Covalent Binding Between Bucillamine Derivatives and Human Serum Albumin

Ryuichi Narazaki,[1] Mizuho Hamada,[1] Kumiko Harada,[1] and Masaki Otagiri[1,2]

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Purpose. To clarify the mechanism of covalent binding between human serum albumin (HSA) and drugs containing thiol groups, we studied the interactions between HSA and bucillamine (BA) and its derivatives.
Methods. To determine the concentration of HSA-drug conjugate, we used columns of N-methylpyridium polymer cross-linked with ethylene glycol dimethacrylate (4VP-Me), and analyzed the reaction between HSA and BA derivatives kinetically. Following pseudo first-order reaction kinetics, the rate constants of reduction of non-mercaptoalbumin (HNA) to mercaptoalbumin (HMA) (k_a) and formation of HSA-drug conjugate (k_c) were determined.
Results. Formation of HSA-drug conjugate was observed only for drugs containing one thiol group. In compound IV, the plots of k_a and k_c against pH were found to be linear. The HSA-drug conjugate was affected by various factors such as pK_a, pH, temparture and the microenviroment of Cys^{34}. The increases in k_a and k_c against pH were mainly due to the increase in mercaptide ion concentration. Further, fatty acid affected the microenviroment of Cys^{34}, which increased HSA-drug formation.
Conclusions. Cys^{34} located in a crevice on the surface of the protein plays an important role on the formation of HSA-drug conjugate. These results may be useful for elucidating the reaction mechanisms between various proteins and thiol compounds.

KEY WORDS: human serum albumin; covalent binding; bucillamine.

INTRODUCTION

Human serum albumin (HSA) is one of the most important plasma proteins involved in drug delivery in the body. HSA is heterogeneous including components derived from Cys^{34}, mercaptoalbumin (HMA) and non-mercaptoalbumin (HNA) (1). HMA contains a free SH group in Cys^{34}, and in HNA this SH group is masked by Cys or glutathione (GSH), or is oxidized (1,2). The ratio of [HMA]/([HMA]+[HNA]), f_{HMA}, is variable with age and in various disease states such as chronic renal failure, nephirotic syndrome, various kinds of hepatitis, liver cirrhosis, etc. (1).

The pK_a of Cys^{34} (pK_a = 5.0) is extremely low compared with those of Cys and GSH (8.5 and 8.9 , respectively) (3). Due to the high reactivity of Cys^{34}, HSA readily reacts with nitric oxide (NO) or thiol compounds including drugs forming S-nitrosoalbumin (HSA-S-NO) (4) or HSA-drug conjugates (5), respectively. These observations suggest that Cys^{34} in HSA might function as a scavenger against various radicals, or as a buffer (or in storage) of thiol compounds and NO.

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862, Japan.
[2] To whom correspondence should be addressed.

Many drugs bind to HSA reversibly. Recently, however, we reported that *in vitro*, the thiol group-containing drug, captopril (Cp), bound to HSA not only reversibly but also covalently. In that report, we analyzed of HSA-Cp conjugate using N-methylpyridium polymer cross-linked with ethylene glycol dimethacrylate (4VP-Me) columns (6). Using ^{14}C-Cp, we demonstrated that this column could detect HSA-Cp conjugate quantitively. In addition, we clarified the reaction mechanism of this covalent binding. As shown in Fig. 1, we also demonstrated that in the HSA-Cp system, Cp was first reduced from HNA to HMA (equation 1), and then HMA formed HSA-Cp conjugate (equation 2).

$$\text{HNA (HSA-SS-Cys)} + \text{Drug-S} \rightleftarrows \text{HMA (HSA-S}^-\text{)} + \text{Cys-SS-Drug} \quad (1)$$

$$\text{HMA} + \text{Cys-SS-Drug} \rightleftarrows \text{HSA-SS-Drug} + \text{Cys}^- \quad (2)$$

Further, this reaction was facilitated by oxygen, free radicals and endogenous thiols. In this continuing investigation, we focused on the anti-rheumatic drug bucillamine (BA) and its derivatives (7) because BA has two thiol residues of different types, one derived from cysteine (R_2) and the other derived from thio-fatty acid (R_1) (Table I). As derivatives of BA, we analyzed the di-thiol form (compound I, II), mono-thiol form (compound III ~ VI), intradisulfide form (compound VII, VIII), and S-methyled form (compound IX, X).

Various adverse hypersensitivity reactions to thiol-containing drugs such as fever, skin rash, proteinurea, etc. have been reported (8). Recently, the hapten hypothesis of drug hypersensitivity was proposed (9); briefly, the hapten hypothesis suggests that drugs or metabolites become covalently bound to a macromolecular carrier such as a protein and that the resulting drug-protein conjugate can be recognized as an immunogen. There have been no reports concerning the relationship between the properties of the thiol groups in drugs and their reactivity. Thus, we studied the relationship between the molecular properties of bucillamine and its binding affinity to HSA, and further examined the effects of pH, temperature, and fatty acids. From these results, we will discuss the mechanism of binding of BA to HSA.

MATERIALS AND METHODS

Materials

HSA was a gift from Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). BA and its derivatives were gifts from Santen Co., Ltd. (Osaka, Japan). HSA was defatted according to the method described by Chen (10), but after neutralization to pH7.0 by addition of 0.2N NaOH the reaction mixture was dialyzed against water to remove NaCl. The mol wt of HSA was assumed to be 66500.

HPLC Conditions

The HPLC system was comprised of LC-4A pump (Shimadzu, Tokyo, Japan) equipped with a gradient programmer and Shimadzu SPD-2AS UV monitor. N-Methylpyridium polymer cross-linked with ethylene glycol dimethacrylate, the column

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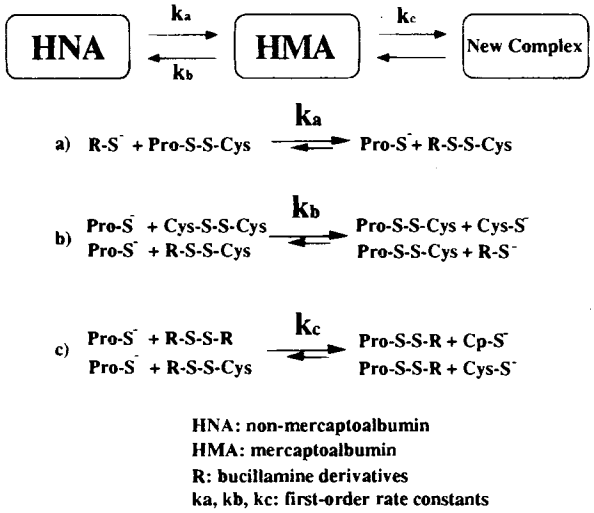


Fig. 1. Possible mechanism for the conformational change of HSA during incubation with BA and its derivatives.

packing material (4VP-Me column), was prepared as described previously (6). HSA was eluted with a 30min linear gradient from 0 to 0.5M sodium acetate in 0.05M Tris-AcOH buffer (pH6.5) at a flow rate of 0.5ml/min at 25°C with detection at 280nm.

Reaction Between HSA and BA Derivatives

HSA was dissolved in phosphate buffer (pH7.4, 0.067M, $\mu = 0.15$) from which endogenous oxygen had been removed by sonication and nitrogen replacement. HSA solution (3.0×10^{-4}M) was preincubated at 37°C for 5 min, and to this an equimolar amount of BA or various derivatives was added. The mixture was incubated at 37°C under anaerobic conditions. After incubation, samples were analyzed by HPLC as described previously.

Definition of HNA

HNA was defined previously (6). Although HNA is a general term which includes HSA-Cys disulfide, HSA-GSH disulfide, and oxidized HSA etc., for convenience, HNA has been tentatively used to refer to HSA-Cys disulfide. Thus, throughout the rest of this report HSA is used to describe HMA, HNA, oxidized form of HSA and HSA-BA conjugate, unless otherwise stated.

Determination of Rate Constant (k_a and k_c)

The reaction mechanism was proposed previously (6), and is shown in Fig. 1. Briefly, on addition of BA derivatives, HNA is reduced rapidly to HMA (equation 1). Next, the produced HMA reacts with BA-Cys mixed-disulfide and forms HSA-BA conjugate. The decreases of HNA (in equation 1) and HMA (in equation 2) follow first order kinetics. Therefore, we could calculate the rate constants (k_a and k_c) from the decreases of HNA (in equation 1) and HMA (in equation 2). In these reactions, no catalytisis was observed.

Determination of Acid Association Constant (pK_a) and Activation Energy (Ea)

The pK_a values of BA derivatives were determined by the titration method; briefly, 5mM BA derivative solutions (20ml) were titrated with 0.1N NaOH

$$pK_a = pH + \ln \{([HA] - [H^+] / ([A^-] + [H^+])\} \quad (3)$$

where, [HA] and $[A^-]$ are the concentrations of undissociated and dissociated drug, respectively, and $[H^+]$ is the proton concentration.

Activation energy was calculated from the Arrhenius equation

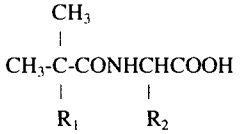
$$K = Ae^{-Ea/RT} \quad (4)$$

where, k is the rate constant, A is the frequency factor, R is the gas constant (1.987 cal mol^{-1} deg^{-1}); and T is temperature (K).

RESULTS

Fig. 1 shows the possible reaction mechanism between HSA and BA derivatives, based upon our previous report (6). Following this scheme, we determined rate constants, k_a and

Table 1. Chemical Structures of Bucillamine and Its Derivatives



Compound		R_1	R_2
I	N-(2-mercapto-2-methyl propanoyl)-L-cysteine (bucillamine)	SH	CH_2SH
II	N-(3,3-dimethyl-1-mercapto butyryl)-L-cysteine	CH_2SH	CH_2SH
III	N-[2-mercapto-2-(methylthio) propanoyl]-L-cysteine	SCH_3	CH_2SH
IV	N-(3,3-dimethyl-1-mercapto butyryl)-S-methyl-L-cysteine	CH_2SH	CH_2SCH_3
V	N-(2-mercapto-2-methyl propanoyl)-S-methyl-L-cysteine	SH	CH_2SCH_3
VI	N-[2-methyl-2-(methylthiomethyl) propanoyl]-L-cysteine	CH_2SCH_3	CH_2SH
VII	6,6,-dimethyl-4,5,-dithia-7-oxo-perhydrocycloheptenepine-2-carboxylic acid	S	$SCH_2(S-SCH_2)$
VIII	7,7,-dimethyl-4,5,-dithia-8-oxo-perhydrocyclooctanociine-2-carboxylic acid	CH_2S	$SCH_2(CH_2S-SCH_2)$
IX	N-[2-mercapto-2-(methylthio) propanoyl]-S-methyl-L-cysteine	SCH_3	CH_2SCH_3
X	N-[2-methyl-2-(methylthiomethyl) propanoyl]-S-methyl-L-cysteine	CH_2SCH_3	CH_2SCH_3

Table 2. Rate Constants of Conformational Changes of HSA with Bucillamine and Its Derivatives

Compound	Rate Constant	
	k_a (h^{-1})	k_c (h^{-1})
I	0.87 ± 0.10	N.D
II	1.15 ± 0.05	N.D.
III	0.59 ± 0.14	1.84 ± 0.64*
IV	0.27 ± 0.04	6.77 ± 1.55*
V	0.50 ± 0.03	0.71 ± 0.44*
VI	0.17 ± 0.07	0.45 ± 0.37*
VII	N.D.	N.D.
VIII	N.D.	N.D.
IX	N.D.	N.D.
X	N.D.	N.D.

Note: N.D.: Not determined (*: × 10^{-3}).

k_c, and the results are shown in Table II. Rate constants k_a were determined only for the derivatives containing free thiol groups. In contrast, k_a could not be estimated in any thiol masked derivatives or intra-disulfide bonded derivatives. Large k_a values were observed for the derivatives with two thiol residues. The rate constants k_c were determined only for derivatives containing one thiol group and compound IV showed the largest k_c value. The acid association constants (pK_a) of these derivatives were determined by the titration method (Table III). pK_a values at R_2 were almost the same, while, those at R_1 were divided into two groups; pK_a values of compounds I and V were about 8.5, and those for compounds II and IV rose to approximately 10.5.

Fig. 2 shows the pH effect of these reaction properties for compound IV. Between pH 6 and 9, the plots of k_a and k_c against pH were linear. Similar findings were also observed for the Cp-HSA conjugate (6).

Thermodynamic parameters for compounds I and IV were calculated using the rate constants k_a and k_c at 25 ~ 45°C. From the slope of ln k_a or k_c against 1/T, we estimated values for activation energies (ΔEa) (data was not shown). ΔEa of k_a for compounds I and IV were almost the same (9.6 and 9.7 kcal/mol, respectively). Interestingly, the ΔEa value of k_c for compound IV was 16.8 kcal/mol, approximately twice that of k_a.

Further, we studied the effects of fatty acids on the formation of HSA-drug conjugates. Various fatty acids, especially oleic acid, effectively increased the formation of HSA-drug conjugate effectively (data not shown). The reactivity was enhanced with increasing concentrations of oleic acid, and a

Table 3. Acid Dissociation Constants of BA and Its Derivatives

Compound	$pK_a(R_1)$	$pK_a(R_2)$
I	8.39	10.22
II	10.80	9.50
III	—	9.86
IV	10.50	—
V	8.56	—
VI	—	10.15

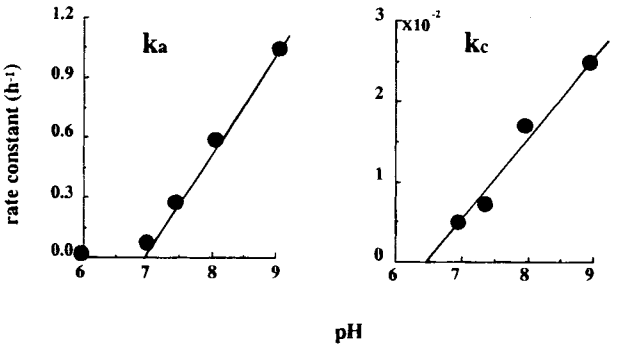


Fig. 2. Rate constants of conformational changes of HSA with Compound IV at various pH.

significant increase was observed at an [oleic acid]/[HSA] ratio of 5 (Fig. 3).

DISCUSSION

We investigated the interactions between HSA and BA derivatives. For compounds I and IV which each have two free thiols, observed k_a values were about twice those of other derivatives. This may have been mainly due to the free thiol content. The k_c values which were found only for derivatives containing one thiol group were quite different depending upon the group and/or position of the substituent. Neither k_a nor k_c values were determined for intra-disulfide-bonded derivatives. However, we previously observed the formation of HSA-Cp conjugate for Cp-disulfide (inter-disulfide) (6). Therefore, the difference between inter- and intra-disulfide bonds might markedly affect conjugation. That is, inter-disul-

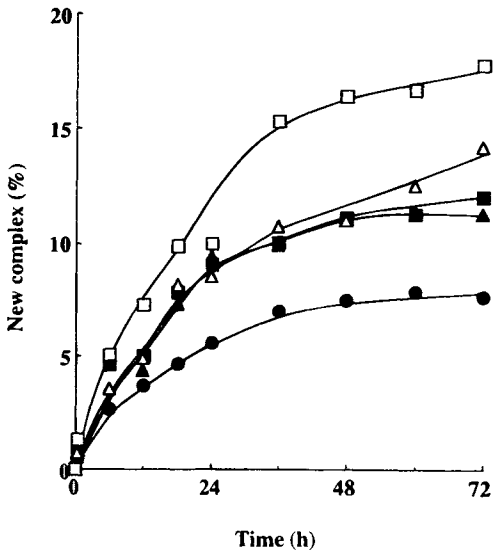


Fig. 3. The effects of oleic acid on the formation of HSA-Compound IV conjugate [HSA]:[oleic acid]:[Compound IV]=1:0:1(●), [HSA]:[oleic acid]:[Compound IV]=1:0.5:1(▲), [HSA]:[oleic acid]:[Compound IV]=1:1:1(■), [HSA]:[oleic acid]:[Compound IV]=1:3:1(△), [HSA]:[oleic acid]:[Compound IV]=1:5:1(□).
All values are means (n = 4).

fide bonds may be much more reactive (less stable) than intra-disulfide bonds.

Furthermore, we examined pK_a values in BA derivatives (Table III). The pK_a values of SH residues were reported generally to be about 8.5, and the presence of carboxyl ions in the vicinity of the SH residue elevates its pK_a value to near 10. The difference in k_a observed between compounds I and II can thus be explained by the distance between R_1 and R_2. In the case of compound II, the distance between SH groups at R_1 and R_2 might be smaller than that for compound I, which facilitates formation of an intra-disulfide bond. This hypothesis was supported by the similar pK_a were obtained for compounds with SH residues located in similar enviroments.

The k_a values varied from $0.173 \sim 0.592$ (h^{-1}) for compounds with one thiol group. Compound III showed the highest k_a values, whereas compound VI showed the lowest. Since these two componds have the same thiol residue in R_2, the difference in the k_a value might be due to the extension of R_1. That is, in the case of compound VI, the SH residue in R_2 was affected by steric hindrance of R_1, which would cause a reduction in reactivity. Compounds V and IV have same the R_2 residue but different R_1 groups. Compound V showed a large k_a value compared with compound IV reflecting the low pK_a of its R_2 groups. Therefore, compound V might have a more reactive SH residue than compound IV. It was previously demonstrated that only the mercaptide ion reacted through SH/SS interchange (11). Thus, the differences in k_a values are considered to be due to differences in pK_a related to the formation of mercaptide ions.

Despite the small differences in pK_a values for compound III, the k_c values were about 4 times higher than for compound VI. This difference may also have been due to a steric hindrance effect similarly to the differences in k_a values. However, in the case of compounds V and IV, the opposite tendency was observed for k_c; compound IV with longer substituents showed a 10-fold larger k_c compared to compound V. This contradictory result was explained as follows. After injection of BA derivatives, HNA was reduced to HMA by the free thiol groups in BA derivaives. Simultaneously, BA derivatives were oxidized to drug-inter-disulfide (or intra-disulfide) or Cp-Cys mixed disulfide (equation (1)). These mixed disulfides reacted with HMA again, and finally formed HNA or HSA-drug conjugate as shown in equation (2) (see Introduction).

In the above reaction mechanism, drugs with low pK_a values easily lost protons and produced the mercaptide form. On the other hand, proton liberation from drugs with high pK_a values was difficult but formation of HSA-drug conjugate was easy. Thus, the high pK_a compound IV would indicate a higher k_c than other derivatives.

Fig. 2 shows the effects of pH on k_a and k_c in compound IV. The k_a and k_c values for compound IV increased linearly with pH. This implies that the reactions between HSA and BA derivatives were mainly affected by the mercaptide ion dependent upon pH. Cater *et al.* reported that Cys^{34} in HSA was located in a crevice on the surface of the protein and that the reactive surface was somewhat protected by several residues (12). In compounds I and IV, ΔEa values of k_a were 9.6 and 9.7 kcal/mol, respectively. Previously, it was reported that ΔEa values of SH/SS interaction between GSH and cystine, or Cys and GSSG, were about 5.1 kcal/mol (13). Such inhibition of the exposure of Cys^{34} would be responsible for the increases in ΔEa values. In compound IV, ΔEa of k_c was 16.8 kcal/mol, the largest value observed in this experiment. The difference in the results was due to the large steric hindrance which affected ΔEa of k_c more strongly than ΔEa of k_a. From these results, the formation of HSA-BA derivative conjugates appeared to be affected by configuration and pK_a of the SH residues.

Takabayashi *et al.* reported that the binding of fatty acids increases the rate of oxidation of Cys^{34} (14). In our experiment, fatty acids increased the formation of HSA-drug conjugate. It was reported that fatty acids bind to HSA strongly and specifically, causing conformational changes in HSA (15). In this study, the largest increase in reactivity was observed in the presence of oleic acid and this effect was dose-dependent.

HSA-acrylodan conjugate (conjugate with Cys^{34}) was prepared in our preliminary experiment. In the presence of oleic acid, relative fluorescence intensity of the dansyl residue was decreased, and λ_{max} was increased (data not shown). Although it is uncertain whether oleic acids affect the pK_a of Cys^{34}, the oleic acid induced a conformational change in HSA and affected the exposure of Cys^{34} and formation of HSA-drug complex. We studied the interaction between thiol-containing drugs and HSA. Recently, the hapten hypothesis of drug hypersensitivity caused by thiol-containing drugs was proposed (9). Our findings may be extrapolated to the binding properties between various thiol compounds and tissue protein. To study the mechanism of adverse drug reactions with thiol compounds, it is important to investigate the reactions occurring between thiol compounds and tissue protein.

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