Aluminum Ions Are Required for Stabilization and Inhibition of Hepatic Microsomal Glucose-6-phosphatase by Sodium Fluoride*

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Stabilization and inhibition of hepatic microsomal glucose-6-P phosphohydrolase (EC 3.1.3.9) by F− requires the presence of Al3+ ions. At millimolar concentrations, reagent grade NaF inhibited glucose-6-P hydrolysis and protected the enzyme against inactivation induced by heat in the presence of 0.025% (w/v) Triton X-100 or by reaction of the catalytic site with the histidine-specific reagent, diethyl pyrocarbonate. The presence of millimolar EDTA in all test systems abolished the effectiveness of NaF, yet EDTA by itself was without significant influence on the kinetics of phosphohydrolase reaction, the thermal stability of the enzyme or its reactivity with diethyl pyrocarbonate. Although ultrapure NaF was ineffectual in all test systems, its potency as a competitive inhibitor or protective agent was markedly increased by micromolar AlCl3 or when assays were carried out in flint glass test tubes. The latter response is explained by the well documented ability of fluoride solutions to extract Al3+ from glass at neutral pH. Our analysis indicates that the effectiveness of fluoride in all test systems derives from the formation of a specific complex with Al3+, most likely Al(F−). The apparent dissociation constant for interaction of the enzyme and Al(F−) is 0.1 μM. The combination of NaF and AlCl3 holds promise as an unusually effective and versatile means to stabilize this notoriously labile enzyme during efforts to purify it. Glucose-6-phosphatase (EC 3.1.3.9) is a multicomponent system in the endoplasmic reticulum of the hepatic parenchyma and renal tubules (1–3). The system consists of (a) the glucose-6-P-specific translocase (denoted T1) which mediates permeation of the hexose phosphate into the luminal compartment, (b) a relatively nonspecific phosphohydrolase situated with its active site in contact with the luminal compartment, and (c) a phosphate translocase (denoted T2) which mediates egress of P, and influx of PP, and carbamyl-P (2). D-Glucose, the other product of glucose-6-P hydrolysis, rapidly equilibrates across the membrane, presumably via a passive, nonmediated process (2).

Despite its important position at the terminus of the gluconeogenic and glycogenolytic pathways in liver and kidney, we know relatively little about the components of the glucose-6-phosphatase system compared with other key enzymes of the gluconeogenic pathways. This situation has arisen in part because of the failure of efforts to purify the phosphohydrolase. The principal complication in the purification historically has been the extreme instability of the enzyme in detergent-dispersed microsomes (4). In more recent studies, Burchell and Burchell (5) were able to make significant progress in the purification following their discovery that sodium fluoride was an unusually effective stabilizer of the "solubilized" enzyme (6).

The present study was initiated to gain insight into the mechanism by which sodium fluoride stabilizes glucose-6-P phosphohydrolase. The literature contains few references describing the interaction of fluoride with glucose-6-phosphatase. It has been reported to be an inhibitor of glucose-6-P hydrolysis (7) and, in more recent studies, it was found that inactivation of the enzyme by the histidine reagent, DEPC,1 could be selectively blocked by added NaF (8). We report here that all of these interactions of fluoride with the microsomal phosphohydrolase are dependent on the presence of Al3+ ions.

EXPERIMENTAL PROCEDURES

Materials—DEPC and NaF were obtained from Sigma and used without further purification. Ultrapure NaF was purchased from Alfa Products (Danvers, MA). Aluminum chloride was obtained from Mallinckrodt. Flint glass culture tubes (10 × 75 mm) were obtained from Fisher Scientific Co. Water used throughout the investigation was purified by reverse osmosis and passage through a series of anion exchange, cation exchange, and activated charcoal columns. Sources or preparative procedures for other chemicals were as described earlier (2, 8).

Animals and Preparations—Young (130 g) male, Sprague-Dawley rats were obtained from Blue Spruce Farms (Altamont, NY) and used in all studies. Rats were fed Purina Lab Chow ad libitum for at least 2 weeks and then fasted for 20 h before death.

Hepatic microsomes were prepared from 10% liver homogenates as described by Nordlie and Arion (9) except the medium for homogenization was buffer A (0.25 M sucrose, 5 mM Hepes, 0.5 mM sodium EDTA, pH 7.4). Microsomes were washed once by centrifugation at 100,000 × g for 60 min in buffer A.

1 The abbreviations used are: DEPC, diethyl pyrocarbonate (also known as ethoxyformic anhydride); T1, a glucose-6-P-specific translocase that mediates the penetration of the hexose phosphate into the endoplasmic reticulum cisternae; T2, a phosphate translocase that mediates efflux of Pi and influx of PPi and carbamyl-P; Al(F−), the first order rate constant for inactivation of the glucose-6-P phosphohydrolase.

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after suspension in buffer B (0.25 M sucrose, 5 mM Hepes, pH 7.4). To prepare fully disrupted microsomes,² microsomes in buffer B (3 mg of protein/ml) were exposed to 0.1% Triton X-100 for 20 min at 0 °C (13). For studies of fluoride inhibition of glucose-6-P phosphohydrolase activity, 1 volume of the Triton-disrupted microsomes was diluted to 9 volumes of ice-cold buffer B (12). In studies involving DEPC inactivation of glucose-6-phosphatase activity, the Triton-disrupted microsomes were underlayered with 2 ml of buffer C (0.5 M sucrose, 5 mM Hepes, pH 7.4) and centrifuged for 60 min at 49,000 rpm in a Beckman 50 Ti rotor. The pellet of washed membranes was resuspended by homogenization in buffer B to a final concentration of about 1 mg of protein/ml.

Analytical Procedures—Protein content of microsomes was determined by a microvolume procedure described by Bickerstaff and Burchell (15). The composition of assay media is specified in the legends to tables and figures. Unless stated otherwise, all incubations of microsomes in media containing NaF were carried out in polystyrene tubes and all transfers of such solutions were made with plastic pipetting devices. The glucose-6-phosphatase activities of intact² microsomal preparations were corrected for the contribution of enzyrnatic activity in the disrupted component as previously described (2). Intactness of microsomal preparations was assessed by determining the proportion of the "low-Kₐ" glucose-6-P phosphohydrolase activity (10, 11). The means of at least three measurements of initial rates are reported in all cases. Initial reaction velocities, designated in the figures as v, are units or milliunits per mg of protein, where 1 unit is the amount of enzyme catalyzing the hydrolysis of 1 pmol of bhexose-6-P per min.

Procedure for Thermal Inactivation of Glucose-6-phosphatase—One volume of microsomes (2 to 3 mg of protein/ml) in 0.1% Triton X-100 (see above) was added to 3 volumes of media, which had been equilibrated at 37 °C and was composed of 25 mM sodium cacodylate, pH 6.5, and the desired concentration of a potential stabilizing agent (e.g. P; or NaF). The incubations were continued at 37 °C for the desired times. To halt the inactivation process, the heated microsomes were diluted with 3 volumes of ice-cold buffer A and stored on ice until assayed.

Inactivation of Glucose-6-phosphatase by DEPC—Microsomes were treated with 4.2 mM DEPC at pH 6.5 as described by Arion et al. (8) except the preparations were not centrifuged after terminating the reaction with 0.1 M imidazole, pH 7.4; rather they were diluted with an equal volume of buffer A and directly assayed for glucose-6-phosphatase activity.

RESULTS

Preliminary Studies with Reagent Grade NaF—In our initial characterizations of the stabilizing and inhibitory actions of NaF, two different commercial batches of reagent grade NaF were used. Qualitatively similar results were obtained in Ithaca with NaF obtained from Sigma (Lot 62F-0791) and in Dundee with NaF purchased from BDH Chemicals (Pro.No. 10246).

The concentration dependence of inhibition of glucose-6-phosphatase activity at pH 6.5 by Sigma NaF in fully disrupted microsomes is shown in Fig. 1. A "mixed" pattern of inhibition was observed or, in Cleland's nomenclature (16), NaF acted as a linear noncompetitive inhibitor, since both the slopes and intercepts of the double reciprocal plots increased linearly with the concentration of inhibitor (see inset in Fig. 1). Supplementary experiments showed that the pattern of inhibition was unchanged over the pH range of 5.5 to 7.5.

At neutral pH, glucose-6-phosphatase of intact microsomes is very stable during prolonged incubations at mild temperatures (25-37 °C), even in the absence of substrate or some stabilizing agent (17). However, in microsomes supplemented with sodium deoxycholate (18-20) or sodium cholate (6), the enzyme is rapidly inactivated when subjected to mild heating. In preliminary studies, the degree of thermal instability of the enzyme was found to be markedly influenced by the concentration of detergent present during heat exposure. For example, when microsomes (2 mg of protein/ml) were exposed to 0.2% sodium deoxycholate or 0.1% Triton X-100, complete inactivation occurred within 5 min at 30 °C. However, enzyme stability comparable to that seen in untreated microsomes (17) or ammonia-disrupted microsomes (19) was restored simply by separating microsomes from the detergent solutions (see "Experimental Procedures"). Enzyme instability was reestablished when washed membrane preparations were again supplemented with detergent.

We have used the finding that detergent concentration can be used to systematically control enzyme instability in efforts to obtain a more quantitative characterization of the ability of NaF to stabilize the enzyme. Fully disrupted microsomes (0.75 mg of protein/ml) were incubated at 37 °C in the presence of 0.025% (w/v) Triton X-100. Under these conditions, the instability of enzyme was reproducible and enzyme inactivation approximated a first order process, as evidenced by the linearity of plots of log enzyme activity against exposure time (Fig. 2, A and B). Thus, we were able to examine the influence of added agents on the first order rate constant for thermal inactivation, k₅₀. The latter was determined from the slopes of semilog plots.

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²In this paper, microsomes isolated from liver homogenates, washed, and assayed without further treatment are referred to as "untreated." As noted previously (2, 5, 10, 11), untreated microsomes are mixtures of an intact vesicle fraction ("intact microsomes") in which the limiting membrane acts as a selective permeability barrier and disrupted structures in which selective permeability is lacking and the enzyme has free access to ionic substrates and inhibitors. The proportion of the two forms is easily quantitated by assays of the low-Kₐ glucose-6-P phosphohydrolase activity that is expressed only in disrupted structures (11). Untreated microsomes are converted to fully disrupted microsomes by treatments with detergents or NH₄OH which completely destroy the selective permeability of the membrane (1, 10-13).

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FIG. 1. The kinetics of inhibition by Sigma NaF of glucose-6-P phosphohydrolase activity in fully disrupted microsomes. Assay media, pH 6.5, contained 20 mM sodium cacodylate buffer, glucose-6-P, and NaF as indicated, and 3 µg of protein of fully disrupted liver microsomes (see "Experimental Procedures").
The secondary plot was linear when Pi was the stabilizing agent. It caused it to be ineffectual at low concentrations. Since fluoride is known to form complexes with a number of divalent metal ions (21), we examined whether EDTA would prevent binding of fluoride. It seemed unlikely that the chelating agent interacted directly against inactivation by heat (data not shown). Therefore, it seemed more likely that a metal ion contaminant was a required cofactor and, as a consequence of chelation of the putative metal ion, EDTA prevented formation of an active complex.

We were able to test this possibility by exploiting the selective permeability properties of the intact microsomal membrane (2).

Previous studies have shown that intact liver microsomes are impermeable to EDTA (11, 22, 23). Therefore, EDTA cannot interact with the catalytic site of glucose-6-phosphatase, which is localized at the luminal aspect of the membrane in intact microsomes (1, 11, 13). The existence of an intact microsomal membrane, however, did not preclude NaF from strongly inhibiting the glucose-6-P phosphohydrolase activity (see Table III). This observation, taken with the earlier finding that inactivation of the enzyme by DEPC in intact microsomes precluded NaF in fully disrupted microsomes. These observations prompted us to examine the mechanistic basis by which EDTA suppressed the effectiveness of fluoride as a stabilizing or inhibitory agent.

The presence of EDTA per se did not significantly influence the thermal stability of the enzyme in Triton-supplemented microsomes (Table I) nor did EDTA alter the kinetics of glucose-6-P hydrolysis by the enzyme of fully disrupted microsomes (Table II). Moreover, EDTA did not interfere with the ability of NaF to inhibit the phosphohydrolase or protect it against inactivation by heat (data not shown). Therefore, it seemed unlikely that the chelating agent interacted directly with the enzyme to prevent binding of fluoride. It seemed more likely that a metal ion contaminant was a required cofactor and, as a consequence of chelation of the putative metal ion, EDTA prevented formation of an active complex.

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### Table I

<table>
<thead>
<tr>
<th>Treatment/additions</th>
<th>Phosphohydrolase activity (nmol P_I formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 4 min at 37 °C</td>
<td>27 ± 1 (8)†</td>
</tr>
<tr>
<td>None</td>
<td>27 ± 1 (8)†</td>
</tr>
<tr>
<td>2 mM NaF</td>
<td>293 ± 13 (82)</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>40 ± 3 (11)</td>
</tr>
<tr>
<td>2 mM NaF + 2 mM EDTA</td>
<td>44 ± 2 (12)</td>
</tr>
<tr>
<td>B. 0 °C control</td>
<td>356 ± 47 (100)</td>
</tr>
</tbody>
</table>

Table II

### Effect of EDTA on inhibition by Sigma NaF of glucose-6-phosphatase in fully disrupted microsomes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Apparent kinetic constant (K_a, V_max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.48 mM units/mg protein</td>
</tr>
<tr>
<td>4 mM NaF</td>
<td>0.44 mM</td>
</tr>
<tr>
<td>4 mM EDTA</td>
<td>1.33 mM</td>
</tr>
<tr>
<td>4 mM NaF + 4 mM EDTA</td>
<td>0.41 mM</td>
</tr>
<tr>
<td>4 mM NaF + 4 mM EDTA</td>
<td>0.70 mM</td>
</tr>
</tbody>
</table>

† Values in parentheses are the percentage of activity remaining relative to the 0 °C control.
some also was blocked by added NaF (8), suggests that the inhibitory species can penetrate the microsomal membrane. Moreover, inhibition of enzyme activity in intact microsomes by NaF was effectively blocked by added EDTA (Table III). Taken together, these observations support the conclusion that EDTA complexed a metal ion which is a necessary cofactor for the inhibitory and stabilizing actions of NaF.

**Use of Ultrapure NaF in Establishing the Requirement for Al**

We were aided in our efforts to identify the metal ion cofactor by a report by Sternweis and Gilman (24), who found that the stimulation of adenylate cyclase by fluoride required Al**. Aluminum fluosilicates are common impurities in commercial preparations of NaF (25). Thus, we obtained an "ultrapure" grade of NaF from Alfa Products (Lot 802384), which according to the manufacturer's analysis contained less than 10 ppm of Al** (i.e., about 16 µg/mol of NaF). The purified NaF was used to confirm the requirement for Al** and to further characterize the interactions of the enzyme with fluoride and Al**.

Fig. 3 illustrates that inhibition of glucose-6-phosphatase activity in fully disrupted microsomes by ultrapure NaF was markedly dependent on added AlCl**. Supplementary studies showed that AlCl** varied from 1-10 µM was totally without effect on the kinetics of the phosphohydrolase reaction and only modest levels of inhibition were seen in test systems containing either 0.4 or 1.0 mM NaF in the absence of AlCl**.

**TABLE III**

Effects of EDTA on inhibition by Sigma NaF of glucose-6-phosphatase activity in intact hepatic microsomes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glucose-6-phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nMol Pi formed/min/mg protein</td>
</tr>
<tr>
<td>None (control)</td>
<td>196 ± 9</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>170 ± 5 (13)*</td>
</tr>
<tr>
<td>5 mM NaF</td>
<td>65 ± 11 (67)</td>
</tr>
<tr>
<td>5 mM NaF + 5 mM EDTA</td>
<td>173 ± 4 (12)</td>
</tr>
</tbody>
</table>

*Values in parentheses are the percentage inhibition relative to the control.

(datum not shown, but see below). In media containing both AlCl** and either 0.4 mM (Fig. 3A) or 1.0 mM NaF (Fig. 3B), the inhibition was purely competitive and responded linearly to added Al** up to at least 5 µM (see insets). The observed value for the slope inhibitor constant, Ks, for Al** varied inversely with the fluoride concentration; specifically, Ks decreased from about 0.8 µM to 0.3 µM when NaF was increased from 0.4 to 1 mM.

Fig. 4 shows the pattern of inhibition of glucose-6-P phosphohydrolase activity in fully disrupted microsomes when AlCl** was held constant at 2 µM and the concentration of ultrapure fluoride was varied from 0.5–2 mM. In this case, noncompetitive inhibition was observed in media containing 1 mM or higher concentrations of NaF. The pattern of inhibition was similar to that seen when higher concentrations of Sigma NaF were studied (cf. Fig. 1).

Fig. 5 shows that the ability of NaF to protect the enzyme against inactivation caused by heat exposure (open circles) or by exposure to 4.2 mM DEPC (closed circles) also required Al**. Inactivation by DEPC is concentration-dependent and, under the conditions described, displays first order kinetics (data not shown, but see Ref. 8). In both test systems, AlCl** was totally ineffective in the absence of NaF and protection of the enzyme was abolished in all situations by the presence of 5 mM EDTA (data not shown). The data in Fig. 5 show that in the presence of 0.5 mM NaF the reciprocals of k inactivation for both treatments increased as a linear function of the concentration of added Al**. It can be calculated from the plots that about 0.5 µM AlCl** was needed to effect a 50% reduction in the first order rate constant for either mode of inactivation.

**Confounding Effects of the Interaction of Fluoride with Glass**—In all experiments described above, care was taken to prevent contact between fluoride solutions and glass surfaces. Plastic tubes, bottles, and pipetting devices were used at all times in all studies. The reason for these precautions is illustrated in Table IV, where we have compared the inhibitory effectiveness of ultrapure NaF when assays were conducted in plastic versus frit glass test tubes. Markedly greater inhibition was seen with NaF when assays were carried out in glass tubes.

Al** is known to be a component of many types of glass.
Interactions of $\text{Al(F)}_3^-$ with Glucose-6-phosphatase

The purpose of the present study was to characterize the interactions of fluoride with the hepatic microsomal glucose-6-P phosphohydrolase system (1). The characterizations included an examination of fluoride’s effectiveness as an inhibitor of the phosphohydrolase activity and its ability to protect the enzyme against inactivation induced by heat in the presence of low concentrations of Triton X-100 or by reaction of the catalytic site with the histidine-specific reagent, DEPC. In all three test systems, added EDTA abolished the effectiveness of “reagent-grade” NaF, yet EDTA by itself had no significant influence on the kinetics of the hydrolytic reaction, the thermal instability of the enzyme or its reactivity with DEPC. Ultrapure NaF was ineffectual in all test systems unless $\text{Al}^{3+}$ was present and all test systems displayed nearly identical concentration dependence on added $\text{Al}^{3+}$. Indeed, when similar concentrations of ultrapure fluoride were used in the three test systems (i.e. 0.4–0.5 mM), about the same concentration of $\text{Al}^{3+}$ was required to double the slope of kinetic plots describing inhibition of the activity (Fig. 3A) and halve the rate of inactivation by heat or DEPC (Fig. 5). This is discussed in more detail below. These observations indicate that the effectiveness of fluoride derives from the formation of a specific complex between it and $\text{Al}^{3+}$ and that the same aluminum-fluoride complex is responsible for the competitive inhibition and the protective or stabilizing actions. It follows that a single binding site on the enzyme must be involved in all responses.

$\text{Al}^{3+}$ and F$^-$ can combine to form six possible complex ions, ranging in composition from $\text{Al}^{3+}(\text{F}^-)$ to $\text{AlF}_3^-$ (27). The absolute concentration of each species as well as the relative proportions of the various forms are determined by the absolute concentrations of fluoride and $\text{Al}^{3+}$ (21, 27). Since the formation constants for each of the complexes has been determined (27), it was of interest to examine whether these could be used to identify which of the complexes was responsible for the competitive inhibition (Fig. 3) and the protective effects (Fig. 5). By using the formation constants in conjunction with the concentration of F$^-$ used, the concentrations of the complexes were calculated for the concentration of $\text{Al}^{3+}$, which (a) doubled the slopes of the reciprocal plots in the inhibition studies (Fig. 3) and (b) halved the rate of inactivation by heat or DEPC (Fig. 5). The results of the calculations are summarized in Table V. The analysis identified $\text{Al}^{3+}(\text{F}^-)_2$ as the most likely candidate, since, among the complexes, only the concentration of $\text{Al}^{3+}(\text{F}^-)_2$ remained essentially constant with the varied combinations of $\text{Al}^{3+}$ and F$^-$ used in the calculations.

If, as indicated, $\text{Al}^{3+}(\text{F}^-)_2$ is the complex ion responsible for the competitive inhibition and stabilizing effects of $\text{Al}^{3+}$ and F$^-$, then the concentrations shown in Table V are in fact

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### Table IV

**Comparison of the inhibitory effectiveness of ultrapure NaF when fully disrupted microsomes were assayed in glass and polyethylene tubes**

Except for the use of glass tubes and ultrapure NaF, assays were carried out as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Additions to assay medium</th>
<th>Flint glass tubes</th>
<th>Polyethylene tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>None</td>
<td>0.69</td>
<td>0.46</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>0.68</td>
<td>0.48</td>
</tr>
<tr>
<td>1 mM NaF</td>
<td>0.69</td>
<td>7.1</td>
</tr>
<tr>
<td>1 mM NaF + 5 mM EDTA</td>
<td>0.70</td>
<td>0.43</td>
</tr>
<tr>
<td>2 mM NaF</td>
<td>0.59</td>
<td>15</td>
</tr>
<tr>
<td>2 mM NaF + 5 mM EDTA</td>
<td>0.68</td>
<td>0.63</td>
</tr>
<tr>
<td>10 mM NaF</td>
<td>0.098</td>
<td>6.2</td>
</tr>
<tr>
<td>10 mM NaF + 5 mM EDTA</td>
<td>0.66</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* $V_{max}$ = units per mg of microsomal protein.
Interactions of Al(F)$_3$ with Glucose-6-phosphatase

Table V

<table>
<thead>
<tr>
<th>Reference experiment</th>
<th>Added NaF</th>
<th>Al(F)$_3$*</th>
<th>Al(F)$_3$</th>
<th>Al(F)$_3$</th>
<th>Al(F)$_3$</th>
<th>Al(F)$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>µM</td>
<td>mM</td>
<td>nM</td>
<td>mM</td>
<td>nM</td>
</tr>
<tr>
<td>Fig. 3A</td>
<td>0.4</td>
<td>0.8</td>
<td>4.3</td>
<td>179</td>
<td>504</td>
<td>110</td>
</tr>
<tr>
<td>Fig. 5</td>
<td>0.5</td>
<td>0.5</td>
<td>1.7</td>
<td>90</td>
<td>319</td>
<td>87</td>
</tr>
<tr>
<td>Fig. 3B</td>
<td>1.0</td>
<td>0.3</td>
<td>0.24</td>
<td>25</td>
<td>175</td>
<td>96</td>
</tr>
</tbody>
</table>

* Al$_3^+$ equals $K_a$ from Fig. 3, A and B, or the concentration of Al$_3^+$ needed for a 50% reduction of $k_{	ext{inact}}$ by heat or DEPC (Fig. 5).

estimates of its dissociation constant. A $K_a = 0.1 \mu M$ makes Al(F)$_3$ by far the most potent inhibitor/stabilizer of glucose-6-phosphatase presently known. For example, from a comparison of the observed values for $K_a$, Al(F)$_3$ is about 20 times more effective as an inhibitor of glucose-6-phosphatase activity than vanadate (28). Given the similarity between the actions of Al(F)$_3$ and P$_i$ (see above and see Refs. 2 and 8), it is reasonable to consider that Al(F)$_3$ functions as a high affinity analogue of H$_2$PO$_4^-$. The findings that NaF plus Al$_3^+$ interact with the enzyme in intact microsomes to inhibit it (Table III) or protect it from inactivation by DEPC (8) can be reconciled by positing that Al(F)$_3$ is transported into microsomes via the fluoride transport, $T_2$ (2).

It has been proposed that glucose-6-phosphatase exists as an active phosphorylated form or as an inactive dephosphorylated form (6). Since fluoride (6, 29, 30), molybdate (6, 31, 32), and glutathione disulfide (6, 33) are all known to be inhibitors of protein phosphatases and since they all prevent inactivation of glucose-6-phosphatase activity when cholate-solubilized microsomes were incubated at 25°C (6), it was postulated that these agents inhibit a microsomal protein phosphatase and thereby prevent inactivation of glucose-6-phosphatase by dephosphorylation.

The present results do not support the earlier interpretation, however, at least as it applies to the stabilizing effect of fluoride. It is highly unlikely that identical values would be obtained for the inhibition constant and the "stabilization constant," if these responses resulted from independent interactions of Al(F)$_3$ with two different enzymes. Moreover, supplementary studies indicated that Al$_3^+$ is not a cofactor in the inhibition by NaF of an endogenous protein phosphatase of hepatic microsomes. Specifically, both reagent grade NaF and EDTA inhibited the activity of the protein phosphatase, and the combination of EDTA and F$^-$ caused more inhibition than either agent by itself.

Further experimentation is required to understand the molecular basis for the "noncompetitive" inhibition that was observed in assay systems containing greater than 1 mM NaF (Figs. 1 and 4, and Table IV). Noncompetitive inhibition requires that the inhibitory species interacts with the enzyme at a site other than the glucose-6-P binding site. The experimental observations to date suggest that Al$_3^+$ also is involved in the noncompetitive inhibition. Specifically, both the competitive and noncompetitive components of the inhibition are abolished by EDTA (see Table IV) and a comparison of the data in Fig. 1 and Table IV reveals that at equal concentrations, reagent grade NaF was a more effective noncompetitive inhibitor than ultrapure NaF. However, since noncompetitive inhibition was never seen at concentrations of F$^-$ below 1 mM, even when 10 µM AlCl$_3$ was present (Fig. 3), we have not been able to correlate the noncompetitive inhibition with the concentration of one of the complexes formed from two ions. Future studies will examine the possibility that at higher concentrations, uncomplexed F$^-$ interacts at the water binding site of the phosphohydrolase in a reaction that is facilitated by Al$_3^+$.

The discovery that added Al$_3^+$ markedly increases the effectiveness of fluoride as a stabilizer of glucose-6-phosphatase represents a promising breakthrough in our efforts to purify this notoriously unstable enzyme. First of all, because of the high potency of the stabilizing complex, significant protection is provided at relatively low concentrations, which permits its inclusion in buffers used, for example, in ion exchange chromatography. Second, the fact that interactions of Al(F)$_3$ with the enzyme can be abolished by EDTA provides a significant advantage over other potential stabilizers of the enzyme, e.g. P$_i$ (Fig. 2), molybdate (20), or vanadate (20). Since all of the latter agents are also potent competitive inhibitors of the phosphohydrolase, enzyme solutions containing them must be substantially diluted before they can be assayed. However, this problem presents the possibility of loss of enzyme activity and there is no way of knowing whether the inactivation occurred in the course of the purification procedure per se or subsequent to the dilution of the stabilizing/inhibitory agent. This problem is readily averted in the case of Al$_3^+$ and fluoride simply by adding EDTA to the assay medium; inhibition is instantaneously abolished and the responsibility for stabilizing the enzyme is simultaneously transferred to glucose-6-P. Thus, preparations of labile phosphohydrolase can always be stored and assayed under conditions which hopefully will minimize loss of activity.

Finally, the concentrations of Al$_3^+$ in liver and kidney of the rat are reported to be in the range of 1–10 µM/kg of wet tissue (34). This is the concentration range over which Al$_3^+$ was highly effective in promoting inhibition and stabilization of the microsomal phosphohydrolase (Figs. 3 and 5). Given the apparent high affinity of the enzyme for Al(F)$_3$ and the strong association between Al$_3^+$ and F$^-$ (27), we believe that future studies should evaluate the possibility that interactions between glucose-6-phosphatase and the aluminum fluoride complex may have either physiological or toxicological significance.

REFERENCES

Interactions of \text{Al(F)}^- with Glucose-6-phosphatase