Hemoglobin Denaturation Caused by Surfactants

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The properties of hemoglobin denaturation caused by two anionic surfactants (sodium dodecylsulfate and sodium lauroylmethyltaurate) were examined using spectrophotometry (multi-plate reader), circular dichroism (CD) and high-performance liquid chromatography (HPLC), and their denaturating action on hemoglobin and sorption to it were examined by various methods. Correlation and factorial analyses were applied to the experimental data and the following results were obtained: (1) High correlations were found among sorption, denaturation and α-helix content and the random structure of hemoglobin. (2) The α-helix of hemoglobin is randomized as the surfactants denature hemoglobin. (3) Factorial analysis indicates that there are two factors involved in hemoglobin denaturation, one related to the destruction of the α-helix and the other related to a change in the β-structure. These two factors are related to change in the environment around the heme group. (4) Destruction of the α-helix seems to be one of the causes of the eye irritation produced by anionic surfactants.

Key words hemoglobin denaturation; eye irritation; surfactant; spectrophotometer; circular dichroism; high-performance liquid chromatography

Animal testing for the evaluation of the irritant effect of chemicals on the eye, *e.g.*, the Draize eye irritation test, has been criticized for years.[1] As part of our program to develop alternative, non-animal test procedures, we have proposed a new method of predicting eye irritancy by measuring the spectrophotometric change of hemoglobin at 418 nm (HDR method), based on the hypothesis that the eye irritation produced by surfactants is a consequence of protein denaturation.[2–6] This hypothesis has been verified by factorial analysis of various alternative eye irritation tests.[7] We have concluded that the correlation between the hemoglobin denaturation ratio (HDR%) and the Draize eye irritation score is high.[6,7] Nevertheless, the physiological significance of hemoglobin denaturation caused by surfactants is still obscure and it was unclear whether or not the HDR method really measured hemoglobin protein denaturation or only the change in the heme moiety. Thus, in this report, we examined the details of the denaturation of hemoglobin by two anionic surfactants: one, sodium dodecylsulfate (SDS), known for its severe irritant effect on the eye and the other, sodium lauroylmethyltaurate (LMT), supposed to be only moderately irritating. The relationship between the denaturation of hemoglobin and eye irritation was also examined.

MATERIALS AND METHODS

Chemicals SDS was purchased from Nacalai Tesque Co., Ltd., Tokyo, Japan. LMT was a gift from Nikko Chemical Co., Ltd., Tokyo, Japan. Hemoglobin and standard phosphate buffer (pH 6.86) were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All chemicals were used without further purification. Water purified by ion-exchange was used throughout the experiment.

Spectrophotometric Measurement (HDR Method) Spectrophotometric measurement (multi-plate reader; BIO-RAD Model 3550) was performed according to the method previously reported.[6] Hemoglobin was dissolved in standard phosphate buffer (pH 6.86) at a concentration of 0.05% (w/w). Surfactant solutions at 12 concentrations in 100 μl water (from 0% to 2%, w/w, serial two-fold dilutions) were placed, 8 replicates each, on a 96-well microplate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). An equal amount of hemoglobin/buffer solution was added to each well in 4 lines, immediately followed by buffer solution in the wells of the remaining 4 lines. The microplate was incubated for 5 min at 25 °C and the absorbance at 418 nm was measured with a multi-plate reader. The absorbance data ($n=4$) were processed in accordance with Eq. 1, and the HDR% at each concentration was calculated.

$$\mathrm{HDR\%} = 100 - \{\mathrm{Abs(SHB)} - \mathrm{Abs(SB)}\}/\{\mathrm{Abs(WHB)} - \mathrm{Abs(WB)}\} \times 100(\%) \quad (1)$$

where Abs(SHB) is the absorbance of surfactant mixed with hemoglobin/buffer solution, Abs(SB) is the absorbance of surfactant mixed with buffer solution, Abs(WHB) is the absorbance of water mixed with hemoglobin/buffer solution, and Abs(WB) is the absorbance of water mixed with buffer solution.

CD Measurement The CD measurements were carried out at room temperature under constant flushing with nitrogen, using a JASCO J-400X spectropolarimeter equipped with a data processor.[8] Hemoglobin (0.5 g/l) was dissolved in standard phosphate buffer (pH 6.86) at a concentration of 0.05% (w/w) to give a hemoglobin/buffer solution. To serial two-fold-diluted surfactant solutions (10 ml) at 12 concentration levels (from 0% to 2%, w/w), an equal amount of hemoglobin/buffer solution was added and the mixture was incubated for 5 min at 25 °C. The CD spectra over the range 184—270 nm were recorded. The data were expressed in terms of molar ellipticity. For measurement of near-UV and far-UV

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spectra, 1 and 0.1 mm cells were used, respectively. The secondary structure of hemoglobin was analyzed by using the four major secondary structures of proteins presented by Brahms and Brahms.[9]

HPLC Measurement A mixture of hemoglobin solution and serially diluted surfactant solution were prepared as described above and, after incubation for 5 min at 25 °C, HPLC analysis was performed under the following conditions, according to Miyazawa *et al.*[2] Apparatus, 880-PU, CO, 851-AS (Jasco); column, TSK G3000SW (Toso); detector, MULTI-340 (220 nm, 418 nm); flow, 1 ml/min; mobile phase, 0.05% phosphate buffer +0.15 M Na_2SO_4; temperature, 30 °C; injection volume, 10 μl. The peak heights of hemoglobin and surfactant were measured, and the HDR% and surfactant sorption to hemoglobin were calculated.

Hemoglobin denaturation measured by HPLC (HDR% by HPLC) was obtained in accordance with Eq. 2.

$$\text{HDR\% by HPLC} = 100 - \{\text{PH(SHB)}/\text{PH(WHB)}\} \times 100(\%) \quad (2)$$

where PH(SHB) is the peak height of hemoglobin mixed with surfactant and buffer solution at 418 nm, PH(WHB) is the peak height of hemoglobin mixed with water/buffer solution at 418 nm.

Sorption was quantified in accordance with the difference in the peak height of the surfactant, with and without hemoglobin, at 220 nm at a given concentration.

Statistical Analysis Correlation and factorial analyses were applied to the results of each assay. Factorial analysis is a statistical method designed to investigate the underlying factors of an event on the basis of the mutual correlation of variables.[10,11] The calculations were done using the Lotus 1-2-3 program with an add-in program for statistical analysis (Lotus 1-2-3 Multi-variate analysis v 1.0) provided by Audemain, Tokyo, Japan.

RESULTS

In the present study, a multi-plate reader, CD spectrophotometer and HPLC apparatus were used to investigate the mechanism of hemoglobin denaturation caused by surfactants.[12-14] SDS and LMT were examined and their sorption properties, denaturation of hemoglobin, and CD spectra were measured. Correlation and factorial analyses were applied to the data obtained.[10,11]

The results are summarized in Table 1. The secondary structure fractions were successfully computed from the CD spectra of hemoglobin in the presence of surfactants. In Figs. 1 and 2, the observed and computed CD spectra in the presence of SDS and LMT are illustrated, respectively. The computed spectra agreed well with the observed ones. In the absence of surfactant, this curve-fitting method gave approximately the same secondary structure fractions as those from X-ray data.[9,15] Here, the standard phosphate buffer used in this study contains about 0.1% of sodium azide which is claimed to be a metal site inhibitor. Therefore, we examined the effect of azide on hemoglobin denaturation and confirmed that it causes a shift of the λmax of the Soret band from 405 to 418 nm, but the α-helix content was not changed. (data not shown).

The calculated correlation coefficients are given in Table 2. High correlations were found between HDR% and α-helix content, and random structure, molar ellipticity at 222 nm, sorption and HDR% by HPLC (correlation

Table 1. Summary of the Results Obtained in Each Assay

Surfactant	Conc. (%)	HDR% (%)	Structure				Molar ellipticity at 222 nm (deg cm^2 dmol^{-1})	Sorption (g/g hemoglobin)	HDR% by HPLC (%)
			α-Helix (%)	Random (%)	β-Turn (%)	β-Sheet (%)			
SDS	1.000	43.025	54	35	0	11	15.686	5.100	100.000
	0.500	37.672	50	33	0	17	15.939	4.900	86.531
	0.250	35.848	50	33	0	17	15.433	2.496	74.966
	0.125	33.009	52	31	3	14	15.939	1.249	65.306
	0.063	17.356	51	31	2	16	16.192	0.625	60.000
	0.031	7.218	53	28	1	18	16.192	0.313	48.163
	0.016	4.704	55	30	0	15	17.204	0.156	3.946
	0.008	0.324	65	23	7	5	19.228	0.078	1.361
	0.004	0.000	76	11	8	5	19.987	0.039	0.000
	0.002	0.000	74	14	2	10	21.252	0.020	2.041
	0.001	0.000	76	11	8	5	20.999	0.010	6.803
	0.000	0.000	74	16	10	0	20.999	0.000	0.000
LMT	1.000	25.574	56	21	9	14	16.192	2.072	92.254
	0.500	18.613	56	23	6	15	15.686	1.115	91.214
	0.250	15.889	56	23	6	15	16.192	1.346	86.358
	0.125	9.575	57	23	4	16	15.939	0.947	37.688
	0.063	4.787	61	25	10	4	18.722	0.562	3.006
	0.031	0.578	64	13	7	16	20.240	0.302	1.605
	0.016	0.000	68	10	8	14	20.999	0.154	0.000
	0.008	0.000	72	4	5	19	20.999	0.078	0.000
	0.004	0.743	71	5	6	18	20.999	0.039	0.000
	0.002	0.454	71	5	6	18	20.746	0.020	0.000
	0.001	0.289	74	11	1	14	20.999	0.010	2.659
	0.000	0.000	74	16	10	0	20.999	0.000	0.000

coefficients of −0.804, 0.711, −0.884, 0.789, and 0.901, respectively). The results of factorial analysis of the data are given in Table 3. Two factors were identified, one related to the α-helix and the other to the β-structure. The contribution ratio suggested that the former has a greater influence on hemoglobin denaturation. Calculated factor scores, showing the effect of the surfactants on the α-helix, are summarized in Table 4. The factor score decreases as the test concentration decreases and the score of SDS at any concentration is smaller than that of LMT.

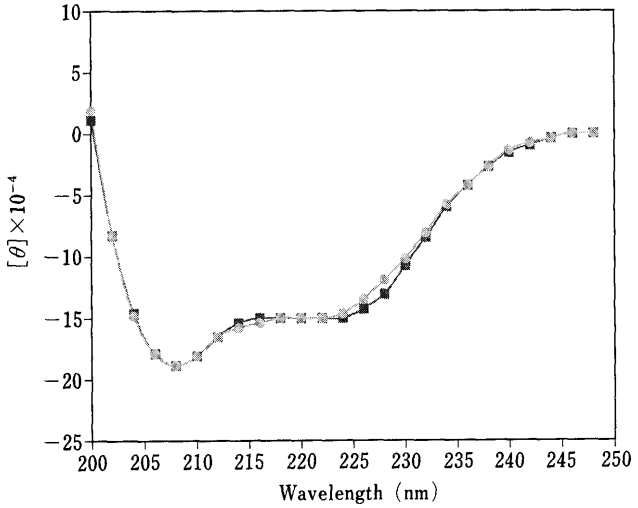

Fig. 1. Comparison of Experimental and Computed CD Spectra of Hemoglobin/Buffer Solution and 1% SDS

■, observed; ●, computed.

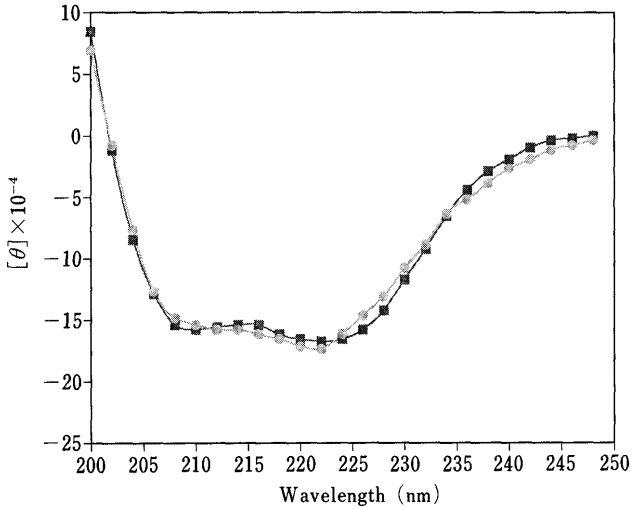

Fig. 2. Comparison of Experimental and Computed CD Spectra of Hemoglobin/Buffer Solution and 1% LMT

■, observed; ●, computed.

DISCUSSION

High correlations between HDR% and α-helix content and random structure, molar ellipticity at 222 nm, sorption and HDR% by HPLC suggest that there may be a relationship among these variables. The correlation between the α-helix content and random structure presumably reflects the fact that the α-helix of hemoglobin becomes randomized as the surfactants denature hemoglobin (Table 2). This result is very similar to the structural change of myoglobin induced by SDS.[16] At the same time, the α-helix content is also related to HDR% and HDR% as shown by HPLC. These findings are consistent with the hypothesis that the α-helix is randomized following sorption of surfactant, and the HDR method is able to follow this process. In this study, the α-helix content and molar ellipticity at 222 nm exhibit a very high correlation, confirming the usefulness of measuring the molar ellipticity at 222 nm to determine the α-helix content. It should be noted that the values of HDR% and HDR% obtained by HPLC in Table 1 are very different. This result may be explained as follows: the HDR method measures the change in the absorbance of heme, which is known to be located near an α-helix region of hemoglobin. Thus, the HDR method can reflect the change in the electronic state of heme and protein denaturation caused by surfactants. HDR% is determined by these two factors. On the other hand, in HPLC, the sample size plays a key role in the determination of the amount of eluted materials. Although the change in α-helix content is also reflected in the absorbance at 418 nm, a change in the size of hemoglobin is not sufficiently reflected in HDR%. Therefore, the effects of the surfactants are clearly manifest in HPLC, resulting in the large values of HDR% obtained by this technique.

The results of the factorial analysis (Table 3) indicate that there are two factors responsible for hemoglobin denaturation, one related to the destruction of the α-helix and one related to the change in β-structure, with the α-helix factor having the more powerful effect. These results suggest that two mechanisms are involved in hemoglobin denaturation and, because the correlation between HDR% and the α-helix content is high, the HDR

Table 2. Correlation Coefficient Matrix of Obtained Data

	HDR%	Structure				Molar ellipticity at 222 nm	Sorption	HDR% by HPLC
		α-Helix	Random	β-Turn	β-Sheet			
HDR%	1.000	−0.804	0.711	−0.402	0.364	−0.884	0.789	0.901
Structure α-Helix	−0.804	1.000	−0.879	0.537	−0.483	0.949	−0.637	−0.792
Random	0.711	−0.879	1.000	−0.529	0.081	−0.871	0.652	0.772
β-Turn	−0.402	0.537	−0.529	1.000	−0.590	0.494	−0.464	−0.511
β-Sheet	0.364	−0.483	0.081	−0.590	1.000	−0.387	0.227	0.308
Molar ellipticity at 222 nm	−0.884	0.949	−0.871	0.494	−0.387	1.000	−0.659	−0.820
Sorption	0.789	−0.637	0.652	−0.464	0.227	−0.659	1.000	0.903
HDR% by HPLC	0.901	−0.792	0.772	−0.511	0.308	−0.820	0.903	1.000

Table 3. Results of Factorial Analysis of the Data

Test item	Factor loading	
	First factor	Second factor
HDR%	−0.9043	0.2665
Structure α-Helix	0.7457	−0.5452
β-Turn	0.3081	−0.6814
β-Sheet	−0.1302	0.7496
Molar ellipticity at 222 nm	0.8259	−0.4294
Sorption	−0.8528	0.1536
HDR% by HPLC	−0.9289	0.2524
Contribution ratio (%)	53.7	23.8
Meaning of the factor	Alteration of α-helix	Alteration of β-structure

Table 4. Calculated Factor Scores of SDS and LMT for the Effect on the α-Helix Factor

Test concentration (%)	Factor score	
	SDS	LMT
1.000	−2.438	−1.500
0.500	−1.864	−0.931
0.250	−1.194	−0.843
0.125	−1.005	−0.060
0.063	−0.380	−0.250
0.031	0.305	0.860
0.016	0.757	0.867
0.008	0.276	1.345
0.004	0.425	1.222
0.002	1.073	1.213
0.001	0.459	1.304
0.000	0.130	0.130

method reflects the major factor. This is consistent with our earlier report that there are two mechanisms for the denaturation of hemoglobin by surfactant.[6] On the other hand, it has been reported that the two mechanisms of hemoglobin denaturation are associated with the structural change in the heme group and the change in the environment around the heme group.[16] The two factors causing hemoglobin denaturation shown in Table 3 are related to the change in the environment. Thus, we can say that this environmental change involves the destruction of the α-helix and the change in β-structure. The higher score of the first factor in HDR% by HPLC compared with HDR% may be due to the influence of hemoglobin size. This is consistent with the large values of HDR% obtained by HPLC shown in Table 1.

According to the factor score (Table 4), SDS has a stronger effect in the destruction of the α-helix than LMT at every test concentration of surfactant. This result is consistent with the well known fact that SDS produces a stronger irritant effect on the eye than LMT.[17-19] The stronger effects of SDS on hemoglobin, compared with LMT, are due to the small cmc (critical micelle con centration) of SDS. The relative values of the cmc of SDS and LMT in phosphate buffer at room temperature were 1.1 and 7.0 mM, respectively. These were estimated from the changes in the molar ellipticity of hemoglobin at 222 nm, which correspond to the isothermal binding curve (data not shown). Thus, it is possible that the destruction of the α-helix is the major cause of eye irritation, although further investigation of a large number of other chemicals is necessary to confirm this.

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