Organic/inorganic hybrid nanomaterials with vitamin B$_{12}$ functions

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Abstract

A hybrid nanomaterial was prepared by human serum albumin (HSA) and vitamin B$_{12}$ derivatives. The incorporation of hydrophobic vitamin B$_{12}$ derivatives, which have ester groups in place of the peripheral amide moieties of the natural cobalamin, into HSA is primarily controlled by the hydrophobicity of the peripheral ester groups. Microenvironmental property around the hydrophobic vitamin B$_{12}$ in HSA was examined by fluorescence and fluorescence polarization measurements. The hydrophobic vitamin B$_{12}$ itself in HSA is in a microenvironmental equivalent in medium polarity to dichloromethane. The molecular motion of hydrophobic vitamin B$_{12}$ in HSA was markedly suppressed under such microenvironmental conditions. Carbon-skeleton rearrangement reaction of an alkyl radical derived from an alkyl ligand bound to the hydrophobic vitamin B$_{12}$ was markedly favored in HSA aqueous solution, relative to the reactions in methanol and benzene. The 1,2-migration of the electron-withdrawing group arises from both the suppression of molecular motion and desolvation effects on the alkylated hydrophobic vitamin B$_{12}$ in HSA.

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1. Introduction

Naturally occurring holoenzymes are typical supramolecules composed of a specific apoprotein and an additional cofactor, such as coenzymes or metal ions. An apoprotein generally provides a binding site for both specific coenzyme and substrate molecules, which are well separated from a bulk aqueous phase. Thus, an enzyme’s active site turns out to be sufficiently hydrophobic and hardly holds water molecules. Under such conditions, the reacting species become efficiently naked so that the reactivity is much enhanced due to thermodynamic reasons. In this work, we designed an artificial enzyme with organic/inorganic hybrid nanomaterials as shown in Fig. 1. We have been interested in vitamin B$_{12}$-dependent enzymes, involving the cobalt species as a catalytic center [1]. We have been dealing with a hydrophobic vitamin B$_{12}$, heptamethyl cobyrinate perchlorate [Cob(II)7C$_7$ester]ClO$_4$, which has ester groups in place of the peripheral amide moieties of the naturally occurring vitamin B$_{12}$ [2]. In order to construct a good catalytic system, we prepared various nanomaterials with vitamin B$_{12}$ activities. One example is a vesicle-type artificial enzyme composed of peptide lipids and hydrophobic vitamin B$_{12}$ [3]. Second one is a silica gel having vitamin B$_{12}$ derivatives by the sol–gel method [4]. In this paper, we reported a new organic/inorganic hybrid nanomaterials; human serum albumin (HSA) containing vitamin B$_{12}$ derivatives. HSA is the most prominent protein in plasma, and serves as a transporter for hydrophobic molecules in vivo. Recently, HSA was used as a protein model of artificial myoglobin or hemoglobin [5,6]. We use HSA as an apoenzyme model for construction of an artificial B$_{12}$ enzyme.

2. Experimental section

2.1. General analyses and measurements

Electronic absorption spectra were recorded on a Hitachi U-3000 spectrometer and a Shimadzu Multispec-1500 apparatus, and fluorescence spectra were obtained with a Hitachi F-4501 spectrofluorometer. Fluorescence polarization measurements were performed with a Union Giken...
FS-501A fluorescence polarization spectrophotometer; emission at $\lambda = 490$ nm was monitored upon excitation at $\lambda = 335$ nm with a slit width of 3.5 nm for both excitation and emission sides. Fluorescence polarization ($P$) was calculated according to a method used previously [7,8].

$^1$H-NMR spectra were taken on a Bruker Avance-500 spectrometer installed at the Centre of Advanced Instrumental Analysis, Kyushu University. GLC analyses were carried out on a Shimadzu GC-9A apparatus with a column of Silicone DC-550 (Shimadzu). GC–MS spectra were taken on a Shimadzu GCMS-QP-5050 with a capillary column of DB-1 (Shimadzu).

2.2. Material

Preparation of 2-(imidazol-4-yl)-N-[5-(dimethylamino)-1-naphthylsulphonyl]ethylamine (dansylhistamine) as a fluorescent probe has been reported previously [9]. 2-Acetyl-2-ethoxy carbonylpropane, 2-acetyl-1-ethoxy carbonylpropane, and 1-acetyl-2-ethoxy carbonylpropane were prepared as a substrate and authentic samples for the reaction products after the procedure reported previously [10]. HSA, F–V extra pure was purchased from Nacalai Tesque. Hydrophobic vitamin B$_{12}$ derivatives; (CN)$_2$Cob(III)7C$_3$ester, (CN)$_2$Cob(III)7C$_2$ester, (CN)$_2$Cob(III)7C$_1$ester, and (CN)$_2$Cob(III)7C$_0$ester, as shown in Fig. 2 were prepared from cyanocobalamin with reference to the methods reported previously [11–13]. The alkylated hydrophobic vitamin B$_{12}$, [(COCH$_3$)$_2$(CO$_2$C$_2$H$_5$)(CH$_3$)CCH$_2$Cob(III)7C$_4$ester]ClO$_4$, was prepared by the previously reported method [3,12,14].

2.3. Equilibrium measurements for incorporation of hydrophobic vitamin B$_{12}$ derivatives into HSA

Gel-filtration chromatography for the incorporation of hydrophobic vitamin B$_{12}$ into HSA was carried out as shown in Fig. 3. Typical experimental procedure is shown as follows: HSA (230 mg, 3.4 $\times$ 10$^{-6}$ mol) and [(CH$_3$)Cob(III)7C$_3$ester]ClO$_4$ (4.6 mg, 3.4 $\times$ 10$^{-6}$ mol) in methanol (5 ml) were dissolved in phosphate buffer (50 ml, 200 mM, pH 7.0). This solution was applied on a column of Sephadex G-50 ($\phi = 1.5$ cm, $L = 15$ cm) and eluted with phosphate buffer. The incorporated complex was eluted first in the column void volume, and its amount was determined from absorbance at 511 and 538 nm.

2.4. Photolysis of alkylated hydrophobic vitamin B$_{12}$ in HSA

HSA (1.00 g, 1.45 $\times$ 10$^{-5}$ mol) was dissolved in an aqueous phosphate buffer (300 ml, pH 7.0). After the solution was deoxygenated with nitrogen gas, a methanol solution (0.2 ml) of [(COCH$_3$)$_2$(CO$_2$C$_2$H$_5$)(CH$_3$)CCH$_2$ Cob(III)7C$_3$ester]ClO$_4$ (1.4 $\times$ 10$^{-5}$ mol) was added to it, resulting in the following final concentrations: [(COCH$_3$)$_2$(CO$_2$C$_2$H$_5$)(CH$_3$)CCH$_2$Cob(III)7C$_3$ester]ClO$_4$, 4.7 $\times$ 10$^{-5}$ M; HSA, 4.8 $\times$ 10$^{-5}$ M. The resulting solution was then
irradiated with a 500 W tungsten lamp at a distance of 30 cm at room temperature. After the alkylated complex was completely decomposed, as confirmed by electronic spectroscopy, the products were extracted with dichloromethane (3 × 20 ml). The dichloromethane solution was evaporated to dryness, and an appropriate amount of diethyl ether (0.5 ml) was added to the residue. The products were identified by means of GLC, with co-injection of authentic samples into column as reported previously [3].
3. Results and discussion

3.1. Incorporation of hydrophobic vitamin B12 derivatives into HSA

The extents of incorporation of the vitamin B12 derivatives into HSA were investigated by gel-filtration chromatography as summarized in Table 1: [(CH₃)Cob(III)₇C₁ester]ClO₄, [(CH₃)Cob(III)₇C₂ester]ClO₄, [(CH₃)Cob(III)₇C₃ester]ClO₄, and [(CH₃)Cob(III)₇C₄ester]ClO₄. All of the hydrophobic vitamins B₁₂ derivatives were completely distributed to the organic layer when these complexes were mixed together with aqueous and dichloromethane phases as shown in Fig. 4. On the other hand, the incorporation behavior of HSA, which provides hydrophobic binding domain toward the hydrophobic vitamin B₁₂, was different from that of organic media. [(CH₃)Cob(III)₇Cₙester]ClO₄ was readily incorporated into HSA when the n-value of Cₙ was equal to two or larger, but [(CH₃)Cob(III)₇C₁ester]ClO₄ was not bound to HSA at all. The latter complex is slightly soluble in water, but [(CH₃)Cob(III)₇C₃ester]ClO₄ and [(CH₃)Cob(III)₇C₄ester]ClO₄ are almost insoluble. Thus, the extent of incorporation of the hydrophobic vitamin B₁₂ into HSA is primarily controlled by the hydrophobicity of the ester groups placed at the peripheral sites of the corrinoid skeleton.

When the ratio of HSA and the hydrophobic vitamin B₁₂ derivative was changed, aqueous HSA was dissolved over 10-times of hydrophobic vitamin B₁₂. When cobalt(II) species of hydrophobic vitamin B₁₂, [Cob(II)₇C₃ester]ClO₄, was dissolved in aqueous HSA solution, the absorption maxima of electronic spectrum was observed at 470, 510, and 540(sh) nm in visible light region. The shoulders at 510 and 540 nm are observed when an axial base is coordinated to cobalt(II) species of hydrophobic vitamin B₁₂ \[12,13\]. According to the electronic spectrum of hydrophobic vitamin B₁₂ derivative in aqueous HSA, an amino acid such as His in HSA is expected to coordinate to hydrophobic vitamin B₁₂ at one axial site. The binding sites of HSA for hydrophobic vitamin B₁₂ are not clear at the present stage.

![Fig. 3. Experimental procedure for the incorporation of hydrophobic vitamin B₁₂ into HSA.](image)

### Table 1

| Complex         | Incorporated complex (%) |
|-----------------|--------------------------|
| [(CH₃)Cob(III)₇C₁ester]ClO₄ | 0 |
| [(CH₃)Cob(III)₇C₂ester]ClO₄ | 20 |
| [(CH₃)Cob(III)₇C₃ester]ClO₄ | 100 |
| [(CH₃)Cob(III)₇C₄ester]ClO₄ | 100 |

*Incorporation was examined by gel-filtration chromatography on a column of Sephadex G-50 with phosphate buffer as eluent.

3.2. Microenvironmental properties around hydrophobic vitamin B₁₂ derivatives in HSA

In order to obtain information about the microenvironment around the hydrophobic vitamin B₁₂ derivatives in HSA, [(CN)Cob(III)₇C₃ester]ClO₄ coordinated at the residual axial site by dansylhistamine as a fluorescent probe was adopted. The microscopic polarity experienced by the dansyl moiety bound to the hydrophobic vitamin B₁₂ is reflected in its fluorescence maxima \[15\]. We used \(E_p^N\) value as a solvent polarity parameters \[16\]. First, the fluorescence maxima of dansylhistamine coordinated to [(CN)Cob(III)₇C₃ester]ClO₄ were measured in various organic solvents as shown in Fig. 5: the fluorescence maximum is shifted to lower wavelength as the solvent polarity decreases. It is clear that HSA provides a microenvironment for the dansyl moiety that is equivalent in medium polarity to dichloromethane. This result leads us to conclude that the hydrophobic vitamin B₁₂ is significantly desolvated in HSA.

The dansylhistamine-coordinated hydrophobic vitamin B₁₂, incorporated into HSA, gave large fluorescence polarization (\(P\)) values at various temperatures, i.e., 0.15–0.31 in the temperature range 5–80°C as shown in...
Fig. 6. This apparently indicates that the molecular motion of the guest molecule in HSA is markedly suppressed, since $P$ values in methanol and benzene are 0.005 and 0.017 at 25.0 °C, respectively. Thus, the microenvironmental effect provided by HSA is quite different from those produced by simple organic solvents which solubilize the hydrophobic vitamin B$_{12}$ derivative homogeneously.

3.3. Photochemical carbon-skeleton rearrangement of alkyl ligand bound to hydrophobic vitamin B$_{12}$ in HSA

Vitamin B$_{12}$-dependent enzymes catalyze various molecular transformations [1]. Especially, the rearrangement reactions are quite interesting. The rearrangement reactions involve the intramolecular exchange of a functional group (X) and a hydrogen atom between neighbouring carbon atoms as shown in Eq. (1). These reactions have attracted much attention from the viewpoints of organic and catalytic chemistry. A carbon-skeleton rearrangement reaction mediated by methylmalonyl-CoA mutase is shown in Eq. (2). This reaction is 1,2-migration of thioester group. In order to simulate the 1,2-migration reaction in such vitamin B$_{12}$-dependent enzyme, we carried out a model reaction which is 1,2-migration of acetyl group as follows.
Hydrophobic vitamin B₁₂ derivative bearing an alkyl ligand at one axial site of the nuclear cobalt was incorporated into HSA in aqueous medium and then irradiated with visible light under anaerobic conditions. Product analyses for the photolysis of the alkylated complex, [(COCH₃)(CO₂C₂H₅)(CH₃)CCH₂-Cob(III)₇-C₃ester]ClO₄, in aqueous HSA, methanol, and benzene were summarized in Eq. (3). The yield of acetyl-migration product was significantly increased in HSA as compared with those in methanol and benzene. This result is similar to that for the vesicle-type or the cyclophane-type artificial B₁₂ enzyme systems as reported previously [3,9]. The 1,2-migration of the electron-withdrawing group arises both from suppression of molecular motion and desolvation effects on the alkylated hydrophobic vitamin B₁₂ in HSA.

4. Conclusion

Various hybrid nanomaterials with B₁₂ functions can be prepared by combination with hydrophobic vitamin B₁₂ and nano-space materials such as vesicles, proteins, silicagels and so on. These softmaterials are expected to be useful as environmental-friendly catalysts. In this work, we presented an artificial B₁₂ enzyme using HSA. This catalytic system can apply various molecular transformations with molecular recognition. This work is a guidepost for construction of organic/inorganic hybrid nanomaterials.

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