Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES)

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The release of GA (mitochondrial glutaminase) from neurons following acute ischaemia or during chronic neurodegenerative diseases may contribute to the propagation of glutamate excitotoxicity. Thus an inhibitor that selectively inactivates the released GA may limit the accumulation of excess glutamate and minimize the loss of neurological function that accompanies brain injury. The present study examines the mechanism of inactivation of rat KGA (kidney GA isoform) by the small-molecule inhibitor BPTES [bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide]. BPTES is a potent inhibitor of KGA, but not of the liver GA isoform, glutamate dehydrogenase or γ-glutamyl transpeptidase. Kinetic studies indicate that, with respect to glutamine, BPTES has a $K_i$ of approx. 3 µM. Moreover, these studies suggest that BPTES inhibits the allosteric activation caused by phosphate binding and promotes the formation of an inactive complex. Gel-filtration chromatography and sedimentation-velocity analysis were used to examine the effect of BPTES on the phosphate-dependent oligomerization of KGA. This established that BPTES prevents the formation of large phosphate-induced oligomers and instead promotes the formation of a single oligomeric species with distinct physical properties. Sedimentation-equilibrium studies determined that the oligomer produced by BPTES is a stable tetramer. Taken together, the present work indicates that BPTES is a unique and potent inhibitor of rat KGA and elucidates a novel mechanism of inactivation.

Key words: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), glutamate excitotoxicity, glutaminase inhibitor.

INTRODUCTION

GA (mitochondrial glutaminase) catalyses the hydrolytic cleavage of glutamine to form glutamate and an ammonium ion. Mammals express three isoforms of GA, which are encoded by two distinct, but structurally related, genes [1,2]. In humans, the liver-type GA gene spans 18 kb on chromosome 12 and is composed of 18 exons [3,4]. The kidney-type GA gene spans 82 kb on chromosome 2, contains 19 exons and uses alternative splicing to produce two isoforms; KGA (kidney GA isoform) and GAC (kidney glutaminase variant) [4,5]. The KGA isoform is abundantly expressed in kidney, brain, intestine, immune system cells and in many transformed cells, whereas GAC is expressed in heart, pancreas, placenta and lung [5]. The KGA and GAC isoforms share identical N-terminal amino acid sequences transcribed from exons 1–14, but have distinct C-terminal regions. The C-terminal amino acid sequence in GAC is derived from exon 15, whereas the KGA C-terminal sequence is derived from exons 16–19. The common N-terminal region of KGA and GAC differs greatly from that of the LGA (liver GA isoform) N-terminus, suggesting that the catalytic domain is located within the central region of the protein. This hypothesis was confirmed by the demonstration that deletion of specific N- and C-terminal regions of KGA has no effect on enzymatic activity [6].

In the brain, KGA expression is important for the intercellular cycle that generates and removes the excitatory neurotransmitter glutamate [7]. In response to a nerve impulse, glutamate is released from secretory vesicles into the synaptic space. Neuronal stimulation is transient, due to rapid and efficient uptake of glutamate by glial cells, which convert glutamate into glutamine by glutamine synthetase. Glial cells then release glutamine, which is taken up by neurons and transported into mitochondria, where it is reconverted into glutamate by KGA. The cycling of glutamate to glutamine is essential to maintain very low (µM) concentrations of extracellular glutamate, as excessive accumulation of glutamate leads to neuronal cell death. A large body of evidence indicates that excitotoxicity contributes to the pathophysiology of neuronal injury, resulting from hypoxic ischaemia, trauma or chronic neurodegenerative diseases [8–11]. Following neuronal injury, there is an immediate release of glutamate from damaged neurons. This is followed by a more pronounced secondary increase in extracellular glutamate [12]. The delayed elevation of glutamate levels exacerbates the primary neuronal injury by causing progressive neuronal death, which can continue for hours or days following brain injury [13]. Consistent with this observation, studies have demonstrated that glutamate receptor antagonists are neuroprotective, even when administered within a few hours following focal or global ischaemia [14,15].

GA is the only brain enzyme known to hydrolyse glutamine to glutamate [16]. Studies with cultured neurons demonstrated that the release of mitochondrial KGA from damaged neurons contributes to the delayed increase in extracellular glutamate and the amplification of excitotoxicity [17]. Additional studies demonstrated that, following brain injury, high levels of KGA activity are maintained in the peripheral regions of the brain and may contribute to the expanding zone of neuronal damage, which evolves for 24–48 h [18]. Thus the gradual release of KGA from disrupted neurons may be a potential contributor to the delayed increase in glutamate. These studies also suggest that pharmacological inactivation of the released KGA may limit the

Abbreviations used: BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide; CK, L-2-amino-4-oxo-5-chloropentanoic acid; DON, 6-diazo-5-oxo-L-norleucine; DTT, dithiothreitol; GA, mitochondrial glutaminase; GAC, kidney glutaminase variant; KGA, kidney GA isoform; LGA, liver GA isoform; rKGA<sub>N</sub>, recombinant N-terminal truncation of rat KGA.

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progressive neuronal damage which is associated with brain injury and prevent extensive disability and death.

A unique catalytic property of KGA is the potent activation by phosphate and other polyvalent anions [19]. The $K_m$ for glutamine decreases in the presence of increasing phosphate concentration, and the extent of phosphate activation correlates with the association of inactive KGA dimers to active tetramers or larger oligomers [20,21]. This suggests that allosteric regulation by phosphate or other bivalent anions is pivotal for the regulation of KGA activity. Furthermore, glutamate reverses enzyme activation and produces a proportional decrease in the extent of KGA tetramer formation. Similarly, the disruption of the tetramer structures by high salt has been associated with decreased catalytic activity [20]. In addition, the efficacies of known KGA inhibitors, DON (6-diaz-o-5-oxo-L-norleucine) and CK (L-2-amino-4-oxo-5-chloropentanoic acid) are also phosphate-dependent. Specifically, DON is more effective at inactivating KGA at high concentrations of phosphate [22], whereas high phosphate concentrations decrease the rate of inactivation by CK [23].

In this study, kinetic and biophysical analyses were performed to characterize the mechanism of KGA inactivation by BPTES [bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide]. BPTES was identified from a screen of a library of chemical compounds for effective inhibitors of glutaminase [24]. In contrast with other known KGA inhibitors, which are affinity labelling reagents or substrate analogues, the structure of BPTES differs greatly from that of glutamine (Figure 1). The resulting data establish that BPTES is a specific and potent inhibitor that causes the formation of a stable, but inactive, tetramer.

**MATERIALS AND METHODS**

**Materials**

Sprague–Dawley rats were obtained from Charles River. Bovine liver glutamate dehydrogenase and all other biochemical reagents were obtained from Sigma. Chromatography and analytical-ultracentrifugation buffers were filtered using 0.22-µm-pore-size filters prior to use. BPTES was synthesized according to the method described previously [24].

**Protein extraction and purification**

Rat KGA was solubilized as described previously [25]. Briefly, kidney mitochondria were prepared by centrifugation at 10000 g for 10 min at 4°C and freeze-dried following addition of 10 mM sodium borate, 100 mM potassium phosphate and 100 mM potassium pyrophosphate buffer, pH 8.4. The freeze-dried pellet was resuspended in water, centrifuged at 100000 g for 30 min at 4°C and then dialysed against a buffer containing 300 mM KC1, 2 mM DTT (dithiothreitol) and 10 mM Tris/HCl, pH 8.0. The post-mitochondrial supernatant was used to measure $\gamma$-glutamyl transpeptidase activity [26]. Rat liver-type glutaminase was solubilized by repeated sonication of isolated rat liver mitochondria and assayed as described previously [27]. Purified glutamate dehydrogenase was assayed by measuring $A_{340}$ to follow the disappearance of NADH during the reductive amination of $\alpha$-oxoglutarate.

rKGA$_{51}$ (recombinant N-terminal truncation of rat KGA), was expressed and purified as described previously [6] with the following modifications. To increase soluble protein expression, rKGA$_{51}$ was expressed in *Escherichia coli* strain BL-21 DE3 Codon Plus RIPL cells (Stratagene). Cultures of the transformed cells were grown at 37°C until the attenuation ($D_{600}$) reached 0.4–0.6. Protein expression was induced by the addition of 2.5 g/litre of ‘The Inducer’, an IPTG (isopropyl $\beta$-D-thiogalactoside) analogue (Molecular Research Laboratories). Cleared cell lysates were first loaded on to a HiTrap chelating column (Amersham Biosciences) containing immobilized Ni$^{2+}$. The protein was eluted using a linear gradient of imidazole (20 mM–1 M) in a buffer containing 10 mM Tris/HCl, pH 8.0, 300 mM KC1 and 10 % (v/v) glycerol. Fractions (0.5 ml) containing rKGA$_{51}$, which eluted at between 200 and 300 mM imidazole, were pooled and diluted 1:10 in the column elution buffer prior to loading on to a HiTrap Q column (Amersham Biosciences), which had been equilibrated in a buffer containing 10 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol and 2 mM DTT. The purified protein was eluted using a linear gradient of KC1 (30 mM–1 M) and protein samples were stored at 4°C for up to 3 weeks in buffer containing 10 mM Tris/HCl, pH 8.0, 300 mM KC1, 10% (v/v) glycerol and 2 mM DTT.

**Kinetic assays**

KGA activity was measured using a two-step assay as described previously [6,28]. To facilitate the collection of multiple data points, the assay was adapted to a microtitre assay format by proportionally reducing reagent volumes to a final reaction volume of 227 µl. Typically, 5 µl of rKGA$_{51}$ was added to 20 µl of an initial assay mix (20 mM glutamine, 150 mM potassium phosphate, 0.2 mM EDTA and 50 mM Tris/acetate, pH 8.6). Samples were incubated at 37°C for 10 min and the reaction was stopped by the addition of 2 µl of 3 M HCl. Next, 200 µl of a second reaction mixture (0.2 µg/ml of purified bovine liver glutamate dehydrogenase, 80 mM Tris/acetate, pH 9.4, 200 mM hydrazine, 2.5 mM ADP and 0.2 mM NAD$^+$/) was added. The samples were mixed and incubated for 40 min at room temperature (22°C). The $A_{227}$ was measured using a Wallac Victor$^\text{V}$ plate reader (PerkinElmer), fitted with a 340 nm excitation filter. Sample absorbance was measured against a blank, in which HCl was added to the first reaction mixture prior to the addition of rKGA$_{51}$. The necessary controls established that the two-step assay is linear with time and that glutamate is quantitatively converted into an equivalent amount of NADH over the range measured in the present study. Glutamate saturation profiles were performed by measuring the activity of rKGA$_{51}$ over a range of glutamine concentrations (2–40 mM) in the presence of 0, 1 and 5 µM BPTES. For assays performed in the presence of BPTES, the appropriate volume of a 10 mM stock of BPTES dissolved in DMSO was added to the glutamine reaction mixtures. Control samples containing equal volumes of DMSO established that DMSO had no effect on enzyme activity. Samples were assayed for activity in quadruplicate at each glutamine concentration. The kinetic constants $K_m$ and $V_{max}$ were determined using KaleidaGraph software (Synergy Software) non-linear least-squares fit for all the data points to the Michaelis–Menten equation. The $K_i$ for BPTES was determined using GraphPad Prism software (Dr E. M. Shepard and Dr D. M. Dooley, Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, U.S.A.) to perform a global non-linear least-squares fit of the data to the
equation for non-competitive inhibition:

$$v = V_{\text{max}} [\text{Gln}]/K_m(1 + [I]/K_i) + [\text{Gln}](1 + [I]/K_i)$$

where $[\text{Gln}]$ is the concentration of the substrate, $[I]$ is the inhibitor concentration, and $K_i$ is the dissociation constant for the binding of inhibitor to the enzyme and the enzyme–substrate complex. Phosphate activation profiles were performed by measuring the activity of rKGAΔ1 over a range of phosphate concentrations (0–200 mM) in the presence of 0, 1 and 3 µM BPTES. Samples were assayed in triplicate at each phosphate concentration. The kinetic constants, $K_m$ and $V_{\text{max}}$, and Hill coefficients were determined by using KaleidaGraph software to perform a non-linear least-squares fit of all the data points to the Hill equation:

$$v_0/V_m = [A]^h/(K_a + [A]^h)$$

where $v_0/V_m$ is the fractional saturation of the enzyme with phosphate; $[A]$ is the concentration of phosphate; $h$ is the Hill coefficient and $K_a$ is the dissociation constant at half saturation.

**Gel filtration**

Gel-filtration chromatography was performed on an AKTA FPLC apparatus using a 14-ml Bio-Silect SEC 250-5 column (Bio-Rad Laboratories). The column was equilibrated in either low (10 mM) or high (200 mM) potassium phosphate buffer containing 10 mM Tris/HCl, pH 8.0, 100 mM KCl and 10% (v/v) glycerol. Following equilibration, 20 µl of 5 µM rKGAΔ1 that had been dialysed against the chromatography buffer was applied to the column and eluted at 0.5 ml/min at 4°C. The retention volumes of the eluted samples were calibrated using a gel-filtration calibration kit (Amersham Biosciences). $K_m$ values for the standards were determined and plotted against the log of the molecular mass. An equation for the linear fit of the data was used to determine the apparent molecular mass of rKGAΔ1.

**Analytical ultracentrifugation**

Experiments were performed in a Beckman XL-I analytical ultracentrifuge using absorbance optics. Sedimentation-velocity measurements were collected using a two-sector, charcoal-filled Epon centrepiece, quartz windows, and 400 µl of sample and 420 µl of reference buffer. All samples were centrifuged using a Beckman An60Ti four-hole rotor at 22°C. Velocity data were edited and analysed using the boundary-analysis method of Demeler and van Holde and Weischet [29] to generate $G(s)$ plots as implemented in Ultrascan version 7.1. The top and bottom 5% of the data were excluded from the analyses due to experimental noise at the highest and lowest absorbances. All sedimentation coefficients ($s_{20,w}$) were corrected to that of water at 20°C. Modeling of hydrodynamic parameters was performed using the Ultrascan program, with $f/f_c$ used to calculate the ratio of the frictional coefficient for the particle of interest to the frictional coefficient of a perfect sphere. Sedimentation-equilibrium experiments were performed at 5°C using the four-hole rotor and six-sector, charcoal-filled Epon centrepieces. In order to span a wide range of loading concentrations (0.87–10.3 µM) while staying within the linear range of the detector, samples were prepared at $A_{280} = 0.21$, 0.46, and 0.63 and $A_{208} = 0.25$ and 0.42. Samples containing 100 µl of rKGAΔ1 and 110 µl of reference buffer were subjected to centrifugation at three different speeds (11000, 14000 and 19000 rev/min). Measurements were collected at $A_{280}$ and $A_{208}$, acquired in 0.001 cm increments and 15 measurements were averaged at each radial position. Overlays of successive scans taken 4 h apart at each speed confirmed that the samples had reached equilibrium. Global fitting of the sedimentation-equilibrium data sets was performed within the Ultrascan program. Data sets were applied to a single ideal species model, a 2-component, non-interacting model, and a monomer–dimer equilibrium model after editing. Direct comparison of the variance from each model confirmed the quality of the fit. The partial specific volume ($\nu$, 0.7458 at 20°C) was calculated from the primary amino acid sequence, and solvent densities ($\rho$) were calculated with the Ultrascan program.

**RESULTS**

**Kinetic studies**

Initial experiments to characterize the specificity of inhibition by BPTES (Figure 1) were performed using solubilized extracts of rat kidney mitochondria and liver mitochondria. The addition of 10 µM BPTES resulted in 80% inhibition of KGA activity, but only a 15% decrease in LGA activity (Figure 2). Furthermore, BPTES had no effect on glutamate dehydrogenase activity and caused only a very slight inhibition of $\gamma$-glutamyl transpeptidase activity. A truncated GA derivative, rKGAΔ1, lacking the sequence encoded by most of the first exon, was used as the source of KGA in order to study the mechanism of BPTES inhibition. The kinetic properties of purified rKGAΔ1 protein containing an N-terminal His6 tag are very similar to those of the native full-length enzyme [6]. To determine the type of inhibition caused by BPTES, glutamine saturation profiles were determined using both 1 and 5 µM concentrations of the inhibitor. The resulting glutamine saturation profiles were hyperbolic (Figure 3A). In accordance with previous studies [6], the $K_m$ for glutamine in the absence of BPTES was 15 mM. Addition of BPTES produced a decrease in the apparent $V_{\text{max}}$, but had little effect on the $K_m$, indicative of non-competitive inhibition. Linear double-reciprocal plots of the glutamine saturation data in the presence of BPTES clearly established that BPTES is not a competitive inhibitor with respect to glutamine (Figure 3B). Non-competitive inhibition occurs when the inhibitor binds to a site distinct from the substrate-binding site and causes a reduction in catalytic activity. For a
classical non-competitive inhibitor, the lines on the double-reciprocal plot would intercept on the 1/[S] axis. The lines on the double-reciprocal plot of the BPTES data intercept slightly below this axis. Thus the current data cannot distinguish between classical non-competitive and a mixed non-competitive inhibition. However, to estimate the $K_i$ for BPTES inhibition, a global fitting of all the data to the equation for non-competitive inhibition was performed using GraphPad non-linear least-squares analysis. This analysis produced a $K_i$ of $2.9 \pm 0.3 \mu M$.

A potential mechanism of inhibition is the blocking of the allosteric activation by phosphate. This could be mediated by direct competition with the phosphate-binding site or through a conformational change that blocks activation by phosphate. To assess the effect of BPTES, phosphate activation profiles were determined in the absence and in the presence of 1 or 3 $\mu M$ BPTES. All of the phosphate activation profiles were sigmoidal, indicating BPTES does not prevent phosphate-induced cooperativity (Figure 4). However, the activation profiles suggested that the addition of BPTES decreased the apparent $V_{max}$. In the absence of BPTES, the Hill coefficient for phosphate activation was 1.8 (Table 1). In the presence of 3 $\mu M$ inhibitor, the Hill coefficient was increased slightly. The derived values for the kinetic constants confirmed the decrease in $V_{max}$, and indicated that BPTES may increase the $K_{0.5}$ for phosphate. Taken together, the data suggests that BPTES does not compete with phosphate, but instead interferes with allosteric activation by phosphate by promoting the formation of an inactive complex that has a reduced affinity for phosphate. Therefore BPTES also functions as a non-competitive inhibitor with respect to phosphate.

**Gel-filtration chromatography**

Gel-filtration experiments were performed as an initial approach to characterize the oligomeric state of rKGA$_{\Delta 1}$. Samples containing 5 $\mu M$ rKGA$_{\Delta 1}$ were applied to a Bio-Silect SEC gel-filtration column. In the presence of 10 mM phosphate, purified rKGA$_{\Delta 1}$ eluted from the Bio-Silect SEC predominantly as a single asymmetric peak (Figure 5A). In contrast, two asymmetric peaks were observed in the sample containing 200 mM phosphate (Figure 5B), suggesting that high phosphate concentration induces...

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**Figure 3** BPTES inhibition with respect to glutamine

(A) Glutamine saturation profiles for rKGA$_{\Delta 1}$ in the absence (●) or presence of 1 $\mu M$ (■) or 5 $\mu M$ (▲) BPTES. Enzymatic activity is plotted as $\mu$mol/min per ml against the glutamine concentration. Saturation profiles represent the non-linear least-squares fit to the Michaelis–Menten equation. Error bars represent the S.E.M. for triplicate activity measurements at each glutamine concentration. (B) Lineweaver–Burk double-reciprocal representation of the glutamine saturation profiles for rKGA$_{\Delta 1}$ in the absence (●) or presence of 1 $\mu M$ (■) or 5 $\mu M$ (▲) BPTES. Lines represent a linear least-squares fit of the mean activity measured at each glutamine concentration.

**Figure 4** BPTES inhibition with respect to phosphate

Phosphate activation profiles for rKGA$_{\Delta 1}$ in the absence (●) or presence of 1 $\mu M$ (■) and 3 $\mu M$ (▲) BPTES. The inset plot is an enlargement of the data obtained at low phosphate concentrations. Enzymatic activity is plotted as $\mu$mol/min per ml against the phosphate concentration. Activation curves represent the non-linear least-squares fit of the data to the Hill equation. Error bars represent the S.E.M. for triplicate activity measurements at each phosphate concentration.

**Table 1** Kinetic constants derived from the characterization of BPTES inhibition with respect to phosphate activation

<table>
<thead>
<tr>
<th>BPTES ($\mu M$)</th>
<th>$K_{0.5}$ (mM)</th>
<th>$V_{max}$ ($\mu$mol/min per ml)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>58 ± 2.3</td>
<td>40 ± 0.7</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>93 ± 9.9</td>
<td>30 ± 1.7</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>88 ± 5.6</td>
<td>14 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>
Mechanism of BPTES inhibition of glutaminase

Figure 5 Gel-filtration profiles of rKGA_{Δ1} in the presence or absence of BPTES

As indicated, 25 µl samples of rKGA_{Δ1} in the absence (broken line) or presence of 10 µM BPTES (continuous line) were injected onto a Bio-Silect column equilibrated with buffer containing 10 mM (A) or 200 mM phosphate (B). Protein elution was monitored at A_{230}.

The oligomerization of rKGA_{Δ1}. When calibrated relative to the elution volumes of standard proteins, the two peaks correspond to proteins that have apparent molecular masses of 155 and 315 kDa, consistent with phosphate-induced dimerization. However, in the presence of 10 and 200 mM phosphate buffers containing 10 µM BPTES, rKGA_{Δ1} was eluted as single asymmetric peaks with apparent molecular masses of 337 and 315 kDa respectively. The results suggest that BPTES may stabilize a unique oligomeric form of rKGA_{Δ1} that is independent of the concentration of phosphate present. Previous data indicated that native glutaminase is asymmetric and therefore yields an anomalous estimate of molecular mass from gel-filtration data [20]. Therefore a more accurate assessment of the oligomeric state of rKGA_{Δ1} was determined using analytical ultracentrifugation.

Analytical ultracentrifugation

The intrinsic self-association properties of rKGA_{Δ1} were initially characterized by sedimentation-velocity analysis in a buffer containing 10 mM phosphate. Three concentrations of rKGA_{Δ1}, ranging from 0.8 to 15 µM were sedimented. The boundaries were analysed using the boundary-analysis method of Demeler and van Holde and Weischet [29] to obtain diffusion-corrected sedimentation-coefficient distributions. At 0.8 µM rKGA_{Δ1}, greater than 80% of the rKGA_{Δ1} sedimented as a homogeneous 6 S species (Figure 6A). At 2.5 µM rKGA_{Δ1}, the sedimentation-coefficient distribution ranged from 6 to 7.5 S and had a shape characteristic of a self-associating system [30]. At 15 µM rKGA_{Δ1}, most of the sample sedimented as a homogeneous 7.7 S species. These results indicate that, in a low phosphate buffer and in the absence of an inhibitor, rKGA_{Δ1} exists in a concentration-dependent equilibrium between 6 S and 7.7 S oligomeric states. Attempts to obtain the molecular mass of the 6 and 7.7 S species using finite solutions to the Lamm equation were inconclusive. Sedimentation equilibrium of rKGA_{Δ1} also proved inconclusive, due to the sample pelleting as the experiment progressed (results not shown). The sedimentation data are consistent with the broad asymmetric gel-filtration profiles and establish that the intrinsic rKGA_{Δ1} self-association pathway minimally involves 6 and 7.7 S structural states, and probably higher-order oligomers as well.

To determine the effects of phosphate on rKGA_{Δ1} self-association, samples containing 2.5 µM enzyme and 0, 50 or 200 mM phosphate...
phosphate were analysed by sedimentation-velocity analysis (Figure 6B). The 0 mM phosphate sample yielded a predominantly 6 S distribution. The sedimentation-coefficient distributions obtained in the presence of 50 and 200 mM phosphate buffers were shifted to higher $s_{20,w}$ values and were biphasic, with a clear break at $\sim 8$ S. In 50 mM phosphate, approx. 30% of the enzyme sedimented between 8–14 S, while the remainder sedimented between 6.0–7.7 S. In 200 mM phosphate buffer, approx. 40% of the sample sedimented between 8–17 S, while the remainder sedimented between 6.0–7.7 S. A comparison of the 6.0–7.7 S material between the 0, 50 and 200 mM phosphate sedimentation-velocity experiments indicated that the distribution is slightly shifted toward the 7.7 S species with an increase in the phosphate concentration. Thus the data in Figure 5(B) indicates that the presence of phosphate alters the intrinsic 6.0–7.7 S association pathway and induces the formation of higher-order rKGA$_{\Delta 1}$ oligomers.

Sedimentation velocity also was used to characterize the effect of BPTES on rKGA$_{\Delta 1}$ self-association (Figure 7A). rKGA$_{\Delta 1}$ was incubated with 10 $\mu$M BPTES in the presence of either 10 or 200 mM phosphate. The sedimentation-coefficient distributions indicated that BPTES induced formation of a single $9.2 \pm 0.3$ S species in 10 mM phosphate and a single $8.5 \pm 0.2$ S species in 200 mM phosphate. The 8.5 S and 9.2 S species are two conformers of the same oligomer, and differ only in their frictional coefficients. Order-of-addition experiments, where BPTES was added to rKGA$_{\Delta 1}$ either before or after the addition of 200 mM phosphate, resulted in the same homogeneous 8.5 S species (results not shown). The data also indicate that BPTES inhibits the formation of higher-order rKGA$_{\Delta 1}$ oligomers by phosphate. In the presence of BPTES, the rKGA$_{\Delta 1}$ forms a single non-interacting hydrodynamic structure.

To determine the molecular masses of the 8.5 and 9.2 S species, five different concentrations of rKGA$_{\Delta 1}$, in the presence of 10 mM phosphate plus BPTES, and four different concentrations of rKGA$_{\Delta 1}$ in the presence of 200 mM phosphate plus BPTES, were centrifuged to equilibrium at three different speeds. On the basis of the sedimentation-velocity experiments, the resulting data sets were globally fitted to a model for a single ideal component. The quality of the fit was judged by a combination of the variance and the randomness of the residuals (Figure 7B). Fitting to a single ideal component model yielded molecular masses in 10 and
200 mM phosphate of 239 and 241 kDa respectively. In both cases the residuals were random and the variances were low (3.6 \times 10^{-5} and 6.5 \times 10^{-5} respectively). The fit to other models for interacting systems did not lower the variance; hence the data are most accurately described by the single ideal component model. The monomer molecular mass of \textit{rKGA}_{\text{a1}} is 60.5 kDa. Therefore the 8.5 and 9.2 S species observed in the presence of BPTES are \textit{rKGA}_{\text{a1}} tetramers (242 kDa).

**DISCUSSION**

These results provide new insight into the oligomerization of \textit{rKGA}_{\text{a1}} and its relationship with the mechanism of BPTES action. Analysis of kinetic inhibition data indicated that BPTES functioned as a non-competitive inhibitor. Non-competitive inhibition demonstrates that the inhibitor associates with both the free enzyme and the enzyme–substrate complex. The biophysical studies, which were performed in the absence of glutamine, confirmed that BPTES binds in the absence of substrate. The \( K_i \) value of 3 \( \mu \)M, obtained from the global fit of the inhibition data to the model for non-competitive inhibition, suggests that BPTES has a very high affinity for both the free enzyme and the enzyme–substrate complex.

In the absence of both phosphate and inhibitor, \textit{rKGA}_{\text{a1}} exists in equilibrium between 6.0 and 7.7 S states. Even the most compact conformation of a 60.5 kDa monomer would not sediment at 6.0 S. Hence, the 6.0 S species is either a dimer with an \( f/f_0 \) of 1.5 or a trimer with an \( f/f_0 \) of 1.9. Given that the 6.0 S species self-associates to form the 7.7 S species and that there were no \textit{rKGA}_{\text{a1}} monomers present (Figure 6A), the simplest interpretation of the data is that the 6.0 S species is a \textit{rKGA}_{\text{a1}} dimer and the 7.7 S species is a tetramer. The data in Figure 6(B) demonstrate that, at low protein concentration, the addition of phosphate shifts the equilibrium toward the 7.7 S tetramer and induces subsequent formation of even higher order (> 15 S) oligomers. The phosphate activation profiles of the recombinant \textit{rKGA}_{\text{a1}} are sigmoidal, with a \( K_{50} \) of 58 mM and a Hill coefficient of 1.8. These properties are very similar to those of the well-characterized phosphate activation of \textit{GPA} purified from rat kidney [31]. Therefore the cooperative phosphate binding and activation of \textit{rKGA}_{\text{a1}} also correlates with the formation of 7.7 S tetramers and the assembly of higher order (> 15 S) oligomers.

Importantly, BPTES was found to inhibit phosphate-dependent assembly of higher-order oligomers. In addition, the sedimentation coefficients of the native (Figure 6B) and BPTES-bound \textit{rKGA}_{\text{a1}} tetramers (Figure 7A) in the presence of 200 mM phosphate were 7.7 S and 8.5 S respectively, indicating that the native \textit{rKGA}_{\text{a1}} tetramer is more compact than the BPTES-bound tetramer. Therefore both the kinetic and the biophysical data argue that BPTES binding alters the conformation of the \textit{rKGA}_{\text{a1}} subunits such that BPTES-bound tetramers are incapable of assembling active higher-order \textit{rKGA}_{\text{a1}} oligomers in the presence of phosphate.

The predominant effect of BPTES inactivation is the formation of an inactive tetramer, which is formed regardless of phosphate concentration. At low phosphate concentrations, BPTES promotes the association of dimers to the inactive tetramer. Likewise, at high phosphate concentrations, active tetramers and larger oligomers of \textit{rKGA}_{\text{a1}} are all converted into the inactive tetramer form. The observed difference in \( s_{20,W} \) values for the tetramer in the presence of 10 or 200 mM phosphate concentrations is too small to reflect a difference in oligomeric state, it may result instead from subtle differences in the calculated \( f/f_0 \) values of 1.4 and 1.5 respectively. Therefore the addition of phosphate to the BPTES-bound tetramer may produce a slight change in conformation. In addition, our results indicate that, once the inactive tetramer is formed, the complex is highly stable. Order-of-addition experiments demonstrated that BPTES predominates over phosphate, as addition of phosphate does not cause the inactive tetramer to revert to the active enzyme. Moreover, the sedimentation properties of BPTES-inactivated \textit{rKGA}_{\text{a1}} does not change when stored at 4 \( ^\circ \)C for 96 h (the time required to perform a sedimentation-equilibrium experiment).

This indicates that BPTES has distinct biochemical properties that are advantageous for therapeutic applications. First, BPTES is highly effective at inhibiting \textit{KGA} with a \( K_i \) of 3 \( \mu \)M. This effective inhibitor concentration is significantly lower than the inhibition constants of other \textit{KGA} inhibitors, which are in the mM range [22,23]. Secondly, in contrast with previously characterized inhibitors, BPTES bears no structural similarity to either glutamine or glutamate and therefore is unlikely to interact with transporters, receptors or other enzymes that recognize glutamine or glutamate as a substrate. This conclusion is supported by previous studies that tested the effect of the addition of BPTES to cultured intestinal epithelial and neuronal cells [24]. The addition of 300 nM BPTES produced a selective decrease in cellular glutamate, whereas addition of up to 10 \( \mu \)M BPTES had no effect on the cellular content of other amino acids or nucleotides. Thirdly, the present study indicates that the binding of BPTES to the enzyme and enzyme–substrate complex is highly specific and requires a defined interaction surface or conformation. The partial inhibition observed with \textit{LGA} suggests that this binding site may be at least partially conserved in the liver isoflorn, which contains a stretch of 123 amino acids that has 80 \% identity with the corresponding segment in \textit{KGA} [32]. In addition, high concentrations of phosphate are not required for effective inhibition by BPTES. The results presented in this study indicate that the BPTES interaction surface is distinct from both the substrate- and phosphate-binding sites. Furthermore, the observed effects of BPTES on \textit{KGA} self-association suggest that BPTES interacts with a solvent-exposed surface of the enzyme that is accessible in both the dimer and tetramer form. In summary, BPTES is a novel and potent inhibitor of \textit{KGA} and thus an attractive prototype for the design of a potent therapeutic agent that may effectively limit progressive neuronal damage associated with a stroke or various types of brain injury.

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