PREPARATION AND CHARACTERIZATION OF AN IMMUNOELECTRON MICROSCOPE TRACER CONSISTING OF A HEME-OCTAPEPTIDE COUPLED TO Fab*

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Molecules possessing peroxidatic activity have found wide usefulness as tracers in electron microscope studies by virtue of the simplicity of performing the histochemical reaction for their detection and the ease of visualizing the reaction product, which usually consists of oxidized diaminobenzidine rendered electron opaque by interaction with osmium (9). Due to the enzymatic nature of the histochemical reaction, the sensitivity of tracer site detection is enhanced compared to techniques where inert tracers such as ferritin or small plant viruses are used. The most commonly used molecules of this type have been horseradish peroxidase (mol wt 40,000) (9), myoglobin (mol wt 17,000) (25), and cytochrome c (mol wt 12,700) (12).

Used by themselves these molecules have served as probes for permeability barriers and as tracers for inter- and extracellular spaces. They have also been coupled covalently to molecules of biologic interest such as antibodies, plant lectins, and hormones where they serve as tags for detection of sites of receptor-ligand interactions (1, 19, 23).

Since all the peroxidatic molecules mentioned possess a Stokes radius $>\sim 15 \AA$ their ability to probe permeability barriers whose pore sizes are below this range is limited. In addition, due to their relatively large size they can be expected to alter the biological activity of smaller ligands to which they may be coupled. This is particularly important for immunocytochemical techniques in which tagged antibodies are required to penetrate several diffusion barriers, such as the plasma membrane and membranes bounding intracellular compartments before interacting with antigenic sites.

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Recently a heme-undecapeptide (11-MP) with a mol wt of 2,000 daltons has been obtained from cytochrome c by limited proteolysis. This molecule which retains peroxidatic activity has been used by Feder as a tracer for permeability studies at the electron microscope level (7, 8). Because of its smaller size (<15 Å in diameter) it should extend the range of pore detection in permeability studies, should result in less alteration of the biologic activity of ligands to which it is coupled, and, in particular, should decrease problems of penetration of tagged antibodies in immunocytochemical studies.

11-MP consists of a heme covalently bound through thioether linkages to a peptide backbone of 11 amino acids. Since the peptide possesses an amino terminal sequence of NH₂-Val-Gln-Lys, random reaction products between the α- and ε-amino groups, and amino groups on acceptor molecules can be expected to occur when covalent coupling is carried out through bifunctional reagents reacting with amino groups. To overcome this problem, we have improved on a procedure to prepare a heme-octapeptide which is derived from the heme-undecapeptide by trypsin cleavage at the lysyl residue (10). This smaller molecule, which carries a single reactive N-terminal amino group, has been successfully coupled to antibody fragments, using a novel two-step conjugation procedure. The coupling reaction is carried out under precisely controlled conditions which result in the formation of a well-defined product in high yield.

Materials and Methods

Materials.—

Reagents:— Horse heart cytochrome c type III and N-hydroxysuccinimide were obtained from Sigma Chemical Co., St. Louis, Mo. and N,N'-dicyclohexyl-carbodiimide (CDI) and p-formylbenzoic acid from Aldrich Chemical Co., Inc., Milwaukee, Wis. Pepsin (EC 3.4.4.1) twice crystallized, trypsin (EC 3.4.4.4) twice crystallized, and papain (EC 3.4.4.10) twice crystallized were obtained from Worthington Biochemical Corp. Freehold, N. J. Other reagents were obtained from the following sources: Sephadex and Sepharose, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; Biogel A 50m, 50–100 mesh, Biogel A 0.5m, 200–400 mesh, and Biogel P6, Bio-Rad Laboratories, Richmond, Calif.

Antigens:— Goat Fab and rabbit Fab were prepared by published procedures (14, 22). Rabbit secretory IgA was prepared from rabbit milk obtained during the first 5 days of lactation (22).

Methods:—

Immunization procedures:— Rabbit antigoat Fab serum and sheep antirabbit Fab serum were produced by weekly injections for 2 mo of 2.5 mg of antigen in complete Freund's adjuvant. Goat antirabbit secretory IgA serum was obtained by 4 weekly injections of 2.5 mg of antigen in complete Freund's adjuvant.

Abbreviations used in this paper: CCD: countercurrent distribution; CDI: N,N'-dicyclohexylcarbodiimide; DAB: 3,3'-diaminobenzidine; 8-MP: 8-microperoxidase or heme-octapeptide; 11-MP: 11-microperoxidase or heme-undecapeptide; SDS: sodium dodecyl sulfate.
Preparation of immunoadsorbents: Antigens were insolubilized on Sepharose 4B or 2B or on Biogel A 50m using published procedures (4).

Preparation of specific antibodies or antibody fragments: 10 ml of specific antiserum or 10 ml of specific antiserum digested with papain were incubated for 2 h at room temperature with 4 g of immunoadsorbent (wet weight). After washing the adsorbent extensively with 0.1 M Tris-HCl, pH 7.5 until the A280 nm was <0.005, the adsorbed antibodies were eluted with 3.0 M KSCN (recrystallized) containing 0.15 M KCl in 0.01 M phosphate buffer, all adjusted to pH 6.0 with HCl. The eluted antibodies (or Fab) were concentrated to 10 mg/ml of protein by ultrafiltration on PM 10 Diaflo membranes (Amicon Corp., Lexington, Mass.) and chromatographed on Biogel A 0.5m. (Sephadex G-100 for Fab) columns, previously conditioned with 1% bovine serum albumin and equilibrated with 0.1 M Tris-HCl, pH 7.5. The columns were 100 cm in length and 1 or 2 cm in diameter depending on the total load of proteins.

Amino acid analysis: After acid hydrolysis, amino acid analysis was performed on a Beckman Model MS instrument (Beckman Instruments, Inc., Fullerton, Calif.) (18). For cysteine determinations, the heme-peptide was freed of the heme group by the method of Paul (21). The remaining peptide was desalted on Sephadex G-25 and the peptide peak was lyophilized. Cysteine was determined as cysteic acid by the method of Hirs (11).

Countercurrent distribution (CCD): CCD was performed on an automatic apparatus containing 1,000 tubes. A two-phase system consisting of butanol, pyridine, and 0.1% acetic acid (5:3:11) was used with 3 ml for the lower phase and 5 ml for the upper phase (3).

Enzyme kinetics: Peroxidatic activity was measured spectrophotometrically at 460 nm with 0.3 mM o-dianisidine as hydrogen donor and hydrogen peroxide as substrate at concentrations specified under results. The reaction was carried out in 0.01 M phosphate buffer, pH 7.0 for 8-microperoxidase (8-MP) and the conjugate, and at pH 6.0 for horseradish peroxidase and cytochrome c. A molar extinction coefficient at 460 nm of $1.13 \times 10^4$ M$^{-1}$ cm$^{-1}$ was used to convert amount of dye oxidized to moles of H$_2$O$_2$ decomposed (29).

Other analytical techniques: Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with a gradient of polyacrylamide ranging from 13% to 5% in a discontinuous buffer system (16). The gel slab (15 X 15 X 0.3 cm) was run for 17 h at 10 mA. Molecular weights were determined by comparison with protein standards.

Double immunodiffusion were performed according to Ouchterlony (20) and immunoelectrophoresis according to the micromethod of Scheidegger (24). Protein concentrations were measured spectrophotometrically using the following extinction coefficients: $E_{280}$ = 13.5 for rabbit secretory IgA (2), 11 for Fab, 12 for IgG.

Gel filtration of the conjugate for determination of the molecular radius (Stokes radius) (15) was performed on Sephadex G 100 columns (1 X 95 cm) and the data are expressed as $K_0 = (V_e - V_0)/(V_i - V_0)$, where $V_e$ is the elution volume corresponding to the peak concentration of the solute and $V_o$ the void volume (30 ml) and $V_i$ the total volume of the gel bed (85 ml).

Immunocytochemical Procedures:

Fixation schedule: Small fragments (1 X 1 X 3 mm) of mammary gland tissue were fixed at room temperature for 4 h in 4% formaldehyde buffered with 0.1 M Na cacodylate pH 7.5 containing 0.2% picric acid. The pieces were rinsed overnight in 0.1 M phosphate buffer pH 7.5. Nonfrozen sections, 50-µm thick, were obtained with a Smith-Farquhar tissue sectioner. Residual aldehyde groups in the tissue were reduced by incubation with 0.1 M Na borohydride in 0.1 M phosphate buffer pH 7.5 for 30 min. The sections were incubated at room temperature for 8 h in the first-step reagent consisting of goat Fab antirabbit secretory IgA dialyzed against 0.1 M phosphate buffer pH 7.5 and containing 1 mg/ml of specific antibodies. After an overnight rinse in phosphate buffer, the sections were incubated for 8 h at room temperature in the 8 MP-rabbit Fab-antigoat Fab solution (2 mg/ml) and rinsed over-
night at 4°C in phosphate buffer. After a 1-h fixation at room temperature with 2% glutaraldehyde in 0.1 M phosphate pH 7.5 (in order to immobilize the immunological reagents), the sections were preincubated 1 h at room temperature in 0.1 M Tris-HCl pH 7.0 buffer containing 0.5 mg/ml DAB followed by 30-45-min incubation at 37°C in 0.1 M Tris-HCl, pH 7.0 containing 0.5 mg/ml DAB and 2 × 10⁻² M H₂O₂. The sections were then postfixed in 2% OsO₄ dehydrated in a graded series of alcohol and propylene oxide and flat embedded in Epon.

**Controls:** Sections incubated with nonspecific Fab as a first-step reagent were devoid of peroxidatic reaction product, except for that contained in erythrocytes and granulocytes.

**RESULTS**

*Preparation of the Heme-Octapeptide.*

Proteolytic digestion of cytochrome c: 20 g of cytochrome c were dissolved in 800 ml of 0.1 N HCl containing 300 mg of pepsin. The solution was incubated at room temperature for 24 h, then adjusted to pH 7.0 with 2 N NaOH and lyophilized. The powder was dissolved in 20 ml of H₂O and 10-ml aliquots were chromatographed on a Biogel P 6 column (4 × 100 cm), equilibrated with 0.1 M NH₄HCO₃, pH 8.0. The elution profile is shown in Fig. 1a. The main peak contains the heme-undecapeptide (11-MP). It is separated from a small

![Elution profiles of proteolytic digestion mix of cytochrome c from a Biogel P 6 column (4 × 100 cm), equilibrated and eluted with 0.1 M NH₄HCO₃ 4°C; flow rate 20 ml/h. The voided and included volumes were 300 and 1,100 ml, respectively. A₂₈₀ nm recorded with an LKB Uvicord II (LKB Instruments, Inc., Rockville, Md.). The heme content was measured at A₄₂₃ nm for each fraction. (a) Pepsin digestion mix. The main peak, containing 11-MP, has a Kₐₘ = 0.06. (b) Trypsin digestion of the main peak from profile shown in a. Kₐₘ of the heme-containing peak is 0.2.](https://example.com/fig1.png)
peak eluted with the void volume which consists of undigested cytochrome c and pepsin. Smaller heme-free peptides are eluted later.

The heme-peptide peaks from several runs were pooled, lyophilized, and dissolved in 50 ml of 0.1 M NH₄HCO₃, pH 8.0. Trypsin was added at a concentration equivalent to 10% of the weight of the heme-peptide, and the solution was incubated for 36 h at 37°C. At 12-h intervals, fresh trypsin was added (5% of the weight of the heme-peptide). The digestion mixture was then lyophilized, dissolved in 10 ml of H₂O, and chromatographed on the same column of Biogel P 6. As shown on Fig. 1 b, the main peak is shifted to the right and is partially separated from undigested 11-MP (left-hand shoulder) and smaller peptides eluted later. This red peak was collected and lyophilized. Amino acid analysis on this fraction indicated that the main constituent was the heme-octapeptide (8-MP).

**Purification of the heme-octapeptide:**  2.5 g of the material obtained from the final Biogel P 6 run was further purified by CCD in the system mentioned under Material and Methods. 2,183 transfers were performed. Each 10th tube (for the peak regions each tube) was measured spectrophotometrically at 423 nm and aliquots were taken for ninhydrin determinations (Fig. 2). The main peak which contains the 8-MP as determined by amino acid analysis has a partition coefficient of 0.20; its profile fits the calculated theoretical

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![CCD profile](image-url)

**Fig. 2** CCD profile. The content of the main peak shown on Fig. 1 b was loaded on a two-phase system consisting of butanol:pyridine: 0.1% acetic acid and 2,183 transfers were performed. The heme content was determined by spectrophotometric measurements at 423 nm and the protein content by ninhydrin reaction. The partition coefficient of the 8-MP peak is 0.20.
curve. It is well separated from 11-MP and from heme-free peptides. The peak was concentrated to dryness by flash evaporation. Amino acids represent 55% of the dry weight of the compound and the composition was as follows: His: 1.01, Thr: 0.98, Glu: 2.15, Ala: 1.01, Val: 1.00, Cys: 1.8. No other amino acids were detected. A single band was observed after paper electrophoresis at pH 2.0 in 20% acetic acid (2 h at 10 V/cm).

Properties of the heme-octapeptide: The presumed structure of the heme-octapeptide is shown in Fig. 3 which is based on published data (10, 17, 28). The molecule consists of a heme (mol wt 613) bound by thioether linkages to the cysteines of the octapeptide (mol wt 944). Its mol wt is 1,555 daltons. The Soret absorption spectrum is characterized by maxima at 360 nm, 405 nm, and 525 nm in the oxidized form. The extinction coefficient $E_{428\text{nm}}$ is 125 and the ratio $A_{428\text{nm}}/A_{380\text{nm}}$ is 3.0.

The peroxidatic activity of the heme-octapeptide was determined using o-dianisidine as hydrogen donor and H$_2$O$_2$ as substrate at concentrations ranging from $10^{-2}$ M to $5 \times 10^{-1}$ M (Table I). 8-MP was maintained at 3 ng/ml in the reaction mixture. An apparent $K_m$ of 0.2 M and an apparent $v_{max}$ of 4 mmol/min per mg of heme-octapeptide were calculated from a double reciprocal (Lineweaver-Burk) plot (Fig. 4). As illustrated in Fig. 5, the pH optimum is 7.0.

Conjugation Procedure.—The overall conjugation procedure consists of two steps which are outlined in Fig. 6.

Derivatization of 8-MP: (a) Preparation of the $N$-hydroxysuccinimide ester of $\rho$-formylbenzoic acid: 4.5 g of $\rho$-formylbenzoic acid and 3.5 g of $N$-hydroxysuccinimide were dissolved in 15 ml of dimethylformamide, which was then diluted with 40 ml of CH$_2$Cl$_2$. After cooling to 0°C, 6.18 g of dicyclohexylcarbodiimide in 6 ml CH$_2$Cl$_2$ was added. After 5 h, dicyclohexyl urea was removed by filtration and filtrate was concentrated to dryness under high vacuum.
TABLE I

Peroxidatic Activity of Free and Conjugated Heme-Octapeptide

|       | 8-MP        | 8-MP-Fab   |
|-------|-------------|------------|
|       | ΔA 460 nm/min | ν0        | ΔA 460 nm/min | ν0       |
| 2 × 10^-2 | 0.010       | 0.295      | 0.010         | 0.0195   |
| 4 × 10^-2 | 0.019       | 0.56       | 0.0195        | 0.0385   |
| 6 × 10^-2 | 0.030       | 0.85       | 0.024         | 0.049    |
| 8 × 10^-2 | 0.036       | 1.05       | 0.034         | 0.067    |
| 1 × 10^-1 | 0.042       | 1.25       | 0.045         | 0.089    |
| 2 × 10^-1 | 0.056       | 1.67       | 0.074         | 0.145    |
| 5 × 10^-1 | 0.078       | 2.30       | 0.025         | 0.250    |

* Δ: moles/liter H₂O₂.

† ΔA 460 nm/minute for 3 ng 8-MP or 45 ng of 8-MP-Fab/milliliter reaction mixture.

§ ν0: millimoles H₂O₂ decomposed/minute per milligram 8-MP. Conversion of dye oxidized to moles of H₂O₂ decomposed is based on a molar extinction coefficient at 460 nm of 1.13 × 10⁴ M⁻¹ cm⁻¹.

Fig. 4 Double-reciprocal (Lineweaver-Burk) plot of 1/ν0 versus 1/[s] for the peroxidatic reaction catalyzed by 8-MP (●) or by 8-MP-Fab (○) at pH 7.0 and 25°C. Values of 1/[s] and 1/ν0 were calculated from data listed in Table I. Each point is the results of three determinations.

in a rotary evaporator. The residue was dissolved in 200 ml of boiling isopropanol, hot filtered, and cooled yielding 3.7 g of the N-hydroxy-succinimide ester of p-formylbenzoic acid (mp 164–166). Elemental analysis indicated the following composition: C 58.37, H 3.82, N 5.64; the calculated composition is C 58.30, H 3.67, N 5.67.

(b) Coupling of the ester to 8-MP: 10 mg of the N-hydroxysuccinimide
Fig. 5 pH optima of 8-MP and 8-MP-Fab. Aliquots of enzymes were incubated at 25°C in substrate solution containing 0.33 mg/ml o-dianisidine, and 2 × 10⁻³ M H₂O₂ in 0.01 M Na phosphate buffer pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.

Fig. 6 Flow sheet for conjugation of 8-MP to Fab fragments. (I) The first step consists of the preparation of N-hydroxysuccinimide ester of p-formylbenzoic acid with dicyclohexyl-carbodiimide (CDI) and its coupling to the N-terminal amino group of the heme-octapeptide, in pyridine. (II) In the second step, the aldehyde group on the derivatized 8-MP is allowed to form a Schiff base with the amino group on the Fab fragment in borate buffer, pH 9.5.
Esther of \( \rho \)-formylbenzoic acid in 500 \( \mu l \) of pyridine was added to 500 \( \mu l \) of pyridine containing 10 mg of 8-MP. The reaction mix was kept at room temperature for 12 h. The solution was then chromatographed on a column (1 X 100 cm) of Sephadex G-25. The fraction containing the derivatized 8-MP was collected and lyophilized and stored at \(-20^\circ C\). The efficiency of coupling was determined by paper electrophoresis in 20\% acetic acid. Approximately 80\% of the 8-MP was coupled as judged by slower mobility when compared with free 8-MP.

**Coupling of the derivatized 8-MP to proteins:** 5 mg of derivatized 8-MP was added to 10 mg of protein (sheep or rabbit Fab) in 1 ml borate buffer \( \mu = 0.1, pH 9.5 \) and the solution was incubated for 5 h at 37\(^\circ C\). The Schiff bases formed between the aldehyde group on the derivatized 8-MP and the amino groups of the protein were subsequently reduced by adding 100 \( \mu l \) of 0.1 M sodium borohydride. This reaction was carried out for 15 min at room temperature.

**Purification of the Conjugate.**—Unreacted 8-MP and unconjugated protein were removed by successive gel filtration and ion exchange chromatography. First the solution containing the conjugate was chromatographed on Sephadex G-100 (1 \( \times \) 95 cm) in 0.1 M Tris-HCl, pH 7.4 to give the elution profile shown in Fig. 7. The conjugate and free Fab are contained in the second peak \( (K_v = 0.27) \) which is well separated from aggregates excluded from the column, and from free 8-MP, eluted with the included volume. Separation of free Fab from the conjugate was achieved by chromatography on DEAE-cellulose

![Fig. 7 Elution profile of unpurified conjugate (8-MP-Fab) from a Sephadex G-100 column (1 X 95 cm), equilibrated and eluted with 0.1 M Tris-HCl, pH 7.5, at 4\(^\circ C\); flow rate 4 ml/h. The voided and included volume were 30 and 85 ml, respectively. \( A_{280} \) nm recorded with an LKB Uvicord II. The heme content was measured at \( A_{423} \) nm for each fraction.](image)
(1 × 5 cm) equilibrated with 0.01 M phosphate buffer, pH 7.4. Free Fab was eluted with running buffer, whereas the conjugate, by virtue of the negative charges of the 8-MP, remained in the column and was eluted with 1.5 N KCl.

**Properties of the Conjugate.**—We have found, by spectrophotometric analysis, that on the average two molecules of 8-MP are attached to each Fab molecule under our conditions. The molecular weight of the conjugate (8-MP-Fab) and free Fab was determined by SDS polyacrylamide gel electrophoresis, using bovine serum albumin, ovalbumin, trypsin, ribonuclease, and cytochrome c as markers. Sheep Fab has a molecular weight of ~40,000 daltons and 8-MP-sheep Fab ~45,000 daltons whereas the molecular weight of rabbit Fab is ~45,000 daltons and 8 MP-rabbit Fab ~50,000 daltons. The Stokes radius of the conjugate (8 MP-rabbit Fab) is 32 Å and of free rabbit Fab 28 Å as calculated from gel filtration experiments according to the correlation between $(K_a$, and Stokes radius determined by Laurent and Killander (15). Proteins of known Stokes radius (cytochrome c, trypsin, ovalbumin, horseradish peroxidase, and bovine serum albumin) were chromatographed on the same column.

The peroxidatic activity of the conjugate was examined with the same assay used to characterize 8-MP. The velocity of the reaction at different substrate concentrations is given in Table I. The apparent $V_{max}$ of the conjugate is 0.4 mmol/min per mg of attached heme-octapeptide and its $K_m$ is 0.4 M (Fig. 4). The pH optimum of the conjugate is 7.0 (Fig. 5).

When the conjugate (8-MP-Fab) is subjected to immunoelectrophoresis, a single line is formed against anti-Fab serum. This is slightly shifted to the anode when compared to that formed with free Fab due to the negative charges of the attached heme-octapeptide. In addition, the precipitin lines formed between the conjugate and the appropriate antiserum stain intensively when incubated with diaminobenzidine and hydrogen peroxide. Quantitation of the antigen-binding capacity of the conjugate was obtained using immunoadsorbents containing the appropriate insolubilized antigen. When 5 mg of conjugate (8-MP-rabbit Fab antigoat Fab) were applied to 1 g (wet weight) of goat IgG-agarose, 3.5 mg were retained on the adsorbent indicating 70% retention of antigen-binding activity. A control adsorbent consisting of insolubilized rabbit IgG did not bind the conjugate.

**Use of the Conjugate for Immunoelectron Microscopy.**—The conjugate was used for the localization of rabbit secretory IgA in the mammary gland of lactating rabbits. The immunocytochemical procedure consists of an indirect localization sequence, with goat Fab directed against rabbit secretory IgA as the first-step reagent. These antibodies react with several antigenic components on the secretory IgA molecule (2), including determinants of the heavy chains and the secretory components and determinants of the light chains which are common to all immunoglobulins. Antibodies against these last determinants were removed by immunoadsorption with insolubilized rabbit IgG. The remaining antibodies react with both the α-chain and the secretory
components of the secretory IgA molecule. The second-step reagent consisted of rabbit Fab directed against goat Fab and coupled to 8-MP as described. Rabbit secretory IgA was detected in two different cell types: in the secretory epithelium of the mammary gland and in plasmacytes located beneath the epithelium in the interstitial space. In the plasmacytes (Fig. 8), the reaction product is confined to the cisternae of the rough endoplasmic reticulum and elements of the Golgi complex. The cytoplasm and the nucleus are free of reaction product. In the epithelial cells reaction product was detected in three different cell compartments which comprise (Fig. 9) the saccules and vesicles of the Golgi complex, small apical vesicles, and large lysosome-like structures located basally.

**DISCUSSION**

The pepsin catalyzed hydrolysis of beef heart or horse heart cytochrome c leads to the production of a heme-undecapeptide, the sequence of which has been elaborated by Tuppy and Paleus (28) and Margoliash et al. (17). (Fig. 3). Trypsin digestion results in the further removal from the undecapeptide of the N-terminal tripeptide Val-Gln-Lys leading to the formation of a heme-octapeptide (10). Their preparative procedure, based on a partition chromatographic method, did not, however, separate completely the heme-octapeptide from contaminants. An additional gel filtration step was introduced later by Tu et al. (27), which improved somewhat the purity of the final product. For conjugation purposes, however, high purity is a requirement, which has been achieved with our countercurrent distribution procedure. In addition large preparative runs can be performed with high yield.

The heme-octapeptide possesses peroxidatic activity as shown by other investigators (7, 27). When compared to peroxidatic enzymes such as horseradish peroxidase, it appears that the heme-octapeptide has a $K_m$ which is about six orders of magnitude larger for the same conditions of assay. At $V_{max}$ the heme-octapeptide has a turnover number of 6,000; but in the range of H$_2$O$_2$ concentration compatible with cytochemistry ($\approx 10^{-4}$ M), it exhibits a specific activity of $\approx 750$ U/mg and a turnover number of 1,100 which, nevertheless, is 350 times higher than that of cytochrome c.

By virtue of its properties of small size (mol wt 1,550 daltons), the presence of a single amino group on the peptide backbone, and peroxidatic activity, it appears that this heme-octapeptide should be particularly suitable as a tracer when conjugated to ligands. In order to minimize random polymerization of reacting partners during conjugation and to increase the yield of products we have used a two-step conjugation procedure, based on the principle introduced by Singer in 1959 (26), who used toluene-2,4-diisocyanate to couple proteins. Since for our purposes, toluene-2,4-diisocyanate was not usable, we have synthesized a bifunctional reagent possessing different functional groups. This compound, the $N$-hydroxy succinimid ester of $p$-formylbenzoic acid, is
characterized by a greater reactivity of the active ester for amino groups compared to its aldehyde group thus satisfying the required selectivity of a bifunctional reagent designed for two-step conjugations. Thus, after the p-formylbenzoyl derivative of 8-MP is formed (activated 8-MP), in a first step, the aldehyde group on the derivative is able to form Schiff bases with functional amino groups on proteins in a second step. Since Schiff bases are reversible, we have formed stable covalent bonds in the conjugate by reduction with NaBH₄ (6). That the linkage is indeed stable is indicated by the absence of dissociation of tracer from the conjugate after treatment with SDS, high salt or acid. We should mention that our coupling reaction is limited in that the first step in the reaction, derivatization of 8-MP, must be carried out under conditions where many peptides and proteins will be denatured. For the future, it will be important to develop similar bifunctional reagents capable of derivatizing sensitive tracer molecules under mild conditions.

The conjugate, 8-MP-Fab, that we have obtained with our two-step coupling procedure consists of an homogeneous population with, on the average, two molecules of 8-MP attached to each Fab fragment. The molecular weights are ~45,000 daltons for 8-MP-sheep Fab, and ~50,000 daltons for 8-MP-rabbit Fab as determined by SDS polyacrylamide gel electrophoresis. This conjugate is the smallest immunological tracer with enzymatic activity available at this time for use in immunoelectron microscopy. Its size cannot be further reduced significantly, since the Fab moiety is the minimal immunological unit which can react specifically with an appropriate antigen.

The apparent $K_m$ of the heme-octapeptide is not significantly changed after conjugation, whereas the calculated apparent $V_{max}$ of the attached 8-MP is only 10% of that of the free 8-MP. At concentrations of $H_2O_2$ ($\approx 2 \times 10^{-3}$ M) used in cytochemistry, the velocity of the reaction is 0.02 mmol/min per mg of bound 8-MP. If one expresses the velocity per milligram of conjugate, a value of 19 $\mu$mol/min per mg is calculated which corresponds to a turnover number of ~400 for a conjugate of 45,000 daltons. This number is ~100 times higher than that of cytochrome c under the same conditions of assay. The peroxidatic reaction product of cytochrome c, used alone as a tracer (12) or coupled to Fab (5, 13), has proven to be useful for electron microscope studies. One can therefore expect that 8-MP-Fab will be much more sensitive for immunoelectron microscopy.

The conjugate retains both immunological (70%) and peroxidatic activity, although our data indicate that these are altered with respect to the unconjugated molecules. Nevertheless, the conjugate has been shown to be of practical use for immunocytochemical procedures. It is important, however, to emphasize that the presence of immunologically inactive conjugates carrying active 8-MP will contribute to background in direct proportion to the concentration at which these molecules are present in the incubation medium. These inactive conjugates have been measured using immunoadsorbent techniques.
Figs. 8-9  Thin sections of rabbit mammary gland fixed for 4 h with 4% formaldehyde, 0.1% picric acid in 0.1 M Na cacodylate pH 7.4. After fixation, the tissue was exposed first to goat Fab directed against rabbit secretory IgA, followed by rabbit Fab directed against goat Fab and coupled to 8-MP. The peroxidatic reaction product of the 8-MP was obtained using diaminobenzidine and H2O2.

Fig. 8  Plasmaocytes in the interstitial space beneath the epithelium. Note the staining of the cisternal spaces of the rough endoplasmic reticulum and of some elements of the Golgi complex. Ep: epithelium; L: lumen; N: nucleus; G: Golgi complex; M: mitochondria. X 9000.

and represent ~30% of the population. On the other hand, immunologically active but enzymatically inactive conjugates will compete with the fully competent conjugates resulting in a decrease of sensitivity. The decrease of peroxidatic activity after the overall conjugation procedure could be the result of
Fig. 9 Epithelial cell with its lumenal surface on the left hand top corner. A myoepithelial cell (MY) containing fibrils is seen on this micrograph. Reaction product is observed in vesicles (arrows), in the Golgi complex (G) and in lysosomal-like structure (L). Nucleus, (N), mitochondria, (M); endoplasmic reticulum, (ER). X 16,000.

partial loss of activity of all the conjugated molecules, or it could reflect the complete loss of activity of some of the molecules. At present these possibilities cannot be assessed.

Comparison of our conjugate with enzyme-labeled antibodies obtained by other conjugation procedures is unfortunately not possible because these conjugates have not been quantitatively checked for retention of immunological or enzymatic activity.
Finally, the good yield of conjugation (approximately 50%) achieved under the conditions described allows coupling of the tracer to ligands available in very small amounts and tends to offset the inactivation of the 8-MP which occurs during conjugation.

Due to its small size and adequate peroxidatic activity, the conjugate should be particularly useful for immunocytochemical techniques where the immunological reagents are allowed to penetrate fixed cells before detection of antigenic sites (preembedding staining). In the past, this type of localization procedure has been severely limited by the relatively large size of the conjugates used (70,000 daltons for cytochrome c-Fab) which were required to penetrate diffusion barriers such as the plasma membrane and membranes delimiting intracellular compartments. As illustrated by our example the smaller conjugate which we have obtained is able to penetrate, in addition to plasmacytes, mammary epithelial cells. Our results show that antigenic determinants of secretory immunoglobulins are located in several different intracellular compartments including the cisternae of the rough endoplasmic reticulum and of the Golgi complex, and the content of small vesicles and lysosomal-like structures.

SUMMARY

A heme-octapeptide (mol wt 1,550) has been obtained from cytochrome c by successive pepsin and trypsin hydrolysis and purified by gel filtration and countercurrent distribution. It possesses peroxidatic activity characterized by an apparent $K_m$ of 0.2 M, an apparent $v_{max}$ of 4 mmol/min per mg of peptide, and a pH optimum of 7.0. Using a novel two-step conjugation procedure, the heme-octapeptide was coupled to rabbit Fab antibody fragments by first derivatizing it with the N-hydroxysuccinimide ester of p-formylbenzoic acid and subsequently allowing it to form a Schiff base with the amino groups of Fab. Stable covalent linkages were then obtained by reduction of the Schiff bases with sodium borohydride. The conjugate consists of $\sim$2 heme-octapeptides attached to each Fab molecule. The molecular weight is 45,000 daltons when coupled to sheep Fab and 50,000 daltons with a Stokes radius of 32 Å, when conjugated to rabbit Fab. Its peroxidatic activity is characterized by an apparent $K_m$ of 0.4 M, an apparent $v_{max}$ of 0.4 mmol/min and per mg of attached heme-octapeptide and a pH optimum of 7.0. The conjugate has been used for the localization at the electron microscope level of secretory immunoglobulins in the mammary gland of lactating rabbits.

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