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Study of the Interaction of Cadmium with Membrane-Bound Succinate Dehydrogenase

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Abstract

Cadmium ions inhibit membrane-bound succinate dehydrogenase with a second-order rate constant of 10.42 $mM^{-1} s^{-1}$ at pH 7.35 and 25°C. Succinate and malonate protect the enzyme against cadmium ion inhibition. The protection pattern exerted by succinate and malonate suggests that the group modified by cadmium is located at the active site. The pH curve of inactivation by Cd^{2+} indicates the involvement of an amino acid residue with p*K*a of 7.23.

Key Words: Mitochondria; phosphorylating electron transport particles; succinate dehydrogenase; heart succinate dehydrogenase; respiratory chain; cadmium; substrate-binding site.

Introduction

It has been known for a long time that succinate dehydrogenase (SDH) (EC 1.3.99.1) is very sensitive to reagents that modify thiol groups (Hopkins and Morgan, 1938; Hopkins *et al.*, 1938). In this regard, evidence has been provided on the presence of a highly reactive sulfhydryl group at the active site of the enzyme (Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984; Phillips *et al.*, 1987; Jay, 1991), which Vinogradov *et al.* (1972) suggested was involved in the tight binding of the inhibitor oxaloacetate. The presence of the sulfhydryl at the active site, however, has been questioned by some authors, based on the finding that the inhibition of the

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enzyme by thiol-binding reagents follows complex kinetics (Sanborn *et al.*, 1971; Felberg and Hollocher, 1972; Lê-Quôc *et al.*, 1981).

During the course of experiments involving the effect of Cd^{2+} on mitochondrial calcium transport, it was found that succinate dehydrogenase was particularly sensitive to the action of this cation (Chávez *et al.*, 1985). As cadmium is reported to be a thiol reagent (Valle and Ulmer, 1972; Ochiai, 1977), such findings were consistent with the important role that –SH groups play in the enzyme.

In order to gain further insight into the effect of Cd^{2+} on succinate dehydrogenase, we studied the kinetics of inhibition of membrane-bound enzyme by cadmium. The results indicate that the binding of the metal at the catalytically active site induces loss of the activity. Inactivation kinetics was used to obtain the pk_a value for the group modified by cadmium. The possible involvement of an –SH group in the binding of Cd^{2+} to the active site is discussed.

Experimental

Beef heart mitochondria were isolated as reported (Jurkovitz *et al.*, 1974). Submitochondrial particles (ETP_H) were prepared as indicated by Lee and Ernster (1966). Since this membrane preparation contained a significant amount of tightly bound oxaloacetate, which inactivates SDH (Wojtczak *et al.*, 1969; Singer *et al.*, 1973), the ETP_H were activated with malonate as previously described (Jay, 1991). Then, in order to achieve complete removal of malonate, the ETP_H were washed twice in 200 mM sucrose–25 mM Hepes (pH 7.3), and 500 mM NaBr. Finally, the ETP_H were washed with the same buffer without NaBr, since the anion (Br^-) itself inhibits at high concentrations (Ackrell *et al.*, 1978). The level of activity reached by the malonate-activated ETP_H was not modified by a subsequent incubation with succinate, indicating that the enzyme was fully activated without any bound oxaloacetate (Kearney, 1957; Ackrell *et al.*, 1974). Also, the catalytic activity measured at 3°C did not show a lag period, indicating that the preparation did not contain malonate (Coles and Singer, 1977).

The inactivation experiments were carried out at 25°C in a standard medium containing 200 mM sucrose, 25 mM Tris (pH 7.35), 1 mM cyanide, 8.5 μM rotenone, and succinate or malonate, at the concentrations indicated in the figures. The reaction was started by the addition of the ETP_H (42 μg protein/ml) followed by the addition of $CdCl_2$, the concentrations of which are indicated in the legends of the figures. Aliquots from these media were withdrawn at the indicated times and the dehydrogenase activity was measured by adding 5.0 mM succinate (or the amount needed to complete

this concentration in the case of the media already having succinate), 0.064 mM dichloroindophenol (DCIP), and 1.4 mM phenazine methosulfate (PMS) (final volume 3 ml) (Mowery *et al.*, 1977). For the determination of the pH dependence of the rate constant of inhibition, the medium was prepared with a mixture of 10 mM MOPS plus 10 mM Tris at all pH values, in order to avoid the effect of different salts at different pH.

All the kinetic constants were determined by fitting the experimental data, by the least-squares method, to the respective equations. Protein concentration was determined by the method of Lowry *et al.* (1951).

Results

Incubation of the ETP_H with cadmium in a sucrose medium at pH 7.35 resulted in a time-dependent loss of succinate dehydrogenase activity. Despite the fact that the amount of Cd^{2+} needed to inactivate the enzyme is low, semilog plots of residual enzyme activity versus time yielded straight lines. Thus, it was not necessary to fit the data to a second-order scheme characteristic of a bimolecular process (Tian *et al.*, 1985). Figure 1a shows the data obtained from inactivation experiments at different concentrations

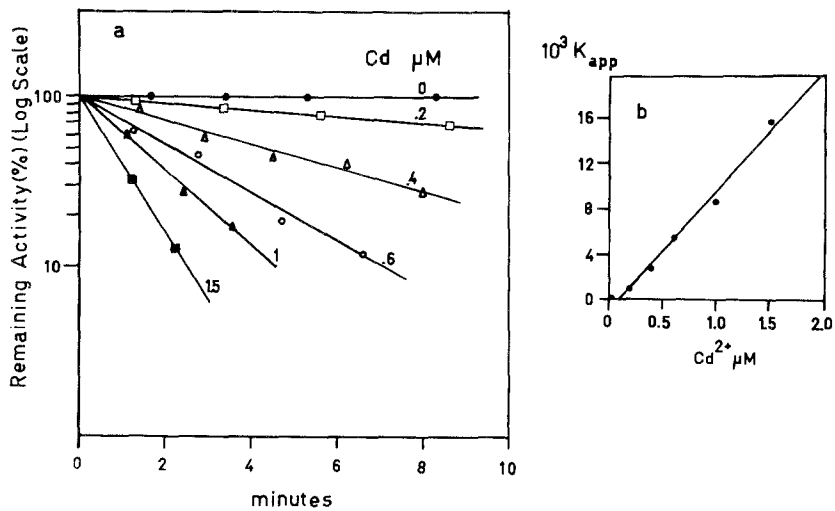

Fig. 1. Inactivation of SDH by cadmium. (a) Semilog plot of percent of remaining activity versus incubation time. Protein (42 μg/ml) from ETP_H was incubated with different concentrations of $CdCl_2$ in a sucrose medium, pH 7.35, at 25°C. At time intervals, aliquots were removed for measurement of the residual enzyme activity (see Experimental). The numbers on the slopes indicate cadmium concentration (μM). (b) Apparent rate constant for inactivation (K_{app}) versus cadmium concentration. The k_{app} was evaluated by multiplying the slopes in (a) by 2.303.

of cadmium. The slopes of these plots were linearly related to the concentration of the reagent (Fig. 1b), suggesting that there is no reversible complex formed prior to the inactivation process (Kitz and Wilson, 1962; Jabalquinto *et al.*, 1983; Mas and Colman, 1983; Dominici *et al.*, 1985). Therefore, the data can be analyzed using a pseudo-first-order scheme for an irreversible inhibitor, where the inactivation velocity is given by

$$-\frac{d[E_\alpha]}{dt} = K_{app}[E_\alpha] \tag{1}$$

where $[E_\alpha]$ is the concentration of active enzyme at time t and K_{app} (apparent pseudo-first-order rate constant) $= K_I[Cd^{2+}]$, where K_I is the second-order rate constant, which can be obtained from the slope of the plot in Fig. 1b ($k_I = 10.42\,mM^{-1}s^{-1}$).

The inactivation of membrane-bound SDH by Cd^{2+} does not directly imply that a specific residue is being modified nor that such residue is present at the active site. However, protection of an enzyme by substrate or by a competitive inhibitor would suggest that the amino acid residues that are

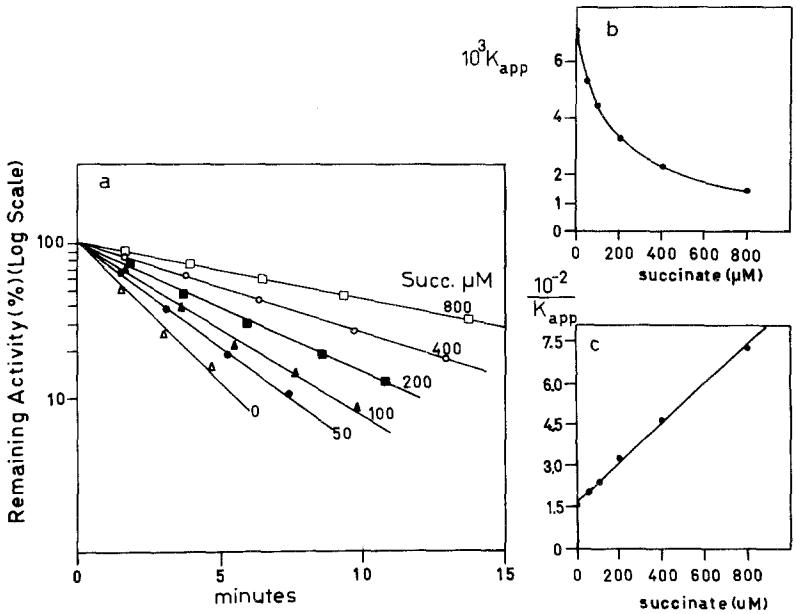

Fig. 2. Effect of succinate on the inactivation of SDH by cadmium. (a) Semilog plot of percent of remaining activity versus incubation time. The incubation conditions were similar to those described in Fig. 1 in the presence of 1 μM Cd^{2+} except for the presence of succinate, the concentrations of which are indicated on the slopes. In the absence of cadmium, the enzyme was perfectly stable in the presence or absence of succinate. (b) Apparent rate constant for inactivation (K_{app}) versus succinate concentration. (c) Plot of $1/K_{app}$ versus succinate concentration.

protected lie at the active site. The observed pseudo-first-order rate constant for the enzyme inactivation in the presence of a ligand of the active site (K'_{app}) is given by (Mildvan and Leigh, 1964)

$$(K'_{app}) = \frac{K_I[I] \times K_D}{K_D + A} \tag{2}$$

and in reciprocal form,

$$\frac{1}{K'_{app}} = \frac{1}{[I]K_I} + \frac{A}{[I]K_I K_D} \tag{3}$$

where A represents either a substrate or a competitive inhibitor of the enzyme, K_D is its dissociation constant, and the irreversible inhibitor (I) will only bind to the free enzyme. Equations (2) and (3) predict a rectangular hyperbola and a straight line when $1/K'_{app}$ is plotted against the concentration of A, thus providing a way to know if I (Cd^{2+}) reacts with the EA complex. When Eq. (3) applies, K_D can be obtained from the extrapolated abscissa intercept of the plot. Figures 2 and 3 show the experiments, with succinate

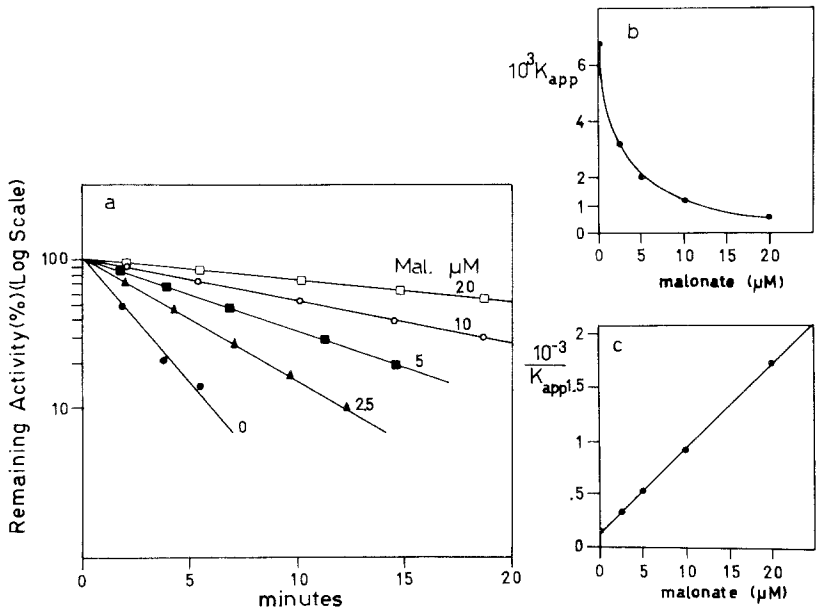

Fig. 3. Effect of malonate on the inactivation of SDH by cadmium. (a) Semilog plot of percent of remaining activity versus incubation time. The incubation conditions were similar to those described in Fig. 1 in the presence of 1 μM Cd^{2+} except for the presence of malonate, the concentrations of which are indicated on the slopes. In the absence of cadmium, the enzyme was perfectly stable in the presence or absence of malonate. (b) Apparent rate constant for inactivation (K_{app}) versus malonate concentration. (c) Plot of $1/K_{app}$ versus malonate concentration.

and malonate respectively, carried out under these considerations. Figure 2a shows that increasing concentrations of succinate progressively diminish (initial value $6.9 \times 10^{-3} \times s^{-1}$) the rate of enzyme inactivation by cadmium. The plot of K'_{app} versus [succinate] was hyperbolicaly descending (Fig. 2b) as predicted by Eq. (2). The plot of $1/K'_{app}$ versus [succinate] was linear (Fig. 2c), in accord with Eq. (3). From this plot a K_D value of 267 μM for succinate was obtained. Similar results, with malonate, are shown in Fig. 3. In this case a k_D value of 1.7 μM was found. Similar values of K_D for succinate (300 μM) and malonate (1.3 μM) have been previously reported (Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984). Thus, succinate and malonate completely protect membrane-bound SDH against inactivation by cadmium.

Information on the residues modified by cadmium was derived from the study of the rate of inactivation as a function of pH. If a cysteine residue is

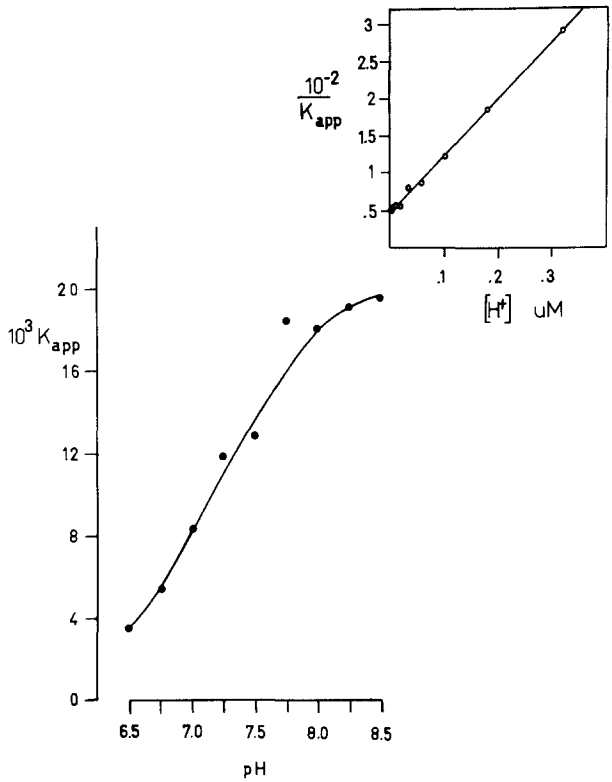

Fig. 4. Inactivation of SDH by cadmium as a function of pH. Apparent rate constant for inactivation (K_{app}) versus pH (1 μM Cd^{2+}). In the absence of cadmium, the enzyme was stable during the assay at all the pH tested. Inset: Plot of $1/K_{app}$ versus $[H^+]$. The incubation conditions are detailed in Experimental.

required for the binding of cadmium, the rate of inactivation of the enzyme will depend on the degree of protonation of the –SH group, provided the unprotonated cysteine is modified at a substantially faster rate than the protonated cysteine. The pseudo-first-order rate constants for inactivation were determined at different pH (Fig. 4) and plotted against $[H^+]$ (Fig. 4, inset) according to the following equation, which is analogous to Eq. (3):

$$\frac{1}{K_{app}} = \frac{[H^+]}{K_{max} K_a} + \frac{1}{K_{max}} \tag{4}$$

where K_{max} is the maximal rate constant and K_a is the ionization constant of the group involved. A plot of $1/K_{app}$ versus $[H^+]$ should thus give a straight line and allows the determination of the apparent K_a. From the data of Fig. 4b, an apparent pK_a value of the inactivation was found to be 7.23. The fact that there does not occur any appreciable deviation from linearity at any pH suggests that just the unprotonated form of the group involved is modified and that just such a group reacts with cadmium to inactivate the enzyme (Cardemil, 1987).

Discussion

The evidence presented here indicates that cadmium inhibited succinate dehydrogenase activity with a pseudo-first-order kinetics (Fig. 1). In addition, the experiments, with fully activated membrane-bound preparations, showed that malonate and succinate provide complete and hyperbolic protection against inhibition by cadmium (Figs. 2 and 3). These results suggest that the inhibitory action of Cd^{2+} was the consequence of the modification of a group located in the region of the catalytically active site of the enzyme. This conclusion is in accord with the proposal that thiol-binding reagents inhibit SDH by a direct interaction with an active-site –SH group (Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984; Jay, 1991). It has been contended (Kenney, 1975; Kotlyar and Vinogradov, 1984) that the finding (Lê-Quôc *et al.*, 1981; Felberg and Hollocher, 1972; Sanborn *et al.*, 1971) of complex kinetics of inhibition by alkylating and mercaptide-forming thiol reagents could be due in part to the presence of a significant amount of oxaloacetate in the incompletely activated preparations utilized.

Another question that arose from the use of Cd^{2+} was the actual number of groups that bound cadmium to the active site. It is known that cadmium forms stable compounds with dithiols (Rasheed *et al.*, 1984), but it has also been reported that it can form one-ligand complexes analogous to the mercury(I) derivatives (Okada *et al.*, 1964; Mundy, 1965). The linear plot of Fig. 1b suggests a stoichiometry of 1 Cd^{2+}/enzyme. However, if two or more

groups simultaneously react with one molecule of inhibitor, a similar stoichiometry would be obtained. At present, therefore, we lack conclusive evidence regarding the actual number of groups that participate in the binding of cadmium during the inactivation process.

Although it is known that cadmium can react with several amino acids in proteins (Perkins, 1961), the stability constants show that the introduction of sulfur into the ligands leads to much stronger complexes, especially when the -SH group is deprotonated (Valle and Ulmer, 1972; Ochiai, 1977; Percovaro *et al.*, 1984; Sillen, 1964; Smith and Martell, 1974, 1975, 1976). From the data of Fig. 4, a pK_a value of 7.23 was obtained for the residue modified by cadmium. The pK_a value for this residue is lower than that for free cysteine. However, it is noteworthy that a similar low pK_a (7.0) value for the active-site sulfhydryl of SDH has been reported (Vinogradov *et al.*, 1976). The increment in the dissociation constant for this group has been taken as evidence for the involvement of the sulfhydryl in the mechanism of succinate oxidation (Vinogradov *et al.*, 1976; Vinogradov, 1986). More recently, however, Hederstadt and Heden (1989) have demonstrated that the active-site cysteine is not essential for either substrate binding or catalysis. The *B. subtilis* succinate dehydrogenase binds succinate and oxalacetate normally; yet it has alanine replacing cysteine. Thus, the cysteine near the active site can influence catalytic activity only indirectly, probably by steric hindrance when a larger group or a charged group, such as cadmium, are attached. On the other hand, the increment in the dissociation constant for this group provides a rational explanation for the great affinity of the enzyme for cadmium.

Further investigation to determine the factors responsible for the anomalous pK_a value of the active-site thiol and the reasons for this phenomenon will produce a better understanding of the structural organization of SDH.

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