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ISOLATION OF A HEME CREVICE PEPTIDE FROM AFFINITY-LABELED HORSERADISH PEROXIDASE

Lynn C. Yeoman[+] and Lowell P. Hager

Biochemistry Department
School of Chemical Sciences
University of Illinois
Urbana, Illinois 61801

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SUMMARY

Apo-horseradish peroxidase was affinity-labeled with the monosulfuric anhydride derivative of mesoheme. The stoichiometry of heme anhydride binding was 1.1 moles of the anhydride per mole of apo-peroxidase.

Tryptic digestion of the affinity-labeled peroxidase yielded a major lysine peptide which corresponded in composition to peptides T8 and T9a in the sequence of horseradish peroxidase (Welinder, K. G., Eur. J. Biochem. 96: 483-502, 1979) which contained one mole of histidine (histidine 170) per mole of peptide.

INTRODUCTION

The use of enzymes reconstituted from apoenzymes and a variety of prosthetic groups has proved to be a valuable approach in the description of heme protein active sites (1,2). Spin-labeled protoheme has been used as a reporter molecule to map the character of the heme environment of hemoglobin, myoglobin, cytochrome c peroxidase and horseradish peroxidase (3). Another

[+]Present address: Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030. Author to whom correspondence should be addressed.

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heme derivative method developed by Warme and Hager (4,5) employs the sulfuric anhydride of mesoheme as applied to sperm whale myoglobin and cytochrome b_{562}.

With the completion of the amino acid sequence of horseradish peroxidase (6), the sulfuric anhydride method for affinity-labeling and its application to horseradish peroxidase can provide information regarding the peptide segments within the heme crevice of the reconstituted enzyme. The ease of reconstitution (2) and peroxidatic properties of this molecule make it a good system in which to introduce a covalently attached prosthetic group. The affinity-labeling of horseradish peroxidase and the composition of a major covalent heme-peptide product are described.

MATERIALS AND METHODS

Preparation of Apo-horseradish Peroxidase - Horseradish peroxidase was purchased from Sigma Chemical Co. (St. Louis, Mo.) and displayed absorbancy ratios (A_{403}/A_{275}) of 2.90 to 3.10. The natural prosthetic group was removed by Teale's acid-butanone method (7).

Reconstitution of Apo-horseradish Peroxidase with Protoheme and Mesoheme Monosulfuric Anhydride - Apo-horseradish peroxidase (0.7 nM) in 1.0 ml of 10 mM $NaHCO_3$ was mixed with 3.0 nmoles of a fresh protoheme solution in 0.1 M NaOH and allowed to stand for 10 minutes at 0° (2). The monosulfuric anhydride of mesoheme was preferentially extracted from crude mixtures of mono- and bis-sulfuric anhydride derivatives as described by Warme and Hager (4). Apo-peroxidase was reconstituted batchwise with a 10% molar excess of mono-anhydride. Covalent linkage was facilitated by incubating at pH 8.2 at 25° for a period of one hour with gentle stirring.

Isolation of Heme Peptides from Affinity-labeled Horseradish Peroxidase - Affinity-labeled horseradish peroxidase was denatured and carboxymethylated according to the method of Hirs (8). The carboxymethylated protein was dissolved in 0.1 M Tris-HCl (pH 8.0) and 0.01 M $CaCl_2$ to give a final concentration of 2% protein (W/V). A total of 10% by weight of trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added in 1 mM HCl initially and at 6 hours. The digestion was carried out at 37° and terminated at 24 hours by freezing. Tryptic heme peptides were purified by high voltage paper electrophoresis in pyridine-acetate buffers

at pH 3.6 and at pH 6.4. Final purifications were achieved by thin layer chromatography on Eastman Chromogram precoated silicic acid sheets (Rochester, N. Y.). Thin layers were developed in either n-butanol-pyridine-acetic acid-water (15:10:3:12) or n-butanol-acetic acid-water (2:1:1). Heme peptides were eluted from paper and silicic acid with 50% pyridine in water.

Amino Acid Analyses - Amino acid compositions of purified heme peptides were determined by the two-column methodology developed by Spackman et al (9) on a Beckman Model 120 amino acid analyzer. Peptides were hydrolyzed at 110° for 21 hours with 5.7 N HCl.

RESULTS

Reconstitution of Apo-horseradish Peroxidase with Mesoheme Monosulfuric Anhydride - Table I demonstrates that apo-horseradish peroxidase retained less than 0.1 percent of its native peroxidatic activity after acid-butanone treatment. Fig. 1 shows that the monosulfuric anhydride of mesoheme can be used to reconstitute a holohorseradish peroxidase. A plot of the increase in absorbance at 392 nm with the addition of equivalents of heme anhydride is shown in Figure 1. A biphasic plot is obtained showing an apparent equivalence point at 4.1 nanomoles of titrant. This equivalence point corresponds to 1.1 moles of heme per 1.0 mole of apo-horseradish peroxidase and agrees with the stoichiometry of 1.0 mole of heme per 1.0 mole of enzyme (2).

The same sample of apo-horseradish peroxidase (0.6 μmole) was reconstituted with 1.3 μmole of mesoheme monusulfuric anhydride, which represents a slightly greater than two-fold excess of heme anhydride.

Non-covalently bound heme was extracted from the affinity-labeled product by the acid-butanone method used to initially prepare the apo-peroxidase. Approximately 30% of the bound heme was extracted with this procedure, which was shown to be

TABLE I

RECONSTITUTION OF APO-HORSERADISH PEROXIDASE WITH PROTOHEME

Sample	Specific Activity (Units/mg)	Percent Activity
Horseradish Peroxidase	765	100
Apo-horseradish Peroxidase	<1	-
Reconstituted Horseradish Peroxidase	695	91

TABLE I A 0.74 nmole sample of apo-horseradish peroxidase was reconstituted in 1.0 ml of 10 mM $NaHCO_3$ by the addition of 3.0 nmole of protoheme. The protoheme solution was prepared immediately before use and the samples all assayed by the o-dianisidine assay in the presence of hydrogen peroxide (10). Specific activities were calculated from linear changes in absorbance at 460 nm which occurred over a 30 second period per 1.0 mg of enzyme in 1.0 ml of assay mixture.

99 percent efficient in the removal of heme from native horseradish peroxidase (Table I). Additional acid-butanone treatments did not remove significant additional amounts of heme.

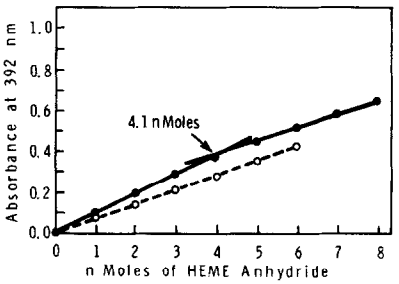

Figure 1 Titration of Apo-horseradish Peroxidase with Mesoheme Monosulfuric Anhydride Extract. A 3.7 μM solution of apo-horseradish peroxidase in 1.0 ml of 10 mM $NaHCO_3$ was titrated in 20 μl increments with the pH 7 extract containing predominantly the monosulfuric anhydride derivative. The increase in extinction at the 392 nm Soret band was plotted versus the equivalents of heme anhydride added. The upper plot (●——●) is the biphasic reconstitution titration and the lower (o-----o) is the buffer blank titration.

Isolation of a Major Heme Peptide from a Tryptic Digest of Affinity-labeled Horseradish Peroxidase - Affinity-labeled horseradish peroxidase was carboxymethylated and digested with TPCK-treated trypsin. Peptide hydrolysates were purified by paper electrophoresis and thin layer chromatography. The amino acid composition of a heme peptide obtained in large yield (II-II) is presented in Table II. It is compared with the amino acid composition of two tryptic peptides (T8 and T9a) which are adjacent in the sequence of horseradish peroxidase (residues 160-178) and are thought to be in the heme crevice of the native enzyme (6,11).

DISCUSSION

Apo-horseradish peroxidase has been affinity-labeled with the monosulfuric anhydride of mesoheme. The reconstituted enzyme took up 1.1 moles of mesoheme monosulfuric anhydride per 1.0 mole of apo-enzyme. Heme extraction procedures revealed that the heme was covalently attached to the protein since repeated acid-butanone treatments could not reduce the R_Z value below 0.9. An R_Z value of 2.46 was measured after a single heme extraction. The covalent binding of heme suggested that electrostatic interactions between the heme propionic acid groups and lysine ϵ-amino groups (lysine 174) play a significant role in the binding of heme to horseradish peroxidase.

A major heme peptide was isolated from tryptic digests of affinity-labeled horseradish peroxidase. The tryptic peptide compositions of the entire horseradish peroxidase sequence were examined (6). Tryptic peptide T8 (residues 160-174) and the arginine containing peptide T9a (residues 175-178) were

TABLE II

AMINO ACID COMPOSITION OF THE MAJOR HEME PEPTIDE FRAGMENT FROM TRYPSIN DIGESTED, AFFINITY-LABELED HORSERADISH PEROXIDASE

Amino Acid	Tryptic Peptides	
	II-II	T8 + T9a
Lys	1.4	1
His	0.5	1
Arg	0.4	1
Asp	2.2	2
Thr	0.9	1
Ser	1.8	3
Glu	2.7	1
Pro	0.9	-
Gly	2.5	3
Ala	1.3	1
Val	0.8	1
Met	-	-
Ile	0.4	-
Leu	1.4	2
Tyr	0.1	-
Phe	0.7	1

TABLE II The values presented represent moles of amino acid per mole of heme based on $\epsilon_{520} = 7.0\ mM^{-1}cm^{-1}$ for mesoheme in 50% pyridine. Both methionine and tryptophan were destroyed in this analysis. Cysteine was not quantitated in this analysis. Peptide II-II was obtained in 28% and 6% final yield from pH 3.6 and pH 6.4 paper electrophoresis, respectively. Additional purification by TLC in n-butanol-acetic acid-water (2:1:1) did not generate additional heme-peptide species. The values presented are not corrected for background since analyses of equivalent areas of the thin layer plate which did not contain peptide were found to contain insignificant amounts of amino acids.

the only tryptic peptides that fit the observed composition. Peptides T8 and T9a are thought to be at or near the heme crevice of this enzyme (6,11). Peptide T8 contains one histidine residue (histidine 170) which is now considered to be the fifth axial ligand of the iron from the ESR studies of Yonetani et al (12) and the photo-oxidation studies of Mauk and Girotti (11). Peptide II-II of this study and "Peptide I" in which Mauk and Girotti have demonstrated an oxidized histidine have the same amino acid composition (11). This evidence would support the localization of Welinder's peptide T8 and T9a (6) at or near the heme binding site of horseradish peroxidase. Lysine 174 is the most likely site of heme attachment since it offers the potential for an electrostatic interaction with one of the propionate side chains of the heme group.

The attachment of the heme anhydride to lysine 174 would be expected to prevent the tryptic cleavage at lysine 174. Hence, the measurement of 1.2 additional aspartic acid residues, 2.7 additional glutamic acid residues and 0.4 additional arginine residues not accounted for in the composition of tryptic peptide T8 is explained by the presence of residues 175-178 (Asn-Gln-Cys-Arg) of peptide T9a at the carboxy terminus of peptide T8 (6). This observation, therefore, provides further support for the attachment of the heme anhydride to the ϵ-amino group of lysine 174.

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