Biochemical and Kinetic Characterization of Xylulose 5-Phosphate/Fructose 6-Phosphate Phosphoketolase 2 (Xfp2) from Cryptococcus neoformans

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Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp), previously thought to be present only in bacteria but recently found in fungi, catalyzes the formation of acetyl phosphate from xylulose 5-phosphate or fructose 6-phosphate. Here, we describe the first biochemical and kinetic characterization of a eukaryotic Xfp, from the opportunistic fungal pathogen Cryptococcus neoformans, which has two XFP genes (designated XFP1 and XFP2). Our kinetic characterization of C. neoformans Xfp2 indicated the existence of both substrate cooperativity for all three substrates and allosteric regulation through the binding of effector molecules at sites separate from the active site. Prior to this study, Xfp enzymes from two bacterial genera had been characterized and were determined to follow Michaelis-Menten kinetics. C. neoformans Xfp2 is inhibited by ATP, phosphoenolpyruvate (PEP), and oxaloacetic acid (OAA) and activated by AMP. ATP is the strongest inhibitor, with a half-maximal inhibition concentration (IC50) of 0.6 mM. PEP and OAA were found to share the same or have overlapping allosteric binding sites, while ATP binds at a separate site. AMP acts as a very potent activator; as little as 20 μM AMP is capable of increasing Xfp2 activity by 24.8% ± 1.0% (mean ± standard error of the mean), while 50 μM prevented inhibition caused by 0.6 mM ATP. AMP and PEP/OAA operated independently, with AMP activating Xfp2 and PEP/OAA inhibiting the activated enzyme. This study provides valuable insight into the metabolic role of Xfp within fungi, specifically the fungal pathogen Cryptococcus neoformans, and suggests that at least some Xfps display substrate cooperative binding and allosteric regulation.

Cryptococcus neoformans, an invasive opportunistic pathogen of the central nervous system, is the most frequent cause of fungal meningitis, resulting in more than 625,000 deaths per year worldwide (1,2). Exposure to C. neoformans is common, as it is an environmental fungus found in the soil and can enter the lungs through inhalation, leading to pulmonary infection. An increased rate of infection occurs in individuals with impaired cell-mediated immunity, particularly those with AIDS and recipients of immunosuppressive therapy.

Acetate has been shown to be a major metabolite released by Cryptococcus during infection (3–5), but the significance of this is not known. Genes encoding enzymes from three putative acetate-producing pathways and two putative acetate transporters have been shown to be upregulated during cryptococcal infection (6), suggesting acetate production and transport may be a necessary and required part of the pathogenic process.

One pathway for acetate production is composed of the enzymes xylulose 5-phosphate (X5P)/fructose 6-phosphate (F6P) phosphoketolase (Xfp) and acetate kinase (Ack). Xfp catalyzes the breakdown of xylulose 5-phosphate (EC 4.1.2.9; X5P + P, ↔ acetyl-phosphate + glyceraldehyde 3-phosphate) or fructose 6-phosphate (EC 4.1.2.22; F6P + P, ↔ acetyl phosphate + erythrose 4-phosphate). Ack utilizes the acetyl phosphate product of the reaction to produce acetate and ATP (acacetate + ATP ↔ acetyl phosphate + ADP; EC 2.7.2.1). These enzymes form a modified pathway, termed the pentose phosphoketolase pathway, in lactic acid bacteria and bifidobacteria (7). This pathway is utilized in the heterofermentative degradation of pentoses and hexoses to the end products CO2, ethanol, acetate, and lactate (8). Xfp can convert X5P generated at the end of the oxidative phase of the pentose phosphate pathway to glyceraldehyde 3-phosphate, which can enter the glycolytic pathway, and acetyl phosphate, which Ack can convert to acetate to generate ATP.

Only the Xfp enzymes from the lactic acid bacteria Bifidobacterium spp. and Lactobacillus plantarum have been purified and kinetically characterized (7,9). The characterized bacterial Xfp enzymes show dual substrate specificity with X5P and F6P and follow Michaelis-Menten kinetics (7,9,10). Here we report the first biochemical and kinetic characterization of eukaryotic Xfp, the C. neoformans Xfp2. Unlike the previously characterized bacterial Xfp enzymes, C. neoformans Xfp2 displays both substrate cooperativity and allosteric regulation. The enzyme is inhibited by ATP, phosphoenolpyruvate (PEP), and oxaloacetic acid (OAA) and is activated by AMP.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma-Aldrich, VWR, or Fisher Scientific. Oligonucleotide primers were purchased from Integrated DNA Technologies. Codon-optimized C. neoformans XFP2 was synthesized by GenScript and supplied in the Escherichia coli expression vector pET21b, which provides for addition of a C-terminal His tag for use in nickel affinity column purification.

Production and purification of C. neoformans Xfp2. The recombinant plasmid pET21b-XFP2 was transformed into Escherichia coli RosettaBlue (DE3) placl (Novagen). The recombinant strain was grown in Luria-Bertani (LB) medium with 50 μg/ml of ampicillin and 34 μg/ml of...
TABLE 1 Apparent kinetic parameters for C. neoformans Xfp2

| Substrate | $K_{0.5}$ (mM) | $k_{cat, app}$ (s$^{-1}$) | $k_{cat, app}/K_{0.5}$ (s$^{-1}$M$^{-1}$) | $h$ |
|-----------|---------------|-----------------|-----------------|---|
| F6P       | 15.9 ± 1.3    | 3.47 ± 0.10     | 0.22 ± 0.01     | 1.41 ± 0.11 |
| X5P       | 6.4 ± 0.2     | 3.76 ± 0.05     | 0.58 ± 0.01     | 1.17 ± 0.06 |
| $P_i$     | 13.3 ± 1.5    | 4.22 ± 0.13     | 0.32 ± 0.03     | 0.59 ± 0.03 |

Values are means ± standard errors of the means. $h$ is the Hill coefficient.

Enzyme reactions were performed at the indicated temperatures in triplicate. (B) pH optima for Xfp2. Enzyme reactions were performed utilizing 50 mM MES or 50 mM morpholinopropanesulfonic acid (MOPS) over a range of pH values. (C) Divalent cation metal specificity for Xfp2. Enzyme reactions were performed in the presence of 5 mM metal (as chloride salt).
into the allosteric regulation of Xfp2 were performed only with F6P. IC50s were determined for all Xfp2 allosteric inhibitors by measuring the decrease in activity as a function of increasing inhibitor concentration. GraphPad Prism 5 software was used to determine IC50s by fitting the data with a log[inhibitor]-versus-response curve. In order to determine if any of the inhibitors bound at the same allosteric site or had overlapping allosteric sites, the IC50 of one inhibitor was measured in the presence of another inhibitor.

RESULTS
Optimization of reaction conditions. An E. coli codon-optimized XFP2 gene (GenScript Inc.) was cloned into pET21b (C-terminal His tag), and the recombinant enzyme was produced and purified by nickel affinity chromatography to electrophoretic homogeneity. Optimal reaction conditions for purified Xfp2 activity were determined. The temperature optimum was found to be 37 to 40°C (Fig. 1A), the temperature to which C. neoformans would be exposed during infection. Xfp2 had highest activity between pH 4.5 and 6.0 (Fig. 1B), and pH 5.5 was used when determining kinetic parameters. Like its bacterial counterpart, C. neoformans Xfp2 requires the cofactor TPP, and 0.5 mM TPP was sufficient for full enzymatic activity (data not shown). Xfp2 prefers Mg2⁺ as the divalent cation, but it can also utilize Ca2⁺, Co2⁺, Mn2⁺, and Ni2⁺ (Fig. 1C).

Kinetic characterization of C. neoformans Xfp. In determining the kinetic parameters in the acetyl phosphate-forming direction, plots of substrate concentration versus velocity were found to be sigmoidal rather than hyperbolic, as would be expected for enzymes that follow Michaelis-Menten kinetics. This is the first demonstration of the existence of substrate cooperative binding among Xfp enzymes; this was not reported in previous characterizations of bacterial Xfps (7, 9, 10). Apparent kinetic parameters (Table 1) were determined by fitting experimental data to the Hill equation (see above), with a Hill constant greater than 1.0 representing positive cooperativity and a Hill constant less than 1.0 representing negative cooperativity (15, 16). Xfp2 exhibited positive cooperativity (h > 1.0) for both X5P and F6P, suggesting that binding of either of these substrates causes a favorable conformational change that facilitates the binding of additional substrate at separate active sites on the enzyme. Xfp2 displayed negative cooperativity (h < 1.0) for Pi, with an average Hill constant of approximately 0.6. The 2.6-fold-higher catalytic efficiency suggested that X5P is slightly preferred over F6P.

Allosteric inhibitors. Since we hypothesized that the Xfp-Ack pathway plays a significant role in ATP production during infection, it would seem likely that this pathway would be regulated, particularly since the F6P substrate of Xfp2 is an intermediate in glycolysis, another key ATP-generating pathway that has been shown to be critical for virulence (17). ATP along with coenzymes and intermediates from glycolysis and the tricarboxylic acid (TCA) cycle were tested to see if Xfp2 is allosterically regulated by these molecules. Of the ligands tested, ATP, PEP, and OAA were found to display the most pronounced inhibition (Fig. 2), while citrate caused slight inhibition (data not shown). Acetyl coenzyme A (CoA), CoA, and pyruvic acid were also tested and had no effect on activity. Progress curves for both substrates Pi and F6P were

FIG 2 Effects of various ligands on Xfp2 activity. Various coenzymes and metabolic intermediates were tested for their effects on Xfp2 activity. Activities are reported as the percentage of maximum activity with no effector present.

FIG 3 Effect of ATP on substrate progress curves. Progress curves were generated in the presence of 0 mM, 3 mM, 6 mM, and 9 mM ATP for the substrates Pi (A) and F6P (B). Activities are reported in micromoles of acetyl phosphate produced, and enzyme reactions were performed in triplicate for each substrate concentration.
generated in the presence of increasing concentrations of ATP (Fig. 3A and B), PEP (Fig. 4A and B), and OAA (Fig. 5A and B).

For each of the three inhibitors, the $K_{0.5}$ for $P_i$ was not significantly affected (data not shown); however, the $K_{0.5}$ concentrations for F6P increased in each case. The $K_{0.5}$ for F6P increased from $15.9 \pm 1.3$ mM (mean ± standard error of the mean) in the absence of inhibitor to $73.9 \pm 3.2$ mM in the presence of 9 mM ATP, to $109.4 \pm 6.9$ mM in the presence of 15 mM OAA, and to $69.3 \pm 9.6$ mM in the presence of 16 mM PEP. The addition of inhibitor also generated a more sigmoidal F6P progress curve, which was reflected in the increase in the Hill coefficient. The Hill coefficient increased from $1.41 \pm 0.11$ to $4.50 \pm 0.20$, $2.32 \pm 0.07$, and $2.40 \pm 0.30$ in the presence of 9 mM ATP, 15 mM OAA, and 16 mM PEP, respectively. This suggests that the binding of ATP, PEP, and OAA has a direct effect on the binding of F6P to the active site in order to more closely regulate Xfp2 activity in response to changing cellular concentrations of these effectors.

The IC$_{50}$, the concentration of inhibitor required to reduce activity to half its maximally inhibited value, was determined for each inhibitor by using the $K_{0.5}$ concentrations of both $P_i$ (13 mM) and F6P (16 mM) (Table 2). The IC$_{50}$ of 0.6 mM for ATP is much lower than those determined for PEP and OAA, which were very similar (Table 2). Since both molecules are similar in size and structure, it is possible that they bind at the same allosteric site. In order to test if the inhibitors share the same allosteric site, reactions were performed in which one inhibitor was held constant at its IC$_{50}$ and the concentration of the second inhibitor was varied. If two inhibitors bind at the same site (or if binding of one inhibitor occludes the binding of the second), then approximately half of the binding sites would be occupied by the first inhibitor, thereby lowering the concentration of the second (varied) inhibitor required to inhibit activity by an additional 50%. The IC$_{50}$ of PEP decreased by approximately half in the presence of OAA; likewise, the IC$_{50}$ of OAA decreased by about half in the presence of PEP (Table 2). This suggests that PEP and OAA share the same allosteric site. Alternatively, PEP and OAA bind at separate over-
lapping sites, with the binding of one inhibitor preventing the binding of the second inhibitor. The IC\textsubscript{50} of PEP in the presence of ATP did not change significantly, suggesting that the PEP/OAA site(s) is separate from the ATP allosteric site.

**Allosteric activator.** *C. neoformans* Xfp2 is activated by the presence of AMP (Fig. 6). The presence of as little as 20 µM AMP resulted in elevated enzymatic activity that reached a maximum at 0.5 mM AMP. The half-maximal activation concentration was determined to be 29.7 ± 1.5 µM. The effect of AMP activation on P\textsubscript{i} and F6P progress curves was evaluated. Increasing amounts of AMP resulted in increased Xfp2 activity at all P\textsubscript{i} concentrations without affecting the overall shape of the curve or the K\textsubscript{0.5} of P\textsubscript{i} (Fig. 6A). Therefore, AMP does not appear to have a direct effect on the binding of P\textsubscript{i} to the active site. The presence of AMP decreased the K\textsubscript{0.5} for F6P from 15.9 ± 1.3 mM in the absence of AMP to 9.1 ± 0.6 mM in the presence of 0.5 mM AMP. Increasing concentrations of AMP resulted in more hyperbolic F6P progress curves (Fig. 6B). The Hill coefficient decreased from 1.41 ± 0.11 in the absence of AMP to 0.97 ± 0.03 in the presence of 0.5 mM AMP, suggesting a more constant affinity for the substrate F6P.

AMP not only causes activation but also alleviates the effects of allosteric inhibitors (Fig. 7). The presence of AMP completely prevents or overrides the inhibitory effect of ATP. However, AMP cannot overcome inhibition by PEP. The results observed are consistent with AMP and PEP acting separately, with AMP fully activating the enzyme, and PEP inhibiting it from this fully activated level. When ATP and PEP are both present, inhibition is slightly additive compared to inhibition by either one alone. Activity decreased from 43.4% ± 0.7% in the presence of PEP to 32.5% ± 0.6% when both ATP and PEP were present. The level of activity observed when AMP was present in addition to PEP was similar to that when AMP was present with both ATP and PEP. In both cases, activity was in the range expected for inhibition of the fully activated enzyme by the IC\textsubscript{50} of PEP.

**DISCUSSION**

Here we report the characterization of *C. neoformans* Xfp2, the first eukaryotic Xfp to be characterized. Xfp functions with Ack in

### TABLE 2 Half-maximal inhibitory concentrations

| Inhibitor used at various concns | Inhibitor used at a constant concn | IC\textsubscript{50} ± SE |
|----------------------------------|-----------------------------------|--------------------------|
| ATP                              | —                                 | 0.61 ± 0.04              |
| PEP                              | —                                 | 8.23 ± 0.09              |
| OAA                              | 4.84 ± 0.07                       |
| ATP                              | 9.85 ± 0.25                       |
| OAA                              | 7.50 ± 0.40                       |
| PEP                              | 3.71 ± 0.07                       |

\textsuperscript{a} Values are means ± standard errors of the means.

\textsuperscript{b} —, no inhibitor was included in the reaction mixture.

**FIG 6** Effect of AMP on substrate progress curves. Progress curves were generated in the presence of 0 mM, 0.02 mM, 0.1 mM, and 0.5 mM AMP for the substrates P\textsubscript{i} (A) and F6P (B). Activities are reported in micromoles of acetyl phosphate produced, and enzyme reactions were performed in triplicate for each substrate concentration.

**FIG 7** AMP activates Xfp2 activity and alleviates inhibition by allosteric inhibitors. Activities are reported as the percentage of the maximum activity with no allosteric effector present. The effectors AMP, ATP, and/or PEP were added to reaction mixtures alone or in combination at final concentrations of 0.5 mM, 0.6 mM, and 8 mM, respectively.
heterofermentative bacteria to form a modified pentose phosphoketolase pathway. This pathway was originally thought to be present only in bacteria but has been more recently identified in euascomycete and basidiomycete fungi (18). Like C. neoformans, many fungi with this pathway have two XFP open reading frames, designated XFP1 and XFP2.

Unlike previously characterized bacterial Xfps, C. neoformans Xfp2 displays both substrate cooperative binding and allosteric regulation. Xfp2 is allosterically regulated by the activator AMP and inhibitors ATP, PEP, and OAA. PEP and OAA appear to share the same allosteric binding site, while ATP and AMP bind at a separate site. The simplest explanation of AMP’s ability to fully overcome ATP inhibition is that they bind at the same site; however, our results do not conclusively rule out separate but interacting binding sites. Xfp2 activation by AMP and inhibition by ATP are consistent with Xfp2 partnering with Ack as part of a modified pentose phosphate pathway to generate ATP and acetate from XSP and F6P. The intracellular ATP:AMP ratio provides an indication of the cellular energy status, and regulation of the Xfp2-Ack pathway by ATP and AMP is a way to modulate ATP production by this pathway. High ATP levels indicate that the energy needs of the cell have been satisfied and thus additional ATP production via the Xfp2-Ack pathway is not necessary, whereas high AMP levels indicate an energy deficit and the need for increased ATP production via the Xfp2-Ack pathway. A possible explanation for Xfp2 inhibition by PEP and OAA is that high concentrations of these intermediates from glycolysis and the TCA cycle, respectively, are also signals that cellular energy needs have been met, and the cell can switch from glycolysis, which produces ATP and utilizes glucose, to gluconeogenesis to synthesize and store glucose.

A ping-pong “bi-bi” mechanism was originally proposed for Lactobacillus plantarum Xfp by Yevenes and Frey (9), who reported that the enzyme-bound TPP first interacts with F6P to form a TPP-F6P complex and releases the product erythrose 4-phosphate (E4P), forming enzyme-bound 2-α,β-dihydroxyethylidene-TPP (DHETPP). DHETPP undergoes a dehydration reaction to form enzyme-bound enolacetyl-TPP, and then ketonization converts it to enzyme-bound acetyl-TPP (AcTPP). AcTPP is phosphorylated and acetyl phosphate is released, leaving TPP available to react with additional F6P substrate and repeat the reaction. Therefore, the product E4P is formed by the interaction of TPP and F6P in the absence of the second substrate, P0. The crystal structure of Bifidobacterium longum Xfp confirmed the reaction mechanism proposed by Yevenes and Frey (19). We suspect that C. neoformans Xfp2 follows the same reaction mechanism as the bacterial Xfps but with the added complexity of allosteric regulation that influences substrate binding affinity. Since all allosteric effectors were found to directly affect F6P binding, the binding of these effectors influences the interaction between F6P and TPP in the first step of the reaction mechanism. We found that the presence of allosteric effectors did not influence the binding of P0, which further confirms and supports the existence of a ping-pong bi-bi mechanism in which the interaction between TPP and F6P to form E4P occurs in the absence of P0 and that it is this first step of the reaction that is allosterically regulated.

Acetate is the most abundant metabolite produced during cryptococcal pulmonary infection, but the role acetate plays in metabolism and infection is unknown (3–5). Genomic expression studies have provided evidence that the Xfp-Ack pathway could be responsible for acetate production. Serial analysis of gene expression by C. neoformans cells collected from the lungs of infected mice showed elevated expression of XFP2 (6). XFP2 was also found to be among the genes upregulated under hypoxic conditions that occur in infected tissue (20). RNA microarray analysis of C. neoformans gene expression within macrophages also indicated that Ack is expressed under this condition, but Xfp2 was not present in that study’s microarray data set (21). In addition, an Xfp2 homolog is required for full virulence in the insect fungal pathogen Metarhizium anisopliae (22). Taken together, these studies suggest that C. neoformans Xfp2 may play a role during infection.

C. neoformans Xfp2 shows maximal activity between 37 and 40°C, which is consistent with a role of Xfp2 during C. neoformans infection (23). The Xfp-Ack pathway could serve as a source of ATP production under the hypoxic and acidic conditions encountered in macrophages, where ATP generation by oxidative phosphorylation is suppressed. In addition, the pH of cryptococcomas has been found to be between 5.4 and 5.6 in vivo (4). It is possible that the production and excretion of acetic acid generated by the Xfp-Ack pathway contributes to the acidic environment of cryptococcomas. This acidic environment can aid in C. neoformans survival outside the macrophage by inducing neutrophil necrosis and decreasing superoxide production (4). Our studies showed that Xfp2 functions best at low pH, with activity decreasing as pH increases. Even in vitro assays of the bacterial Xfps were performed at a pH below neutral, i.e., between pH 6.0 (9) and 6.5 (7), and an optimal pH was not reported. The intracellular pH of C. neoformans during infection is unknown, but even though C. neoformans cells are tolerant to low pH (24), it is unlikely that Xfp2 is exposed to low pH in vivo. The presence of AMP can increase Xfp2 activity from 13.4% ± 0.7% to 117.0% ± 2.0% in the presence of 0.5 mM AMP at pH 7, with 100% activity considered the activity at pH 5.5 when using K0.5 substrate concentrations (K. Glenn and K. Smith, unpublished data), so pH could provide an additional means of regulating Xfp2 activity.

The allosteric regulation that has evolved for C. neoformans Xfp2 versus its bacterial homolog seems to support a more complex role for this enzyme within fungal metabolism and during infection. However, allosteric regulation by PEP and OAA but not AMP and ATP may occur with at least some bacterial Xfps (K. Glenn and K. Smith, unpublished data). We believe that Xfp2 partners with Ack to generate ATP, which is supported by the findings that Xfp2 is regulated by both ATP and AMP levels. The biochemical properties of this enzyme suggest that it utilizes its partnership with Ack to generate ATP during infection and within the human macrophage, where hypoxic conditions are encountered that limit ATP production by the electron transport chain. Xfp2’s involvement in C. neoformans metabolism, especially during infection, lends support to future studies that focus on this enzyme as a possible drug target in the treatment of cryptococcal infection.

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