**SUMMARY**

This laboratory has previously reported the use of 4,4'-bis-dimethylaminodiphenylcarbinol as a highly sensitive reagent for the quantitative determination of sulfhydryl residues in proteins. The reagent dissociates in buffers below pH 6.5 to form a resonance-stabilized "carbonium-immonium" ion which exhibits an absorption maximum centered around 606 nm. Sulfhydryl residues react with this cation from the reagent to form stable S-(4,4'-bis-dimethylaminodiphenylmethyl-) derivatives with a concomitant quantitative loss in absorbance. Initiation of mitochondrial malate dehydrogenase (1-malate:NAD+ oxidoreductase, EC 1.1.1.37) from porcine heart with a 20-fold molar excess of the reagent over enzyme leads to a time-dependent loss of enzymatic activity which is complete within approximately 30 min. Inactivation is coincident with the incorporation of approximately 1 mole of reagent per enzymatic active center of the enzyme. The presence of the coenzyme, NAD+, fully protects the enzyme from inactivation and the modification of cysteine residues, suggesting interaction at or near the coenzyme binding site. The modified mitochondrial malate dehydrogenase was digested with trypsin and a peptide containing the active center cysteine was isolated. Due to a unique aggregation phenomenon exhibited by the peptide modified with this reagent, isolation was achieved in a single step of chromatography on Sephadex G-100. These studies are suggestive that this reagent may be widely applicable for the selective chemical modification of active center cysteines in proteins and that peptides containing this reagent can be isolated in an extremely simple manner.

When 4,4'-bis-dimethylaminodiphenylcarbinol (BDC-OH) is dissolved in aqueous buffers in the pH range 3 to 6, an equilibrium is established in which at least three species are present. The actual concentration of each species present is determined by the hydrogen ion concentration of the solution. This equilibrium is illustrated in Fig. 1. The three species present are BDC-OH+, 4,4'-bis-dimethylaminodiphenylmethyl cation (BDC+), and a dication (BDCH22+) which we postulate has the structure indicated in which one amino group is protonated. The use of BDC-OH as an analytical reagent for the determination of sulfhydryl residues (1) is dependent upon the unique spectral properties of the BDC+ cation. BDCH22+ exhibits a visible absorption with a λmax of 606 nm while BDC-OH and BDCH2+ are transparent in this region. The reaction of BDC+ with sulfhydryl residues leads to a complex devoid of visible color.

Preliminary investigations reported recently (2) suggested the potential usefulness of BDC-OH as a highly specific chemical modifying reagent. The reagent was shown to be easily synthesized with an isotopic label to facilitate quantitative modification studies. In order to establish the suitability of this reagent for such use, it is necessary to define more clearly certain of its properties under conditions to be used in chemical modification work.

It is the purpose of this communication to report on the feasibility of the use of BDC-OH as a site-specific modifying reagent. This laboratory (3) has previously reported the specific chemical modification of an active center sulfhydryl residue of porcine heart mitochondrial malate dehydrogenase through the use of the alkylating reagent N-ethylmaleimide. In this present investigation an active center sulfhydryl residue of each of the 2 identical subunits has been modified with BDC-OH and a single peptide containing the modified residue isolated and characterized.

**EXPERIMENTAL PROCEDURE**

Materials—4,4'-Bis-dimethylamino benzophenone purchased from J. T. Baker Chemical Co., was recrystallized from absolute methanol. Diphenylcarbamoyl trypsin, NAD+, and l-malic acid were purchased from Sigma. Aquasol, a universal liquid scintillation mixture, was purchased from New England Nuclear. NaBH₄ was purchased from International Chemical and Nuclear Corporation. AG-60X8 and Bio-Gel P-2 were purchased from Bio-Rad and Sephadex G-25, G-50, and G-100 were obtained from Pharmacia. Porcine heart mitochondrial malate dehydrogenase was isolated and purified as previously described (3). ³H-Labeled BDC-OH³ (630 μCi per mmole) was prepared by the procedure previously described (2).

Enzymatic Assays—Enzymatic assays were performed on a Unicam SP 1800 recording spectrophotometer equipped with a cell holder thermostated at 25°C. The standard assays were conducted for porcine malate dehydrogenase as previously described (3). Protein concentrations were determined spectrophotometrically at 280 nm utilizing the extinction coefficient, E₅₄₀, of 2.5.

³BDC-OH can be purchased from Pierce Chemical Co., Rockford, Illinois.
Chemical Modification—Porcine heart mitochondrial malate dehydrogenase was dialyzed against 100 mM sodium acetate buffer, pH 5.20, for approximately 2 hours. The enzyme (3.0 mg per ml) was incubated with 0.02 mM of [3H]BDC-OH at 4°C and at a pH of 5.2. The course of inactivation was followed by assaying for enzymatic activity as a function of time with the use of the previously described assay system. A control sample of enzyme, incubated under identical conditions, but in the absence of BDC-OH, was measured for enzymatic activity at each time the incubation sample was assayed.

^3H Incorporation—Incorporation of [3H]BDC^+ into the protein investigated was determined by standard isotopic dilution methods by use of a Nuclear-Chicago Mark 11 liquid scintillation counter. An aliquot (500 µl) of each sample was placed into 13 ml of Aquasol scintillation fluid and monitored for radioactivity. A blank sample of 500 µl of the final dialysis buffer and an external source ^3H were counted with each group of samples. Counting times were 10 min with three repetitions, and an average of the counts, corrected for quench, was used to calculate moles of BDC^+ bound per mole of enzyme. The exact protein concentrations in each inactivated sample was determined by the method of Lowry et al. (4).

Amino Acid Analysis—The amino acid composition of the BDC^+-labeled peptide isolated from malate dehydrogenase was determined by the method of Spackman et al. (5). All determinations were performed on a Glenco model 10@AS amino acid analyzer. Determination of cysteine was performed by the method of Moore (6), in which cysteine is estimated as cysteic acid after performic acid oxidation.

Tryptic Digestion of BDC^-labeled Protein—Samples of inactivated ^3H-labeled malate dehydrogenase were digested with trypsin essentially as described by Liu et al. (7). Protein samples were exhaustively dialyzed against 25 mM ammonium bicarbonate (pH 8.0) and subsequently lyophilized. The dried material was dissolved in a volume of 100 mM Tris-Cl buffer (pH 8.0) containing 0.2% (v/v) thioglycol and 8 M urea to yield a protein concentration of 4% (w/v). The solution was incubated at 25°C for 30 min, followed by dilution with 100 mM Tris-Cl buffer (pH 8.0) containing 0.2% (v/v) thioglycol to yield a final protein concentration of 1% (w/v). Trypsin was added in a ratio by weight of 1:30 trypsin to protein. The digest was incubated at 25°C for 4 hours followed by a second addition of trypsin to yield a ratio of 1:25. The digestion was allowed to proceed for 16 hours. The digestion mixture was desalted and small peptides were removed by chromatography on a column (2.5 x 90 cm) packed with Sephadex G-25 and equilibrated with 80 mM ammonium bicarbonate (pH 8.0) containing 0.2% (v/v) thioglycol. Fractions were monitored for absorbance at 230 nm and radioactivity. Fractions exhibiting radioactivity were pooled and lyophilized. Peptide mapping was performed according to the method described by Katz et al. (8).

Column Chromatography—Chromatography of peptides derived from the tryptic digestion of malate dehydrogenase inactivated with [3H]BDC-OH was performed on columns packed with either Sephadex G-25, G-50, or G-100 or Bio-Gel P-2 as noted under "Results." In each case the column was equilibrated with 50 mM ammonium bicarbonate (pH 8.0) containing 0.2% (v/v) thioglycol. The column eluants were monitored for radioactivity and for peptide material by either ninhydrin or absorbance at 230 nm.

Removal of [3H]BDC-Label from Peptide—The [3H]BDC-labeled peptide was dissolved in 1 ml of 100 mM phosphoric acid containing 0.2% (v/v) thioglycol. The solution was adjusted to pH 3.0 with sodium hydroxide. The mixture was incubated for 30 min to facilitate removal of the [3H]BDC^+ from the peptide and the mixture was applied to a column (0.9 x 10 cm) packed with Dowex 50-X8 equilibrated in the above-mentioned buffer. The column was washed with 30 ml of this equilibrating buffer followed by 10-ml applications each of 500 mM phosphoric acid, adjusted to pH 4.0 and 5.0, respectively.

RESULTS

The effect of BDC-OH on the enzymatic activity of porcine heart mitochondrial malate dehydrogenase has been reported previously (2). The inactivation is accompanied by the incorporation of approximately 1 molecule of BDC-OH per polypeptide chain. The addition of 37 mM cysteine, NAD^+, was observed to prevent completely the inactivation of the enzyme, suggesting interaction at or near the coenzyme binding site.

A sample (65 mg) of a tryptic digest of malate dehydrogenase inactivated with [3H]BDC-OH was chromatographed on a column (2.5 x 90 cm) packed with Sephadex G-25. A single radioactive peak (Fig. 2a) was observed in a region corresponding to the void volume of the column. The total recovery of radioactivity applied to the column was 90%. The fractions containing the major radioactive material (Fractions 23 to 28) were combined and lyophilized. These fractions accounted for 73% of the radioactivity applied to the column.

A sample of 4 ml representing the pooled radioactive fractions from the Sephadex G-25 column was applied and chromatographed on a column (2.5 x 84 cm) of Sephadex G-100. As before a single radioactive peak (Fig. 2b) was observed in a region corresponding to the void volume of the G-100 column. The total recovery of radioactivity applied to this column was 84%.

The fractions (Fractions 51 to 89) containing radioactivity, accounting for 65% of the radioactivity applied to the column, were again combined, lyophilized, and the [3H]BDC label was removed as described under "Experimental Procedure." The yield of peptide based upon the radioactivity retained after all steps of purification was approximately 47%. Only one peak positive to ninhydrin or Folin's reagent was eluted from the Dowex 50-X8 column used for this purpose. The radioactive [3H]BDC-OH was retained by the column under these conditions and requires extensive washing with 1 N sodium hydroxide in order to be eluted from the column.

In order to compare the chromatographic properties of the peptide derived from malate dehydrogenase before and after removal of [3H]BDC^+, a column (1.5 x 70 cm) was packed with Sephadex G-50. A sample (15 mg) of the tryptic digest of malate dehydrogenase labeled with [3H]BDC-OH was applied to the column and the fractions were monitored for A_280 and radioactivity (Fig. 3). The [3H]BDC-peptide was observed to elute from the column in 38 ml (void volume). Subsequent removal of the [3H]BDC from the peptide on the Dowex 50 column described above led to an elution volume of 50 ml for the peptide when chromatographed on the same Sephadex G-50 column.

A portion of the fractions containing ninhydrin-positive material from the Dowex 50-X8 column was lyophilized and desalted on a column (1.5 x 25 cm) packed with Bio-Gel P-2. The

![Diagram](https://via.placeholder.com/150)
Purification of labeled peptide from a tryptic digest of malate dehydrogenase inactivated with [3H]BDC-OH. A sample of the tryptic digest of malate dehydrogenase (15 mg) labeled with [3H]BDC-OH was applied to a column (2.5 x 90 cm) of Sephadex G-25 equilibrated with 50 mM ammonium bicarbonate (pH 8.0), containing 0.2% (v/v) thiodiglycol. Fractions (7.5 ml) were collected at a flow rate of 90 ml per hour and monitored for peptide material (A*w) (O-O) and SH (O---O). The fractions containing 3H from the above Sephadex G-25 column were lyophilized and dissolved in 4 ml of 50 mM ammonium bicarbonate (pH 8.0), containing 0.2% (v/v) thiodiglycol. The sample was applied to a column (2.5 x 84 cm) of Sephadex G-100 equilibrated in the above-mentioned buffer. Fractions (4 ml) were collected at a flow rate of 24 ml per hour and monitored for peptide material (ASH) (O-O) and aH (O- - -O).

The peptide-containing fractions from this desalting column contained a single ninhydrin-positive spot as demonstrated by peptide mapping. The amino acid composition of the peptide derived from malate dehydrogenase was determined as described above and the values obtained are shown in Table I.

Preliminary attempts to reverse the BDC-OH inactivation of the enzyme malate dehydrogenase from porcine heart have also been successful. Removal of the BDC moiety should yield the cysteinyl residues in the form of free thiols. Approximately 10 mg of [3H]BDC-malate dehydrogenase (74% active, containing 0.75 mole of [3H]BDC per mole of enzyme) were incubated in 8 ml of 100 mM sodium acetate buffer (pH 5.25) containing 2-mercaptoethanol at 4°C. The addition of 2-mercaptoethanol was not essential for reversal of the BDC moiety; however, this enzyme is generally more stable in the presence of this reagent and thus accounts for its addition in this instance. Aliquots were removed at 5, 30, and 60 min and chromatographed on a column (1.5 x 30 cm) packed with Sephadex G-25 and equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The fractions were monitored for protein (A280) and enzymatic activity. The pooled fractions containing malate dehydrogenase were assayed for enzymatic activity and content of [3H]BDC. As seen in Table II the results indicated a direct correlation between loss of aH and increase in enzymatic activity. At the end of 1 hour of incubation, approximately 65% of the radioactivity

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount found</th>
<th>Fondy peptide T</th>
<th>Devenyi peptide T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>19.5 (1.0)^c</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Histidine</td>
<td>14.4 (0.8)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>22.5 (1.2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cm-Cysteine</td>
<td>43.0 (2.3)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cysteine^e</td>
<td>35.2 (1.9)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>18.7 (1.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>21.2 (1.1)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Proline</td>
<td>17.9 (1.0)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.8 (0.6)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>45.0 (2.4)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Valine</td>
<td>27.4 (1.5)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Methionine</td>
<td>37.3 (2.0)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.4 (0.5)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

^a Fondy et al. (11).  
^b Devenyi et al. (10).  
^c Values in parentheses are molar equivalents.  
^d Determined as cysteic acid after performic acid oxidation (see text).  
^e Arbitrarily set at 1.0 M eq.
Dehydrogenase was incubated in 100 mM sodium acetate buffer (pH 5.2) containing 10 mM 2-mercaptoethanol at 4°C. Aliquots were removed at times indicated and chromatographed on a Sephadex G-25 column as detailed in text. Enzymatic activity and radioactivity were determined as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Per cent Active</th>
<th>[H]BDC per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>73</td>
<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>78</td>
<td>0.42</td>
</tr>
<tr>
<td>30</td>
<td>85</td>
<td>0.31</td>
</tr>
<tr>
<td>60</td>
<td>87</td>
<td>0.27</td>
</tr>
</tbody>
</table>

was removed and a concomitant regain in enzymatic activity was observed.

**DISCUSSION**

The purpose of this work has been to investigate the usefulness of the reagent BDC-OH for chemical modification studies and, if possible, for isolation of an active center peptide of malate dehydrogenase containing the essential sulfhydryl residue. This enzyme has previously been shown to possess one essential sulfhydryl residue per enzymatic active center (3).

As illustrated in Fig. 1, when 4,4'-bis-dimethylaminodiphenylcarbinol is dissolved in aqueous buffers, an equilibrium is established in which three species are present: the carbinol (BDC-OH), the cation (BDC+), and the dication (BDCH2+). The species which reacts with the sulfhydryl residue and which exhibits a visible absorption (λmax 606) is BDC+ (1). The molar absorption coefficient for BDC+ is 128,000 ± 4,000 M−1 cm−1 while the apparent molar absorption coefficient for the reagent BDC-OH at pH 5.2 in aqueous buffers is 10,000 M−1 cm−1 (9). Thus, the actual concentration of effective reagent (BDC+) at any one time is considerably less than would be expected from the molar excess values reported, indicating the high effectiveness of this reagent.

Conditions (pH, temperature, and molar excess of reagent) for inactivation were chosen such that the incorporation of [H]-BDC+ was kept to a minimum in the NAD+ protected sample and such that the enzymatic activity of the control sample remained constant. As previously reported (2) the addition of the coenzyme, NAD+, to the incubation mixture completely prevented the inactivation by BDC-OH. This protection of the enzyme from the inactivating effects of BDC-OH suggests that the residues undergoing modification in the absence of the coenzyme are located at or near the enzymatic active center of the enzyme. It should be noted that the reduced form of the coenzyme (NADH) cannot be utilized for similar protection studies. Addition of NADH to the incubation mixture causes the reduction of the BDC+ "carbonium-imonium" ion to yield the insoluble 4,4'-bis-dimethylaminodiphenylmethyl derivative of the reagent, thus depleting it from the incubation mixture.

In order to isolate tryptic peptides containing the active center sulfhydryl residues of porcine heart malate dehydrogenase, the enzyme was inactivated with [H]BDC-OH as described above. The enzyme used in these experiments was only approximately 50% inactivated so as to minimize random reaction of sulfhydryl groups, which may become more significant upon prolonged incubation with the reagent (2).

Following tryptic digestion of the [H]BDC+-labeled malate dehydrogenase, chromatography on Sephadex G-25 indicated that 90% of the radioactivity resided in fractions corresponding to the void volume of the column. This finding was somewhat unexpected as this protein contains approximately 60 arginine and lysine residues per 70,000 molecular weight (3). Work by Devenyi et al. (10) has suggested that this enzyme is composed of two identical, or very similar, subunits with a molecular weight of about 35,000. Thus if these residues (30 per subunit) are randomly distributed, digestion with trypsin should yield peptides which would be expected to be retarded by this column to some extent. Application of a sample of the tryptic-digested material on a column packed with Sephadex G-100 (Fig. 2b) led to a similar finding. As before, the majority of the radioactive material was observed to be in the fractions associated with the void volume of this column. A similar finding was made when the material was chromatographed on a column packed with Sephadex G-200.

Amino acid analysis of the radioactive material from the Sephadex G-100 column indicated that the composition was almost identical with that of a cytochrome containing trypptic peptide previously isolated from malate dehydrogenase (10, 11). Thus a peptide containing approximately 19 residues was chromatographed on the Sephadex G-25 and G-100 columns in an irregular manner. One explanation of this phenomenon could be that due to the highly hydrophobic nature of the BDC modifying group, those peptides containing the reagent, once freed by trypsin digestion, are associating in such a manner as to form an aggregate structure. A material of this nature would be expected to pass through a Sephadex G-100 column (molecular weight exclusion limit 100,000) rather than being retarded as biffing a peptide of this size (19 residues).

This conclusion is further supported by the chromatographic properties of the peptide on a Sephadex G-50 column (Fig. 3). When the labeled peptide was chromatographed on a column packed with Sephadex G-50, the radioactive material was eluted in fractions corresponding to the void volume; however, subsequent removal of the [H]BDC label led to a peptide which now was eluted from the same column in a position more consistent with its size as deduced from amino acid analysis.

It is possible that the fractions representing the void volumes of these Sephadex columns could all contain some aggregated or undigested protein material in addition to the [H]BDC-labeled peptide. However, the homogeneity of this material was established by the presence of a single ninhydrin-positive spot when analyzed by peptide mapping techniques. Thus, if aggregated or undigested protein were present its amount must be minimal.

As outlined in the previous section, the BDC+ label can be easily removed from peptides by incubation at pH 3.0 and passage of the sample over a column of Dowex 50-X-8. This procedure is useful in chemical modification and peptide isolation studies. [H]BDC-OH is freed from the sulfhydryl residue during the conditions of acid hydrolysis. The regenerated sulfhydryl residue is observed to be present as cysteine during the normal course of elution from the analyzer column. The BDC-OH moiety in some instances has been found to be irreversibly bound to the upper portion of the amino acid analyzer column during analysis of the hydrolyzed peptide. It has therefore become routine practice in this laboratory to remove the [H]BDC label just prior to acid hydrolysis and the amino acid analysis.

The amino acid composition of the peptide isolated is provided in Table I. Its amino acid composition is almost identical with one of the seven peptides isolated previously by other investiga-
tors studying all of the sulphydryl-containing peptides of this enzyme (10, 11). Fondy et al. (11) used a novel approach in attempting to identify an active center cysteine in this enzyme. They carboxymethylated all of the cysteine residues of malate dehydrogenase in the presence of 8 M urea. Tryptic digestion was followed by peptide mapping. A single alkylated peptide common to three species of malate dehydrogenase was observed. These authors suggested that this peptide was a portion of the active center conserved throughout evolution. However, no direct proof of the essentiality of that residue was established by that approach. It is interesting to note that their suggestion is most probably correct in that the composition of their peptide fits well with the composition of the peptide isolated in this work after selective chemical modification.

The last portion of the “Results” section includes preliminary data dealing with the reversibility of the inactivation of malate dehydrogenase by BDC-OH. This information is included for the benefit of those readers who might find it advantageous to utilize this reagent in a reversible manner. If the enzyme under investigation can tolerate conditions in the area of pH 4.5 for short periods of time, the optimum rate of reversal will probably be achieved due to the formation of BDC+ and free sulphydryl residue. Malate dehydrogenase from porcine heart is unstable under these conditions; however, limited success can be achieved by incubation of the labeled enzyme at pH 5.2 for up to 1 hour.

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Bruce A. Humphries and John H. Harrison


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