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Isolation and Identification of Cysteinyl Peptide Labeled by 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate in the Reduced Diphosphopyridine Nucleotide Inhibitory Site of Glutamate Dehydrogenase†

Surendra P. Batra and Roberta F. Colman*
Department of Chemistry, University of Delaware, Newark, Delaware 19716
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ABSTRACT: 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TADP) has been shown to react at the reduced diphosphopyridine nucleotide (DPNH) inhibitory site of bovine liver glutamate dehydrogenase with incorporation of 1 mol of reagent/mol of enzyme subunit [Batra, S. P., & Colman, R. F. (1984) *Biochemistry 23*, 4940–4946]. The modified enzyme had lost one of the six free sulfhydryl groups per enzyme subunit as detected by 5,5′-dithiobis(2-nitrobenzoate). In the unmodified enzyme digested with trypsin, six cysteinyl peptides labeled with [^{14}C]iodoacetic acid were detected by high-performance liquid chromatography (HPLC), whereas only five were observed in the 6-BDB-TADP-modified enzyme. A cysteinyl peptide has been isolated from modified enzyme digested with trypsin and chymotrypsin. Purification of the nucleotidyl peptide was accomplished by chromatography on phenyl boronate–agarose, followed by gel filtration on Sephadex G-25 and Bio-Gel P-4 in 50 mM ammonium bicarbonate, pH 8.0. The modified peptides were finally purified by HPLC on a C_{18} column using 0.1% trifluoroacetic acid with an acetonitrile gradient. By comparison of the amino acid composition and N-terminal residue of the isolated peptide with the known amino acid sequence of the enzyme, the peptide in the DPNH inhibitory site labeled by 6-BDB-TADP has been identified as the 19-membered fragment from Glu-311 to Lys-329. A unique residue, Cys-319, was identified as the reactive amino acid within the DPNH inhibitory site.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme whose activity is modulated by purine nucleotides: adenosine 5′-diphosphate (ADP)[1] activates, GTP inhibits, and relatively high concentrations of DPNH inhibit by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972; Eisenberg et al., 1976). The enzyme is composed of six identical polypeptide chains, each of which has been reported to have a site for ADP, two sites for GTP, and two sites for DPNH (Sund et al., 1975; Pal & Colman, 1979). The amino acid sequence of glutamate dehydrogenase has been established (Julliard & Smith, 1979). For the identification of essential amino acid residues in the regulatory sites of glutamate dehydrogenase, the reactions of several purine nucleoside affinity labels with the enzyme have been studied (Colman, 1983). For example, the affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) has been reported to react specifically, with 0.5–1.0 mol of reagent incorporated per enzyme subunit, at the DPNH inhibitory site of glutamate dehydrogenase (Pal et al., 1975; Saradambal et al., 1981). Schmidt and Colman (1984) demonstrated that the residues in the DPNH inhibitory site modified by 5′-FSBA are Lys-420 and Tyr-190.

We have recently shown that the new reactive adenine nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TADP) also acts as an affinity label of the DPNH inhibitory site of glutamate dehydrogenase with incorporation of about 1 mol of reagent per peptide chain (Batra & Colman, 1984). The modified enzyme has been shown to be catalytically active, and many

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[1] Abbreviations: 6-BDB-TADP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate; ADP, adenosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; DPNH, reduced diphosphopyridine nucleotide; CM, carboxymethylated; DTNB, 5,5′-dithiobis(2-nitrobenzoate); PBA, phenyl boronate–agarose; Tris, tris(hydroxymethyl)aminomethane; 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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of its kinetic properties have been examined (Batra & Colman, 1984). The present paper describes the isolation and characterization of the peptide of glutamate dehydrogenase modified by 6-BDB-TADP and identifies Cys-319 as a participant in the DPNH regulatory site. A preliminary version of this work has been presented (Batra & Colman, 1985).

Experimental Procedures

Materials. Bovine liver glutamate dehydrogenase was purchased from Boehringer Mannheim Corp. as an ammonium sulfate suspension. The enzyme (15–50 mg) was dialyzed overnight at 4 °C against 100 mM potassium phosphate buffer, pH 7.1. The dialyzed material was centrifuged at 4 °C for 30 min at 15000 rpm to remove precipitated, denatured protein. The concentration of enzyme was determined from the absorbance at 280 nm by using $E^{1\%} = 9.7$ (Olsen & Anfinsen, 1952). A molecular weight of 56000 per peptide chain was used in the calculations (Smith et al., 1970). Chymotrypsin and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin were purchased from Worthington. HPLC-grade acetonitrile was obtained from Burdick & Jackson Laboratories. Trifluoroacetic acid and dansyl chloride were supplied by Pierce Chemical Co. Phenyl boronate–agarose Matrex Gel PBA-30 was from Amicon, and Bio-Gel P-4 (100–200 mesh) was obtained from Bio-Rad Laboratories. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate (6-BDB-TADP) was synthesized according to the procedure of Colman et al. (1984) by reaction of 1,4-dibromobutanedione with 6-mercaptopurine ribonucleotide 5′-diphosphate. The β-^{32}P-labeled 6-mercaptopurine ribonucleoside 5′-diphosphate was synthesized with [^{32}P]phosphoric acid (Batra & Colman, 1984). [1-^{14}C]Iodoacetic acid, [^{32}P]orthophosphoric acid, and NaB^3H_4 were supplied by New England Nuclear Corp.

Preparation of 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Diphosphate Modified Enzyme. Glutamate dehydrogenase (2 mg/mL) was incubated with 0.25 mM 6-BDB-TADP at 25 °C in 0.05 M potassium phosphate buffer, pH 7.1, containing 10% (by volume) methanol. After 2 h, the modified enzyme was isolated by the column centrifugation technique described by Penefsky (1979) using Sephadex G-50,80 equilibrated with 0.05 M potassium phosphate buffer, pH 7.1. Two successive centrifuge columns followed by an overnight dialysis against 0.05 M ammonium bicarbonate, pH 8.0, were required to completely remove any noncovalently bound reagent. The amount of covalent incorporation was determined either by measurement of the ^{32}P of the radiolabeled reagent or by assay of the phosphorus content of the enzyme modified with the nonradioactive 6-BDB-TADP, as described previously (Batra & Colman, 1984). The incorporation was approximately 1 mol of reagent/subunit.

Titration of Enzyme Sulfhydryl Groups with 5,5′-Dithiobis(2-nitrobenzoate). Aliquots of glutamate dehydrogenase (0.2 mL of 1 mg/mL in 50 mM sodium phosphate buffer) were added to 0.6 mL of 200 mM Tris-HCl buffer, pH 8.0, in a 1.0-mL cuvette. Sodium dodecyl sulfate (0.1 mL 10% w/v) was added to denature the protein. The absorbance of the solution was read at 412 nm against a protein-free buffer blank which was otherwise identical with the test solution. A freshly prepared 10 mM DTNB solution (0.1 mL) was added to each cuvette, and the concentration of free –SH groups in the enzyme was calculated from the change in absorbance. A molar extinction coefficient of 13600 at 412 nm for thionitrobenzoate was used in the calculations (Ellman, 1958).

Tryptic Digestion of ^{14}C-Labeled Carboxymethylated 6-BDB-TADP-Modified and Unmodified Enzyme. 6-BDB-TADP-modified glutamate dehydrogenase containing 1 mol of reagent per peptide chain was denatured in 6 M urea. A 10-fold molar excess of dithiothreitol was added over the moles of sulfhydryl groups in the enzyme. After 1 h, [1-^{14}C]iodoacetic acid was added in a 2-fold excess over the number of moles of dithiothreitol, followed 10 min later by the addition of excess β-mercaptoethanol to quench the reaction. Native glutamate dehydrogenase was carboxymethylated in a similar manner. The carboxymethylated modified and unmodified proteins were dialyzed against 0.05 M ammonium bicarbonate, pH 8.0, to remove excess reagents and were then digested with trypsin at 37 °C for 3 h in the same buffer by using a trypsin/glutamate dehydrogenase ratio of 1/20 (w/w). Following addition of a second portion of the same amount of trypsin, the digestion was continued at 37 °C for another 3 h, after which the sample was lyophilized.

Separation of [^{14}C]CM-Cysteine Tryptic Peptides by High-Performance Liquid Chromatography. These tryptic peptides were separated on a Varian Model 5000 HPLC system equipped with a Vydac C_{18} column (0.46 × 25 cm). Each digest (200 μg) was dissolved in 0.8 mL of 0.1% trifluoroacetic acid, and filtered through 0.45-μm membrane filters (Schleicher & Schuell) prior to injection. Separation of peptides was carried out by elution at 1.5 mL/min with 0.1% trifluoroacetic acid, pH 2.0 (solvent A), from 0 to 10 min followed by a linear gradient from solvent A to 60% solvent B (0.08% trifluoroacetic acid in acetonitrile) from 10 to 130 min. UV absorbance of the effluents was monitored at 220 nm. Fractions (1.5 mL) were collected, and aliquots (1.4 mL) were assayed for radioactivity in 15 mL of ACS scintillation liquid (Amersham) by using a Packard TriCarb liquid scintillation counter, Model 3330.

Reduction of 6-BDB-TADP-Modified Enzyme by NaB^3H_4. Glutamate dehydrogenase was modified with nonradioactive 6-BDB-TADP under the conditions described earlier. In order to introduce a radioactive label into the nonlabeled 6-BDB-TADP–enzyme complex, NaB^3H_4 was used to reduce the dioxo groups of the reagent.[2] The modified enzyme (30 mg) was cooled on ice and reduced with two additions of aliquots of 180 mM NaB^3H_4 [specific activity (8–16) × 10^{12} cpm/mol containing four hydrogens] in 0.02 N NaOH followed by a 1-h incubation after each addition. The final concentration of NaB^3H_4 was 10 mM. A control was also run consisting of the native enzyme reduced with NaB^3H_4 under the same conditions as the modified enzyme. The reduced, modified, and unmodified enzymes were dialyzed extensively for several days against six to eight changes of 6 L of 0.05 M NH_4HCO_3, pH 8.0.

Proteolytic Digestion of 6-BDB-TADP-Modified and NaB^3H_4-Reduced Enzyme. After extensive dialysis, the modified, NaB^3H_4-reduced enzyme was successively digested with trypsin and chymotrypsin. (In some cases, prior to proteolytic digestion, the reduced enzyme was treated with nonradioactive iodoacetate to produce the carboxymethylated modified enzyme, as described earlier.) Digestion with trypsin was carried out as described earlier. After the tryptic digest was maintained at room temperature for 15 h, chymotrypsin was added in a ratio of 1/20 (w/w) of the total amount of modified enzyme and incubated at 37 °C for 3 h. After addition of a second portion of chymotrypsin of the same amount, the incubation was continued at 37 °C for another 3 h. The digest was then lyophilized.

[2] The radioactivity has been used as a tracer for the peptides rather than as a quantitative measure of the modified residue in the peptide, because of the likelihood of isotopic selection in the reduction reaction.

Separation of Modified Peptides on a Phenyl Boronate–Agarose Column. The first step in the purification of nucleotidyl peptide was chromatography on a phenyl boronate–agarose (PBA-30) column, 1 × 28 cm, equilibrated with 0.05 M potassium phosphate buffer, pH 8.0. The total tryptic–chymotryptic digest was dissolved in 2.5 mL of 0.05 potassium phosphate buffer, pH 8.0, and applied to the column. The column was eluted with the same buffer, until the absorbance at 220 nm of the eluant returned to zero, thereby removing a large number of the unmodified peptides. Subsequently, the column was eluted with deionized water which eluted the nucleotidyl peptides. Aliquots (10–30 μL) of fractions were used to measure radioactivity in 10 mL of ACS scintillation liquid. Fractions were pooled on the basis of radioactivity and A_{220nm} and were lyophilized.

Gel Filtration of Peptides. The water pool from the PBA column was dissolved in 1 mL of 0.05 M NH_4HCO_3, pH 8.0, and applied to a Sephadex G-25 column (1 × 95 cm) equilibrated with the same buffer. The column was eluted with 0.05 M NH_4HCO_3, pH 8.0, at a flow rate of 20 mL/h, and 1-mL fractions were collected. Fractions were monitored for A_{220nm} and radioactivity. Appropriate radioactive fractions were pooled and lyophilized. To standardize the column, the following peptides were separately applied to the column and their elution volumes measured: bradykinin (1240 daltons); insulin, chain A (2500 daltons); insulin, chain B (3480 daltons).

Peptides were subsequently purified by gel filtration on a column of Bio-Gel P-4 (1 × 100 cm) equilibrated with 0.05 M NH_4HCO_3, pH 8.0. Fractions (1 mL) were collected at 14 mL/h and were monitored for A_{220nm} and radioactivity. Radioactive fractions which absorbed at 220 nm were pooled and lyophilized.

Fractionation of 6-BDB-TADP-Labeled Peptides by Reverse-Phase HPLC. The radioactive pool from the Bio-Gel P-4 column was concentrated and fractionated by HPLC using the same column and solvents as used for the CM-cysteinyl peptides. Initial separation of peptides was carried out by elution at 1 mL/min with 0.1% trifluoroacetic acid from 1 to 10 min, followed by a linear gradient from solvent A to 30% solvent B from 10 to 130 min, and 1-min fractions were collected.

Amino Acid Analysis of Peptides. Peptides (1.2–3.0 nmol) were hydrolyzed in 6 N HCl (after flushing with nitrogen) at 110 °C for 19 h in evacuated, sealed tubes. The hydrolysate was dried, diluted in 0.2 N sodium citrate, pH 2.2, and analyzed with a Beckman Model 120C amino acid analyzer, using the single column procedure described by Likos and Colman (1981). Under these conditions, tyrosine, phenylalanine, histidine, and lysine elute at 171, 176, 201, and 209 min, respectively. For hydrolysates of radioactive peptides, 1.5-min fractions (2.0 mL) of the effluent were collected from the amino acid analyzer after passage through the photometer. Fractions were assayed by adding concentrated HCl (0.2 mL of 12.1 N) to 1.0 mL of each fraction prior to the addition of 10 mL of ACS scintillation liquid (Amersham).

N-Terminal Analysis. The amino terminal of peptide (0.1–0.4 nmol) was dansylated by using the procedure described by Gray (1972). The dansyl amino acid was identified by two-dimensional thin-layer chromatography on 5 × 5 cm micropolyamide sheets (Schleicher & Schuell) in the solvent system described by Gray (1972).

Synthesis of NaB^3H_4-Reduced Product of Reaction of 6-BDB-TADP and Cysteine. The reagent 6-BDB-TADP (2.5 mM) and cysteine (50 mM) were incubated for 30 min at room temperature in 0.05 M potassium phosphate buffer, pH 7.1, followed by two additions (45 min apart) of aliquots of 100 mM NaB^3H_4 in 0.02 N NaOH to yield a final concentration of 10 mM. The borohydride-reduced cysteine–nucleotide derivative was subsequently separated on a phenyl boronate–agarose column equilibrated with 50 mM potassium phosphate buffer, pH 8.0. Elution with 50 mM phosphate buffer, pH 8.0 (2 L), until the radioactivity returned to zero removed the unreacted NaB^3H_4 and cysteine. Subsequent washing with deionized water eluted the cysteine–nucleotide product.

Table I: Incorporation of [^{14}C]Iodoacetic Acid into Tryptic Peptides of Native and 6-BDB-TADP-Modified Glutamate Dehydrogenase[a]

CM-cysteinyl peptides	native enzyme (cpm)	modified enzyme (cpm)
I	593	621
II	599	581
III	624	550
IV	619	549
V + VI	1075	624

[a] CM-cysteinyl peptides correspond to the numbered peaks illustrated in Figure 1. The total radioactive count listed for each peak represents the average of four HPLC runs.

The product yielded a single UV-absorbing, ninhydrin-positive spot on thin-layer chromatography using precoated cellulose sheets without fluorescent indicator (E. Merck) and the solvent system butanol–acetic acid–water (5:1:2). The R_f of the product spot was 0.35, which may be compared with R_f 0.33 for the UV-absorbing, ninhydrin-negative spot of 6-BDB-TADP and the ninhydrin-positive spots of R_f 0.40 for cysteine and R_f 0.09 for cystine.

This radioactive, reduced product of 6-BDB-TADP and cysteine was hydrolyzed in 6 M HCl at 110 °C for 19 h and subsequently applied to the amino acid analyzer, as described earlier. It yielded two radioactive peaks eluting at 183 and 194 min.

Results

Evidence for Reaction of 6-BDB-TADP with a Cysteinyl Residue in Glutamate Dehydrogenase. Bovine liver glutamate dehydrogenase is known to contain six cysteine residues per subunit (Goldin & Frieden, 1972). The number of free sulfhydryl groups in native and modified enzymes were determined by reaction with 5,5′-dithiobis(2-nitrobenzoate) as described under Experimental Procedures. Native enzyme exhibited 5.96 –SH groups per mole of enzyme subunit, and modified enzyme containing 1 mol of 6-BDB-TADP incorporated/mol of enzyme subunit was found to contain 5.09 free cysteine residues. This result suggests that modified enzyme had lost one of the six free –SH groups per enzyme subunit.

That a cysteine residue is the site of attack by 6-BDB-TADP was further tested by carboxymethylating the cysteines of both modified and unmodified enzymes with [^{14}C]iodoacetic acid. The carboxymethylated enzymes were digested with trypsin, and the resultant peptides were separated by HPLC as described under Experimental Procedures. Figure 1A typifies the separation of the tryptic peptides by HPLC. The separations of the (carboxymethyl)cysteine-containing peptides for native and 6-BDB-TADP-modified enzymes are illustrated by the elution profiles of radioactivity in parts B and C of Figure 1, respectively. Table I summarizes the results of HPLC separations of each type of digest.

For native enzyme, peaks I–IV each exhibit similar amounts of radioactivity, while the fifth peak has almost twice the amount of radioactivity; thus it is proposed that the fifth peak

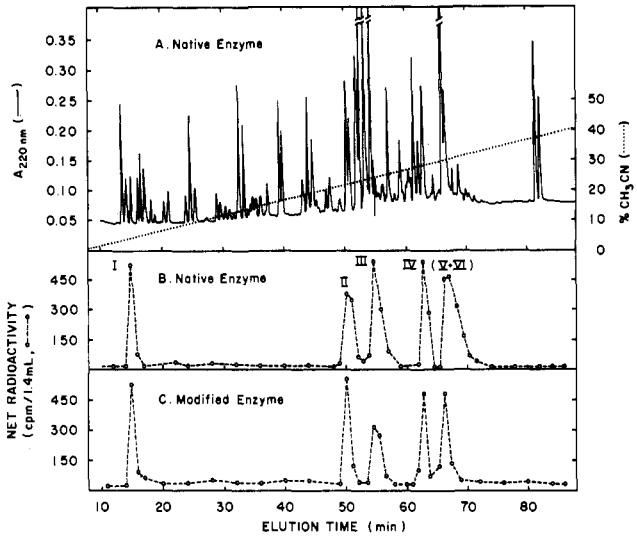

FIGURE 1: Separation of [^{14}C]carboxymethylcysteinyl peptides by HPLC. The tryptic digests (250 μg) from carboxymethylated modified and unmodified glutamate dehydrogenase were separated by HPLC on a Vydac C_{18} HPLC column. Peptides were eluted with 0.1% TFA, pH 2.0, followed by a linear gradient of acetonitrile, as described under Experimental Procedures. Absorbance (—) was monitored at 220 nm. The linear gradient of acetonitrile is indicated by dotted line (···), as shown in (A). A 1.4-mL aliquot of each fraction was counted for radioactivity (O). (B) and (C) show the radioactive peaks corresponding to CM-cysteinyl peptides from native and 6-BDB-TADP-modified enzyme, respectively.

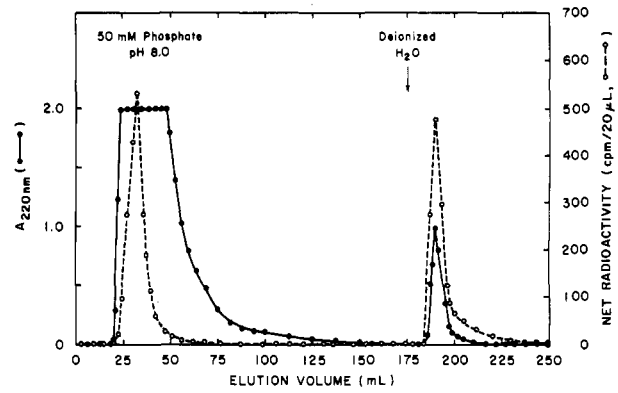

FIGURE 2: Separation of tryptic–chymotryptic peptides on phenyl boronate–agarose. An aliquot of the total tryptic–chymotryptic digest of 30 mg of 6-BDB-TADP-modified glutamate dehydrogenase was applied to a PBA-30 column equilibrated with 50 mM potassium phosphate buffer, pH 8.0. The column was washed with phosphate buffer followed by deionized water, as indicated by the arrow. Fractions (1.25 mL) were collected and analyzed for absorbance at 220 nm (●) and for radioactivity (O). Fractions from 182 to 215 mL elution volume were pooled for further purification steps.

includes two cysteine peptides and is designated as (V + VI). In contrast, in the case of modified enzyme, all five peaks show similar amounts of radioactivity. It appears that only five (carboxymethyl)cysteine peptides are present in the modified enzyme: there is one less in the (V + VI) region than in native enzyme. This result indicates that 6-BDB-TADP reacts with one cysteine and prevents subsequent reaction with [^{14}C]-iodoacetic acid.

Isolation of 6-BDB-TADP-Modified Peptides. The initial attempts to isolate the labeled peptides were carried out by using modified enzyme prepared by incubation with 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-[β-^{32}P]-diphosphate. Since the β-phosphate proved to be labile under the conditions used for purification, this approach was not feasible. As an alternate strategy, the bromodioxobutyl moiety of the modified enzyme was reduced with NaB^3H_4, and the modified peptides were separated and detected as tritiated, reduced peptides.

Chromatography on Phenyl Boronate–Agarose. The first step for purification of the nucleotidyl peptide was accomplished by chromatography on a phenyl boronate–agarose (PBA) column, which is based on the formation of a complex between the cis–diol moiety of the nucleotidyl peptide and the dihydroxyboryl group. Thus, nucleotidyl peptides are selectively retained by the column. A large number of peptides in the tryptic–chymotryptic digest of BDB-TADP-modified enzyme were eluted in the void volume as a broad peak during the initial elution with 0.05 M potassium phosphate buffer, pH 8.0, as indicated by the A_{220nm} profile shown in Figure 2. The modified peptides were eluted as a sharp peak, when the salt concentration of the buffer was lowered by elution with deionized water. The water eluate also contained some unmodified peptides that were retained on the column, possibly by hydrophobic interactions with the phenyl groups of the PBA matrix or by nonspecific ionic interactions with the boronate ions. The total yield of the modified peptides in the water eluate was 38–44% of the amount of radioactivity applied to the column. These fractions were pooled, lyophilized, and saved for the subsequent purification procedures.

In addition, 40–55% of the radioactivity applied to the column was eluted in the first peak with 0.05 M phosphate buffer. It is proposed that this radioactivity is attributable to a small amount of reduction of many peptide bonds by NaB^3H_4 throughout the protein on the basis of the following experiment: Native glutamate dehydrogenase was treated with NaB^3H_4 under the same conditions used for the modified enzyme and was similarly digested with trypsin and chymotrypsin. The digest from this control sample was applied to the PBA column. Most of the radioactivity was eluted with 0.05 M phosphate buffer, pH 8.0. For the same amounts of total protein, the amount of radioactivity eluted by phosphate buffer was the same in the native and the 6-BDB-TADP-modified enzymes. The difference between the two samples was seen exclusively in the water eluate, and hence, the water peak is considered to be the only one containing the 6-BDB-TADP-modified peptides.

Separation of Modified Peptides by Gel Filtration. The peptides from the water pool of the PBA column were fractionated on a Sephadex G-25 column as shown in Figure 3. Comparison of the elution volume of the radioactive peak with that of the standard peptides of known molecular weight led to an estimate of about 2300 daltons as the molecular weight of the modified peptide(s). This molecular weight is consistent with a peptide of 17–19 amino acids. Almost all of the radioactivity applied to the column was found in fractions 30–57, and these were pooled, lyophilized, and chromatographed on a Bio-Gel P-4 column to give a small additional purification.

Fractionation of Peptides by HPLC. The radioactive peptide pool from the Bio-Gel P-4 column was fractionated by HPLC in 0.1% trifluoroacetic acid, pH 2.2, with a linear gradient of acetonitrile as illustrated in Figure 4. Two major peaks of radioactivity were detected in fractions 96–97 (peptide a) and 105 (peptide b), corresponding to 21% and 23% acetonitrile, respectively.

Peptide pools a and b, when rechromatographed separately by HPLC, each gave a single radioactive peak eluting at its original position. Pool a exhibited two very close UV-absorbing peaks eluting at 21% acetonitrile (Figure 5A); while peptide b exhibited a single UV-absorbing peak centered at 23% acetonitrile. The same peptide peaks were observed whether or not the original 6-BDB-TADP-modified enzyme was car-

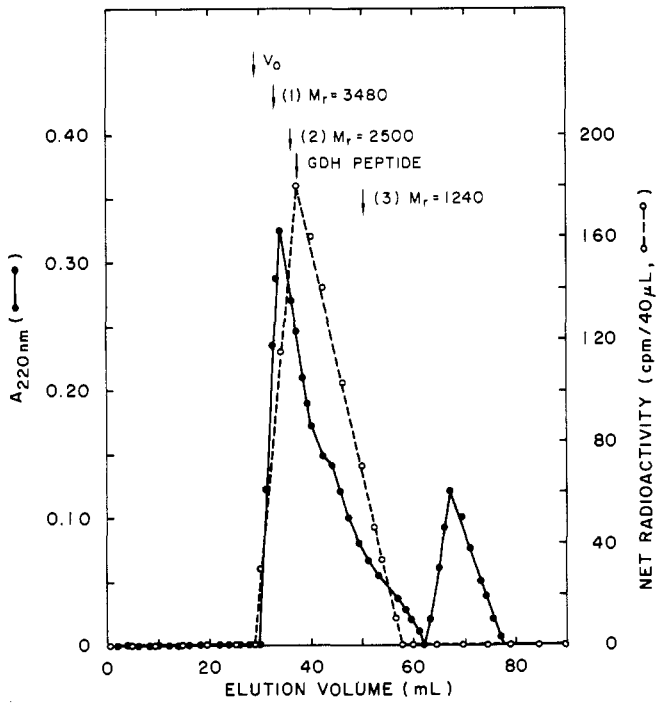

FIGURE 3: Fractionation of peptides on Sephadex G-25,80. The water eluate from the PBA column was lyophilized, dissolved in 50 mM NH_4HCO_3, pH 8.0 (0.8 mL), and applied to the column (95 × 1 cm) equilibrated with the same buffer. The void volume was 29.2 mL, and total volume was 67.3 mL. Elution was carried out with 50 mM NH_4HCO_3. Fractions (1 mL) were collected and monitored for absorbance at 220 nm (●), and the radioactivity (○) was measured. Fractions from 30 to 57 mL were pooled.

boxymethylated prior to digestion by trypsin and chymotrypsin.

Analysis of Isolated Tryptic–Chymotryptic Peptides. Peptide b and the two A_{220nm} peaks of peptide a, separately or pooled, were hydrolyzed in 6 N HCl at 110 °C for 19 h

Table II: Amino Acid Composition[a] and N-Terminal Identification of Peptides a and b

amino acid	peptide a[b] amount measured (nmol)	peptide a[b] mol/mol of peptide[d]	peptide b[c] amount measured (nmol)	peptide b[c] mol/mol of peptide[d]
Lys	1.62	1.00 (1)[e]	1.72	1.00 (1)
His	0.68	0.42 (0)	0.59	0.36 (0)
Arg	0.52	0.32 (0)	0.70	0.43 (0)
Asp	2.94	1.81 (2)	3.33	1.94 (2)
Thr	0.70	0.43 (0)	1.08	0.63 (0–1)
Ser	3.54	2.18 (2)	3.34	1.94 (2)
Glu	4.10	2.53 (3)	4.49	2.61 (3)
Pro	1.61	0.99 (1)	1.87	1.09 (1)
Gly	5.14	3.17 (3)	5.78	3.36 (3)
Ala	3.09	1.90 (2)	3.21	1.87 (2)
Val	1.38	0.85 (1)	1.73	1.01 (1)
Met	0.11	0.07 (0)	0.14	0.08 (0)
Ile	2.38	1.47 (1–2)	2.51	1.46 (1–2)
Leu	2.12	1.31 (1–2)	2.81	1.63 (2)
Tyr	0.51	0.31 (0)	0.34	0.20 (0)
Phe	0.61	0.37 (0)	0.61	0.35 (0)
N-terminal analysis[f]	dansyl-Glu		dansyl-Glu	

[a] Composition was determined after hydrolysis for 19 h at 110 °C in 6 N HCl. [b] Average of five analyses. [c] Average of four analyses. [d] Each amino acid residue is normalized to one lysine. [e] The values in the parentheses are the nearest integers. [f] By dansylation and thin-layer chromatography on polyamide plates.

prior to amino acid analysis. No difference in amino acid composition was discerned between fractions containing the two peaks derived from peptide a. Furthermore, as recorded in Table II, the amino acid compositions of peptides a and b appear almost identical. The actual amount of each amino acid measured was normalized to the amount of lysine, since it is probable that lysine is the carboxyl-terminal residue of the peptides on the basis of the specificity of cleavage by trypsin. The amino-terminal residue of each peptide was

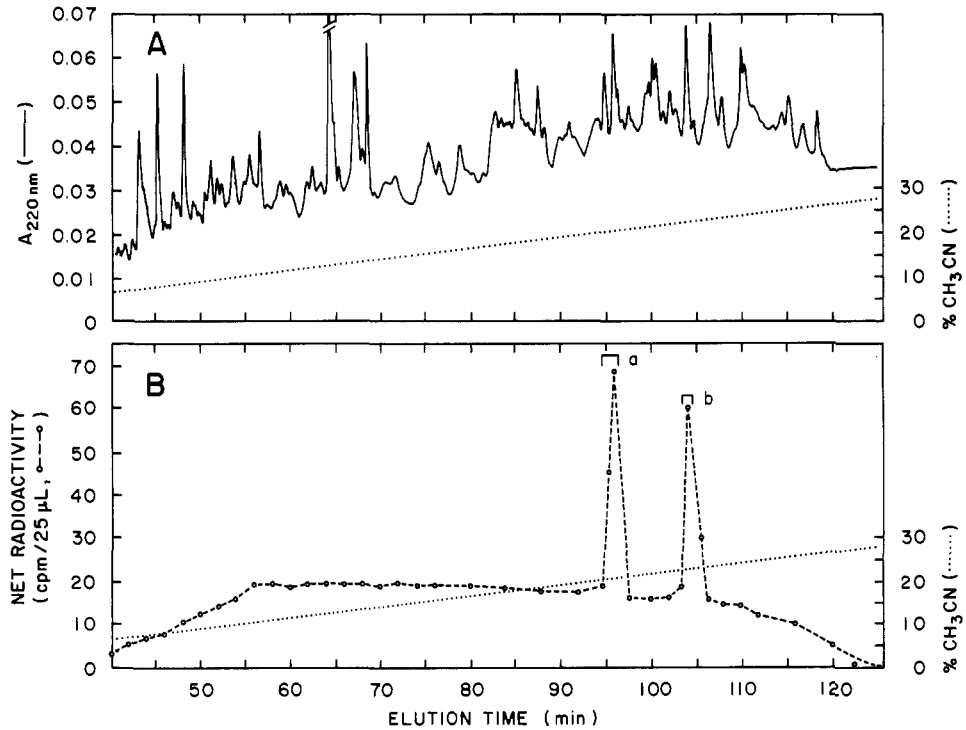

FIGURE 4: Separation of peptides by reverse-phase high-performance liquid chromatography in trifluoroacetic acid. The A_{220nm}-absorbing radioactive fractions from the Bio-Gel P-4 column were applied to a Vydac C_{18} HPLC column. Peptides (containing 20–25 nmol of ^3H label) were eluted with 0.1% trifluoroacetic acid, pH 2.0, followed by a linear gradient of acetonitrile, as described under Experimental Procedures. Absorbance (—) was monitored at 220 nm. A 25-μL aliquot of each fraction was counted for radioactivity (○). The linear gradient of acetonitrile is indicated by the dotted line (···). Peptide peaks a (fractions 96–97) and b (fraction 105) were pooled as indicated and lyophilized.

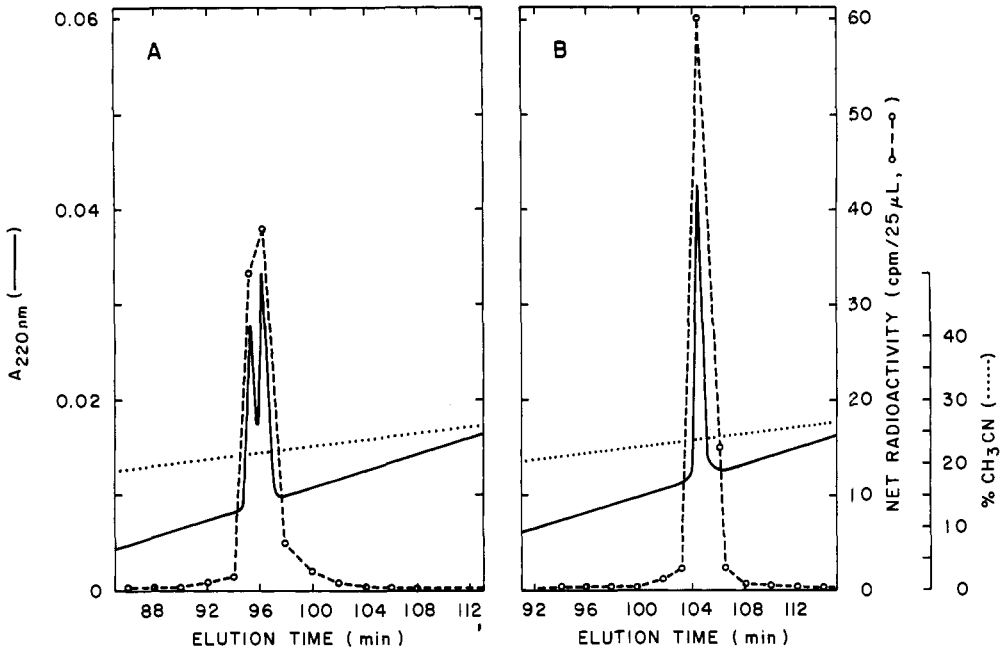

FIGURE 5: Rechromatography of pools a and b. Pools a and b from HPLC (Figure 4) were reapplied separately to HPLC in the same solvent system as that used for peptide separation in Figure 4. Fractions 96–97 (A) and fractions 105–106 (B) were collected for analysis.

determined after reaction with dansyl chloride and separation of dansyl amino acids by two-dimensional thin-layer chromatography on polyamide sheets. Peptides a and b were each shown to have glutamic acid as the amino-terminal residue.

Both peptides a and b yielded two radioactive peaks eluting from the amino acid analyzer between phenylalanine and histidine (Figure 6). The elution positions of products prepared by reacting 6-BDB-TADP with cysteine, followed by NaB^3H_4 treatment and acid hydrolysis, were the same as the radioactive peaks arising from the peptides. These results indicate that both peptides contain 6-BDB-TADP-modified cysteine and provide further confirmation of the reaction of 6-BDB-TADP with a cysteine of glutamate dehydrogenase.[2]

Comparison of the isolated peptides with the known amino acid sequence of glutamate dehydrogenase (Julliard & Smith, 1979) reveals that only one of the cysteine peptides can be considered to be a good candidate on the basis of the specificity of trypsin and chymotrypsin, the molecular weight, the N-terminal, and amino acid composition. The sequence of this peptide is

Glu^{311}-Gly-Ser-Ile-Leu-Glu-Val-Asp-Cys^{319}-Asp-Ile-Leu-Ile-Pro-Ala-Ala-Ser-Glu-Lys^{329}

This peptide would result from cleavage following Tyr^{310} by chymotrypsin and Lys^{329} by trypsin, in accordance with the known specificity of these proteolytic enzymes. The molecular weight of the derivatized peptide would be about 2500 daltons, consistent with the elution position from Sephadex G-25. The amino terminal was identified as glutamate. The amino acid composition is as expected, except that Gly is high and Ile and Leu are somewhat low. Three moles of glycine per mole peptide were detected upon the acid hydrolysis of the isolated peptide, whereas the 19-membered fragment from Glu-311 to Lys-329 has only one glycine residue. We have observed 1 mol of glycine upon acid hydrolysis of 6-BDB-TADP, presumably a breakdown product of the adenine moiety of 6-BDB-TADP. The residual extra glycine may be attributed to minor contamination from the glassware, since a blank containing only buffer, upon acid hydrolysis followed by amino acid analysis, showed the presence of glycine in about the same amount as the excess found in the peptides. The lower values

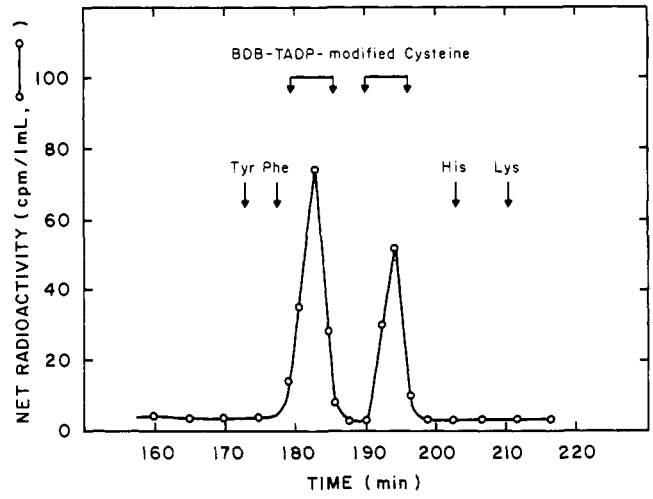

FIGURE 6: Amino acid analysis of sodium borohydride reduced modified peptide. 6-BDB-TADP-modified enzyme was treated with NaB^3H_4, and the modified-tritiated peptide was isolated, acid-hydrolyzed, and applied on an amino acid analyzer as described under Experimental Procedures. The elution positions of NaB^3H_4 reduced amino acid residues from the isolated peptides are shown by radioactive peaks at 183 and 194 min. The elution positions of model compound prepared by reacting cysteine with 6-BDB-TADP followed by sodium borohydride treatment and hydrolysis are indicated by arrows. The elution position of standards are Tyr (173 min), Phe (177 min), His (203 min), and Lys (210 min).

of isoleucine and leucine found in the peptide (Table II) may be due to incomplete cleavage of Ile–Leu bonds on hydrolysis with 6 M HCl at 110 °C for 19 h. Indeed, Cosson et al. (1976) have also observed lower values of isoleucine and leucine on amino acid analysis of the glutamate dehydrogenase peptide 309–329, which was hydrolyzed for 24 h with 6 M HCl. In the presence study the only cysteine peptide of glutamate dehydrogenase that is consistent with all the data is residues 311–329.

The two peptides a and b eluted as distinct peaks at different concentrations of acetonitrile (21% and 23%, respectively) but have the same amino acid compositions and N-terminals. This observation may be attributed to the loss of one phosphate from the BDB-TADP moiety of the modified peptide during the

purification procedure, which would result in a different retention time on HPLC. It is notable that when attempts were made to follow the isolation of the 6-BDB-TADP-labeled peptides using reagent labeled with β^{32}-P, loss of radioactivity occurred during the purification. We do not have a conclusive explanation for the observation of the same amino acid composition for the two UV-absorbing peaks in the "Peptide a" region of HPLC. However, others have also observed single peptides that give two peaks, apparently due to an artifact of the separation procedure (Hollemans et al., 1983; Jacobson & Colman, 1984); this may be the result of a differential interaction of the trifluoroacetic acid with the peptide.

We conclude that 6-BDB-TADP reacts with only one residue in glutamate dehydrogenase. That residue is Cys-319.

Discussion

We have previously presented evidence that the nucleotide analogue 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate reacts to the extent of about 1 mol per subunit of glutamate dehydrogenase at the DPNH inhibitory site (Batra & Colman, 1984). Modified enzyme completely loses its normal ability to be inhibited by high concentrations of DPNH (>100 μM) but retains its full activity when assayed at 100 μM DPNH and is still measurably activated by ADP and inhibited by GTP. Saturating concentrations of DPNH alone or DPNH in the presence of GTP protect glutamate dehydrogenase against reaction with 6-BDB-TADP, while GTP alone does not prevent the reaction (Batra & Colman, 1984). We have now shown that 6-BDB-TADP reacts with a cysteine in glutamate dehydrogenase and have isolated a single type of derivatized peptide from the tryptic–chymotryptic digest of 6-BDB-TADP-modified glutamate dehydrogenase: the 19-membered peptide Glu-311 to Lys-329. These results indicate that Cys-319 in the DPNH inhibitory site is labeled by 6-BDB-TADP.

This is the first evidence for the involvement of cysteine in the DPNH inhibitory site of glutamate dehydrogenase. However, it has been reported that reaction of methylmercuric iodide with a cysteine residue causes decreased response to the allosteric activator ADP (Nishida & Yielding, 1970), although the cysteine residue was not identified. Cysteine-319 was shown to react with *p*-mercuribenzoate (Cosson et al., 1976), and methylmercuric chloride decreases the incorporation of radioactive *p*-mercuribenzoate (Cosson & Pantaloni, 1976). Furthermore, it was observed that glutamate dehydrogenase labeled at Cys-319 with *p*-mercuribenzoate mimics some regulatory properties normally associated with ADP (Cosson et al., 1978). In none of these studies, however, were any regulatory compounds evaluated for their ability to protect against modification by mercurials; nor were the mercurial-enzymes tested for their sensitivity to inhibition by DPNH.

An interaction between the ADP and DPNH inhibitory sites has been reported on the basis of various binding studies that show that ADP competes with inhibitory concentrations of DPNH (Pantaloni & Dessen, 1969; Cross & Fisher, 1970; Koberstein & Sund, 1973; Koberstein et al., 1973; Pantaloni & Lecuyer, 1973), although chemical modification studies indicate that the ADP and DPNH sites are not identical (Pal et al., 1975). We have observed that the 6-BDB-TADP-modified glutamate dehydrogenase exhibits a decrease in the maximum activation by ADP but have concluded that the site of attack by 6-BDB-TADP is the DPNH regulatory site and that the altered response to ADP activation is an indirect result of modification of the reduced coenzyme site (Batra & Colman, 1984). Thus, our conclusions about the effect of reaction of Cys-319 with 6-BDB-TADP are not inconsistent with the reported characteristics of the enzyme modified at Cys-319 with mercurials.

The adenine nucleotide analogue 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (5′-FSBA) has also been shown to react at the DPNH inhibitory site of glutamate dehydrogenase (Pal et al., 1975; Saradambal et al., 1981) and to label in equal amounts Tyr-190 and Lys-420 (Schmidt & Colman, 1984). If one assumes that 5′-FSBA is bound in an extended conformation, in analogy to that often assumed by bound DPNH in dehydrogenases, then the reactive sulfonyl fluoride moiety will occupy the enzyme subsite for the nicotinamide ribose or the pyrophosphate. Both Tyr-190 and Lys-420 may be in this region of the DPNH subsite. In contrast, for 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate, since the reactive bromoketo moiety is located adjacent to the 6-position of the purine moiety, it would be expected that reaction would occur with an amino acid residue (identified now as Cys-319) that participates in the adenine subsite of the DPNH inhibitory site. Thus, affinity labeling has strongly implicated three amino acid residues from different parts of the linear amino acid sequence as participants in the binding site of one regulatory nucleotide, DPNH. This type of data may provide important constraints for assessing in solution the three-dimensional structure of glutamate dehydrogenase.

On the basis of secondary structure predictions from the known primary sequence and comparisons with the coenzyme binding domains previously identified in other dehydrogenases (Rossmann et al., 1975), Wootton (1974) and Austen et al. (1980) have predicted two coenzyme binding domains in bovine glutamate dehydrogenase: (1) consisting of residues 9–128 and (2) consisting of residues 245–356. The two amino acid residues Tyr-190 and Lys-420, identified by reaction with 5′-FSBA as part of the DPNH inhibitory site (Schmidt & Colman, 1984), are not included in either of the two predicted coenzyme binding domains. One possibility suggested by Schmidt and Colman (1984) is that the DPNH binding site labeled by 5′-FSBA is an allosteric site and hence may differ from the active sites of dehydrogenases studied by X-ray crystallography (Rossmann et al., 1975). In the present study, the implication of Cys-319 (which is included in the second predicted coenzyme domain) indicates that the inhibitory DPNH site of glutamate dehydrogenase includes at least part of the predicted coenzyme binding domain.

Registry No. EC 1.4.1.3, 9029-12-3; 6-BDB-TADP, 90269-32-2; DPNH, 58-68-4; L-Cys, 52-90-4.

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Enthalpy and Heat Capacity Changes for Formation of Compound I with Horseradish Peroxidase and of Complexes with Benzhydroxamic Acid and Benzhydrazide

Hiroshi Nakatani*
Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
Katsutada Takahashi
Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan
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ABSTRACT: Calorimetric measurements were performed on the formation of compound I and of complexes of horseradish peroxidase with benzhydroxamic acid and benzhydrazide at pH 7.0 and at 9.4, 15, and 25 °C. The enthalpy and heat capacity changes for the formation of compound I are −111.2 kJ/mol and −1.75 kJ/(K mol) at 25 °C, respectively. By combining these results with other available data for related reactions, it can be shown that the heme-coordinated oxygen atom is energetically stabilized only about 10 kJ/mol compared to the oxygen atom in its molecular state. Thermodynamic quantities for the binding of benzhydroxamic acid to the enzyme were determined from calorimetric results and equilibrium titration data. At 25 °C, the values are $\Delta G°$ = −31.1 kJ/mol, $\Delta H°$ = −58.3 kJ/mol, $\Delta S°$ = −0.09 kJ/(K mol), and ΔC_p = 0.02 kJ/(K mol). The corresponding values for the binding of benzhydrazide to peroxidase are $\Delta G°$ = −21.7 kJ/mol, $\Delta H°$ = −42.4 kJ/mol, $\Delta S°$ = −0.07 kJ/(K mol), and ΔC_p = 0.94 kJ/(K mol) at 25 °C. It is evident that the most striking difference between the two processes is the large difference in their heat capacity changes.

Compound I is an active intermediate formed during reaction between native peroxidase and hydrogen peroxide or organic peracids:

$$E + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

where E is native peroxidase (Dunford & Stillman, 1976). Compound I formation reactions with hydrogen peroxide or aromatic peracids are very fast. The bimolecular rate constants are around diffusion controlled (Dunford & Hewson, 1977; Nakatani & Dunford, 1979). During reaction, an oxygen atom is trapped into the heme iron, which is then converted to ferryl from ferric, and the cation radical of hemin is produced (Fujita et al., 1983). Compound I reacts as an electron acceptor with second substrates (Dunford & Stillman, 1976). Although many spectroscopic studies of compound I have been reported, its formation has not as yet been thermodynamically characterized. Compound I is not a very stable intermediate, but its formation is practically irreversible. This enables the enthalpy change in the reaction to be determined by direct calorimetric measurement, although the Gibbs energy data cannot be readily determined.

Specific ligands such as aromatic hydroxamic acids and azides bind strongly to horseradish peroxidase (Shonbaum, 1973). Their dissociation constants are measurable by spec-

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