Notes

Effect of the Steric Hindrance and Alkyl Chain of a Biotin Derivative to Avidin-Biotin Binding

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Many biological molecules show excellent properties of recognition for molecules. Enzymes have active sites to catalyze selective reactions, and antibodies bind antigens specifically. Neurotransmitters and hormones relate to the control and/or modulation of every cellular and physiological process. The biospecific binding of ligand sites becomes a trigger or modulation of their function. Avidin-biotin binding is known to be one of the strongest bindings between a protein and a ligand. It has been reported that tryptophan and lysine-forming avidin relate to the binding.¹ Many studies of this binding have been reported. The interaction can be applied to wide fields, such as chromatography²⁻⁴, sensors^{5,6} and spectrophotometry.^{7,8}

On the other hand, studies with bisbiotinyl compounds have reported that the binding site for the biotin of avidin was 9 Å below the surface of the avidin molecule.^{9,10} It was also described that the strength of the avidin-biotin binding decreased in the case of short spacer arms of the bisbiotinyl compounds. This was due to the effect of a steric hindrance. When compounds having appropriate spacer arms were used, the steric hindrance was improved. In the present study, the effects of a spacer and a steric hindrance of the terminal of a biotin derivative were investigated. To examine the influences, three biotin derivatives with Nile Blue A (Biotin-NB) having different types of spacers were prepared. The avidin-biotin binding was evaluated using the association of enzyme-labeled avidin with biotin immobilized on the wall of a microtiter plate well. The competitive reaction between immobilized biotin and biotin dissolved derivatives for the biotin binding sites of avidin enabled an estimation of the binding strength. Furthermore, the effect of the steric hindrance

of Nile Blue A was compared with that of the iodine moiety of *N*-Iodoacetyl-*N*-biotinylhexylenediamine.

Experimental

Apparatus

The visible-spectra of Biotin-NB were measured by a Shimadzu UV-2400 UV-VIS recording spectrophotometer. For shaking of the samples, an Iwaki V-S model shaker was used.

Reagents

Sulfosuccinimidyl D-biotin, sulfosuccinimidyl *N*-(D-biotinyl)-6-aminohexanoate, sulfosuccinimidyl *N*-[*N*-(D-biotinyl)-6-aminohexanoyl]-6'-aminohexanoate as biotinylation reagents, Nile Blue A, avidin and biotin were purchased from Wako Pure Chem. Co. (Osaka, Japan). *N*-Iodoacetyl-*N*-biotinylhexylenediamine (IB) was obtained from Pierce (IL, USA). Biotinamidocaproyl-bovine serum albumin (Biotin-BSA; ca. 9.6 mol of biotin per mol of BSA), bovine-serum albumin fraction V (BSA) and 3,3', 5,5'-tetramethylbenzidine (TMB) were supplied by Sigma (MO, USA). Horseradish peroxidase-avidin D conjugate (Av-P, 1.5 mol dm⁻³ peroxidase/1 mol dm⁻³ avidin) was purchased from Vector Labs (CA, USA). All other reagents were of analytical reagent grade.

The preparation of Biotin-NB

The preparation of the Biotin-NB and the determination of the concentrations were described in ref. 11 in detail. Nile Blue A of 1 mM and 1 mM biotinylation reagent were mixed in a 50 mM phosphate buffer (pH 7.0) and DMF, and the solution was stirred for 48 h at 4°C. The Nile Blue A which reacted with sulfosuccinimidyl D-biotin, sulfosuccinimidyl *N*-(D-biotinyl)-

Fig. 1 Structures of biotin with Nile Blue A (Biotin-NB).

-6-aminohexanoate, sulfosuccinimidyl *N*-[*N'*-(D-biotinyl)-6-aminohexanoyl]-6'-aminohexanoate was Biotin-NB-S, Biotin-NB-M and Biotin-NB-L, respectively. The product was separated by thin layer chromatography. Figure 1 shows their structures.

Procedure of the enzyme assay

Phosphate buffer with 0.002% biotin-BSA was added to the wells of the microtiter plates (Wako Pure Chem. Co., Osaka, Japan). The sample, PBS and Av-P (0.12 nmol dm⁻³) were incubated in the wells. Next, the TMB solution was added to each well. The mixture was shaken with 0.01% hydrogen peroxide and the reaction was stopped by 0.1 mol dm⁻³ sulfuric acid. Details concerning the procedure were described in a previous report.¹² The absorbance of the oxidized TMB was measured at 450 nm.

Results and Discussion

Effect of spacer in Biotin-NB

To investigate the strength of the binding of the Biotin-NB for avidin, an enzyme-linked ligand-sorbent assay was used. The principle is shown in Fig. 2. The uptake of avidin peroxidase onto the biotin plate is inhibited by the addition of Biotin-NB or biotin into the solution, because of the competition between immobilized biotin and free biotin for the limited number of biotin binding sites of the avidin conjugate. When four of the biotin binding sites in avidin are occupied by free Biotin-NB or biotin, the avidin is unable to complex with immobilized biotin. This method demonstrates the utility of a solid-phase avidin-biotin binding assay for the determination of biotin and Biotin-NB conjugates.

Fig. 2 Scheme of the solid-phase avidin-biotin binding assay.

Fig. 3 Dependence of the avidin-biotin binding on the length of spacer of the Biotin-NB. ●: Biotin, ▲: Biotin-NB-S, ◆: Biotin-NB-M, ▼: Biotin-NB-L. Measured at 450 nm.

The results of the assay for biotin derivative and biotin are shown in Fig. 3. The detection limit of biotin, estimated as three-times the standard deviation of the blank, was 2×10^{-10} mol dm⁻³. The relative standard deviation at 2×10^{-9} mol dm⁻³ biotin was 5.5% (n=5). When the strength of binding was compared to that of biotin, it was found that the apparent strength of the binding depended on the length of the spacer. The IC₅₀ values, where IC₅₀ was the concentration of the dis-

Fig. 4 Structures of biotin with Daunomycin (Biotin-D).

Fig. 5 Effect of the terminal group of Biotin-NB-M and IB on the avidin-biotin binding. ●: Biotin, ◆: Biotin-NB-M, ▲: IB. Measured at 450 nm.

placing ligand required to inhibit 50% of the probe ligand binding, were evaluated.¹³ IC_{50} of Biotin-NB was compared to that of biotin. The order of the strength of the avidin-biotin binding was as follows: Biotin-NB-L (2×10^{-8} M) > Biotin-NB-M (3×10^{-8} M) > Biotin-NB-S (5×10^{-8} M) > biotin (1×10^{-7} M). The reason is that the binding site of avidin for biotin has a hydrophobic environment, while the surface of the avidin molecule is hydrophilic.¹⁴ Hence, the hydrophobic property of the spacer contributes to the strength of the binding in addition to the avidin-biotin interaction. The order of the binding is mainly due to the length of the spacer. In a previous study, the same enzyme assay

was carried out by biotin with Daunomycin (Fig. 4)¹⁵, where the strength of the binding was Biotin-D-M > Biotin-D-L > biotin > Biotin-D-S (D denotes Daunomycin). Because Daunomycin has a sugar part, it is expected that this part affects the binding by a steric hindrance.

Comparison of the strength of the binding for Biotin-NB-M and IB

On the other hand, the strength of the binding of an IB was examined because the length between the biotin part and iodine of IB was similar to the length between the biotin part and Nile Blue A of Biotin-NB-M. It is expected that the iodine part at the terminal of IB does not influence the steric hindrance for the avidin-biotin binding. Figure 5 shows the relationship between the concentration of the biotin derivative (biotin) and the enzyme activity. The curve of Biotin-NB-M was similar to that of IB. Accordingly, this suggests that a steric effect based on the Nile Blue A part is small. When the strength of the binding of Biotin-NB-M was compared to that of IB, the binding between the biotin derivative and avidin was not affected by the terminal of the spacer. The order of the binding is mainly due to the length of the spacer.

To clarify the hydrophobic effect, small molecules, such as amino acid, should be used. At present, an investigation by using biotin modified with cysteine is under way. Furthermore, a study will be carried out by labeled biotin with an alkyl chain not containing a NH-CO group in the spacer.

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