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Circular Dichroism Quantitative Estimate of α-Helix Content of Human Serum Albumin in the Presence of NaSCN, Urea and KCl at Various pH

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ABSTRACT. Conformational changes in human serum albumin (HSA) in the presence of several concentrations of NaSCN, urea and KCl at various pH were examined quantitatively on the basis of rotational strength at 208 nm by means of circular dichroism (CD). The α-helix content of HSA was markedly dependent on concentrations of NaSCN and urea, but not on KCl. However, when these salts coexisted with a concentration of hydrogen ion in the albumin solution, the α-helix content of HSA was markedly dependent on all these salts. Among these salts, the distortion power of NaSCN on the conformational stability of the peptide backbone was undoubtedly several hundred times stronger than that of the other salts. Conformational changes in HSA were scarcely observed at pH 4.8-10.0 under a constant concentration of each salt, but were more dependent on pH outside of the above range regardless of salt. The α-helix content of highly denaturated HSA in solution containing a high salt concentration was less dependent on the hydrogen ion concentration, while at a low concentration of each salt, it was pH dependent, as if there was no salt.

Key words : Circular dichroism — Human serum albumin — Conformational alteration — Sodium thiocyanate

Hofmeister[1] was the first to recognize specific ion effects on macromolecules, and gave the name "chaotropic salts" to those ions which specifically lead to unfolding, extension and dissociation of proteins.[2] More recently, Sawyer *et al.* demonstrated that NaSCN does not cause a major shift in protein conformations at low concentrations.[3] NaSCN has been successfully employed in the study of *in vitro* steroid receptors.[4-8] In addition, it has been recently reported that *in vitro* administration of NaSCN showed an inhibitory effect on normal and neoplastic mammary development through thyroid hormone modification in female mice and rats,[9,10] and that it also prevented the gastrotoxicity of aspirin and related drugs in rats.[11] To clarify the effects and mechanism of *in vitro* and *in vivo* NaSCN on the receptor proteins involved in these biological reactions, it would be very useful to obtain information regarding the effect of NaSCN on secondary conformational change in the protein affecting the drug-protein interaction, since it is generally supposed that the biological response of a drug action is closely related to the intensity of the interaction between the drug and the protein. Notwithstanding, most approaches described previously have not

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proved appropriate for following the effects of NaSCN on conformational change. Therefore, little information is available on the relationship between conformational change and salts.[12,13] Circular dichroism appears to be a more suitable tool for monitoring the conformational change in proteins under a variety of conditions. Human serum albumin was used here to answer questions regarding the relationship between salts and conformational change, because it is well known that HSA binds a variety of drugs and that this drug binding ability is dependent on conformational alteration of this protein.[14–16]

The present paper attempts to answer the above questions on the basis of the detailed CD spectral behavior of HSA in phosphate buffer when altered by pH and/or various concentrations of NaSCN, and describes the marked effects of NaSCN concentration on the secondary structure as compared with KCl and urea.

MATERIALS AND METHODS

Reagents. Redistilled, deionized water was used throughout this investigation. All other chemicals used were analytical grade reagents and were employed without further purification. Essentially fatty acid-free human serum albumin was obtained from Sigma, St. Louis, MO. The albumin concentration was 13.1 μM (0.09%), and all solutions were made with 1/15 M phosphate buffer and adjusted to the desired pH by addition of small aliquots of 1.0 M HCl or 1.0 M NaOH.

Circular Dichroism Measurement. CD measurements were made at 25°C with a Union Giken Mark II spectropolarimeter calibrated with(+)-D-camphorsulfonic acid. All spectra were recorded in a square quartz cell with a 1.0 mm path length using a full scale deflection of 0.02 and a spectral band width of 5.0 nm. Results are expressed as molar ellipticities, $[\theta]$ (deg cm^2 dmol^{-1}), calculated with reference to the HSA concentration, using a molecular weight of 69000. Each CD spectrum reported is the average of 20 scans. α-helix content was estimated from the molecular ellipticity at the 208 nm band as described elsewhere by Greenfield *et al.*[17] It has been reported that no difference larger than the experimental error has been observed among various samples of HSA with respect to the CD between monomeric bovine serum albumin and unchromatographed protein.[18] Therefore, essentially fatty acid-free HSA from the Sigma Chemical Co. was used throughout the experiment without any further treatment.

RESULTS

There were two peaks present in the CD spectrum of HSA in pH 7.4 phosphate buffer at 208 and 220 nm (strong skeletal absorption of the peptide) in the 200–260 nm range as has previously been reported,[19] and these can be seen in Figure 1. The amplitude of curves at pH 3.0 and 12.0 showed a remarkable difference in their dependence on the hydrogen ion concentration in comparison with that of the band at pH 7.4. In the present investigation, the CD peak at 208 nm was mainly used, since the amplitude of this band correlates with the α-helix content of HSA. The influences of NaSCN, urea and KCl

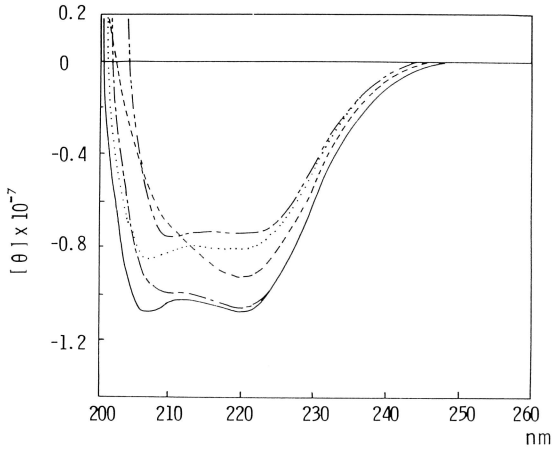

Fig. 1. CD spectra of HSA at various pH in the presence of various concentrations of NaSCN in 200–260 nm region at pH 7.4. CD curves of pH 4.8 and 10.0 were quite similar to that of pH 7.4. HSA : pH 7.4 ; (——), pH 3.0 ; (……) and pH 12.0 ; (— — —). HSA-NaSCN : 2.0 mM ; (— — —) and 40 mM ; (— — —).

on HSA in phosphate buffer at pH 7.4 over concentration range from zero to 40 mM, 6.0 and 3.0 M, respectively, were investigated at room temperature. CD curves showing the effect of NaSCN on the optical rotation of the protein are presented in Figures 1 and 5. Figures 2-4 are plots of α-helix content calculated by the 208 nm band strength for HSA showing the influences of pH and/or those salt concentrations. The rotational strength of 208 nm at pH 7.4 reflected significant decreases in α-helix content with increases in the concentrations of NaSCN (Fig. 1) and urea (Fig. 3), while KCl showed a constant value of ellipticity regardless of its concentration (Fig. 4). When there were changes in the pH in the buffer containing the respective salts of a constant concentration, all titration curves of α-helix content vs pH showed a more or less extended

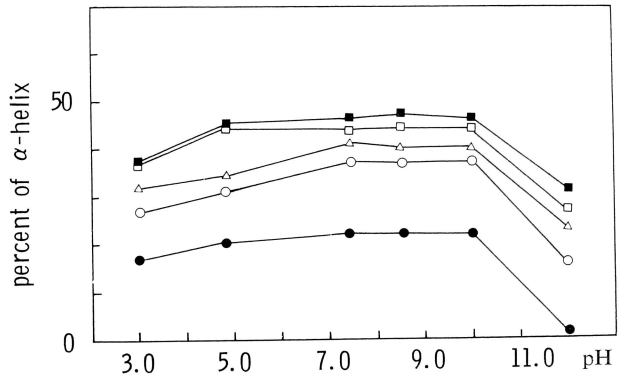

Fig. 2. The plots of α-helix content (%) vs pH. The α-helix content (ordinate) of HSA was estimated from the ellipticity strength of 208 nm in the presence of various concentrations of NaSCN at various pH (abscissa). no-salt ; (■), 65 μM ; (□), 2.0 mM ; (△), 10.0 mM ; (○) and 40.0 mM ; (●).

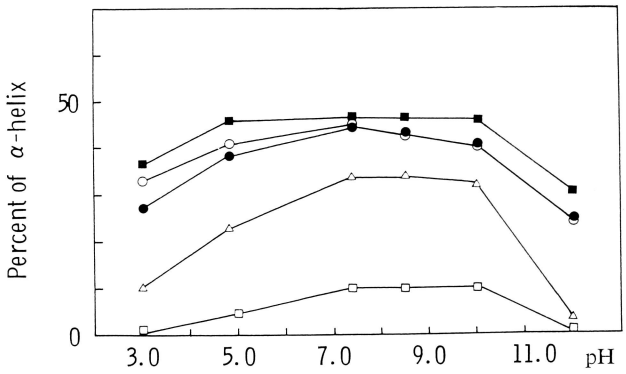

Fig. 3. The plots of α-helix content (%) vs pH in the presence of various concentrations of urea. 0.5 M ; (○), 1.0 M ; (●), 3.0 M ; (△) and 6.0 M ; (□). Conditions were as in Fig. 2.

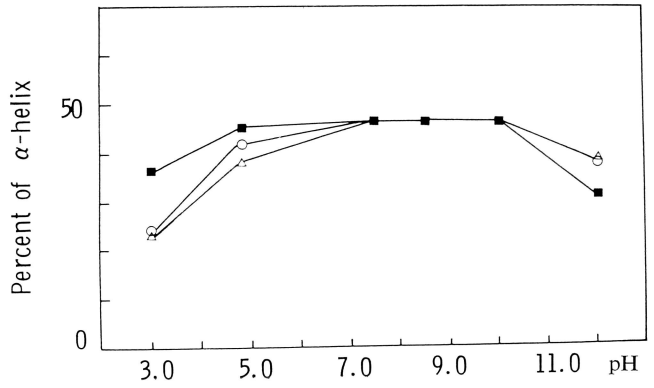

Fig. 4. The plots of α-helix content (%) vs pH in the presence of various concentrations of KCl. 1.0 M ; (○) and 3.0 M ; (△). Conditions were as in Fig. 2.

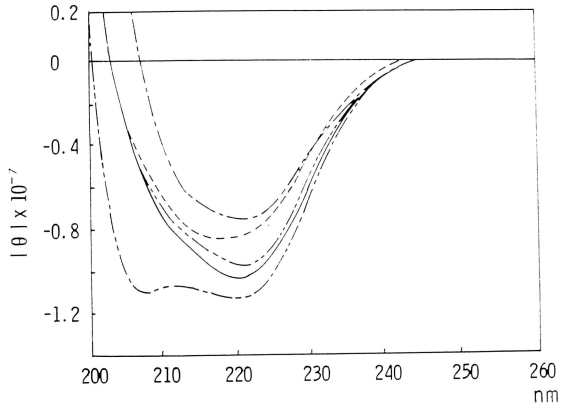

Fig. 5. CD spectra of HSA in the presence of a constant concentration of 40 mM NaSCN at various pH in 200–260 nm. pH 7.4 ; (——), pH 4.8 ; (— - - —), pH 3.0 ; (- - - - -), pH 12.0 ; (— - —) and HSA only at pH 7.4 (— - - - - —).

plateau through the isoelectric region. However, they changed moderately at acid and alkaline pH values, as shown in Figures 2–4. In general, upon denaturation by salt or pH, the negative band at 208 nm became weaker with increasing concentrations of salt on both sides of the isoelectric region. NaSCN displayed some different curves marked by a decreased rate in α-helix content in acid as compared with those of urea and KCl. Urea, on the other hand, showed a remarkable pH-dependence of α-helix content at 3.0 M in alkaline and acid pH values, but not at 6.0 M (Fig. 3). KCl did not display any concentration dependence of salt in an isoelectric region, but displayed slight dependence in other pH regions (Fig. 4). The curves of α-helix content vs pH for only albumin were quite different from those found for the protein when the salts coexisted. The α-helix content of highly denaturated HSA in solution containing a high salt concentration was no longer dependent on the hydrogen ion concentration, while at a low concentration of each salt, it was pH-dependent as if there was no salt. A part of the spectra in Figure 1 shows the effects of NaSCN on HSA at pH 7.4 in concentrations of 2 and 40 mM and that in Figure 5 also shows the effect of various pH at a constant concentration of 40 mM NaSCN. The rotational ellipticity of 208 nm was much more dependent on NaSCN concentration and pH than that of 220 nm. The degree of weakness of whole curves in the presence of NaSCN over corresponding wavelengths greatly differed from those of urea and KCl (spectra not shown). This strongly suggests that NaSCN has a major effect on α-helix rather than on β-sheet or random conformations responsible for absorption (220 nm) near to, but outside of the 208 nm band, in contrast with urea and KCl. In addition to this special effect of NaSCN on the 208 nm band, it was very clearly demonstrated that the destruction power of α-helix in HSA due to NaSCN was a hundred times stronger than that of the other salts at 40 mM concentration. To make sure that NaSCN of a low concentration modifies the conformation of HSA, the effect of NaSCN on α-helix structure was investigated using poly-L-lysine at pH 11.0, under which conditions it demonstrates one hundred percent α-helix conformation. The effects of millimolar concentrations of the salt on conformation as contrasted to those of urea and KCl will be presented in a separate paper.

This represents an extension of some of the work done by investigators studying the effect of denaturation on the rotatory ellipticity of HSA.[12,13] Our results on the CD spectra of HSA in phosphate buffer at different pH or concentrations of urea and KCl are essentially the same as theirs and are included here for the sake of completeness and confirmation of those authors' findings.

DISCUSSION

The ellipticity power corresponding to α-helix content at 208 nm of optical active HSA will change in an acid or alkaline solution according to pH titration curves.[12] This is brought about as a result of salt formation, which alters the magnitude of the partial ellipticity contribution of the substituents involved. Since the isoelectric point of HSA is 4.8, it will show a pH dependence of ellipticity at both sides of this point. The protonized basic groups, such amino groups as arginine, lysine and histidine, in HSA increase as a result of decreases

in the pH value from 4.8, while the deprotonized acidic groups of aspartic and glutamic acids increase as a result of increases in pH from the isoelectric point of the protein.[20] Thus, it is proposed that the ratio of ionized to free groups may be easily changeable on both sides of the isoelectric point. It appears possible to account for the changes in the molecular ellipticity of the protein in terms of the binding of anion (pH 4.8>) or cation (pH 4.8<) to the ionized groups of the polypeptide chain. Consequently, the pH titration curve in HSA showed a more or less defined plateau in the neighborhood of the isoelectric point (Figs. 2, 3), but more dependence on the hydrogen or hydroxy ionic concentrations at other points because of the asymmetric center disturbance caused by rupture of the intramolecular hydrogen bond formed by amino or carboxyl groups responsible for the stabilization of secondary conformation and for a partial contribution to the overall optical activity of the protein. In addition, HSA in acid solution showed a somewhat smaller amount of α-helix content than in alkaline solution, where it is to be expected that such basic groups as the amino, imidazol and guanidyl groups will remain free judging from the isoelectric point of HSA. These findings suggest that the conformational stability of the protein is responsible for the ionized basic group as well as for the salt formation of the carboxyl group with Na^+ or K^+.

On the other hand, the molecular ellipticity corresponding to the α-helix content of HSA in the presence of various concentrations of NaSCN, urea or KCl diminished continuously with increases in those salt concentrations in comparison with those of respective pH. This decrease was more depedent on the acid than on the alkaline side (Figs. 2, 3). The interaction between the protonized amino group of the protein and anions of each salt, and the interaction between the deprotonized carboxyl group and cations might be the essential feature of salt formation leading to changes in the ellipticity strength in acid and alkaline media, respectively. By way of illustration, a free NH_2 group in HSA arising from arginine, lysine and/or histidine which ionizes to NH_3^+ at a pH of around 3.0 forms respectively, a $NH_3^+SCN^-$ and/or $NH_3^+Cl^-$ complexes with NaSCN and KCl in addition to $NH_3^+OH^-$, whereon a free COOH group may remain unmodified. By this means, the formation of such salt results in neutralization of the positive charges on the polypeptide. This added steric and mutual repulsion of the neutralized groups, and interaction of the electrical asymmetry in the optical active center, make a partial contribution to the overall ellipticity of HSA. Concomitantly, the bigger the interactive groups are, the more the asymmetric center will be distorted. This suggests obviously that the distinct difference between NaSCN and KCl in the effect on absorption at 208 nm may be due to disorder of the asymmetric center in the protein caused by more bulky linkage and abundance of $NH_3^+SCN^-$ than with $NH_3^+Cl^-$ in the case of KCl. On the other hand, urea is a weak base and partially protonized in acid pH. Therefore, in acidic solution, the chlorine ion is the only anion for formation of the complex with the protonized base group of the polypeptide and/or urea like $NH_3^+Cl^-$ (eg. Cl^- from added HCl). The neutralization of a part of the basic group leading to rupture of the intramolecular hydrogen bonds would allow the entrance of urea between the secondary structure in the polypeptide chains to eventually reduce molecular ellipticity through disturbance of the asymmetric center.[21] In this manner, it appears possible to account for the remarkable decrease in α-helix content

in Figure 2 as being due to the distance of the acid side from the isoelectric regions under the increase in the urea concentration.

A question of considerable interest here is why the molecular ellipticity of HSA was markedly decreased by such a low concentration of NaSCN. Unfortunately, at this time, only a very tentative suggestion can be offered in partial explanation of the phenomenon. Fundamentally, denaturation is an intramolecular change in which there is a dislocation and relocation of certain constituent atoms and groups such as NH_2 and COOH, and it involves the exposure of nonpolar groups, together with a spatial redistribution of the polar group. It is evident that the appearance of any chromophore group in closer proximity to an asymmetric center due to NaSCN will lead to decreased molecular ellipticity through vicinal action. It may be supposed that the flexible conformation of HSA permits many potentially active chromophore groups to interact with each other, or with other asymmetric centers. Especially, on general unfolding of the protein structure through NaSCN action, many of the groups become physically free to come within the range of action of current asymmetric centers, and thereby make decreased contributions to the overall optical ellipticity of the molecule. The destructive effect of millimolar concentrations of NaSCN on the conformation of HSA was quite different from the results of Sawyer *et al.*[3] showing that salt did not cause a major conformational alteration in a protein. They demonstrated that NaSCN provided a highly effective means for dissociation in multienzyme complexes and that it brought about an improvement in the solubility of membrane-bound proteins.[3] This NaSCN action is apparently due to the rupture of the hydrophobic interactions and hydrogen bonds in the proteins which normally function to stabilize the protein complexes. When a salt with such action is applied to a free peptide molecule, which is stabilized by the same forces as the protein complexes, the intramolecular force of stabilization within the protein should be disrupted by NaSCN as is the case with intermolecular forces. Accordingly, the modification of such a force intramolecularly results in a conformational change. Therefore, the NaSCN effect, without causing major changes in the secondary structure of the protein as previously reported, may be found to be the only peptide mainly composed of a random structure or which has a rigid structure.

As mentioned above, an attempt was made to correlate the pH-dependence of the molecular ellipticity on HSA under various concentrations of NaSCN, urea and KCl. The contribution to the ellipticity of the protein is attributed to distortion of the secondary helix by complex formation with base or acid groups on the polypeptide chain of HSA. In particular, the extremely strong effect of NaSCN on the secondary conformational modification of HSA might be one factor causing the biological responses described in the introduction. The conformational alteration responsible for a concentration of a few millimolar orders of NaSCN should affect the interaction of ligands and proteins such as receptors or enzymes composed of α structure.

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