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S-Nitrosylation of Sulfhydryl Groups in Albumin by Nitrosating Agents

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The reaction of sulfhydryl groups in human serum ablumin with bacteriostatic and hypotensive nitrosating agents such as sodium nitroprusside and sodium nitrite has been examined. The low reactivity of sodium nitroprusside to sulfhydryl groups in albumin has been observed and the sterical inaccessibility of this agent to the site which sulfhydryl group resides was implicated. The reaction of sodium nitrite with albumin was highly influenced by pH and little reactivity was observed at physiological pH. On the other hand, the reaction between albumin and S-nitrosoglutathione, an intermediate induced from the reaction of glutathione and nitrosating agents, resulted in the rapid decrease of free sulfhydryl groups in albumin. S-Nitrosylation of the sulfhydryl group by S-nitrosoglutathione and the subsequent production of mixed disulfide is the probable route of modification. In the physiological system, S-nitrosoglutathione may act as an active intermediate in expressing reactivity of nitrosating agents to sulfhydryl groups in albumin.

Key words: S-nitrosoglutathione, S-nitrosylation, Hypotensive agent, Bacteriostatic agent

INTRODUCTION

The vasodepressor action of compounds that contain nitroso moiety such as sodium nitroprusside (SNP), glyceryl nitrate, sodium nitrite, and nitric oxide (NO) gas has been known for long time (Genest *et al.*, 1983). Especially, SNP, an inorganic nitroso compound, has been employed as an intraveously administered hypotensive drug for over 50 years. Despite the widespread clinical use of such vasodilators, their mechanisms of action remain uncertain (Needleman *et al.*, 1973). Recent studies demonstrate that NO can activate guanylate cyclase, which may be the responsible mechanism for the reduction of blood pressure; however, the molecular mechanism of which NO is transferred from nitroso compound to guanylate cyclase has not been elucidated clearly. On the other hand, the bacteriostatic effect of sodium nitrite has been studied extensively (Hansen and Levine, 1975; Morris and Hansen, 1981), but the mechanism of action is also far from clear.

There is no question that nitrosating agents are reactive molecules which can modify many cellular components. From studies in several laboratories (Mittal and Murad, 1977; Craven and DeRuberis, 1978; Braughler *et al.*, 1979; Morris *et al.*, 1984), it has been inferred that sulfhydryl (SH) groups of proteins could play a role in both bacteriostatic and hypotensive effects. But the reactivity of SH groups in proteins to nitrosating agents has not previously been studied in detail.

Because the chemistry and potential sites of action of nitrosating agents are complex, we have been studying simple model system which may act by the same or similar mechanism as nitrosating agents on SH groups of cellular proteins (Park, 1988). The protein chosen for this study is human serum albumin, referred hereafter as HSA. HSA was chosen because of its presence at high level in serum and possessing a well studied single free SH group per molecule. Furthermore, intravenously administered nitrosating agents will primarily react with simple thiols such as glutathione (GSH), cysteine, and SH groups in HSA.

In this report, we study the reaction of SH groups in HSA with nitrosating agents and a probable intermediate S-nitrosoglutathione (GSNO), which is induced from the reaction of GSH and nitrosating agents, to acertain whether GSNO may function as an active intermediate in expressing the reactivity of SNP and sodium nitrite to SH groups in HSA. Recently, there has been a report suggesting that S-nitrosylation of SH groups

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in albumin may serve as intermediates in the cellular metabolism of endothelium-derived NO and raise the possibility of an additional type of cellular regulating mechanism (Stamler *et al.*, 1992).

EXPERIMENTAL METHODS

Materials

HSA (Fraction V), GSH, 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) were obtained from Sigma. Sodium nitrite was purchased from Aldrich. All reagents used were of reagent grade commercially available.

Reaction of SNP with HSA

The reaction of 10 mM SNP and 2 mM GSH at pH 7.4 and 23℃ was followed by visible spectroscopy at 522 nm. The reaction of 100 μM HSA with SNP at pH 7.4 and 23℃ with or without 8 M urea were followed at 522 nm and total free SH groups were determined by the Ellman reaction (Ellman, 1959).

Reaction of sodium nitrite with HSA

The reaction of HSA with sodium nitrite was carried out by mixing 100 μM HSA and various concentrations of sodium nitrite at pH 7.4 and 3.0. Quantitative conversion of HSA to S-nitroso-HSA was followed by an increased absorbance at 330 nm with time and by employing the Ellman reaction.

Formation of GSNO from GSH and SNP

One ml of 100 mM SNP, in the appropriate O_2-free buffer, pH 7.4, was prepared under a continuous flow of N_2 gas. To this solution, 1 ml of 200 mM GSH, prepared in O_2-free H_2O, was added by means of a Hamilton gas-tight syringe, in a pyrex tube with a serum bottle stopper. A N_2 gas flow maintained through a 20-gauge needle, vented to the atmosphere. After incubation, the reaction mixture was applied to an anion exchange (Bio-Rad, AG 2-X) column (1.5×5 cm) with a UV monitor at 254 nm. GSNO was eluted at low pH (~3).

Preparation of GSNO

GSNO was prepared according to the method of Saville (1958). The method was slightly modified by the dropwise addition of HCl to a solution containing an equimolar amount of GSH and sodium nitrite until a pH 1.5 was attained. After standing for 5 min, the red GSNO solution was neutralized with NaOH. GSNO displays absorption maxima at 544 (ε=15.0 $M^{-1}cm^{-1}$) and 332 nm (ε=750 $M^{-}cm^{-1}$).

Reaction of GSNO with HSA

The reaction of GSNO and HSA in 0.1 M phosphate, pH 7.4 was followed by decrease of absorbance at 544 nm and by employing the Ellman reaction. Prior to determine free SH groups, reaction mixtures were applied to a Sephadex G-25 column.

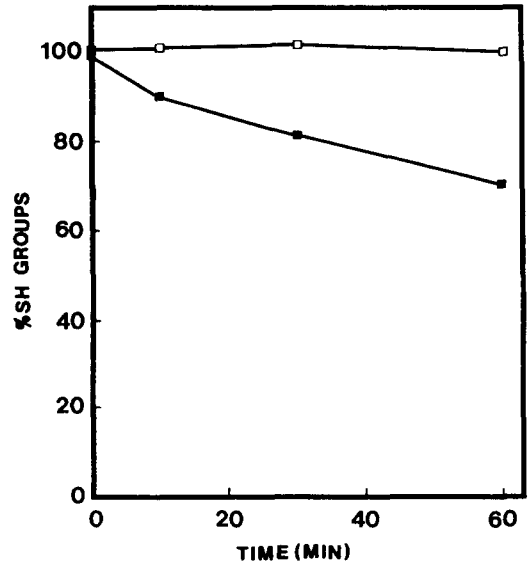

Fig. 1. Reaction of 100 μM HSA and 4 mM SNP in the presence (■) and in the absence (□) of 8 M urea. The remaining SH groups were determined by Ellman reaction and expressed as a percentage of the value from the zero time incubation.

RESULTS AND DISCUSSION

Reaction of SNP with HSA

The reaction of SNP with GSH at pH 7.4 gave rise to an intense red color which developed instantaneously and then faded gradually. The spectrum of the colored intermediate showed a maximum at 522 nm. It has been known that the brightly colored adduct having the structure, $[(CN)_5FeN(O)GS^{3-}]$, may be induced by the addition of anion GS^- to the coordinated NO moiety (Johnson and Wilkins, 1984).

On the other hand, HSA did not give coloration with SNP even at higher pH, although this protein gave a positive reaction with Ellman's reagent. The total SH content of the HSA determined with DTNB was not changed with a higher concentration of SNP (20 mM), even with longer incubation time (1 hr). To examine whether the low reactivity of SNP to HSA may be due to sterical inaccessibility the reaction was carried out in 8 M urea solution where HSA is supposed to be unfolded. As shown in Fig. 1, the reaction of 100 μM HSA with 4 mM SNP at pH 7.4 generated an easily measureable decrease in absorbance at 412 nm due to the reaction of SH groups in HSA with SNP. The effect of urea on the reaction of SNP with HSA suggesting that SH groups in HSA are in a sterically restricted environment that has hydrophobic character.

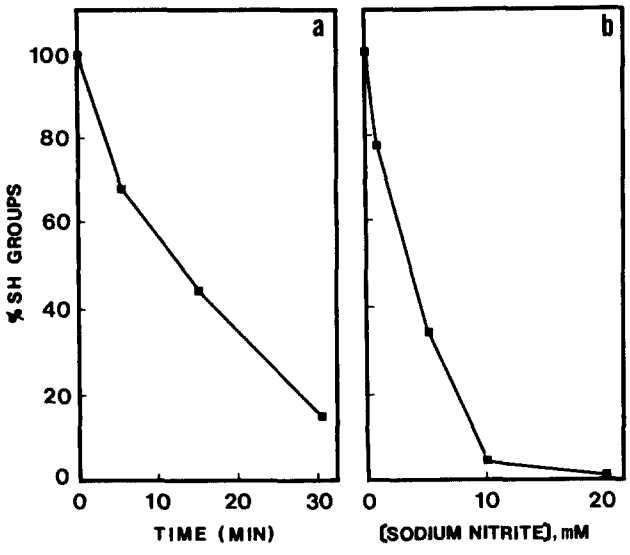

Fig. 2. (a) Time course of the reaction between 100 μM HSA and 2.5 mM sodium nitrite at pH 3.0 and 23℃. (b) Reaction of 100 μM HSA with various concentrations of sodium nitrite at pH 3.0 for 10 min. The remaining SH groups were determined and expressed as described in Fig. 1.

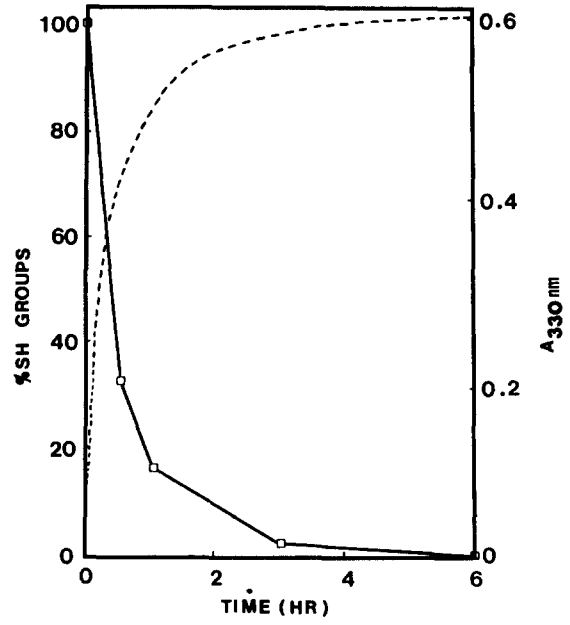

Fig. 3. Increase in the absorbance at 330 nm (---) and the concomitant loss of SH groups in HSA as determined by Ellman reaction (□) upon the reaction with 0.5 mM HSA and 0.5 mM sodium nitrite at pH 3.0.

There is also evidence for the existence of negatively charged group nearby SH groups in HSA (Wilson *et al.*, 1980).

Reaction of nitrite and HSA

Another nitrosating agent sodium nitrite did not react readily with thiols at neutral pH. The reactivity of sodium nitrite with GSH varies considerably depending on the pH. The reaction between HSA and sodium nitrite at pH 3.0 and 23℃ was followed by the Ellman reaction (Fig. 2). Fig. 2a shows a time course of the reaction of 100 μM HSA with 2.5 mM sodium nitrite and Fig. 2b shows reactions of 100 μM HSA with various concentrations of sodium nitrite for 10 min incubation. The absorbance at 330 nm, characteristic for S-nitrosothiols, increased with reaction time and the Ellman reaction showed a concomitant loss of free SH groups (Fig. 3). A conformational change known as the N-F transformation occurs in HSA at pH 3.6-4.0. This could possibly influence the reactivity of the SH group in part. The most important factor for the nitrite reaction has been assumed that the protonation of nitrous acid, which appears necessary for initiating all nitrosation reaction. But it seems likely that the intracellular pH would not be low enough to maintain significant level of protonated nitrous acid (pKa=3.4) and to induce conformational change, so direct modification of SH groups in HSA could be minimal and slow.

Reaction of GSNO with HSA

There is information available which suggests that the bacteriostatick activity of sodium nitrite and the hypotensive activity of sodium nitrite and SNP can be markedly enhanced by the help of thiols such as cysteine and GSH (Incze *et al.*, 1974; Ignarro and Gruetter, 1980; Brandwein *et al.*, 1981). In view of these findings, it can be assumed that simple thiols may serve as a nitrose group carrier in the mechanism which the activity of proteins is subject to regulating through modification of critical SH groups.

Due to the extremely fast reaction between GSH and SNP and the high cellular concentration of GSH it can be postulated that one of the reaction products, we propose GSNO, could be an active species for the biological effect of nitrosating agents including SNP. S-Nitrosothiols have been known as a bacteristatic agent which interfere with the development of bacterial spores into vegetative forms (Incze *et al.*, 1974; Hansen and Levine, 1975). They also have been implicated to involve relaxation of vascular smooth muscle (Ignarro *et al.*, 1980, 1981). We have been shown that S-nitrosothiols can act as hypotensive agents as effective as SNP (Means and Park, 1990). There has been a recent upsurge of interest in S-nitrosothiols, largely owing to the demonstration that S-nitrosothiols may be a precursor of endothelium-derived relaxing factor (EDRF) (Furchgott and Vanhoutte, 1989; Ignarro, 1989; Myers *et al.*, 1990).

Formation of GSNO from SNP with GSH was difficult to monitor by visible spectroscopy because of the necessity of removing the interfering red-colored complex before spectral analysis of reaction mixture. This can be separated by an ion-exchange column from the

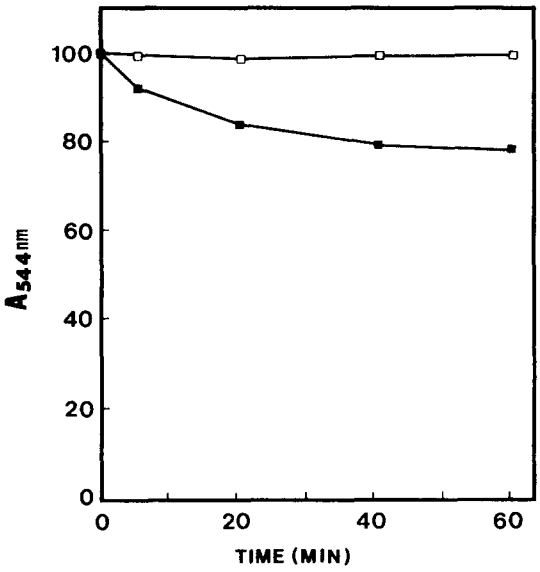

Fig. 4. Decrease in the absorbance at 544 nm upon the reaction of 1 mM HSA and 2.5 mM GSNO (■) and GSNO control (□).

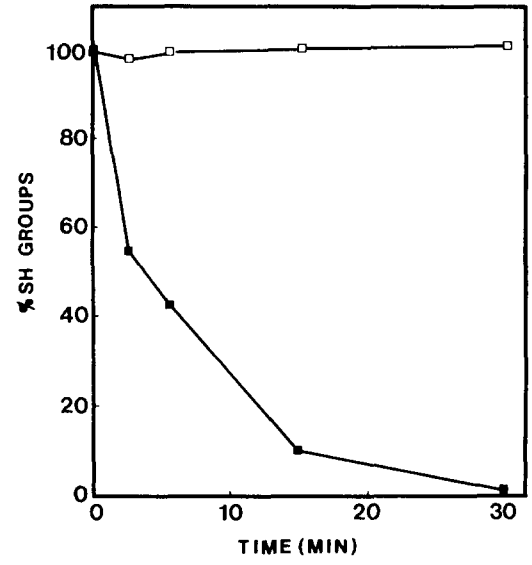

Fig. 5. Total SH groups in an untreated 100 μM HSA (□) and loss of SH groups in HSA determined by Ellman reaction upon the reaction of 100 μM HSA and 1 mM GSNO at pH 7.4 and 23℃ (■) with various incubation times.

mixture of anaerobically incubated GSH and SNP. GSNO was observed to elute from the column monitoring their absorbance at 544 nm. GSNO solution has been easily prepared by the reaction of GSH and sodium nitrite at pH 1.5. GSNO formation from sodium nitrite occurred best at pH below 5.0, although little product formation was evident at pH 7.4. Unlike the reactions involving sodium nitrite, formation of GSNO induced by SNP occurred best at neutral pH. This result indirectly explains that much lower biological activity of sodium nitrite compared to SNP at physiological pH (Mittal and Murad, 1977; Ignarro *et al.*, 1981).

The reaction of 1 mM of HSA with 2.5 mM GSNO at pH 7.4 caused decrease in absorbance at 544 nm (Fig. 4). In order to establish if the reaction was due to the modification of SH groups in HSA, Ellman reaction was performed to determine the concentration of free SH groups after treatment with GSNO was carried out. Incubation of GSNO-modified HSA with DTNB revealed a significant decrease in the concentration of free SH groups when compared to an untreated control (Fig. 5). The reaction of GSNO with thiols revealed that GSNO is a nonselective nitrosating agent which acts by transferring its nitroso group directly to other thiols (transnitrosation). This reaction is extremely fast and reaches equilibrium instantaneously (Park, 1988). On the basis of previous finding, it can be postulated that nitrosylation of the SH groups in HSA by GSNO and the subsequent production of the protein mixed disulfide as a possible route of modification (Albumin-SH + GSNO $\rightleftarrows$ Albumin-SNO + GSH $\rightarrow$ Albumin-S-SG + HNO). The effectiveness of GSNO to react with SH groups in HSA is consistent with the suggestion that this species might act as an active intermediate in experessing the reactivity of nitrosating agents to SH groups in proteins in the biological system (Park, 1988; Stamler *et al.*, 1992). Recently, it has been shown that S-nitrosoalbumin induces similar, but slower and much more persistent biological effects compare to simple S-nitrosothiols and NO. Furthermore, it has been suggested that protein S-nitrosylation may serve as a signal transduction mechanism analogous to phosphorylation and this reaction mediated thiol-disulfide exchange may involve several regulatory mechanism (Stamler *et al.*, 1992).

Although this study does not define any physiological role for the SH groups in HSA on the nitrosating agents, the reactivity of GSNO reflects the ability of nitrosating agents react with SH groups of proteins in a hydrophobic environment. It can be assumed that many proteins which contain reactive SH groups may also react with nitrosating agents in a similar manner.

ACKNOWLEDGEMENTS

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