Effect of N–B Transition on the Microenvironment Surrounding ^{34}Cys in Human Serum Albumin

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The effect of pH on the microenvironment surrounding ^{34}Cys in human serum albumin (HSA) has been studied using acrylodan, a Cys-specific fluorescence probe. The reactivity of ^{34}Cys with 5,5′-dithiobis(2-nitro benzoic acid) (DTNB) followed a pseudo-first-order reaction, and the increase in reactivity was dependent on pH and oleate content. Compared with the N-form of HSA–acrylodan conjugate (pH 6.2), the B-form (pH 8.4) has a blue-shifted Em_{max} and enhanced fluorescence intensity derived from acrylodan covalently attached to ^{34}Cys suggesting that the exposure around ^{34}Cys in the B-form was less than that in the N-form. The conformational change induced by fatty acid increased the exposure around ^{34}Cys, while that induced by an increase in pH decreased it. Further, since the effect of oleate on the fluorescence of acrylodan was nearly the same for both conformers, the effects of pH and oleate on the microenvironment surrounding ^{34}Cys should be independent and additive. We concluded that the increase of reactivity of ^{34}Cys as a function in increasing pH may well be related to an increase in mercaptide ion content.

Key words human serum albumin; ^{34}Cys residue; N–B transition; acrylodan; fluorescence

Human serum albumin (HSA) is characterized by a unique arrangement of disulfide double loops which exist as a series of triplets.[1,2] HSA contains nine such loops, produced as a result of 17 disulfide bonds. Only one cysteine residue (^{34}Cys) exists as a free SH group. Except for salmon albumin, this ^{34}Cys residue is highly conserved in all albumin molecules for which sequences have been determined.[1] ^{34}Cys is part of site V, one of the major ligand binding sites, for endogenous and exogenous thiol containing compounds such as ^{34}Cys, glutathione and captopril, as well as various metals such as Cd, Au, Hg and Ag.[1,3] Recently, Meyer reported that ^{34}Cys is also capable of binding superoxide and nitric oxide (NO).[4] In an earlier study, Wayner reported that ^{34}Cys in HSA possessed radical-trapping antioxidant activity, similar to vitamin E, urate and ascorbate.[5] These results suggest that the ^{34}Cys in HSA may play an important functional role as a scavenger. As a result, recent studies have focused on the chemistry of ligand binding associated with ^{34}Cys.

We previously proposed a mechanism for the formation of HSA–thiol drug conjugates with ^{34}Cys through SH/SS interchange and examined the effects of pH, fatty acid and metal ions on this interchange.[3,6] We examined the microenvironment in the vicinity of ^{34}Cys using the fluorescence probe, acrylodan, which covalently binds only to ^{34}Cys.[7] Our data indicated that the reactive SH residue of ^{34}Cys is located in a crevice on the surface of the albumin molecule and that this reactive SH residue was protected from the solvent, to some extent, by adjacent amino acid residues.[1] We predicted that saturation of high affinity oleate binding sites would be expected to induce a microenvironment change in the vicinity of ^{34}Cys by increasing the exposure of the region in the vicinity of ^{34}Cys. We further proposed the possibility that fatty acid binding might provide a method of regulating the radical-trapping antioxidant activity of this residue.[7]

The reactivity of ^{34}Cys with thiol drugs (the formation of HSA–thiol drug conjugates) is significantly influenced by pH.[3,6] Serum albumin is known to undergo a conformational change in the neutral region, roughly between pH 6 and 9, the so called "N–B transition."[8–10] However, the effect of pH on the microenvironment around ^{34}Cys has not been extensively studied. The present paper reports some studies of the effect of N–B transition on the microenvironment around ^{34}Cys using the HSA–acrylodan conjugate. In addition, the effect of oleate on the N- and B-forms of HSA was also investigated.

MATERIALS AND METHODS

Materials Acrylodan was obtained from Molecular Probes Inc. (Eugene, OR). HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). HSA was defatted using methodology originally described by Chen but with some modifications as described previously.[3] The concentration of albumin was determined using an absorption coefficient (at 279 nm) of 5.31.[11] Oleate (sodium salt) was obtained from Sigma (St. Louis, MO). 5,5′-Dithiobis(2-nitro benzoic acid) (DTNB) was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were reagent grade or better.

Reactivity of ^{34}Cys with DTNB HSA solution (1.0×10^{-4} M, 2 ml 0.067 M phosphate buffer) was preincubated at 37 °C. The absorbance increase at 412 nm was monitored against time after the addition of DTNB (final concentration 2.0×10^{-4} M).[12] The reactivity of the SH residue is expressed following pseudo-first-order reaction.

$$\text{Pro-SH} + \text{DTNB} \rightarrow \text{Pro-SS-TNB} + \text{TNB}^- \quad (1)$$

In Eq. 1, the observed first-order rate constant (k) was calculated from the slope of Eq. 2

$$\ln([\text{TNB}^-]_{t=\infty} - [\text{TNB}^-]_t) = A - kt \quad (2)$$

Preparation of the HSA–Acrylodan Conjugate The HSA–acrylodan conjugate was prepared using Wang's method with minor modifications.[13] A stock solution containing 50 μM HSA was prepared in 0.1 M phosphate buffer (pH 7.0). To 40 ml of this solution, sufficient acrylodan (in the minimum amount of CH_3CN) was added to give a molar ratio of HSA to acrylodan of 1 : 1.

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This mixture was gently stirred and maintained at room temperature for 10 h and then dialyzed at 4 °C against 500 ml 1:20 (v/v) acetonitrile–phosphate buffer. After 12 h, the initial dialysate was replaced with 500 ml phosphate buffer. This solution was changed at 12 h intervals over a 4 d dialysis period. Charcoal was used to remove the remaining acrylodan in the same manner as for the removal of fatty acids.[7]

Fluorescence Measurements Steady-state fluorescence experiments were performed at 25 °C on a Jasco FP-770 fluorometer (Tokyo, Japan). The HSA–acrylodan conjugate was prepared at a concentration of 2.0×10^{-5} M in 0.067 M phosphate buffer. The excitation wavelength for the HSA–acrylodan conjugate was 351 nm using a 5 nm slit width.[13] Emission was then scanned from 450 to 550 nm with slit widths between 5 and 10 nm.

Although probe concentrations were maintained at low levels to minimize inner-filter effects, the intensities were further corrected by Eq. 3.[14]

$$F_{cor} = F_{obs} \times \text{anti log}\left(\frac{A_{em} + A_{ex}}{2}\right) \quad (3)$$

where, F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, and A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

Albumin molecules in aqueous solution are not static structures and undergo a variety of conformational changes.[15] Numerous researchers have investigated the effects of pH and fatty acids on these conformational changes.[8,16–22] Such conformational changes affect drug binding on site I and site II of HSA, but little is known about the effect on site V in the region of ^{34}Cys. We, therefore, studied the effects of fatty acid and pH on this region.

An increase in pH as well as the presence of oleate induced an increase in the observed first-order rate constant for the reactivity of ^{34}Cys with DTNB, as shown in Fig. 1. In all cases, a linear relationship was observed between pH, oleate content and reactivity. We previously examined the microenvironment around ^{34}Cys using the fluorescence probe, acrylodan, which only binds to ^{34}Cys covalently.[7] In that study, we demonstrated that oleate induced an increase in the exposure of ^{34}Cys. From these findings, we proposed that the enhancement of the reactivity of ^{34}Cys might be related to an increase in exposure of ^{34}Cys and that this is induced by oleate.[7]

Serum albumins undergo the N–B transition. It is evident from Table 1 that the HSA–acrylodan conjugate of the B-form of HSA (pH 8.4) showed a blue-shifted Em_{max} and an enhanced fluorescence intensity, compared with the N-form (pH 6.2). This suggests that the exposure around the ^{34}Cys in the B-form was less than that in the N-form. This effect was clearly different from the effects of oleate. In addition, as shown by the previous results and Fig. 2 of this report, an increase in pH induces formation of the mercaptide ion and, as a result, the reactivity of ^{34}Cys increases with increasing mercaptide ion content.[6] If one of the roles of ^{34}Cys in HSA is regulating radical-trapping antioxidant activity, the effect of conformational change as a function of pH may reduce the excess reactivity of ^{34}Cys.

We next studied the effect of oleate on the microenvironment around ^{34}Cys in the N-form or B-form HSA–

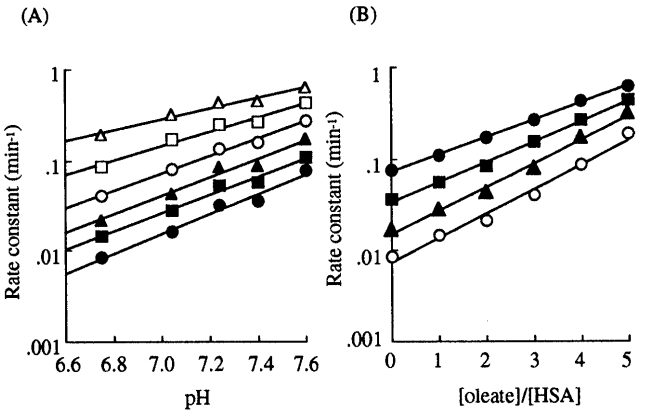

Fig. 1. Effect of Oleate on Enhancing the Reactivity of the ^{34}Cys in HSA at Various pH Values

The reactivity was obtained from first-order analysis of the increase in absorbance at 412 nm. (A) [oleate]/[HSA], the ratio was 0 (●), 1 (■), 2 (▲), 3 (○), 4 (□), 5 (△). (B) the pH was 6.75 (○), 7.04 (▲), 7.40 (□), 7.60 (●).

Table 1. Effect of pH on Fluorescence Parameters of the HSA–Acrylodan Conjugate

pH	λ_{max} (nm)	Relative F.I.[a]
6.2 (N-form)	476.7	1
7.4	475.8	1.035
8.4 (B-form)	470.6	1.103

a) These values are the relative fluorescence intensity at pH 6.2 (N-form).

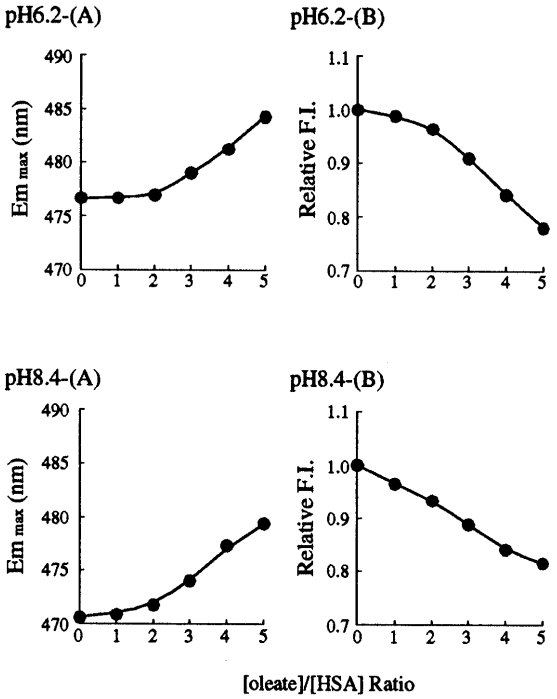

Fig. 2. Effect of Oleate on the Fluorescence Emission Maximum Wavelength (A) and Relative Fluorescence Intensity (B) of ^{34}Cys in the HSA–Acrylodan Conjugate at pH 6.2 (N-Form) and pH 8.4 (B-Form)

acrylodan conjugate. A red-shift of Em_{max} and a decrease in fluorescence intensity was observed for both conformers (Fig. 2). The effects, however, were independent of the N–B transition of HSA. This suggests that the effect of pH and oleate on the microenvironment around ^{34}Cys might be independent and additive (not multiplicative).

The X-ray crystal structure of HSA has provided an extremely valuable model for understanding the structure-function relationship of albumin as a carrier protein,[1] while HSA in solution undergoes a conformational change, the so-called N–B transition, between pH 6 and 9. This N–B transition is important because this conformational change has a great effect on the function of HSA, *e.g.* ligand binding affinity[9] and interaction with membranes.[2,3] We, therefore, studied the effect of the N–B transition on the region surrounding ^{34}Cys. In general, the effect of a conformational change induced by fatty acid on ligand binding, especially for site I (in domain IIA) and site II (in domain IIIA), was parallel to that induced by an increase in pH. However, the effects of fatty acid and pH on ^{34}Cys (in domain IA) were opposed. This suggests that the conformational change induced by fatty acid and pH may affect the microenvironment of ^{34}Cys in HSA independently.

These data suggest that N–B transition would be expected to reduce the reactivity of ^{34}Cys with DTNB. If one of the functions of ^{34}Cys in HSA is as a scavenger of oxygen radicals in the body, this N–B transition might well depress the reduce reactivity of ^{34}Cys.

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