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Inhibition of pyruvate dehydrogenase complex by moniliformin

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The mechanism for the inhibition of pyruvate dehydrogenase complex from bovine heart by moniliformin was investigated. Thiamin pyrophosphate proved to be necessary for the inhibitory action of moniliformin. The inhibition reaction was shown to be time-dependent and to follow first-order and saturation kinetics. Pyruvate protected the pyruvate dehydrogenase complex against moniliformin inactivation. Extensive dialysis of the moniliformin-inactivated complex only partially reversed inactivation. Moniliformin seems to act by inhibition of the pyruvate dehydrogenase component of the enzyme complex and not by acting on the dihydrolipoamide transacetylase or dehydrogenase components, as shown by monitoring the effect of moniliformin on each component individually. On the basis of these results, a suicide inactivator mechanism for moniliformin on pyruvate dehydrogenase is proposed.

INTRODUCTION

Moniliformin is a highly toxic fungal metabolite produced by several species of *Fusarium*, most of which are commonly found on basic foodstuffs (Rabie *et al.*, 1982). It is produced in large quantities (approx. 10 g/kg of growth medium) by some fungal isolates under laboratory conditions (Kriek *et al.*, 1977) and has also been shown to occur naturally on maize (Thiel *et al.*, 1982). Moniliformin is normally isolated as the potassium or sodium salt of semi-squaric acid (1-hydroxycyclobut-1-ene-3,4-dione) (Springer *et al.*, 1974) (Fig. 1). It is acutely toxic to ducklings (LD_{50} = 3.68 mg/kg, orally), rats (LD_{50} = 50.0 and 41.6 mg/kg, orally, for males and females respectively) (Kriek *et al.*, 1977), cockerels (LD_{50} = 4 mg/kg, orally) (Cole *et al.*, 1973) and mice (LD_{50} = 24 mg/kg, intraperitoneally) (Farb *et al.*, 1976). Ducklings and rats treated with moniliformin at the above-mentioned LD_{50} values undergo 'progressive muscular weakness, respiratory distress, cyanosis, coma and death' (Kriek *et al.*, 1977).

These clinical symptoms led Thiel (1978) to study the effect of moniliformin on energy yielding processes by measuring oxygen consumption after the addition of various substrates to isolated rat liver mitochondria. Micromolar concentrations of moniliformin were found to inhibit mitochondrial pyruvate and α-oxoglutarate oxidation selectively.

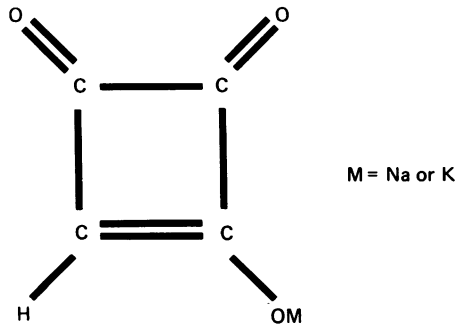

Fig. 1. Chemical structure of moniliformin

The overall reaction of the α-oxo acid dehydrogenases can be represented as:

$$R\text{–}CO\text{–}COO^- + CoA + NAD^+ \rightarrow R\text{–}CO\text{–}SCoA + CO_2 + NADH + H^+$$

where R = CH_3 (pyruvate) or CH_2–COO^- (α-oxoglutarate) (Stanley & Perham, 1980). The reaction is dependent on five cofactors, i.e. lipoate and FAD (both of which are bound to the complex), thiamin pyrophosphate, CoA and NAD^+.

Pyruvate dehydrogenase complex from bovine heart consists of pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoamide transacetylase (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (EC 1.8.1.4) [for reviews see Reed (1974) and Hucho (1975)].

Preliminary results of a study of the molecular mechanism of moniliformin inhibition led Hofmeyr *et al.* (1979) to propose that moniliformin acts as a suicide enzyme inactivator, which requires chemical activation by the target enzyme. A chemical reaction then occurs between the inhibitor and the enzyme, resulting in irreversible inhibition of the enzyme (Rando, 1974). The present paper describes experiments designed to test the hypothesis that moniliformin acts as a suicide enzyme inactivator.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical reagent grade. Bio-Gel A-150m was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Moniliformin was isolated from pure cultures of *F. moniliforme* var. *subglutinans* grown on maize (Steyn *et al.*, 1978).

Enzyme isolation

The α-oxo acid dehydrogenase complexes were isolated from bovine heart by the procedure described by Stanley & Perham (1980). This involved homogenization of the heart tissue, fractionation of the complexes with poly(ethylene glycol) and chromatographic purification of the partially purified complexes by gel filtration. The

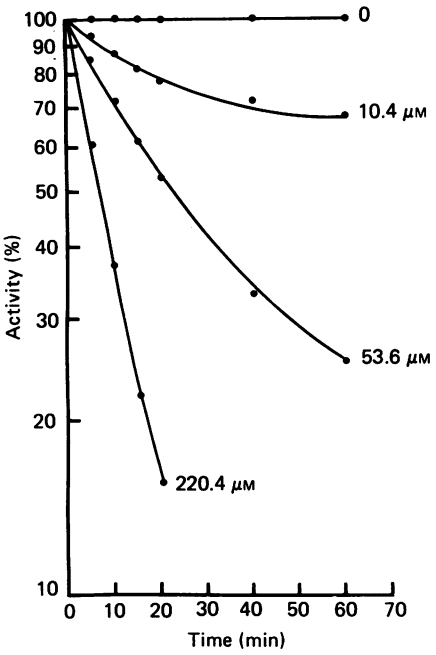

Fig. 2. Time-dependence of the inactivation of pyruvate dehydrogenase complex by moniliformin

Pyruvate dehydrogenase complex (0.39 mg/ml) was incubated in potassium phosphate buffer (50 mM, pH 6.8) containing $MgCl_2$ (1 mM), EDTA (0.5 mM), thiamin pyrophosphate (2 mM, pH 6.8) and dithiothreitol (2 mM) in the absence or presence of moniliformin at the concentrations shown on the Figure.

following minor modifications were made. Phenylmethanesulphonyl fluoride, a proteinase inhibitor and an inhibitor of pyruvate dehydrogenase phosphatase, was omitted from the initial homogenization step, thereby increasing by 47% the specific activity of the crude preparation of pyruvate dehydrogenase complex obtained after phosphatase activation. Replacing Sepharose 2B with Bio-Gel A-150m for gel filtration of the partially purified pyruvate dehydrogenase complex increased the recovery in this step from 29 to 68%. The specific activities of the purified pyruvate dehydrogenase and α-oxoglutarate dehydrogenase complexes were 5.7 and 0.23 μmol/min per mg of protein respectively.

Enzyme assays

Assays for pyruvate dehydrogenase and α-oxoglutarate dehydrogenase complex activities and for dihydrolipoamide dehydrogenase activity were based on the methods of Brown & Perham (1976). For the assay of pyruvate dehydrogenase and α-oxoglutarate dehydrogenase activities, cysteine was replaced by dithiothreitol and the pH of thiamin pyrophosphate stock solution was adjusted to 8.0. Thiamin pyrophosphate was omitted from the dihydrolipoamide dehydrogenase assay. Dihydrolipoamide transacetylase activity was determined by the method of Hayakawa *et al.* (1966). Pyruvate dehydrogenase activity was determined by the reduction of 2,6-dichlorophenol-indophenol as described by Khailova *et al.* (1977). All enzyme activities are given in units of μmol of substrate consumed or product produced/min.

RESULTS

Inhibition of pyruvate dehydrogenase complex by moniliformin

Pyruvate dehydrogenase complex was incubated at 30 °C with and without moniliformin for 60 min. In the absence of moniliformin, pyruvate dehydrogenase complex showed no loss of activity over 60 min. However, in the presence of moniliformin the inactivation of pyruvate dehydrogenase complex was time-dependent and followed first-order kinetics for at least 15 min (Fig. 2). Subsequent experiments were done by determining the percentage inactivation after 5 min incubation. The inhibition reaction was shown to follow saturation kinetics (Fig. 3).

These results were analysed by the theoretical model developed by Kitz & Wilson (1962). In the equation

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E_I$$

E = free enzyme, I = free inhibitor, EI = enzyme–inactivator complex and E_I = inactivated enzyme.

Enzyme inactivation proceeds by a two-step mechanism in which equilibrium is maintained in the first step throughout the reaction. For each moniliformin concentration, the observed rate constant for inactivation (k_{obs}) could be calculated from the half-time of inactivation ($k_{obs} = \ln 2/t_{\frac{1}{2}}$) obtained in Fig. 2. A secondary plot of $1/k_{obs}$ versus 1/moniliformin concentration (Fig. 4) gave

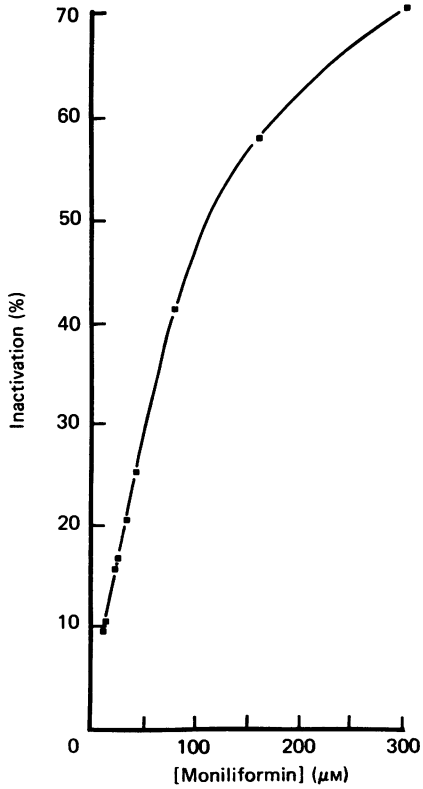

Fig. 3. Effect of moniliformin concentration on the percentage inactivation of pyruvate dehydrogenase complex after 5 min incubation

Conditions were as described in Fig. 2.

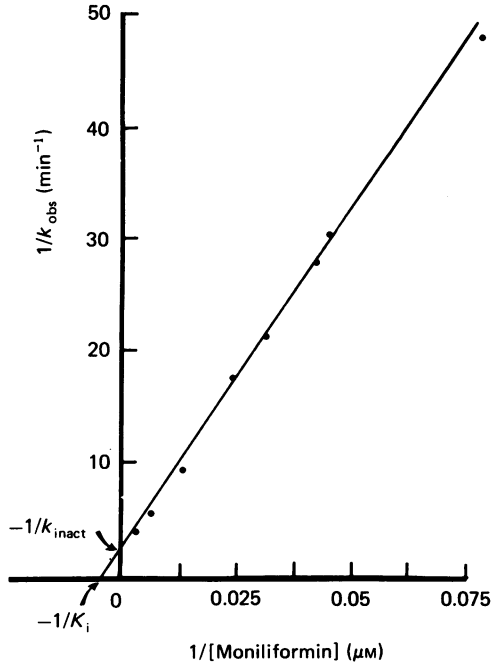

Fig. 4. Determination of kinetic constants for the inactivation of pyruvate dehydrogenase complex by moniliformin

Conditions were as described in Fig. 2.

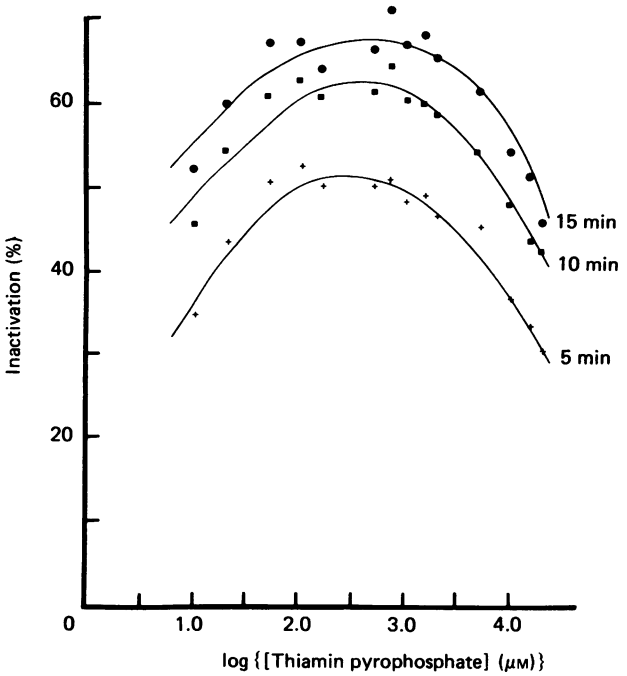

Fig. 5. Inhibition of pyruvate dehydrogenase complex by moniliformin in the presence of various thiamin pyrophosphate concentrations

Pyruvate dehydrogenase complex (0.39 mg/ml) was incubated for 5, 10 and 15 min in potassium phosphate buffer (50 mM, pH 6.8) containing $MgCl_2$ (1 mM), EDTA (0.5 mM) dithiothreitol (2 mM), moniliformin (0.1 mM) and various concentrations of thiamin pyrophosphate (0–20 mM, pH 6.5)

the K_i value of 0.24 mM and a rate constant for inactivation (k_{inact}) of 0.38 min^{-1}.

Varying the thiamin pyrophosphate concentration from 0 to 20 mM during incubation of pyruvate dehydrogenase complex with moniliformin indicated that the degree of moniliformin inhibition is dependent on the presence of thiamin pyrophosphate in the reaction mixture, but that the percentage inactivation decreases as the thiamin pyrophosphate concentration is increased from 0.5 to 20 mM (Fig. 5).

Effect of pyruvate on moniliformin inhibition

Pyruvate dehydrogenase complex was treated with moniliformin in the presence of increasing concentrations of pyruvate. The inhibitory effect of moniliformin on pyruvate dehydrogenase complex was decreased with increasing concentrations of pyruvate (Fig. 6), indicating a possible competition of moniliformin and pyruvate for the same site on the enzyme.

Reversibility of moniliformin inhibition

Pyruvate dehydrogenase complex was incubated both with and without moniliformin for 60 min at 30 °C. The activity of the complex was determined directly after incubation, after 6 h dialysis at 4 °C and again after a further 18 h dialysis at 4 °C (Table 1). The uninhibited enzyme complex lost 15% of its activity during dialysis. The inhibited enzyme complex, which lost 95% of its activity, regained only 10% of its original activity during dialysis, indicating a degree of irreversible binding of moniliformin to the complex.

Identification of the enzyme component(s) involved in moniliformin inhibition

The activities of the individual enzyme components of pyruvate dehydrogenase complex were determined in the

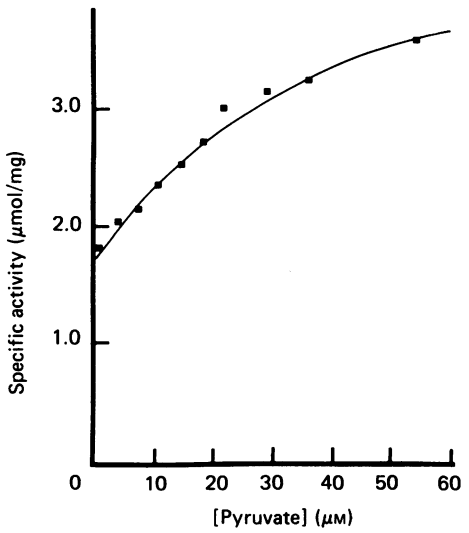

Fig. 6. Effect of pyruvate on the inhibition of pyruvate dehydrogenase complex by moniliformin

Pyruvate dehydrogenase complex (0.39 mg/ml) was treated with increasing concentrations of pyruvate (0–60 μM) in a reaction mixture containing potassium phosphate buffer (50 mM, pH 6.8), $MgCl_2$ (1 mM), EDTA (0.5 mM), thiamin pyrophosphate (2 mM, pH 6.8), dithiothreitol (2 mM) and moniliformin (0.078 mM).

Table 1. Effect of dialysis on pyruvate dehydrogenase complex inhibited by incubation with moniliformin

The enzyme mixtures were dialysed against 400 vol. of potassium phosphate buffer (50 mM, pH 6.8) containing $MgCl_2$ (1 mM), EDTA (0.5 mM) and dithiothreitol (2 mM). The enzyme mixtures, in a total volume of 5 ml, were as described in Fig. 2 legend.

	− Moniliformin		+ 303.4 μM-Moniliformin	
Time	Specific activity (μmol/mg)	Activity (%)	Specific activity (μmol/mg)	Activity (%)
0 min	1.70	100	1.73	100
After 60 min incubation (30°C)	1.64	96	0.09	5
After 6 h dialysis (4 °C)	1.64	96	0.18	10
After 24 h dialysis (4 °C)	1.44	85	0.26	15

Table 2. Effect of moniliformin on the individual enzymes forming the multi-enzyme complex

	Activity (%)		
Incubation mixture	Dihydrolipoamide dehydrogenase	Dihydrolipoamide transacetylase	Pyruvate dehydrogenase
− Moniliformin	100	100	100
+ Moniliformin (303.4 μM)	102	91	18

presence and absence of moniliformin (Table 2). Moniliformin had a negligible effect on dihydrolipoamide transacetylase and dihydrolipoamide dehydrogenase. However, pyruvate dehydrogenase activity was decreased by 82% in the presence of moniliformin.

DISCUSSION

Despite repeated attempts to improve the final yield and specific activity of the purified α-oxo acid dehydrogenase complexes, the specific activity of purified pyruvate dehydrogenase complex was only 50% of that reported by Stanley & Perham (1980). The specific activity of purified α-oxoglutarate dehydrogenase complex was extremely low compared with reported values, which prevented an extensive study of the effect of moniliformin on this enzyme complex. The lower yield of the α-oxo acid dehydrogenase complexes could have resulted from an inherently lower content of these two enzyme complexes in the heart muscle used.

The inhibition of pyruvate dehydrogenase complex by moniliformin was dependent on the presence of thiamin pyrophosphate in the incubation mixture (Fig. 5). The small amount of inhibition found when no thiamin pyrophosphate was added to the incubation mixture could be due to residual thiamin pyrophosphate bound to the enzyme complex. On the basis of structural similarity between moniliformin and pyruvate, as well as the dependence of inhibition on thiamin pyrophosphate, one can speculate that complex-formation between thiamin pyrophosphate and moniliformin on the active site of the enzyme is analogous to complex formation between thiamin pyrophosphate and pyruvate. At thiamin pyrophosphate concentrations above 2 mM, the excess of thiamin pyrophosphate could exchange with the moniliformin-bound thiamin pyrophosphate associated with the active site before moniliformin reacts with the enzyme and thereby decrease the inhibitory effect of moniliformin.

Burka *et al.* (1982) have previously shown from spectroscopic and chromatographic data that a non-enzymic reaction between moniliformin and the primary amine group of thiamin hydrochloride does not take place.

Rando (1975) pointed out that small-molecule toxins almost never function as simple isosteric competitive inhibitors of enzymes. These molecules often function by mechanisms which require them to be substrates for the target enzymes, leading to irreversible inhibition of the enzyme. There are a number of experimental criteria by which suicide enzyme inhibitors can be identified (Abeles & Maycock, 1976):

(1) the loss of enzyme activity is time-dependent and first-order;

(2) the reaction follows saturation kinetics;

(3) the rate of inactivation decreases as the substrate concentration is increased;

(4) enzyme inactivation is irreversible because of covalent binding of the inhibitor to the enzyme.

The inhibition of pyruvate dehydrogenase complex by moniliformin is time-dependent and follows first-order kinetics (Fig. 2). Time-dependence provides good, but not definitive, evidence that covalent modification has taken place. The inactivation of pyruvate dehydrogenase complex follows saturation kinetics. The rate of inactivation at a given moniliformin concentration decreases as the pyruvate concentration increases (Fig. 6), indicating a protective effect by pyruvate against moniliformin inactivation. Substrate protection against

inactivation confirms the involvement of the enzyme's active site in the inactivation process.

Determination of the activity of the individual component enzymes of the pyruvate dehydrogenase complex in the presence and absence of moniliformin indicated that moniliformin acts on pyruvate dehydrogenase and not on dihydrolipoamide dehydrogenase or dihydrolipoamide transacetylase.

Preliminary experiments indicated that radioactivity was irreversibly bound to the enzyme complex after inactivation with [^{14}C]moniliformin and subsequent removal of unbound moniliformin by dialysis. However, the radioactivity was too low to be detected by autoradiography in the individual components of the complex after separation by SDS/polyacrylamide-gel electrophoresis. Final evidence for the covalent binding of moniliformin to pyruvate dehydrogenase complex would require the use of radioactively labelled moniliformin with a much higher specific radioactivity. It should then also be possible to show to which component enzyme the radioactivity becomes irreversibly associated. The use of labelled inhibitor will also allow the stoichiometry of labelling to be established.

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