4,4'-BIS DIMETHYLAMINODIPHENYLCARBINOL: A NEW REAGENT FOR SELECTIVE CHEMICAL MODIFICATION. INTERACTION WITH PORCINE MALATE DEHYDROGENASE

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SUMMARY: 4,4-bis Dimethylaminodiphenylcarbinol (BDC-OH) has recently been reported to be a highly sensitive reagent for the quantitative determination of sulfhydryl residues in biological materials (1). In this communication the effectiveness of BDC-OH as a reagent for selective chemical modification of "active center" cysteine residues was investigated. The supernatant and mitochondrial forms of malate dehydrogenase were chosen for investigation by this reagent. Supernatant malate dehydrogenase which has never been found to contain an "active center" cysteine is unaffected by this reagent. Mitochondrial malate dehydrogenase (L malate: NAD^+ oxidoreductase, EC 1.1.1.37) from porcine heart can be irreversibly inactivated by a 20 fold M excess of the reagent. Chemical modification of two essential sulfhydryl residues is prevented by the presence of the coenzyme, NAD^+, suggesting that the site of interaction is located at or near the coenzyme binding site and hence at or near the enzymatic center of this enzyme.

INTRODUCTION: We have recently reported the utilization of 4,4'-bis-dimethylaminodiphenylcarbinol (BDC-OH) as a sensitive and specific reagent for the quantitative determination of sulfhydryl residues in biological materials (1,2). When BDC-OH is dissolved in aqueous buffers of pH 4 to pH 6, an equilibrium is established between BDC-OH and the resonance stabilized "carbonium-immonium" ion, BDC^+, shown in Figure 1. The species which interacts with the sulfhydryl residue is the BDC^+ form of the reagent. The supernatant and mitochondrial form of porcine heart malate dehydrogenase contain 10 and 14 sulfhydryl residues respectively. Sulfhydryl residues (one per active center) have been previously shown to be essential in porcine heart mitochondrial malate dehydrogenase (m-MDH) through the use of the modifying reagent N-ethylmaleimide (3). The

sulfhydryl residues of the supernatant form of this enzyme (s-MDH) however are resistant to selective chemical modification (4,5). In order to elucidate the usefulness of BDC-OH as a site specific modifying reagent we have investigated its effectiveness as an inactivator of these enzymes.

MATERIALS AND METHODS: Porcine m-MDH was purified from acetone powders of fresh pig hearts (3) and exhibited a specific activity of 450 units/mg. Supernatant malate dehydrogenase was purified according to the method of Gerding and Wolfe (6). Enzymatic assays were performed on a Unicam S. P. 800 recording spectrophotometer equipped with a cell holder thermostatted at 25°. The standard assay consisted of 90 mM sodium pyrophosphate buffer (pH 10.6), 1.5 mM NAD^+, and 33 mM sodium L-malate; the enzyme was added at zero time, and the absorbance increase at 340 nm was measured. One unit of enzyme is defined as the quantity which catalyzed the reduction of 1 μmole of NAD^+ per min. Protein concentrations were determined spectrophotometrically at 280 nm, utilizing an extinction coefficient of $E^{1\%}_{1cm}$ of 2.5 for the mitochondrial enzyme, and 1 mg/ml = 1 absorbance unit for the supernatant enzyme. Following chemical modification, protein concentrations were determined by the method of Lowry et. al. (7) using the appropriate enzyme as standards.

4,4'-bis-Dimethylaminobenzophenone purchased from Baker Chemical, was recrystallized from absolute methanol. [^{3}H] labeled BDC-OH was prepared by a modification of the procedure previously described (2). A sample of 250mCi of [^{3}H] $NaBH_4$, purchased from International Chemical and Nuclear Corp., was dissolved in 100 ml of absolute ethanol along with 1.5 gm (39.4 m moles) of unlabeled $NaBH_4$. 2.0 gm (7.46 m moles) of 4,4'-bis-dimethylaminobenzophenone was added and the solution stirred at a reflux for 90 min. After cooling to room temperature, 25 ml of 50% of NaOH was added and the mixture again refluxed for 60 min. The reaction mixture was filtered (paper) into 200 ml of ice water, producing a yellow precipitate which was filtered under nitrogen, washed with cold water, and dried in vacuo at room temperature. The pale yellow solid was recrystallized from cyclohexane, affording a white solid

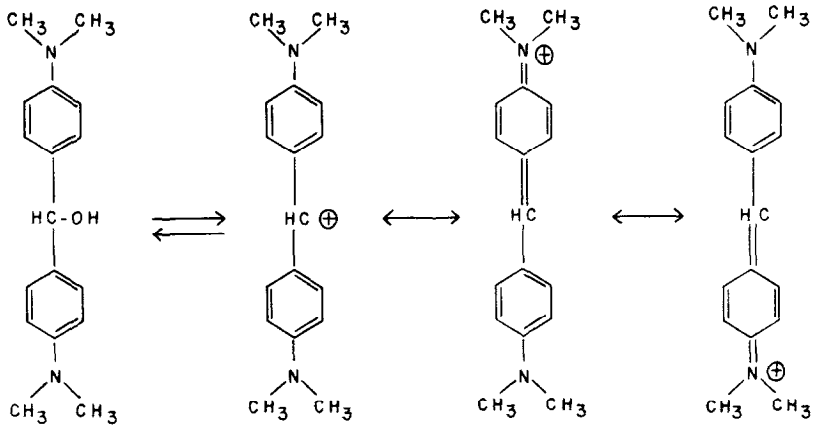

Figure 1

Equilibrium between BDC-OH and BDC^+ and resonance forms of BDC^+ (carbonium-immonium ion) in sodium acetate buffer pH 5.1.

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with melting point 103-104° (2), specific radioactivity 0.63 m Ci/m mole.

Incorporation of [^{3}H] labeled BDC-OH into malate dehydrogenase was assayed by standard isotopic dilution methods by use of a Nuclear-Chicago Mark II liquid scintillation counter. A fractional aliquot (500µl) of each sample was placed in 15 ml of Aquasol scintillation cocktail (New England Nuclear) and monitored for radioactivity. A blank sample of an equal volume of the final dialysis buffer and an external [^{3}H] source were counted with each group of samples. Counting times were 10 min. with five repetitions, and an average of the assays, corrected for quench, was used to calculate moles of BDC^+ bound per mole of enzyme.

RESULTS AND DISCUSSION: Samples of m-MDH 2.9×10^{-5} to 6.0×10^{-5} M were incubated with a 23 fold M excess of BDC-OH at 25° in 0.1 M sodium acetate, pH 5.2. A control sample with an equivalent amount of buffer but containing no sulfhydryl reagent was also incubated under the same conditions. The effect of BDC-OH on the enzymatic activity of the mitochondrial enzymes is shown in

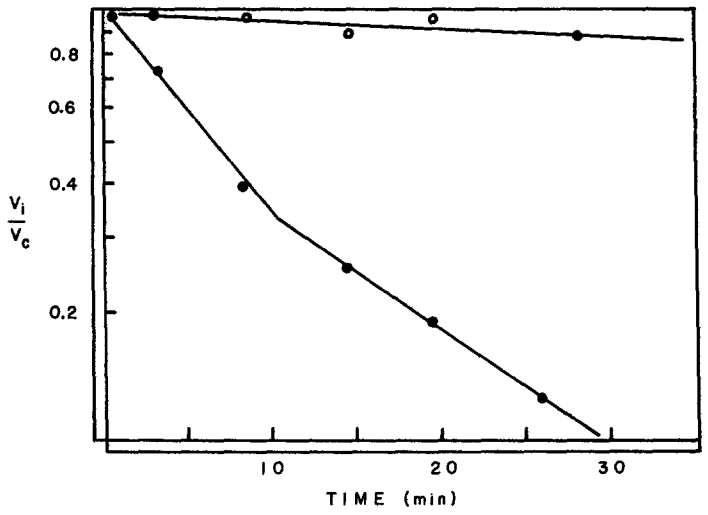

Figure 2

The effect of the coenzyme, NAD^+, on the inhibition of m-MDH by BDC^+. Enzyme (2.0 mg) was incubated with a 23 fold molar excess of BDC-OH at 25° in 0.1 M sodium acetate buffer (pH 5.2). (●), no NAD^+ added. (○), 0.37 mM NAD^+ added. Aliquots were removed at times indicated and assayed as discussed in "Materials and Methods".

Figure 2. The inactivation is represented as the log of the ratio $v_i:v_c$ (velocity of inactivated sample of to velocity of a control sample) as a function time.

The addition of the coenzyme, 0.37 mM NAD^+, to the incubation mixture completely prevented the inactivation by BDC-OH (Figure 2). It is of interest to note that the reduced coenzyme, NADH, cannot be utilized for similar protection studies because its addition to the incubation mixture causes the reduction of the BDC^+ "carbonium-immonium" ion to yield the highly insoluble 4,4'-bis dimethylaminodiphenyl methyl derivative of the reagent.

In order to determine the effect of BDC-OH upon the supernatant form of MDH, samples of s-MDH 2.7×10^{-5} M were incubated with 10-75 fold M excesses of BDC-OH at 25° in 0.1 M sodium acetate, pH 5.2, for periods up to 2 hours. In contrast to the inactivation observed with the mitochondrial form of MDH, no inactivation of s-MDH was observed under these conditions.

To determine quantitatively the interaction of BDC^+ with m-MDH, the incorporation of $[^3H]$ BDC^+ was determined by means of standard isotopic

dilution techniques following inactivation of the enzyme at pH 5.2 and 4°C. A sample of 16 mg m-MDH was incubated with a 75 fold M excess of [^{3}H] BDC-OH.

Aliquots of the reaction mixture were removed at intervals during the inactivation and the reaction terminated by the addition of 0.57 m moles of 2-mercaptoethanol. The samples removed during the time course of inactivation were applied to a 1.5 x 35 cm column of Sephadex G-25 to remove excess reagent. Fractions collected from these chromatography columns containing protein were exhaustively dialyzed against 50 mM sodium phosphate buffer (pH 7.0). A control sample was treated in an identical fashion minus the addition of BDC^+. Following dialysis each sample was assayed for enzymatic activity and analyzed for [^{3}H] and free sulfhydryl content according to the method of Ellman (8).

Figure 3 represents the loss of enzymatic activity as a function of the moles of BDC^+ incorporated per mole of m-MDH. As indicated in this figure extrapolation of the line generated by all of the experimental points to a position indicating total inactivation of m-MDH indicates that approximately four moles of BDC^+ are incorporated during the inactivation.

While the total number of residues modified at full inactivation is slightly higher than the expected two residues, it is apparent that extrapolation of the initial points of this figure (dotted line) does indeed indicate the essentiallity of not more than two residues, an observation consistent with previous studies (3). The additional residues which were modified in the absence of the coenzyme, NAD^+, were not modified in its presence thus suggesting that they are also located in the general area of coenzyme binding however are not essential for enzymatic activity. NAD^+ thus renders specific residues (approximately one/center) at or near the coenzyme binding center, and thus, at or near the enzymatic active center, inaccessible for chemical modification by this reagent. Similar incorporation studies performed on a sample of s-MDH indicated that less than 0.2 moles of BDC were incorporated into that fully active enzyme even when the enzyme was incubated for 2 hours with a 100 fold M excess of reagent. Sulfhydryl analysis of the mitochondrial and supernatant enzyme indicated the

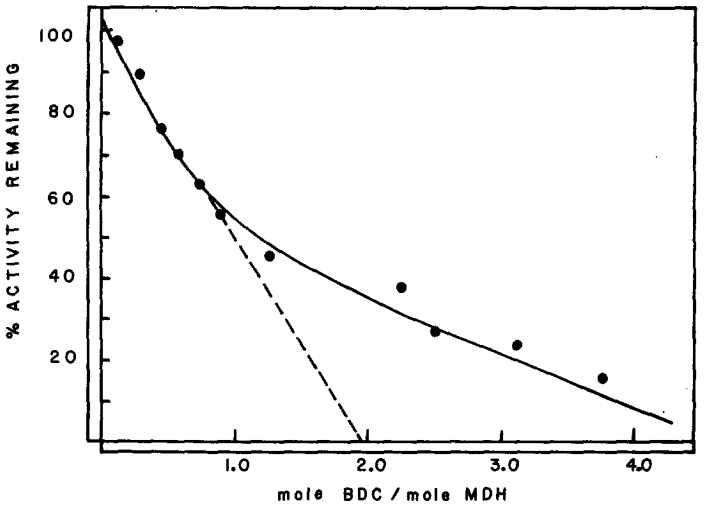

Figure 3

The incorporation of BDC^+ into m-MDH as a function of % activity remaining. A sample of m-MDH (16 mg) was incubated with a 75 fold molar excess of [^{3}H] BDC-OH. Aliquots were removed from the reaction mixture and excess reagent was removed as discussed in "Results". Incorporation of BDC was determined by standard isotopic dilution techniques and enzymatic activity as previously described.

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loss of sulfhydryl residues concomitant with the incorporation of tritiated BDC.

Thus it is apparent that BDC^+ can be effectively used as a reagent not only for the quantitative determination of sulfhydryl residues in biological materials, but also has the potential for usefulness as a means of chemically modifying active center cysteine residues. Under the conditions employed in this work the sulfhydryl residues of s-MDH were unaffected by the reagent as were the nonessential residues m-MDH. In this particular instance only a 20 fold molar excess of reagent was required to totally inactivate m-MDH in the period of 30 minutes. As a contrast total modification of the active center cysteine residues with N-ethylmaleimide required a 500 fold molar excess and a period of 50 minutes (3). Furthermore the reagent has been shown previously to be specific for sulfhydryl residues under these conditions (2), and is easily synthesized with an isotopic label [^{3}H] to facilitate quantitative chemical modification studies.

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