}F is greater than to either ^{31}P or ^{51}V, and several F atoms will be bound per site instead of one P or V. The potential for ^{27}Al studies with nuclear magnetic resonance also exists.

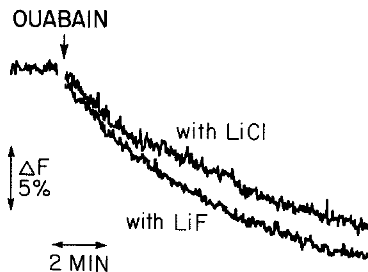

Fig. 2. Fluoride-stimulated ouabain binding. Fluorescence changes in the enzyme covalently labeled with FITC are shown after addition of 30 μM ouabain (arrow) in the presence of 50 mM Tris/HCl (pH 7.5), 0.1 mM (EDTA, 0.5 mM $MgCl_2$, and either 1.5 mM LiF or LiCl. (LiCl was added to control for any effects of Li^+ on fluorescence or ouabain binding.)

Studies with Beryllium

Beryllium inactivation of the (Na + K)-ATPase closely resembles that due to fluoride, including an apparent first-order time course, a requirement for divalent cation, potentiation by K^+, and antagonism by Na^+ and by ATP (Toda *et al.*, 1967; Robinson, 1973). Sternweis and Gilman (1982) noted that beryllium, alone of all the metal cations tested, could substitute for aluminum to permit stimulation of adenylate cyclase by fluoride. This substitution reflects the close similarities in chemical properties between these metals, for their nearly identical charge–radius ratio correlates with their tendency to form bonds of a highly covalent character (Everest, 1964).

In those earlier experiments with beryllium no fluoride was added, and it is an unlikely contaminant. Still, if beryllium is to inactivate through a complex mimicking phosphate, then some anion must participate. Chloride, present in those experiments, seems a plausible participant. However, when the effects of beryllium were tested in media lacking all halides, the only anions being nitrate and HEPES, the extent of inactivation was the same as in the presence of chloride (Table V). (These experiments were performed like those with fluoride: an initial inactivating incubation was followed by dilution into an assay medium, and the extent of inactivation did not change during the assay incubation. The assay medium also contained only nitrate and HEPES anions.)

Table V. Factors Influencing Inactivation by Beryllium[a]

Additions	Percent loss of activity		
	At pH 6.5	At pH 7.5	At pH 8.1
$Be(NO_3)_2$, 3 μM	–	-4 ± 3	–
$+KNO_3$, 5 mM	–	-1 ± 2	–
$+Mg(NO_3)_2$, 1.5 mM	9 ± 2	35 ± 3	54 ± 4
$+KNO_3$ and $Mg(NO_3)_2$	12 ± 2	48 ± 2	71 ± 3
$+KCl$, 5 mM, and $Mg(NO_3)_2$	–	53 ± 3	–
$+Mg(NO_3)_2$ and NaCl, 10 mM	–	9 ± 2	–
$+Mg(NO_3)_2$ and ATP, 3 mM	–	-3 ± 2	–
$+CaCl_2$, 0.1 mM	–	12 ± 1	–
$+MnCl_2$, 0.02 mM	–	29 ± 4	–
$BeCl_2$, 3 μM + $MgCl_2$, 1.5 mM	–	31 ± 3	–

[a]The enzyme was first incubated for 8 min in media containing either 20 mM PIPES/triethylamine (pH 6.5), 20 mM HEPES/triethylamine (pH 7.5), or 20 mM HEPES/triethylamine (pH 8.1), plus the additions noted. The residual activity was then measured in the standard assay medium with nitrate salts instead of chlorides; the buffer pH in the assay media was 7.5 in all cases. The percent loss of activity (or gain: negative numbers) is calculated relative to the corresponding control lacking $Be(NO_3)_2$ or $BeCl_2$. Where the dash appears no experiment was performed.

If the hypothesis is correct, that beryllium inactivates through a complex resembling phosphate, then $Be(NO_3)_2$ seems an unlikely species, especially since it must be essentially equipotent with $BeCl_2$ (Table V). There remains, however, another possible candidate, hydroxide ion. At pH 7.5 the concentration is 0.32 μM, which is only 10-fold lower than the $Be(NO_3)_2$ concentration used. Moreover, beryllium forms hydroxides that are highly amphoteric, with coordination to form tetrahedral $Be(OH)_4^{2-}$ species that can polymerize to form chains analogous to polyphosphates (Everest, 1964; Cotton and Wilkinson, 1962).

To test the possibility that beryllium hydroxides are the active species, the effect of pH on inactivation to beryllium was examined, over the range 6.5 to 8.1 (hydroxide concentrations from 0.03 to 1.3 μM). At pH 8.1 inactivation was increased, whereas at pH 6.5 inactivation was almost completely abolished (Table V). An alternative explanation is that basic pHs induce in the enzyme a more reactive configuration, but if beryllium does interact as a phosphate analog, then it would be expected to do so more readily at acidic than basic pHs. This was the case with fluoride inactivation (Table II), and is attributable to lower pHs favoring the E_2 conformational family (Skou and Esmann, 1980) to which phosphate and vanadate bind preferentially (Cantley *et al.*, 1978; Robinson and Mercer, 1981).

As in experiments on inactivation by fluoride, vanadate preincubation also protects against inactivation by beryllium: an initial 10 min incubation with vanadate prevents inactivation by beryllium added to the second 10 min incubation (Table VI).

The argument that a beryllium complex inactivates the (Na + K)-ATPase as a phosphate analog, like AlF_4^-, thus rests on: (i) the similar requirements for divalent cations, potentiation by K^+ and antagonism by

Table VI. Protection by Vanadate against Inactivation by Beryllium[a]

Initial inactivating medium	Subsequent addition	Percent loss of activity
$Be(NO_3)_2$, 3 μM	None	50 ± 1
$Be(NO_3)_2$, 3 μM	Vanadate, 0.01 mM	42 ± 2
Vanadate, 0.01 mM	None	5 ± 5
Vanadate, 0.01 mM	$Be(NO_3)_2$, 3 μM	9 ± 6

[a]The experiments were performed and the data are presented analogously to those in Table III. The enzyme was first incubated at 37°C for 10 min in media containing 20 mM HEPES/triethylamine (pH 7.5), 1.5 mM $MgCl_2$, and either 3 μM $Be(NO_3)_2$ or 0.01 mM vanadate; then to half the media containing $Be(NO_3)_2$ was added vanadate and to half the media containing vanadate was added $Be(NO_3)_2$ and the incubation continued for a further 10 min. As described above, the media were then diluted and centrifuged, and the pellets resuspended and assayed after preincubation for 8 min at 37°C.

Na^+ and by ATP; (ii) the pH dependence of inactivation, consistent with formation of tetrahedral beryllium hydroxides, resembling orthophosphate or oligophosphates; and (iii) protection against inactivation by beryllium through prior incubation with vanadate. The potential for using beryllium compounds as experimental reagents is, nevertheless, limited by the serious toxicity of these substances: all direct contact must be strictly avoided.

Conclusions

These experiments describe properties of two reagents, fluoride in the presence of aluminum and beryllium at basic pHs, that bind to the (Na + K)-ATPase with high affinity and dissociate only slowly. The characteristics of their interactions with the enzyme resemble those of phosphate and vanadate, indicating a mode of action for the two reagents whose inactivation of the enzyme was previously explained. The parallels with vanadate further support the argument that that reagent acts as a phosphate analog. Moreover, the interactions with the enzyme impose additional constraints on models of the phosphate-binding locus of the enzyme active site; in particular, the tighter binding of these reagents, compared to vanadate, requires explanation. Finally, AlF_4^- is potentially a valuable reagent for future structural studies on the active site.

Acknowledgments

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