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# REACTIVITY OF ESSENTIAL CYSTEINE AND LYSINE RESIDUES PRESENT AT THE CATALYTIC DOMAIN OF PIG HEART MITOCHONDRIAL MALATE DEHYDROGENASE

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Pig heart mitochondrial malate dehydrogenase was inactivated very rapidly by *o*-phthalaldehyde as compared to 5,5′-dithio bis(2-nitrobenzoic acid) and pyridoxal 5′-phosphate. The *o*-phthalaldehyde reaction followed pseudo first order kinetics, and a second order rate constant of 38 $M^{-1}$ $S^{-1}$ was obtained. Cysteine and lysine residues participating in the *o*-phthalaldehyde reaction are located in the NADH binding region of malate dehydrogenase as shown by protection experiments. The decrease in the rate of inactivation in the presence of NADH was used to determine the dissociation constant of the enzyme-NADH complex. pH dependent studies and molar transition energy calculations of the *o*-phthalaldehyde-inactivated enzyme have indicated that cysteine and lysine residues involved in the isoindole derivative formation are located in a hydrophobic environment at the coenzyme binding site.

KEY WORDS: *o*-phthalaldehyde, malate dehydrogenase, cysteine, lysine

## INTRODUCTION

Mitochondrial malate dehydrogenase (EC 1.1.1.37) is an essential enzyme of the citric acid cycle. It catalyzes the reversible reduction of oxaloacetate by NADH to yield L-malate. This mitochondrial enzyme differs from the cytosolic form in as much as that during translocation from cytosol to mitochondrial matrix, it loses 24 amino acids from the amino terminus region of the polypeptide chain.[1] The enzyme is a dimeric molecule composed of identical[2] and catalytically independent subunits.[3]

X-ray diffraction and chemical modification studies have reported the presence of histidine, cysteine, lysine, arginine and aspartic acid at the catalytic center of malate dehydrogenase.[4–10] *o*-Phthalaldehyde, a fluorescent reagent had been used for the characterization of the essential residues of many enzymes.[11–15] Preliminary results showed that malate dehydrogenase was inhibited by *o*-phthalaldehyde and that cysteine residues were shown to be crucial for enzyme activity.[16] The studies reported in this paper give additional insight into the involvement of cysteine and lysine at

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the catalytic domain of malate dehydrogenase. Comparative inhibition studies have established that *o*-phthalaldehyde is a highly effective inhibitor for the enzyme.

## EXPERIMENTAL PROCEDURE

### *Materials*

Pig heart mitochondrial malate dehyrogenase and oxaloacetate were purchased from Boehringer Mannheim GmbH, Germany. 5,5′-dithio bis (2-nitrobenzoic acid) [DTNB], NADH, *o*-phthalaldehyde and buffer components were procured from Sigma Chemical Company, USA. Pyridoxal 5′-phosphate [PLP] was from Calbiochem, USA. All other reagents were of the highest purity grade commercially available.

### *Methods*

*Enzyme preparation and activity measurements.* The enzyme was prepared according to the method described earlier.[16] Malate dehydrogenase activity was determined on a Gilford UV/vis spectrophotometer, Model 260 equipped with a Gilford recorder. The assay was monitored as a function of decrease in absorbance of NADH at 340 nm in 1 min. The solutions of 4 mM NADH and 10 mM oxaloacete were prepared in 100 mM phosphate buffer (pH 7.5) 0.50 $\mu$l of each of these were added to the assay mixture in a final volume of 990 $\mu$l. The reaction was initiated by the addition of 10 $\mu$1 of malate dehydrogenase (2.88 $\mu$g protein/ml) to the assay mixtures.

*Inactivation studies of malate dehydrogenase.* The inactivation experiments were carried out in 100 $\mu$l reaction mixtures containing enzyme and inhibitors (DTNB or PLP or *o*-phthalaldehyde) at the indicated pH and temperature. After designated time intervals, aliquots were withdrawn from the incubation mixture and the residual activity was determined. Parallel control sets were run under identical conditions but without inhibitors. DTNB and PLP solutions were prepared in the assay buffer, while *o*-phthalaldehyde was dissolved in 1% methanol prior to use. All the control samples contained the same amount of methanol which did not show any effect on the enzyme activity. The inactivation of enzyme by *o*-phthalaldehyde followed pseudo-first order kinetics. The first order rate constants ($k_{obs}$) were calculated from the slope of the plot according to equation 1.

$$-\ln\left[\frac{[E]_t}{[E]_o}\right] = k_{obs}\, t \qquad (1)$$

where $E_o$ and $E_t$ represent the enzyme activities at zero and any given time t, respectively.

*Determination of dissociation constant of malate dehydrogenase and NADH complex.* The protection behavior of NADH was examined kinetically to determine the dissociation constant of malate dehydrogenase-NADH complex. In this case, the enzyme was incubated with variable concentrations of NADH followed by the addition of *o*-phthalaldehyde. At different time intervals, aliquots were withdrawn to measure

the rate of protection. The dissociation constant for NADH in the enzyme-NADH complex was determined using the following equation 2,[17]

$$\frac{k_{obs}}{k^{R}_{obs}} = 1 + \frac{[R]}{K_d}\left[1 - k_2\frac{[I]}{k^{R}_{obs}}\right] \quad (2)$$

Where $k_{obs}$ and $k^{R}_{obs}$ are the pseudo-first order rate constants in the absence and presence of the protecting reagent [R], respectively; $k_2$ is the bimolecular inactivation rate constant for the enzyme-NADH complex wih inhibitor. [I] and [R] are the concentrations of inhibitor and protecting reagent, respectively.

*pH-dependent inactivation of malate dehydrogenase by o-phthalaldehyde.* Effect of pH on the inactivation profile of the reaction was observed over a pH range of 6.0–7.8 using 0.1 M potassium phosphate buffer. Appropriate control measurements were also made under similar pH conditions. The effect of pH on enzyme inactivation was analyzed according to the equation 3.[17]

$$\frac{1}{k} = \left[\frac{1}{k_{max}}.K\right][H^+] + \frac{1}{k_{max}} \quad (3)$$

Where k is the second order rate constant at each pH, $k_{max}$ is the maximal second order rate constant, K is the ionization constant of the group involved in the catalysis and $[H^+]$ is the hydrogen ion concentration.

## RESULTS

### *Differential Inactivation Studies of Malate Dehydrogenase*

When malate dehydrogenase was incubated with DTNB, PLP or *o*-phthalaldehyde separately at pH 7.5, differential patterns of inhibition were obtained, as shown in Figure 1. The enzyme lost only 28% and 50% of the activity at 4 mM concentration of PLP and DTNB, respectively. By contrast, *o*-phthalaldehyde was found to be very effective, resulting in 73% loss of activity at 0.25 mM concentration. This rapid inactivation may be attributed to the bifunctional nature of *o*-phthalaldehyde which enables it to bind with sulfhydryl as well as to $\varepsilon$-amino groups simultaneously.

### *Kinetics of Inactivation of Malate Dehydrogenase with o-Phthalaldehyde*

A typical inactivation rate profile of malate dehydrogenase was obtained when treated with *o*-phthalaldehyde (Figure 2). The rate of inactivation was found to be dependent on the ratio of *o*-phthalaldehyde to the enzyme, and inactivation increased with increasing concentrations of *o*-phthalaldehyde. A linear plot was obtained when pseudo-first order rate constants were plotted against different concentrations of *o*-phthalaldehyde (Figure 2, inset), where the second order rate constant for the enzyme inactivation was found to be 38 $M^{-1}s^{-1}$.

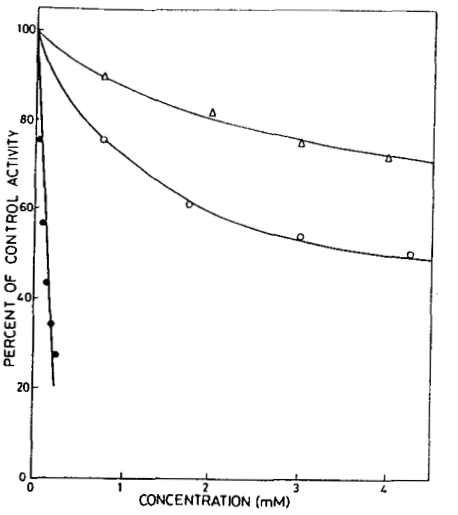


FIGURE 1 Inactivation of mitochondrial malate dehydrogenase as a function of inhibitor concentration. Malate dehydrogenase (2.88 μg/ml) was incubated with 0–4 mM concentrations of pyridoxal 5′-phosphate (△), 5,5′-dithio bis-(2-nitrobenzoic acid) (○), or *o*-phthalaldehyde (●). The reactions were carried out at 25°C in 0.1 M potassium phosphate buffers at pH 7.5. The inhibitor and the enzyme were incubated for 5 min in each case. The enzyme activity was determined as described in "Experimental Procedure".

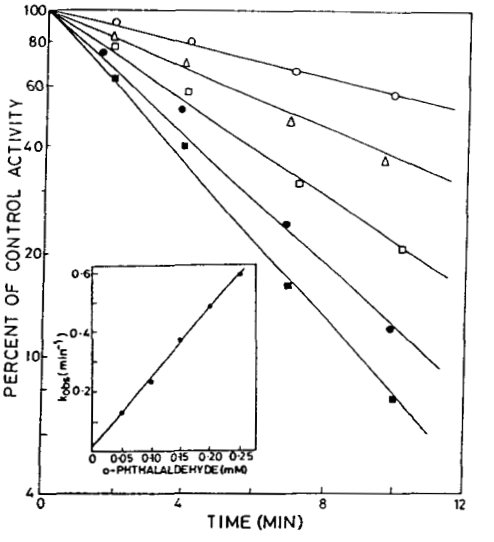


FIGURE 2 Kinetics of inactivation of mitochondrial malate dehydrogenase by *o*-phthalaldehyde. The enzyme (2.88 μg/ml) was incubated with, (○) 0.05 mM, (△) 0.1 mM, (□) 0.15 mM, (●) 0.2 mM, (■) 0.25 mM *o*-phthalaldehyde for different time periods, and then the residual enzyme activity was measured. The inset shows a plot of the first order rate constant versus *o*-phthalaldehyde concentration.

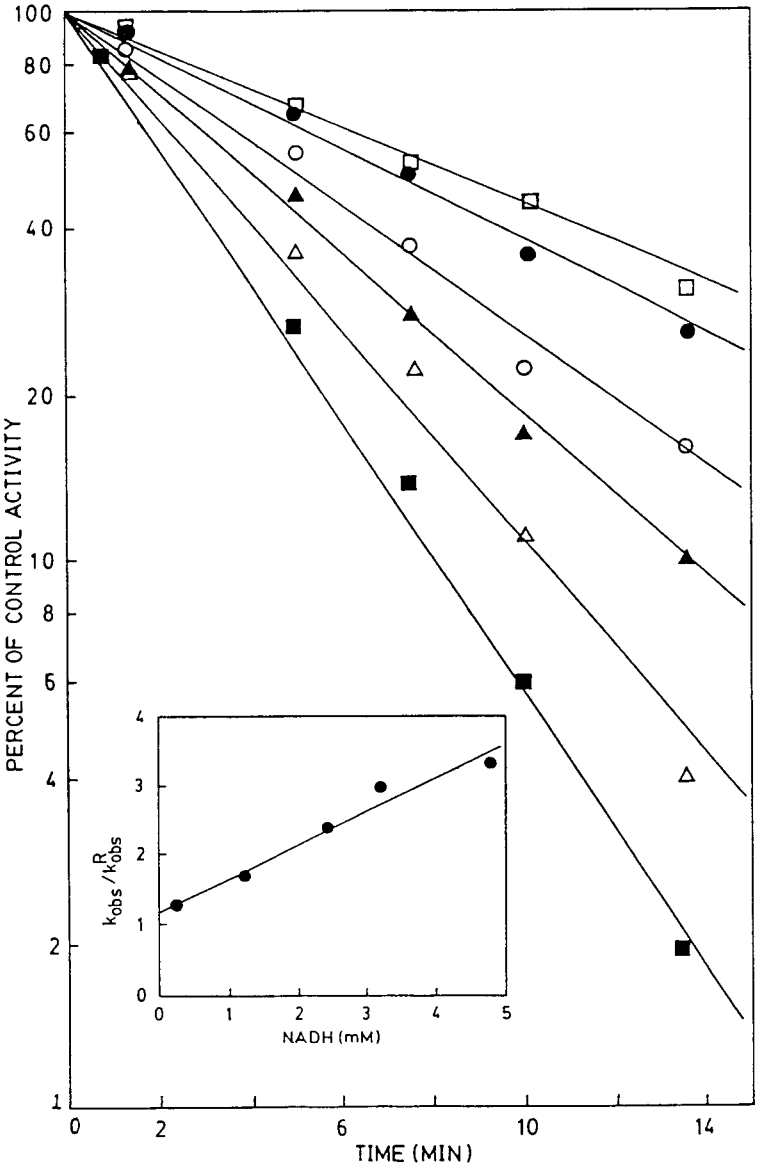


FIGURE 3 Protective effect of NADH on *o*-phthalaldehyde inactivation of malate dehydrogenase. The enzyme (2.88 μg/ml) was incubated in 0.1 M potassium phosphate buffer (pH 7.0) for 5 min with the following concentrations of NADH: (■) 0 mM, (△) 0.2 mM, (▲) 1.2 mM, (○) 2.4 mM, (●) 3.2 mM, and (□) 4.8 mM. These samples were then treated with 0.3 mM *o*-phthalaldehyde and then the residual activity was determined. The inset shows a plot of the ratio of $k_{obs}$ and $k^{R}_{obs}$ which are the pseudo-first order inactivation rate constants in the absence and presence of NADH respectively, as a function of NADH concentrations.

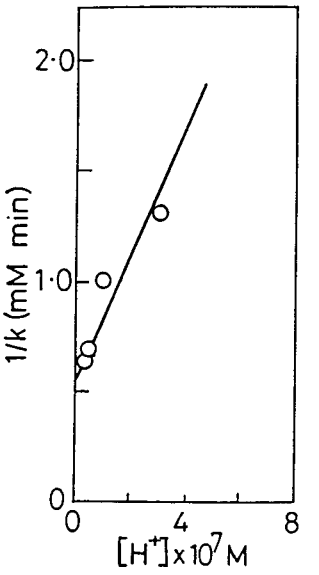


FIGURE 4 Mitochondrial malate dehydrogenase inactivation by *o*-phthalaldehyde as a function of pH. The enzyme (2.8 $\mu$g/ml) was incubated with 0.2 mM *o*-phthalaldehyde in 0.1 M potassium phosphate buffer over the pH range 6.0–7.8. The reciprocal of the second order rate constant (k) for inactivation was plotted against $[H^+]$. For second order rate constant (k) determination see "Experimental Procedure".

*Protective Effect of NADH on Inactivation of Malate Dehydrogenase by o-Phthalaldehyde*

The coenzyme protected the enzyme against *o*-phthalaldehyde inactivation. A decrease in the rate of inactivation was observed with an increase in NADH concentration. A linear relationship was obtained when $k_{obs}/k^R_{obs}$ values were plotted against concentrations of NADH as shown in Figure 3, representing complete protection against *o*-phthalaldehyde inactivation. A dissociation constant of 2 mM was obtained from the slope of the straight line. These investigations suggested that the loss of enzyme activity results from the binding of *o*-phthalaldehyde at the coenzyme (NADH) binding site.

*Effect of pH on Malate Dehydrogenase Inactivation by o-Phthalaldehyde*

The determination of the rate of modification of an enzyme by an inhibitor as a function of pH allows one to identify the pK of the reactive group in the enzyme. The inactivation pattern of the enzyme due to *o*-phthalaldehyde in the range pH 6.0–7.8 was analyzed. A linear plot was obtained when the reciprocal of the second order constant was plotted against $[H^+]$ (Figure 4). The slope showed that the group on malate dehydrogenase participating in the *o*-phthalaldehyde reaction has a $pK_a$ value of 6.8 which can be assigned to the $\varepsilon$-amino group of lysine as discussed later.

## DISCUSSION

Pig heart mitochondrial malate dehydrogenase was inactivated differentially when incubated with monofunctional (DTNB and PLP) and bifunctional (*o*-phthalaldehyde) reagents. DTNB is a highly specific sulfhydryl reagent and binds to the -SH group of cysteine, whereas PLP selectively binds to the $\varepsilon$-amino group of lysine. The extent of inhibition obtained separately by these two reagents was much lower than the inhibition by *o*-phthalaldehyde. This can be expected for the bifunctional reagent, *o*-phthalaldehyde which binds with both the -SH group of cysteine and the $\varepsilon$-$NH_2$ group of lysine, thus, resulting in a faster rate of inactivation. It has been shown earlier that the reactions of DTNB and PLP with malate dehydrogenase can be prevented by the presence of NADH.[9,10] NADH also protected malate dehydrogenase against *o*-phthalaldehyde inactivation. Thus, cysteine and lysine residues participating in DTNB and PLP binding may be involved in the reaction with *o*-phthalaldehyde. The plots of $K_{obs}/K^{R}_{obs}$ against NADH concentration gave a straight line which indicated that E-NADH does not allow additional binding of *o*-phthalaldehyde. Hence, it can be concluded that *o*-phthalaldehyde binding to the enzyme leads to the modification of cysteine and lysine residues present at the coenzyme binding region. Kinetic investigation showed that the *o*-phthalaldehyde reaction followed pseudo-first order kinetics. Replot of the values yielded a second order rate constant of 38 $M^{-1}s^{-1}$, which is favorably comparable with the previous reports on *o*-phthalaldehyde reaction.[18–19]

There is considerable evidence regarding the nature of the product formed as a result of the *o*-phthalaldehyde reaction with the enzyme. It has been earlier shown that the *o*-phthalaldehyde reaction with malate dehydrogenase leads to the formation of isoindole derivative with $\lambda_{em}$ of 405 nm.[16] The pH dependence of inactivation by *o*-phthalaldehyde shows that a group with pK 6.8 is essential in this reaction. As the reaction involves a cysteine and lysine pair, the pK should correspond to one of them. It has been reported earlier that the pK value obtained for the *o*-phthalaldehyde reaction must be assigned to the pK value of the $\varepsilon$-amino group of the lysine residue as the group in a deprotonated state is used for isoindole derivative formation.[19,20] The pK value of essential lysine is reported to be lower than those of the other lysine residues in many enzymes as a result of difference in the environment at the catalytic center.[21,22] For example, the pK value of 7.7 was obtained by Chen *et al.*[19] for the *o*-phthalaldehyde inactivation of phosphoenolpyruvate carboxykinase. This higher value than that for malate dehydrogenase is probably due to the less hydrophobic nature of the catalytic site of phosphoenolpyruvate carboxykinase. The difference in the nature of the environment where the *o*-phthalaldehyde reaction takes place can be understood by comparing the molar transition energy with those of the model isoindole derivatives. The relationship between the fluorescence emission maximum and the molar transition energy in kJ/mol was determined by Palaczewski *et al.*[12] The molar transition energy ($E_T$) is calculated from the value of the fluorescence emission maximum ($\lambda_{em}$) of the isoindole product by equation 4.

$$E_T = 2.985\lambda_{em} - 1087.28 \quad (4)$$

The $E_T$ value for the malate dehydrogenase MDH-*o*-phthalaldehyde adduct was found to be 121.65 kJ/mol, whereas, the $E_T$ for phosphoenolpyruvate carboxy-kinase reported by Chen *et al.*[19] was 187 kJ/mol, showing that the latter reaction site is comparatively more hydrophilic than the former in nature since it has been observed that as the polarity increases, the higher the value for the molar transition energy.[12]

The results presented here conclusively demonstrate the involvement of cysteine and lysine residues in the catalytic activity of malate dehydrogenase. They also provide evidence regarding the nature of the environment at the catalytic site which reflects the physiological status of the functional groups involved in the catalysis.

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