Cofactor-Directed, Time-Dependent Inhibition of Thiamine Enzymes by the Fungal Toxin Moniliformin

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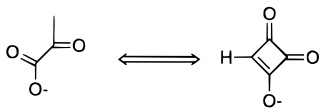
The mode of action of the fungal toxin moniliformin[1] (**1**), produced by a pathogen common in stored grains, *Fusarium moniliforme*, has been controversial. It acts *via* inhibition of α-ketoglutarate and pyruvate dehydrogenase complexes, essential activities linking glycolysis and the Krebs cycle, but whether it attacks cofactors or protein is unproved. Burka[2] was unable to establish any reaction between the thiamin pyrophosphate cofactor and moniliformin, as might be postulated, but Thiel and Hofmeyr[3] have shown that inactivation of the pyruvate decarboxylase subunit of the pyruvate dehydrogenase complex is time dependent, follows saturation kinetics, is dependent on thiamine pyrophosphate, is prevented by excess substrate or cofactor, and is irreversible. A key step in the mechanism for the decarboxylase is the nucleophilic attack of the thiamine pyrophosphate ylide on the ketone carbonyl of pyruvate. Given the structural similarity of pyruvate and moniliformin, it is reasonable to postulate that the thiamine pyrophosphate ylide also attacks one of the carbonyls of moniliformin (eq 1).



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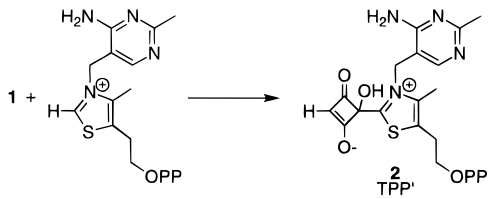
In order to gain support for the hypothesis that moniliformin inhibits pyruvate dehydrogenase by acting as a "molecular metaphor"[4] for pyruvate, we have studied its action on three other thiamine-dependent enzymes, α-ketoglutarate dehydrogenase complex,[5] yeast pyruvate decarboxylase,[6] and aceto hydroxy acid synthase.[7]



2 TPP'

The kinetics of the inactivation of α-ketoglutarate dehydrogenase were determined by preincubating enzyme with concentrations of **1** from 0 to 100 μM and assaying the loss of enzymatic activity over time (Figure 1).[8] Data were fit to a Kitz−Wilson model[9] (eq 2)

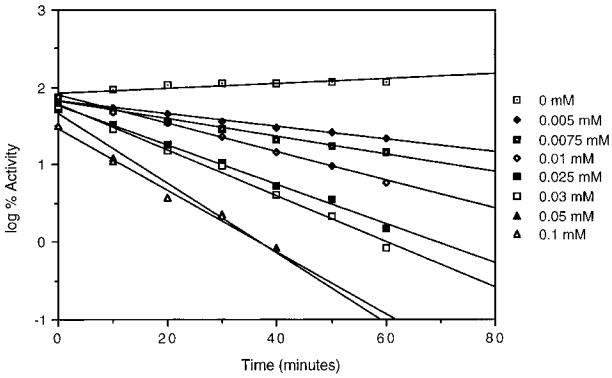


Figure 1. Loss of KGDH activity over time. α-Ketoglutarate dehydrogenase was incubated with varying concentrations of moniliformin (0−0.1 mM) for 1 h and then assayed for remaining activity. Each assay was carried out at 30 °C and contained potassium phosphate buffer (50 mM, pH 8), NAD (2.5 mM), TPP (0.2 mM), $MgCl_2$ (1.0 mM), CoA (0.13 mM), cysteine (2.6 mM), enzyme (2.3×10^{-5} U), moniliformin (varying), and saturating α-ketoglutarate (2 mM) for a total volume of 2 mL.

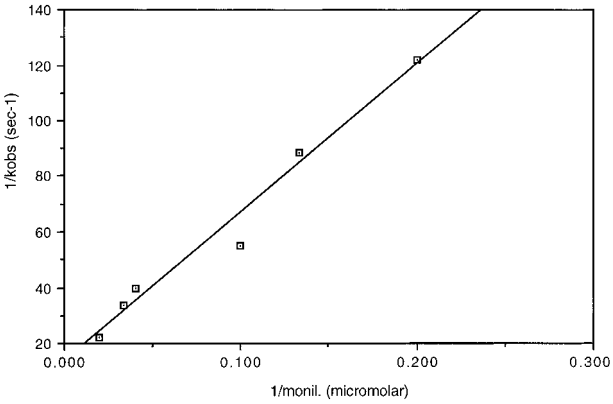


Figure 2. Replot of KGDH inactivation data.

$$\mathrm{E{-}TPP} + \mathrm{M} \overset{K_i}{\rightleftharpoons} \mathrm{E{-}TPP{-}M} \xrightarrow{k_{inact}} \mathrm{E{-}TPP'} \quad (2)$$

assuming the tightly bound enzyme-cofactor complex[10] is in facile equilibrium with moniliformin, and the ternary complex undergoes an irreversible step to produce inactivated enzyme (possibly bearing the modified cofactor TPP′). The kinetics give saturation behavior with respect to **1**; the parameters derived from the plot of rate of loss of activity versus inhibitor concentration (Figure 2) are as follows: $K_i = 40\ \mu M$, $k_{inact} = 1.25 \times 10^{-3}\ s^{-1}$. Ketoglutarate protects the enzyme from inactivation during the preincubation (supporting information) and

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(8) We have also reproduced the kinetics of inactivation of pyruvate dehydrogenase complex as reported by Theil and Hofmeyr: $K_i = 107\ \mu M$, $k_{inact} = 1.25 \times 10^{-3}\ s^{-1}$.

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(10) While excess cofactor (∼5 mM) is present in this experiment, it is in only slow exchange with tightly-bound TPP compared to the rate of the inactivation reaction. Removal of the cofactor requires denaturation, from which activity could not be reconstituted.

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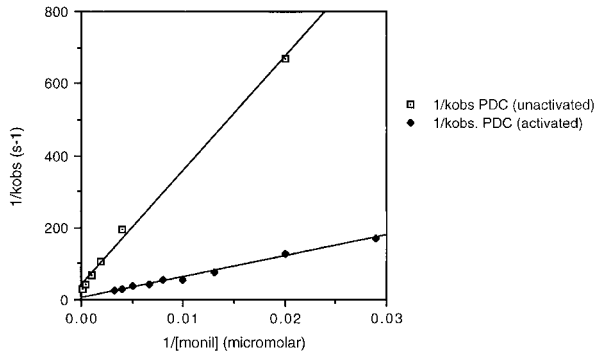


Figure 3. Replot of PDC inactivation data, with and without pyruvamide activation.

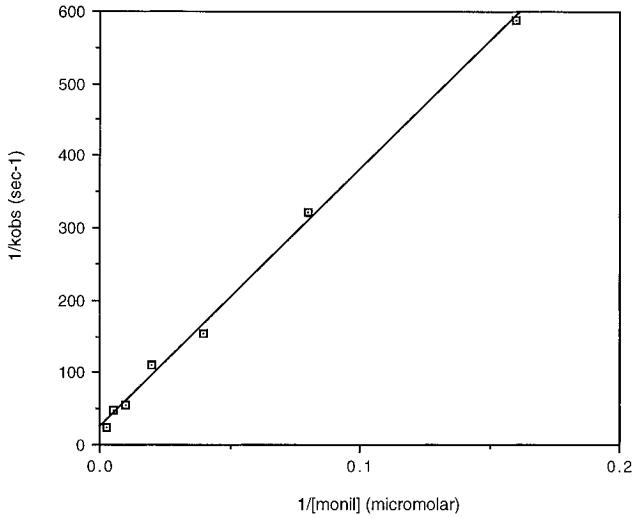


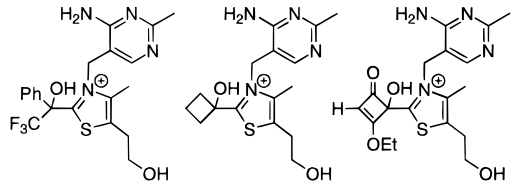
Figure 4. Replot of AHAS inactivation data.

inactivation requires thiamine pyrophosphate (data not shown). Controls and earlier work[2] show that there is no reaction between free cofactor and moniliformin in this assay mixture or in a basic aqueous solution.

With pyruvate decarboxylase, the parameters derived from the kinetic plot (Figure 3) are as follows: $K_i = 1$ mM, $k_{inact} = 4.2 \times 10^{-4}$ s^{-1}. The kinetic analysis of the influence of substrate on the inactivation is complex because this is a hysteretic enzyme. As the concentration of pyruvate in the preincubation mixture increases, the rate of loss of enzymatic activity also increases. This behavior is precedented[11] and can be explained by pyruvate binding at a regulatory site, increasing catalytic activity and facilitating inactivation, rather than competing at the active site and preventing inactivation. To further investigate this point, the enzyme was activated by 10 mM pyruvamide.[12] Preincubating enzyme over 15 min with concentrations of **1** from 0 to 300 μM and assaying the loss of enzymatic activity give by Kitz–Wilson analysis $K_i = 1$ mM, $k_{inact} = 2.9 \times 10^{-3}$ s^{-1}. The correspondence between the K_i's when the enzyme is activated and unactivated demonstrates that only the rate of the inactivation step is affected by the activator and suggests that binding is active-site directed.

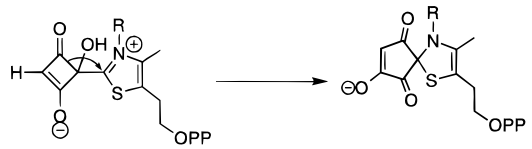
With acetohydroxyacid synthase, the parameters derived from the Kitz–Wilson plot (Figure 4) are as follows: $K_i = 140$ μM, $k_{inact} = 7 \times 10^{-4}$ s^{-1}. Kinetic studies under steady-state conditions show that pyruvate competes with moniliformin for binding to the enzyme with an IC_{50} of 76 μM compared to the pyruvate K_m of 5 mM (supporting information).

The molecular basis for inactivation of thiamine enzymes by moniliformin is proposed involving formation of an adduct between cofactor and the reactive carbonyl in **1**. This process is unlikely to reverse because of the strain relief provided by incorporation of an sp^3 carbon into the cyclobutenedione ring and possible following reactions (*vide infra*). Support for this idea is provided by chemical model reactions of thiamine[13] with three reactive ketones, trifluoroacetophenone, cyclobutanone, and the isopropyl ester of **1**. Condensations with thiamine hydrochloride promoted by anhydrous sodium ethoxide followed by anhydrous HCl in ethanol give adducts **3**–**5** (FAB MS, NMR).[14] Diagnostic signals for adduct formation include an AB pattern for the now-diastereotopic methylene bridging the two heterocycles of the cofactor in both **3** and **5**.



3 *m*/*z* = 439 **4** *m*/*z* = 335 **5** *m*/*z* = 392

This study demonstrates that moniliformin meets criteria for an active site-directed, irreversible affinity label for a diverse group of enzymes utilizing thiamine. It provides support for the idea that inhibition of pyruvate dehydrogenase, the basis for moniliformin's toxic action, is cofactor-directed. It provides a possible molecular mechanism for inhibition involving cofactor modification and supports the proposal by preparation of adducts of thiamine with reactive ketones, including a moniliformin ester. One speculation concerning the potency of this inhibitor is that the moniliformin-TPP adduct **2** undergoes an irreversible pinacol-like rearrangement to produce **6**, a process that is precedented in recent synthetic work on the addition of acyl anion equivalents to squarate esters.[15]



2 TPP' **6**

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Supporting Information Available: Kinetic plots and mass spectra (7 pages).

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(14) **3**: ^{1}H NMR (300 MHz, D_2O) δ 2.37 (3H, s), 2.42 (3H, s), 3.24 (2H, t, J = 4.6 Hz), 3.91 (2H, t, J = 4.6 Hz), 5.2 (1H, s), 5.26 (1H, s), 7.96 (1H, s). FAB-MS MH$^+$ 439. **4**: ^{1}H NMR (300 MHz, D_2O): δ 1.878 (2H, m), 2.07 (2H, m), 2.370 (3H, s), 2.54 (3H, s), 3.17 (2H, t, J = 6.5 Hz), 3.87 (2H, t, J = 6.5 Hz), 4.15 (2H, q, J = 7.4 Hz), 5.48 (2H, s), 7.32 (1H, s); FAB-MS MH$^+$ 335.

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