Evaluation of the Numbers of Functional Groups Introduced into Horseradish Peroxidase in Reactions with Four Heterobifunctional Reagents

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Numbers of functional groups introduced into horseradish peroxidase in reactions with four heterobifunctional reagents, *S*-acetylmercaptosuccinic anhydride, *N*-(ε-maleimidocaproyloxy)succinimide, *N*-succinimidyl 3-(2-pyridyldithio)propionate and *N*-(3-bromoacetamido-*n*-propionoyloxy)succinimide, were investigated. In the reactions at large molar ratios of the reagents to the enzyme in neutral and slightly alkaline media, maximum numbers of the incorporated functional groups were approximately 2.2: two reactive amino groups are present in the enzyme molecule.

Keywords reagent heterobifunctional; horseradish peroxidase; *S*-acetylmercaptosuccinic anhydride; *N*-(ε-maleimidocarproyloxy)succinimide; *N*-succinimidyl 3-(2-pyridyldithio)propionate; *N*-(3-bromoacetamido-*n*-propionoyloxy)succinimide; functional group-introduction number; enzyme-labeling; cross-linking

Horseradish peroxidase (EC 1.11.1.7, HRP) has been utilized as an enzyme for the preparation of HRP-conjugates of proteins such as antibodies and antigens, which are required in histochemical and cytochemical studies,[1,2] enzyme immunoassays[3,4] and enzyme-linked immunospot assays.[5,6]

In the preparation of HRP-conjugates, recently, heterobifunctional reagents have been preferably used as cross-linkers. The well controlled introduction of functional groups into HRP is a key step in the reproducible preparation of HRP-conjugates, because of the numbers of functional groups introduced into HRP affect bulkiness, immuno- and enzyme-activities and/or homogeneity of the conjugates. The relationships between the numbers of functional groups introduced into HRP molecule and the molar ratios of heterobifunctional reagents to HRP have been discussed in the reactions with *S*-acetylmercaptosuccinic anhydride (SAMS) and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Fig. 1), at pHs 7.0—8.0.[7-9] However, no systematic investigations of the numbers in the reactions of HRP with several heterobifunctional reagents have heretofore been made.

The purpose of this study is to evaluate the average numbers of functional groups introduced into HRP (Fig. 1) in the reactions with four representative heterobifunctional reagents (Fig. 1) in neutral and slightly alkaline media (pHs 7.0—7.5).

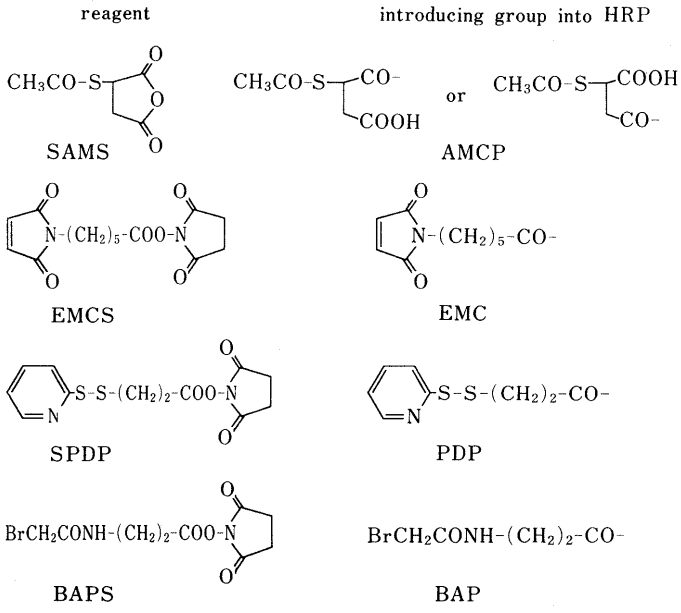

Fig. 1. Investigated Heterobifunctional Reagents and Their Introducing Functional Groups

Experimental

Materials and Apparatus SAMS[7,10] and HRP (285 purpurogallin units/mg, type VI) were purchased from Sigma (St. Louis, U.S.A.), SPDP[8,9] and Sephadex G-25 from Pharmacia LKB Biotechnology AB (Uppsala, Sweden), *N*-(ε-maleimidocaproyloxy)succinimide (EMCS)[11] from Dojindo Laboratories (Kumamoto, Japan). *N*-(3-Bromoacetamido-*n*-propionoyloxy)succinimide (BAPS)[12] and Gly(A1), Phe(B1)-dicitraconyl insulin[13] were prepared as described previously. All other chemicals were of reagent grade. Deionized water was passed through a Milli-QII system (Japan Millipore, Tokyo, Japan). Cellulose dialyzer tubings (molecular weight cutoff, approximately 8000; Nacalai Tesque, Kyoto, Japan) and molecular membrane tubes (Spectra/Por 3; molecular weight cutoff, approximately 3500; Spectral Medical Ind., Los Angeles, U.S.A.) were used for dialyses of reaction mixtures containing HRP and Gly (A1), Phe (B1)-dicitraconyl insulin, respectively. Dialyses were carried out at 4 °C for 12 h. Absorbances were measured with a Hitachi 150—20 spectrophotometer in semimicro quartz cells (10-mm optical path length, 1 ml).

Amino acid analyses were performed with a Hitachi 835 amino acid analyzer after hydrolyses of protein samples in 6 M HCl *in vacuo* at 110 °C. for 24 h. A stepwise elution of amino acids was carried out according to the handling manual[14] except that the concentrations of benzyl alcohol in eluants III and IV described in the manual (0 and 0.5% (v/v), respectively) were replaced with 0.5 and 1.0% (v/v), respectively.

Reaction of SAMS with HRP To HRP (1 mg, 25 nmol) dissolved in 1 ml of 100 mM Na phosphate buffer (pH 7.0) containing 100 mM NaCl was added various amounts of SAMS (5—500-fold molar excess to HRP) in 50 μl of *N*,*N*-dimethylformamide (DMF) with vigorous stirring at 30 °C, and the mixture was kept stirring at 30 °C for 1 h.

Determination of Sulfhydryl Group from AMCP Group The number of 2-(or 3-)acetylmercapto-3-carboxypropanoyl (AMCP) groups introduced into HRP was estimated based on that of sulfhydryl group yielded by the deacetylation of the AMCP group with hydroxylamine[10]: to 1.05 ml of the resulting mixture mentioned above was added an evacuated mixture of 150 μl of 100 mM Tris–HCl buffer (pH 7.0), 15 μl of 100 mM ethylene diaminetetraacetic acid disodium salt (EDTA·2Na) in the buffer and 300 μl of 1 M hydroxylamine hydrochloride with stirring at 30 °C for 5 min. The solution was dialyzed against 1 l of evacuated 100 mM Na phosphate buffer (pH 6.0) containing 5 mM EDTA·2Na and 100 mM NaCl. The resulting dialysate was immediately subjected to the determination of the sulfhydryl group by the 4,4′-dithiopyridine method.[15]

Reactions of EMCS with HRP and Gly(A1), Phe(B1)-dicitraconyl Insulin To HRP (6 mg, 150 nmol) or Gly(A1), Phe(B1)-dicitraconyl insulin (1 mg, 167 nmol) dissolved in 1 ml of 100 mM Na phosphate buffer (pH 7.0) was added EMCS (2—300- or 10—100-fold molar excess to HRP

or the insulin, respectively) in 50 μl of DMF with vigorous stirring at 30 °C. The mixture was kept stirring at 30 °C for 1 h. The resulting mixture was dialyzed (3 times) against 2 l of 100 mM Na phosphate buffer (pH 6.7) containing 100 mM NaCl.

Determination of EMC Group The numbers of ε-maleimidocaproyl (EMC) groups introduced into HRP and Gly(A1), Phe(B1)-dicitraconyl insulin were determined by back titration of 2-mercaptoethanol with 4,4′-dithiopyridine according to the method of Grassetti *et al.*[15] with some modifications: the reaction mixture mentioned above (80 μl each) was mixed with 100 μl of 0.25 mM 2-mercaptoethanol and the mixture was allowed to stand at 30 °C for 15 min. To the mixture, 820 μl of 0.2 mM 4,4′-dithiopyridine in 100 mM Na phosphate buffer (pH 6.5) (solution S) or the same buffer (solution B_2) was added and the mixture was allowed to stand at 30 °C for 20 min. To the buffer (80 μl) were added 100 μl of 0.25 mM 2-mercaptoethanol and 820 μl of 4,4′-dithiopyridine (solution B_1), and the mixture was left standing at 30 °C for 20 min. The buffer (900 μl) was mixed with 100 μl of 2-mercaptoethanol (solution B_3). The absorbances of the four solutions (A_S, A_{B1}, A_{B2}, and A_{B3}) were measured at 324 nm. The numbers of sulfhydryl groups into HRP and the insulin were calculated from the equation $(A_{B1}-(A_s-(A_{B2}-A_{B3}))/19800)C_p$, where C_p represents the concentration of HRP or the insulin, and the value of 19800 is the molar absorptivity of 4-thiopyridine which is formed in the reaction.[15]

Reactions of SPDP with HRP and Gly(A1), Phe(B1)-dicitraconyl Insulin To HRP (5 mg, 125 nmol) or Gly(A1), Phe(B1)-dicitraconyl insulin (1 mg, 167 nmol) dissolved in 500 μl of 100 mM Na phosphate buffer (pH 7.5) containing 100 mM NaCl was added SPDP (3—50- or 1—30-fold molar excess to HRP or the insulin, respectively) in 80 μl of ethanol with vigorous stirring at 23 °C. The mixture was kept stirring at 23 °C for 30 min for HRP or for 40 min for the insulin. The resulting mixture was subjected to gel-chromatography on Sephadex G-25 (35 g, 100 cm × 1.5 cm i.d.) using 100 mM Na phosphate buffer (pH 7.5) containing 100 mM NaCl as a mobile phase. The eluates containing the proteins were collected and dialyzed (3 times) against 1 l of water and lyophilized.

Determination of PDP Group The numbers of 3-(2-pyridylithio)-propionoyl (PDP) groups introduced into HRP and Gly(A1), Phe(B1)-dicitraconyl insulin were measured according to the method of Carlsson *et al.*[8]

Reactions of BAPS with HRP and Gly(A1), Phe(B1)-dicitraconyl Insulin To HRP (2.5 mg, 62.5 nmol) dissolved in 500 μl of 100 mM Na phosphate buffer (pH 7.5) or Gly(A1), Phe(B1)-dicitraconyl insulin (1 mg, 167 nmol) dissolved in 1 ml of the buffer was added BAPS (3—200- or 10—200-fold molar excess for HRP or the insulin, respectively) in 100 μl of acetonitrile with vigorous stirring at 23 °C for HRP or at 30 °C for the insulin. The mixture was kept stirring for 1 h at 23 °C for HRP or at 30 °C for the insulin. The resulting mixture was dialyzed (7 times) against 1 l of water and lyophilized. The lyophilizate from the insulin was dissolved in 1 ml of 100 mM Na phosphate buffer (pH 7.5) containing 100 mM NaCl and the solution was dialyzed against 1 l of the buffer, and the resulting solution was subjected to the removal of the citraconyl group by dialyzing against 1 l of 1 M acetic acid at 30 °C for 24 h, followed by dialysis against 3 changes of 1 l of water and lyophilization.

Determination of BAP Group The numbers of 3-bromoacetamido-*n*-propionoyl (BAP) groups introduced into HRP and Gly(A1), Phe(B1)-dicitraconyl insulin were calculated from the amounts of β-alanine produced by acid hydrolysis from the introduced BAP groups, respectively.[12]

Results and Discussion

HRP consists of 308 amino acid residues: a hemin group, eight neutral carbohydrate side chains, four disulfide bridges, a blocked α-amino terminal in the form of a pyridone carboxyl residue and six lysyl residues.[16] The lysyl residues were candidates of the reactions with succinic anhydride moiety of SAMS and *N*-hydroxysuccinimide ester moieties of EMCS, SPDP and BAPS.

The average number of AMCP group introduced into HRP at the molar ratios of SAMS to HRP from 5 to 500 was approximately 0.2—2.2 at pH 7.5 at 30 °C for the reaction period of 1 h (Fig. 2, curve 1). Additionally, when the reaction was performed at the molar ratios of 200 and 300 at pH 7.5 for the reaction period of 1.5 h, the same values of 2.1 were obtained in triplicate and duplicate determinations, respectively. The average number of AMCP groups introduced into HRP was less than three.

These facts suggest that two fairly reactive amino groups to SAMS are present in the HRP molecule. However, Weston *et al.* reported that the number was 0.3—9.0 when the molar ratios of 7.5—120 was applied at pH 8.0 at ice-cold temperature.[7] HRP has six free amino groups, thus their data apparently showed the coupling of SAMS with group(s) other than the amino groups. They used a dropwise-addition of 1 M sodium hydroxide to maintain the reaction mixture at pH 8.0. This procedure may temporarily cause a strongly alkaline reaction medium, and this is a possible reason for the high degree of introduction.

The reaction of the *N*-succinimidyl ester side of EMCS with amino group of HRP was performed at pH 7.0, at which pH EMCS was fairly stable (the decomposition was 4.5% at 30 °C for 20 min).[17] The average number of EMC groups introduced into HRP was apporoximately 2.2 at the molar ratio of 300 (Fig. 2, curve 2). The validity of the number was proved by determining the maximum num-

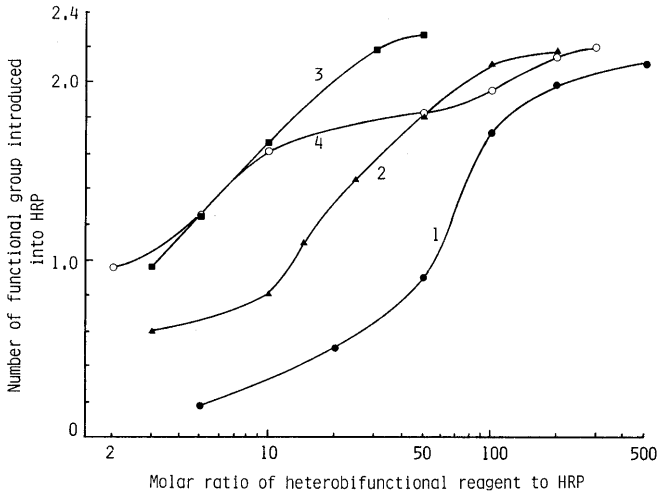

Fig. 2. Effect of the Molar Ratios of Bifunctional Reagents on the Average Numbers of Functional Groups Introduced into HRP Molecule

Curves: 1, SAMS; 2, EMCS; 3, SPDP; 4, BAPS. Each plot represents the mean value of duplicate determination.

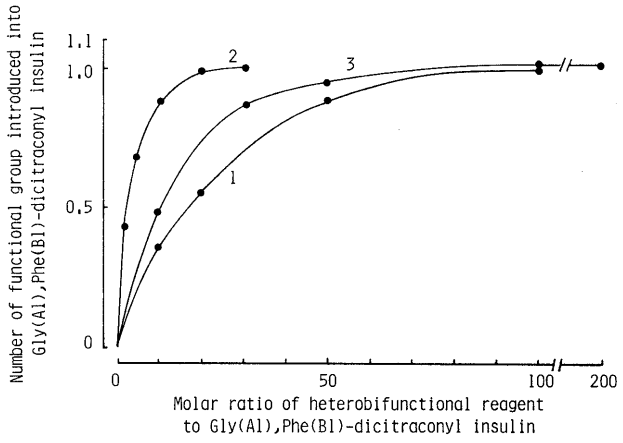

Fig. 3. Effect of the Molar Ratios of Bifunctional Reagents on the Numbers of Functional Groups Introduced into Gly(A1), Phe(B1)-dicitraconyl Insulin

Curves: 1, EMCS; 2, SPDP; 3, BAPS.

ber of EMC groups introduced into Gly(A1), Phe(B1)-dicitraconyl insulin, which has one free amino group in the molecule, at a large molar ratio of EMCS to the insulin (100). The maximum number of EMC groups introduced into the insulin was 1.0 (Fig. 3, curve 1). These observations suggest that the number obtained by the procedure for the determination of EMC groups is reliable. It is clear that two amino groups of HRP readily react with EMCS.

The average number of PDP group introduced into HRP was 2.3 (Fig. 2, curve 3) in the reaction with SPDP at the molar ratio of 50, at pH 7.5 and at 23 °C for the reaction period of 30 min. Under similar conditions to the reaction of HRP with SPDP, Carlsson *et al.* reported that the numbers were 0.5, 0.7 and 2.3 at the molar ratios of 4.8, 9.6 and 25, respectively.[8] The number (2.3) obtained in this study at the molar ratio of 50 was compatible with their data (2.3) obtained at the ratio of 25. Imagawa *et al.* reported that the number was 2.5—2.7 at the molar ratio of 50.[9] The maximum number of PDP group introduced into Gly (A1), Phe (B1)-dicitraconyl insulin was 1.0 (Fig. 3, curve 2). This indicates that the values obtained by the method for the determination of PDP groups is reasonable. It is thought that two amino groups of HRP react more readily with SPDP than another amino group, which reacts at high concentrations of SPDP.

The maximum number of BAP groups introduced into HRP was 2.2 at the molar ratio of BAPS of 200 at 23 °C for the reaction period of 1 h (Fig. 2, curve 4) and that of BAP groups introduced into Gly (A1), Phe (B1)-dicitraconyl insulin was approximately 1.0 at the same molar ratio at 30 °C for the reaction period of 1 h (Fig. 3, curve 3).

In conclusion, this study showed that only two reactive amino groups of HRP, possibly ε-amino groups of the lysyl residues, could react with SAMS, EMCS, SPDP and BAPS even in the reactions at high molar ratios of the reagents in neutral and slightly alkaline media. The HRPs introduced with functional groups described here should be useful for the preparation of HRP-conjugates of proteins, which have low heterogeneity.

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