Color Change of New Coccine in a Lysozyme–Alimezine–Trimetoquinol Syrup: Optical Spectroscopic and Kinetic Analyses

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A color change of New coccine in an alimezine syrup after the mixing of commercial alimezine, lysozyme and trimetoquinol syrups was studied by means of spectroscopic methods. No color change of New coccine in the alimezine syrup was observed by an addition of either lysozyme or trimetoquinol syrup. Yet both lysozyme and trimetoquinol were required for drastic changes in absorption and circular dichroism spectra of New coccine. From these spectral changes, it was considered that the color change of New coccine resulted from the binding of New coccine to lysozyme. In this case, it seems that New coccine molecules are placed in a hydrophobic lysozymal region with a specific configuration. Since the color change was observed also by a mixing of pure New coccine, lysozyne and sodium sulfite, the color change of the admixture mentioned above likely resulted from the addition of sulfite groups to New coccine molecules. Sodium sulfite is contained in commercial syrups as a stabilizer. From the thermodynamic parameters calculated from the spectral changes, a possible role of lysozyme in the color change of New coccine was demonstrated. The values of the parameters suggest also that lysozyme fixes New coccine molecules in a specific conformation.

Keywords New coccine; alimezine; lysozyme; trimetoquinol; sodium sulfite; absorption spectrum; CD spectrum; thermodynamic parameter

An admixture of alimezine, trimetoquinol and lysozyme syrups is widely used to treat the common cold in children. The alimezine (10-[3-(dimethylamino)-2-methylpropyl] phenothiazine tartrate (2:1)) syrup used for antihistaminics[1] is colored red by the addition of a food additive, New coccine (3-hydroxy-4-[(4-sulfo-1-naphthalenyl)azo]-5,7-naphthalenedisulfonic acid trisodium salt). It is well known, however, that the red color of this syrup tend to change to yellow several hours after admixing of trimetoquinol (1-1-(3,4,5-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) syrup for bronchodilation[2-4] and lysozyme syrup for mucolytic enzyme. Such a color change has caused patients to feel anxious about its safety.

In this report, we described the properties of the color change of the alimezine syrup to clarify the mechanism from a view point of quality control.

Experimental

Materials Lysozyme (Leftose) syrup was obtained from Nippon Shinyaku Co., Ltd. (Kyoto, Japan). Alimezine syrup which contains New coccine as a coloring matter was from Daiichi Seiyaku Co., Ltd. (Tokyo, Japan). Trimetoquinol hydrochloride (Inolin) syrup was purchased from Tanabe Seiyaku Co., Ltd. (Osaka, Japan). Simple syrup used as solvent was from Hoeiyakko Co., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis). All other chemicals used were of an analytical grade purity.

Preparation of Samples A lysozyme syrup was mixed with equal volumes of either alimezine syrup containing New coccine and a simple syrup (system (a)) or a trimetoquinol syrup (system (b)). The mixtures were allowed to stand at room temperature and their spectra were measured at appropriate times after preparation.

Spectral Measurements A Hitachi 220 spectrophotometer and a Jasco J-400X spectropolarimeter equipped with a data processor were used for absorption and circular dichroism (CD) spectral measurements, respectively. The measurements were carried out at room temperature. The observed absorption and CD spectra were expressed in terms of absorbance and molar ellipticity, $[\theta]$, respectively. The values were calculated on the basis of the initial concentration of New coccine. For measurements of absorption and CD spectra, the cells used were of 1 cm path length. In the CD measurement, the sensitivity was 0.5 m°/cm.

Results

We examined the effects of lysozyme on New coccine in alimezine syrup in two systems: (a) a lysozyme–alimezine–simple syrup and (b) a lysozyme–alimezine–trimetoquinol syrup. As shown in Fig. 1a, New coccine in symple syrup shows a band at about 510 nm with a shoulder at about 420 nm. When a lysozyme syrup was mixed with an alimezine syrup containing New coccine (system (a)), the peak at 510 nm shifted slightly to the longer wavelength side with a slight decrease in intensity. After standing for 24 h, the intensity of the 510 nm band decreased and the shoulder increased. When lysozyme syrup was mixed both with the alimezine syrup containing New coccine and the trimetoquinol syrup (system (b)), the absorption spectra of

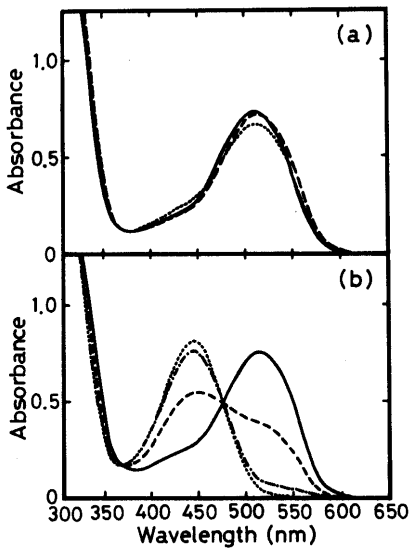

Fig. 1. Absorption Spectra of New Coccine in the Lysozyme–Alimezine–Simple Syrup (a) and the Lysozyme–Alimezine–Trimetoquinol Syrup (b)

(a) ——, New coccine in simple syrup; — —, New coccine in the lysozyme–alimezine–simple syrup immediately after preparation; ------, New coccine in the lysozyme–alimezine syrup 24 h after preparation. The concentration of both New coccine and alimezine syrups was 0.01%. The lysozyme concentration was 5 mg/ml. (b) ——, immediately after preparation; — —, 1 h after preparation; —·—, 5 h after preparation; ------, 24 h after preparation. The concentration of New coccine and alimezine was 0.01%. Trimetoquinol concentration was 1 mg/ml. The lysozyme concentration was 5 mg/ml.

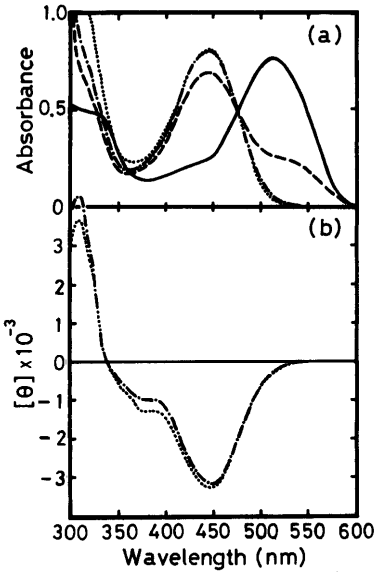

Fig. 2. Absorption (a) and CD (b) Spectra of New Coccine in the Lysozyme–Sodium Sulfite System

(a) ——, New coccine–sodium sulfite system; — —, immediately after preparation; —·—, 20 min after preparation; ------, 20 h after preparation. (b) —·—, 15 min after preparation; ------, 20 h after preparation. The concentrations of New coccine, lysozyme and sodium sulfite were 0.01%, 5 mg/ml and 0.1%, respectively.

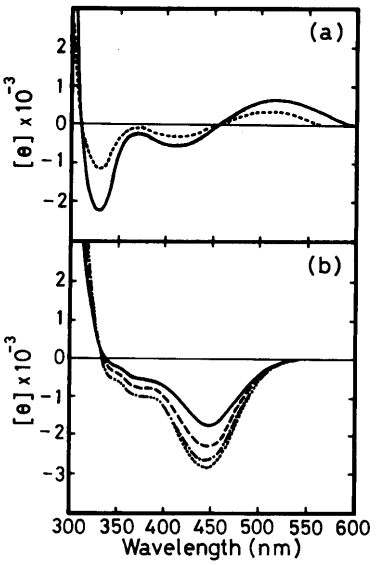

Fig. 3. CD Spectra of New Coccine in the Lysozyme–Alimezine Syrup (a) and the Lysozyme–Alimezine–Trimetoquinol Syrup (b)

(a) ——, 5 h after preparation; ------, 24 h after preparation. The concentrations of New coccine, alimezine and lysozyme are the same as in Fig. 1. (b) ——, 1.5 h after preparation; — —, 3 h after preparation; —·—, 5 h after preparation; ------, 24 h after preparation. The concentrations of New coccine, alimezine, trimetoquinol and lysozyme were the same as in Fig. 2.

New coccine was drastically changed (Fig. 1b). After 24 h, the 510 nm band of New coccine disappeared and a new band appeared at about 445 nm. In this system, two isosbestic points were observed at about 371 and 475 nm, indicating that there are two species of New coccine molecules. The color change from red to yellow could be recognized even after 30 min. Since commercial alimezine and trimetoquinol syrups contain sodium sulfite as a stabilizer, the color change was examined also in the mixture of lysozyme (5 mg/ml), New coccine (0.01%) and sodium sulfite (0.1%). In this system, New coccine changed its color from red to yellow in a similar manner as above, but more rapidly (Fig. 2a).

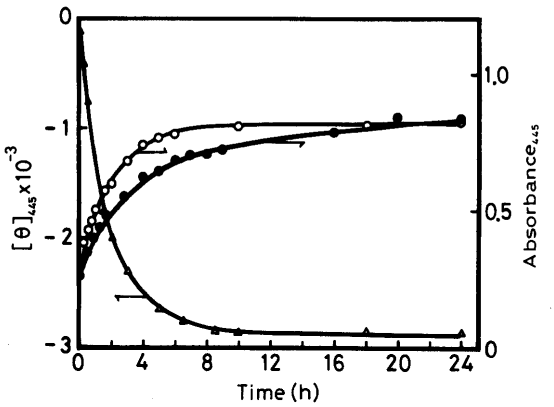

Fig. 4. Time Courses of Intensity Changes of Absorbance and CD at 445 nm

●, change of absorbance at 4 °C; ○, change of absorbance at 35 °C; △, change of molar ellipticity at 25 °C. The curves were drawn by means of the equation $y = ae^{-kt} + b$. The values of k, a and b were determined so as to give the smallest mean-square error between the observed and theoretical values.

TABLE I. Rate Constants and Activation Parameters of New Coccine Change in Lysozyme–Alimezine–Trimetoquinol System

Temperature (°C)	k (h^{-1})	E_a (kcal/mol)	ΔF^* (kcal/mol)	ΔS^* (cal/mol·degree)
4	0.25			
25	0.37	3.48	18.01	−48.75
37	0.49			

According to these results, the color change of the syrup is due to an interaction of New coccoine with lysozyme in the presence of sodium sulfite. A similar reaction proceeded in the mixture of BSA (aqueous solution of 5 mg/ml), New coccine (0.01% solution) and sodium sulfite (0.1% solution), though the color change in the BSA system was slower than that in system (b) (data not shown). The concentrations of sodium sulfite in the alimezine and trimetoquinol syrups were 0.01% and 0.1%, respectively. It seems that the amounts of sodium sulfite were too small to cause a color change in system (a).

In order to examine the environmental characteristic of the New coccine binding site, we examined the CD spectra of systems (a) and (b). The CD spectra of system (a) are presented in Fig. 3a. Although New coccine in the alimezine syrup is optically inactive when it is free in solution, an induced CD was observed in the presence of lysozyme. A positive CD band at about 510 nm and two negative bands at about 420 and 330 nm were observed. The crossover point was at 455 nm.

Although the magnitude of these bands was constant for a couple of hours after preparation, they decreased gradually.

The CD spectra of system (b) at various times after preparation are shown in Fig. 3b. The spectra are quite different from those observed in system (a).

A negative CD band was observed at about 445 nm with shoulders at about 370 and 340 nm. As time elapsed, these bands gradually increased in intensity without any shift. An isoelliptic point was observed at about 329 nm.

In Fig. 2b, the CD spectra of the mixture of lysozyme, New coccine and sodium sulfite are shown. CD spectrum

similar to system (b) was observed. However, in the New coccine–sodium sulfite system, the color change and induced CD were not observed.

It is thus obvious that the band at 445 nm originates from New coccine molecules bound to lysozyme in the presence of sodium sulfite in the trimetoquinol syrup. The change of the amplitude of the 445 nm band in system (b) was plotted as a function of the incubation time (Fig. 4). In Fig. 4, the time courses of the intensity change of the 445 nm band in the absorption spectra of system (b) were also shown. The time courses of the amplitude changes could be explained by the single exponential equation ($y=ae^{-kt}+b$). By the use of this equation, the rate constants of the color change of New coccine were calculated at various temperatures (Table I). The values of the rate constant obtained from the change in CD spectra were the same as those from the absorption spectra. From these rate constants, thermodynamic parameters were shown also in Table I as the result of calculation using the Arrhenius plot.

Discussion

Spectroscopic Properties of New Coccine–Lysozyme Complexes From the present data, it seems that the color change of New coccine in the alimezine–trimetoquinol syrup results from a structural change in New coccine. It can be postulated that New coccine molecules bound to lysozyme in the presence of sodium sulfite, which is contained in the trimetoquinol syrup. From the standpoint of a non-specific interaction between dye molecules and hydrophobic regions of lysozyme, aromatic dye molecules tend to bind preferentially to the active-site regions of globular proteins.[5] New coccine may bind to the active site of lysozyme. The CD spectrum of the bound New coccine molecules to lysozyme contains information describing a dissymmetric conformation of the binding molecules.

The absorption spectrum of New coccine can be characterized on the basis of the spectroscopy of azo compounds.[6,7] The long-wavelength band is actually composed of two transitions. The lowest energy transition is allowed while the higher energy one is forbidden. These n–π^* transitions of the long-wavelength bands have small oscillator strength but may have significant rotational strengths. At the wavelength corresponding to the absorption band and shoulder of New coccine, there are two CD bands which are opposite in sign (Figs. 1—3). The positive CD band appears at the longer wavelength side. Based on these predictions, the properties of New coccine bound to lysozyme can be considered as follows. The induced CD may arise from an asymmetric conformation of the bound New coccine and/or from an interaction of the bound New coccine with an asymmetric environment of lysozyme.[7,8] In system (a), the CD should be caused mainly from the former, namely, a dipol-coupling type CD between two naphthalene skeletons of New coccine, because the 510 and 420 nm CD bands correspond to the absorption bands and their signs are opposite from each other. The signs of the CD bands show that R-chirality is applicable to New coccine molecules bound to lysozyme.

In the presence of trimetoquinol syrup containing sodium sulfite, the CD spectrum was drastically changed (Fig. 3). The positive band of the lowest energy transition disappeared, though the absorption band at 510 nm remained as a shoulder (Fig. 1b). This indicates that one naphthalene ring of New coccine is fixed in a particular environment and the other one is rather free. Such a different situation concerning the two naphthalene rings of New coccine molecules in systems (a) and (b) resulted from an interaction between sodium sulfite and New coccine. The fixed ring seems to undergo a change of structure. This naphthyl ring should be a hydroxy group-substituted one because of its ability to form a hydrogen bond.

From these considerations, it can be said that the color change of New coccine is not due to just metachromasy but to chemical change. The important event for the chemical change of New coccine should be the location of molecules in the hydrophobic protein pockets with a specific configuration in the presence of sodium sulfite. New coccine molecules that are placed in the special environment seem to undergo the addition of a sulfite group of sodium sulfite in the presence of lysozyme.

Thermodynamic Properties of Color Change in the Lysozyme–Alimezine–Trimetoquinol System From Table I, it seems that the value of the activation energy, E_a, is smaller than that of the general hydrolytic reaction of chemicals (*ca.* 20 kcal/mol). The color change of New coccine in the syrup is due to a relatively fast reaction. From a small value of activation entropy change, ΔS^*, it can be said that the increase of degree of freedom is small compared to the initial state. This should result from the fact that New coccine molecules are rigidly fixed in a specific configuration, which is appropriate to the reaction. Comparable values of ΔF^* to the present data can be found in the interaction between proteins and small molecules,[9] the degradation of riboflavine,[10] enzymatic reactions such as the deacylation of an acylenzyme intermediate in an α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan amide.[11] In particular, in an interphase transfer of sulfonamides, the values of other thermodynamic parameters are also comparable to those calculated in the present study.[12] Thus, these thermodynamic parameters indicate a facility of the color change of New coccine in the system (b). It was reported that lysozyme activity decreased to about 80% after 7 d of mixing of the lysozyme syrup with trimetoquinol syrup.[13] This is due to the sodium sulfite in the trimetoquinol syrup. At present, however, it is uncertain whether or not there is a structure change in lysozyme in the syrup mixed with alimezine syrup containing New coccine. Therefore, more detailed study concerning the pharmacology and pharmaceutics of the alimezine syrup should be investigated. The chemical structure of the yellow product in the syrup will be reported in the following paper.

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