Heparin–Selenocystamine Conjugate with Selenol Groups

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Heparin–selenocystamine conjugate, which was intended to mimic the heparin–selenoprotein P complex, was prepared. The conjugate had glutathione peroxidase-like activity and activity was observed toward hydrogen peroxide, tert-butyl hydroperoxide, and cumene hydroperoxide. The ultraviolet spectrum of an aqueous solution of the conjugate was stable and had a similar shape to that observed transiently when selenocystamine was reduced by sodium cyanoborohydride; this suggests that the diselenide bond of selenocystamine introduced into heparin was cleaved during conjugate preparation and the selenol group is preserved. The conjugate reacted to the same degree as cysteine with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) releasing thionitro-benzoic acid, which indicated that the selenium in the conjugate is present as selenol. However, the reaction rate of the conjugate was slower than cysteine which may be due to partially restricted access of DTNB to the selenol group in the conjugate. This conjugate had 1,1-diphenyl-2-picryl-hydrazyl(DPPH) radical scavenging activity as well as superoxide anion scavenging activity. These results indicate that the conjugate serves as a useful model compound with a stable selenol group having a range of biological activities, and suggest a possible antioxidant defensive role for the complex of endogenous heparin-like substance and selenoprotein P.

Key words selenium; heparin; selenocystamine–heparin conjugate; selenoprotein P; antioxidant activity

It has been suggested that heparin and related compounds act in the body as antioxidants and inhibit the harmful effects of oxygen free radicals. On the other hand, selenium has a variety of biological activities in addition to its antioxidant properties and is known to be located at the active center of glutathione peroxidase(GPx). Accordingly, selenium is closely associated with the antioxidant defense system of living cells.

Although some of the biological activities of selenium may be explained by the chemical reactivity of the element and reactions catalyzed by selenium-containing enzymes, the function of selenoprotein P(SeP), the most abundant selenium-containing component in plasma, remains to be clarified. SeP has high affinity for heparin, and a heparin–Sepharose affinity column can be used to separate the protein. Recently it was suggested that SeP may play a protective role towards cells by binding to the cell surface where heparan sulfate proteoglycans with heparin-like moieties are present. In this study, we introduced selenocystamine(SeCyst) covalently into heparin and investigated the characteristics of the resulting conjugate. The purpose of this study was to obtain a high molecular weight selenocompound with biological activity and to clarify the function of SeP by mimicking the heparin–SeP complex under the assumption that SeP plays a role in providing selenol groups for heparin-like substance making it an antioxidant-active complex.

MATERIALS AND METHODS

Materials Heparin (sodium salt), sodium periodate, sodium cyanoborohydride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1,1-diphenyl-2-picrylhydrazyl(DPPH), reduced glutathione, hydrogen peroxide, cumene hydroperoxide, N-1-naphthylethenediamine dihydrochloride, hydroxylamine-o-sulfonic acid, hypoxanthine, and NADPH were purchased from Nacalai Tesque Co., Ltd., Kyoto, Japan. tert-Butyl hydroperoxide was purchased from Katayama Chemical Ind.

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Co., Ltd., Osaka, Japan. Sulfanilic acid, hydroxylamine hydrochloride, EDTA disodium salt, and selenium standard solution(1000 ppm) were purchased from Wako Pure Chemical Co., Ltd., Osaka, Japan. SeCyst dihydrochloride, selenomethionine, sodium selenite, glutathione reductase, and xanthine oxidase (grade I, from buttermilk) were purchased from Sigma Chemical Co., Ltd., St. Louis, MO. 2-Phenyl-1,2-benziselenazol-3(2H)-one(ebselen) was a gift from Daiichi Seiyaku Co., Ltd., Tokyo, Japan.

Preparation of the Conjugate Heparin–SeCyst conjugate was prepared according to the method described by Bernstein et al. for dextran–daunomycin conjugate with some modifications. The sodium salt of heparin (0.3 g) was incubated with sodium periodate (0.048 g) in 0.1 M sodium phosphate (pH 4.4) for 2 h at room temperature in the dark. The mixture was then dialyzed against 0.1 M phosphate buffer (pH 7.5) overnight at 4°C. SeCyst (10 mg as the dihydrochloride) was added to the oxidized heparin solution, followed by sodium cyanoborohydride (23 mg). The reaction mixture was held for 24 h at room temperature. The solution was adjusted to pH 4 with 1 M acetic acid and dialyzed against distilled water for 48 h at 4°C.

Heparin–SeCyst conjugate was obtained by lyophilization of the dialyzed solution. The selenium content of the conjugate was measured by graphite furnace atomization atomic absorption spectrometry, where Rh(NO3)3 was used as a matrix modifier.

To prepare a reference compound for the heparin–SeCyst conjugate not containing SeCyst, heparin was treated with sodium periodate and subsequently sodium cyanoborohydride in the absence of SeCyst; we called this treated heparin.

Spectral Measurement Ultraviolet spectra of intact heparin (4.0 mg/ml), treated heparin (4.0 mg/ml), SeCyst (26.7 μg Se/ml), and heparin–SeCyst conjugate(4.0 mg/ml, i.e. 26.7 μg Se/ml) were measured in aqueous solution. Ultraviolet spectra of SeCyst (0.5 mg/ml) solution were also measured before and immediately after reduction by sodium cyanoborohydride. A UV-visible recording spectrophotome-
ter (model Ubest-50, Jasco Corporation, Tokyo) was used for spectral measurements.

**Reaction with DTNB** Absorbance at 412 nm based on the release of 5-thio-2-nitrobenzoic acid (TNB) was recorded to monitor the reaction of the conjugate with DTNB in 0.05 M phosphate buffer (pH 8.0). Concentrations of the conjugate and DTNB were 0.64 mg/ml, i.e., 6.73 μg Se/ml (=0.085 mM as for Se) and 0.5 mM, respectively. The reaction of cysteine, SeCyst, or heparin with DTNB was also studied under the same conditions.

**GPx-like Activity** The GPx-like activity was measured using the method described by Müller et al. Hydrogen peroxide, tert-butyl hydroperoxide, and cumene hydroperoxide were used as substrates (0.3 mM). The concentrations of heparin–SeCyst conjugate were 0.1, 0.5, 1.0, 5.0 μg Se/ml and the pH of the reaction mixture was adjusted to 7.0 using phosphate buffer. The activity was expressed in units which correspond to 1 μmol NADPH change per minute.

**DPPH Scavenging Activity** The conjugate and DPPH were dissolved in distilled water and ethanol, respectively. The concentrations of heparin–SeCyst conjugate were 0.04, 0.2, 0.4, 2.0, 4.0 μg Se/ml and the conjugate was incubated with 0.083 mM DPPH at 37 °C for 30 min. The volume ratio in the mixture of conjugate solution/DPPH solution was 1/5. The DPPH radical scavenging activity of the conjugate was evaluated by monitoring the DPPH concentration at 517 nm. The activity was expressed as the reduction in absorbance.

**Superoxide Anion Scavenging Activity** Superoxide anion scavenging activity was assayed by the method of Oyanagi. The activity was expressed as a percentage of the inhibition of the color arising from the superoxide anion. The concentrations of heparin–SeCyst conjugate were 0.5, 1, 5, 2.0, 10.0 μg Se/ml. Experiments on the other compounds were performed under conditions corresponding to those for the conjugate.

In the measurement of conjugate activities, the concentrations of the compounds not containing Se are expressed by weight. These activities were measured for heparin, SeCyst, as well as ebselen. Data are given as an average of three determinations.

**RESULTS**

**Preparation of the Conjugate** The yield of heparin–SeCyst conjugate (0.24 g) was 80% for heparin and 50% for selenium, and the selenium content of the conjugate was 10.5 μg/mg. No unconjugated free SeCyst was observed when the conjugate was analyzed by gel filtration using a Sephadex G-10 column (data not shown).

**Spectral Measurement** Absorbance at 200—300 nm was markedly enhanced by conjugate formation between heparin and SeCyst (Fig. 1). When SeCyst was reduced by sodium cyanoborohydride, a similar, but transient, increase in absorbance was observed, but this returned to the original level within 30 min (Fig. 1, inset). Treated heparin showed higher absorption over this wavelength range than intact heparin, but its absorption at around 300 nm was much lower than that of the conjugate.

**Reaction with DTNB** Absorbance at 412 nm increased gradually and reached a plateau at around 180 min when the conjugate was incubated with DTNB (Fig. 2). In the case of cysteine, whose concentration was the same as that of selenium in the conjugate used for this experiment, the absorbance instantly reached a plateau and remained at a level which was almost the same as that observed in the conjugate. Absorbance remained at very low levels in intact heparin and SeCyst, although a slight increase was observed in treated heparin.

**GPx-like Activity** The GPx-like activity of the conjugate was observed toward hydrogen peroxide (Fig. 3A), tert-butyl hydroperoxide, or cumene hydroperoxide. The activity was 1.9, 1.4, 1.1 μU for hydrogen peroxide, cumene hydroperoxide, tert-butyl hydroperoxide, respectively, when the conjugate concentration was 5 μg Se/ml. No activity was observed with intact heparin, treated heparin, or SeCyst. Ebselen had several times the activity of the conjugate at the same selenium concentrations.

**DPPH Radical Scavenging Activity** The conjugate had DPPH scavenging activity (Fig. 3B). Treated heparin and SeCyst had a low level of this activity while intact heparin did not. No activity was observed for ebselen.
Superoxide Anion Scavenging Activity The conjugate had activity about half that of ebselen (Fig. 3C). Treated heparin had a similar level of activity, while intact heparin and SeCyst did not.

DISCUSSION

The facts that 1) a similar increase in the absorbance at 200—300 nm was observed in the formation of heparin–SeCyst conjugate (Fig. 1) and in the reduction of SeCyst (Fig. 1, inset) and 2) the increase was transient and returned to the original level in the latter case suggest that the conjugate contains selenol groups in its structure. This speculation was supported by the difference in the reactivity of the conjugate and SeCyst with DTNB (Fig. 2). The release of TNB means that the conjugate contains a selenol group, or groups convertible to selenol, and that a selenol-disulfide exchange reaction occurred between the conjugate and DTNB. The selenol group may be formed during the reduction of the Schiff-base by sodium cyanoborohydride. The chemical form of almost all the selenium atoms introduced into the conjugate is considered to be selenol because the plateau absorbance value for the conjugate was nearly the same as that for cysteine at the corresponding concentration (Fig. 2). The rate of TNB release was much slower in the conjugate than in cysteine (Fig. 2) indicating that access of DTNB to the selenol groups in the conjugate is partly restricted and the selenol is also protected from rapid oxidation, since selenol is usually susceptible to oxidation in air. Therefore, this conjugate may serve as a model compound with a comparatively stable selenol group which is known to be essential for active selenoproteins.

The conjugate showed GPx-like activity (Fig. 3A) and this result also supports the presence of a selenol group in the conjugate since selenol is the active center of the enzyme. The potency of the activity per selenium atom was lower than that of ebselen, which is known to mimic of GPx, or the enzyme prepared by Luo et al., which is reported to have activity close to that of the native enzyme. The weak activity of the present conjugate may be due to the same reason as that for the slow reaction rate of the conjugate with DTNB, and it may also be that the pKₐ of the introduced selenol group is increased by the negatively charged sulfate groups surrounding it resulting in low reactivity.

The conjugate had DPPH radical scavenging activity (Fig. 3B) while ebselen did not, and this is consistent with the report by Noguchi et al.. An electron or hydrogen atom transfer is considered to occur during the scavenging reaction, and the selenol in the conjugate would be more suitable than the selenium in ebselen for carrying out the reaction. Taking into account the fact that the conjugate has scavenging activity toward DPPH and ebselen has not, this conjugate does not simply mimic GPx and it may be expected to serve as a scavenger for both oxygen and non-oxygen radicals.

The conjugate had superoxide anion scavenging activity, which was lower than that of ebselen (Fig. 3C). However, this activity was not due to the introduced selenol group, since treated heparin had similar activity (Fig. 3C). This activity is actually considered to be due to the partially cleaved heparin structure which is common to the conjugate and treated heparin, although the precise mechanism is not clear.

Although it is known that the biological activity of heparin as an anticoagulant is dependent on its molecular weight, unfractionated commercial heparin was used in this study. Therefore, the obtained conjugate will be a mixture of molecules with different molecular weights. It is possible that the activities observed in this study may also be dependent on the molecular weight of the conjugate and further studies with conjugates prepared from fractionated heparins are necessary to clarify this point.

SeP is one of the major selenoproteins in plasma and was first isolated as a 57 kDa species from rat plasma by Yang et al.. Although it has been suggested that SeP may serve as a selenium carrier protein or may be involved in an antioxidant defense system in the extracellular space, this has not been completely demonstrated and its function may not be a simple one, for chemical heterogeneity of SeP has been reported in rat plasma.

The affinity of SeP for heparin is high enough for a heparin-fixed affinity column to be required to purify the protein. Considering that heparin-like substances are present in the body, it is no wonder SeP should play its role by interacting with such substances. SeP contains selenocysteine residues and may have a role in supplying selenol groups to heparin-like substances on the surface of cells. If SeP is not working simply as a selenium carrier, it could be involved in oxidoreductive reactions, especially in antioxidative defense systems. However, the antioxidant function of SeP itself has not been established until recently. Therefore, we assumed...
that the function of SeP is revealed when it forms a complex with heparin-like substances. We believe that the heparin–selenocompound conjugate may have activities which are not exhibited by heparin or low molecular weight selenocompound alone. The heparin–SeCyst conjugate prepared in this study has a selenol group in the molecule and is effective in scavenging active and harmful chemical species. We postulate that such activity is one of the major functions of SeP.

REFERENCES