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The Induction of Metachromasia and Circular Dichroism of Coomassie Brilliant Blue R-250 with Collagen and Histone H1 Is Due to the Low Content of Hydrophobic Amino Acid Residues in These Proteins

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Collagen and histone H1 are stained a pink-red color with Coomassie Brilliant Blue R-250 (Coomassie R) instead of the blue color of most proteins after SDS-PAGE. Spectrophotometrically, this metachromasia was characterized by an increase in the absorbance at 535 nm and a decrease in the absorbance at 600 nm. The ratio of the absorbance at 535 nm to that at 600 nm ranged from 1.5 to 2.5 for the pink-red-stained proteins and was about 1 for the blue stained proteins. In their amino acid composition, the pink-red-stained proteins collagen and histone H1 contained less than 11% of hydrophobic amino acid residues, whereas the five blue-stained proteins examined contained more than 25% of such residues. Collagen and histone H1 also induce circular dichroism (CD) of Coomassie R in the visible region with a different CD spectrum. In the case of native collagen, a CD (+) band at 530 nm with 10^5 molar ellipticity was observed, while the denatured collagen showed a CD (−) band at 530 nm. When the amino groups of the amino acid residues in collagen and histone H1 were converted into hydrophobic groups by fluorescamine treatment, these proteins stained bluer than pink-red and the induced CD was a lower intensity. This is the first report of the metachromatic interaction between a protein and Coomassie R that is accompanied by CD induction. This report also suggests that the induction of metachromasia and CD of Coomassie R was due to the low content of hydrophobic amino acid residues in the peptides.

Key words: circular dichroism, collagen, Coomassie Brilliant Blue, hydrophobic amino acid residues, metachromasia.

Collagen is known to stain pink-red with Coomassie R after SDS-PAGE (*1, 2*). A few other proteins, including histone H1 (*3*), parotid saliva protein (*4*), and rubrophilin (*5*), have likewise been described as pink-red-staining proteins. Although the reason for the metachromatic staining of these proteins remained uncertain, this phenomenon has been used as a convenient marker for the detection of collagen in tissue homogenate (*1, 2*). McCormick *et al.* (*1*) discussed the possibility that the collagen-specific repeating glycine tripeptide structure caused the metachromasia of the collagen-Coomassie R complex. Rosenthal *et al.* (*5*) reported that contaminants in the Coomassie R dye were responsible for the pink-red-staining of rubrophilin. Later, Duhamel *et al.* (*3*) showed that histone H1, which does not have a collagen-like structure, stained a pink-red color. From the fact that both collagen and histone-H1 are proline-rich proteins (containing more than 11% of proline residues), they proposed that closely-spaced proline residues in the sequence facilitate dye stacking, which induces the metachromasia of Coomassie R.

On the other hand, the induction of CD accompanied with metachromasia when the dye interacts with the polypeptide or polymer has also been reported. Pal and Mandel (*6*) reported that carbocyanine dye pinacyanol exhibits metachromasia and CD in the visible region when bound to poly-L-glutamic acid. Pal and Pal (*7*) also reported the induction of CD in anionic dyes by the addition of cationic chitosan. Salter *et al.* (*8*) reported the CD induction of acridine orange by its binding with glycosaminoglycans. In those cases, they supposed that the systematic aggregation of the dye along with the helical conformation of the polymer induced the CD of the dye.

In this report, we describe the characteristic properties of metachromasia and CD of Coomassie R after forming complexes with proteins in solution and in polyacrylamide gel. We also show that the hydrophobic amino acid residue contents of the polypeptides relate to the induction of metachromasia and CD of Coomassie R with an experiment using proteins whose basic amino acid residues were converted to hydrophobic groups.

MATERIALS AND METHODS

Materials—Sodium dodecylsulfate (SDS), Coomassie Brilliant Blue R-250 (Coomassie R), dimethyl sulfoxide (DMSO), methanol, acetic acid, and glycerol were obtained from Nacalai Tesque, Kyoto. The Bio-Rad protein assay kit, acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, and *N,N,N',N'*-tetra methylenediamine

Abbreviations: Coomassie G, Coomassie Brilliant Blue G-250; Coomassie R, Coomassie Brilliant Blue R-250; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; SCE, collagen type I (bovine) solubilized with the enzyme Proctase; SCL, collagen type I (bovine) solubilized with alkali.

were purchased from Bio-Rad Laboratories, Richmond, CA. Kimwipe (wiping paper) was received from Jujo Kimberly, Tokyo. Histone III-S (bovine; as the histone III-S fraction was composed of histone H1, we treated the histone III-S as histone H1 in this study), β casein (bovine), $\varkappa$ casein (bovine), and fluorescamine were purchased from Sigma Chemical, St. Louis, MO. Ovalbumin (chicken) and chymotrypsinogen A (bovine) were obtained from ICN Biomedicals, Cleveland, OH. Serum albumin (bovine) fraction V (BSA) was purchased from Miles, Kankakee, IN. Collagen type I (bovine) solubilized with an enzyme Proctase (SCE) and collagen type I (bovine) solubilized with alkali (SCL) (*9*) were obtained from Nippi, Tokyo. Ninhydrin and dansyl chloride were purchased from Wako Pure Chemical Industries, Osaka.

Polyacrylamide Gel Staining—A protein solution at a concentration of 1 mg/ml in 0.05 M Tris buffer, pH 6.8, containing 0.2 M NaCl and 2% SDS was boiled for 2 min, then an aliquot of the protein solution was mixed with 10% acrylamide solution in 0.375 M Tris buffer, pH 8.8, containing 0.1% SDS. The 10% acrylamide solution containing 0-200 μg/ml of the proteins was polymerized by addition of ammonium persulfate in a slab gel assembly with a thickness of 0.75 mm. The polyacrylamide gel was stained with a freshly prepared solution of 0.25% Coomassie R in 10% acetic acid and 50% methanol at 20°C for 20 min. The ratio of Coomassie R solution volume (15 ml) to gel volume (5 ml) was kept constant. The stained gels were destained at 20°C for 24 h in 40 ml of a solution of 7.5% acetic acid and 5% methanol. The unbound dye in the destaining solution was absorbed using a Kimwipe paper.

Estimation of the Coomassie R Bound to the Proteins in the Polyacrylamide Gel—A piece of the stained gel with a volume of 75 μl was removed and immersed in 925 μl of an extraction solvent that consisted of 80% DMSO, 10% methanol, and 2% acetic acid. The extracted Coomassie R was spectrophotometrically quantified at 600 nm with a Hitachi Spectrophotometer Model U-3200. The staining background value was determined with a gel that did not contain the protein, which was subjected to the process of staining and destaining with Coomassie R. From the calibration curve, an absorbance at 600 nm of 1.0 corresponded to 15.5 μg Coomassie R/ml.

Spectrophotometry and CD—For spectrophotometry of the gel, the stained polyacrylamide gel was cut into a 8×30 mm rectangular piece and placed in a cuvette containing water. The absorption spectra of the Coomassie R (0.0015%)-protein solution and the stained gels were recorded from 400 to 700 nm at a scan rate of 60 nm/min with a 2 nm band path using a Hitachi Spectrophotometer Model U-3200. For CD spectroscopy, the protein dissolved in PBS (phosphate buffered saline) was pipetted into the Coomassie R solution in the destaining solution (7.5% acetic acid and 5% methanol). The CD spectrum of the Coomassie R (0.005%, 6.05×10^{-5} M)-protein (0.03%) solution was recorded with a spectropolarimeter Jasco-600 (Japan Spectroscopic) using 1-cm cuvette at 20°C.

Amino Group Blocking Using Fluorescamine and Dansyl Chloride—Fluorescamine (*10*) and dansyl chloride, which are fluorogenic and hydrophobic reagents used to assay primary amine of peptides (*10*), were used to convert amino groups to hydrophobic groups. One milliliter of the protein solution (2 mg/ml) in PBS (in the case of fluorescamine) or 0.5 M sodium hydrogen carbonate solution (in the case of dansyl chloride) was added to 300 μl of the fluorescamine solution in DMSO (30 mg/ml) or 300 μl of dansyl chloride in acetone and incubated at 20°C for 1 h. The protein solution was then dialyzed against PBS to remove DMSO and unengaged fluorescamine. A ninhydrin assay (*11*) which omits the hydrolysis procedure of the polypeptides was used to estimate the number of unreacted groups. The fluorescamine treatment had blocked 90% of the amino groups in histone H1, 80% in BSA and 70% in collagens (SCE, SCL).

Detection of Collagen Conformational Change Using a Proteolytic Enzyme—Trypsin digestion was used to test for conformational change in fluorescamine-treated collagen (*12*). As native collagen is resistant to trypsin digestion, trypsin can be used as a probe for the triple helical conformation of collagen. In testing for conformational change in the stained collagen in the polyacrylamide gel, the trypsin digestion method was modified as follows. A solution of native or heat-denatured collagen (2 min at 100°C) was mixed with the acrylamide solution and polymerized in a slab gel assembly. A 100-mg piece of the stained gel containing 10 μg of collagen was incubated with 50 μg of trypsin dissolved in 1 ml of 0.1 M Tris-HCl, pH 7.5, for 30 min at 20°C. The enzymatic reaction was terminated by adding 100 μl of 20% SDS solution and followed by analysis using SDS-PAGE.

RESULTS

Relation between Coomassie R Metachromasia and Amino Acid Composition of Proteins in Polyacrylamide Gel—We compared the pink-red-stained gels containing collagen or histone H1 with the normal blue-stained gels by measuring the differences of the absorption spectra against the BSA-containing gel (Fig. 1). The binding capacities of the proteins to Coomassie R in the polyacrylamide were also measured (Table I). The number of bound dyes was roughly matched to the number of positively charged residues in the protein sequences as described by Tal *et al.* (*13*). As the binding capacity of collagen (SCE, SCL) for Coomassie R was about half that of BSA, the contents of collagen and SCL in the gel were twice that of BSA to keep the absorbance of the proteins at similar levels in the range of 400-700 nm. The difference spectra of the pink-red-stained gels against blue-stained gel showed a (+) band at 535 nm and a (−) band at 600 nm. As the absorbance ratio at 535 and 600 nm showed a constant value for each protein, the ratio of 535/600 nm is used as the metachromatic index in Table II. The collagens (SCE, SCL)-Coomassie R complex, which appeared to be the most reddish in color, showed the highest metachromatic indices, with values of 2.5 and 2.4, respectively (Table II). On the other hand, the metachromatic index of the histone H1-Coomassie R complex, which stained reddish with a trace of blue, was 1.6. The index values of orthochromatically blue-stained proteins, those including proline-rich β casein and $\varkappa$ casein, were around 1.

Native collagen, which maintains the triple helical conformation (resistant to trypsin digestion), and heat-denatured collagen stained the same reddish color and had the same index value (Table II). This result implies that the primary structure or amino acid composition is responsible

for the metachromasia rather than the specific conformation of collagen. As Hayashi and Nagai (*14, 15*) had pointed out that the abnormal behavior of collagen in SDS-PAGE is due to the low content of hydrophobic amino acid residues, we examined the content of hydrophobic amino acid residues in each protein (Table II). We counted Ile, Leu, Phe, Tyr, Val, and Met [described by Hayashi and Nagai (*14, 15*)] plus Trp residues as hydrophobic amino acid residues. All of the metachromatic stained proteins that were examined or had been described in other reports (*16, 17*) contained less than 11% hydrophobic amino acid residues. The pink-red-stained collagens (SCE, SCL) contain only 7% hydrophobic amino acid residues and have the highest metachromatic indices, while histone H1 contains 11% hydrophobic amino acid residues and shows moderate metachromasia. Screening of the SWISS-PROT database (Release 23) revealed that only 26 of the 12,000 proteins listed meet the criterion of containing less than 11% of hydrophobic amino acid residues. The blue-stained proteins contain more than 25% hydrophobic amino acid residues and did not show any significant difference in their metachromatic indices.

Coomassie R Metachromasia in Protein Solution—We recorded the absorption spectra of Coomassie R in solutions of different compositions (Fig. 2). The Coomassie R solution (0.0015%) in 100% methanol showed a bluer color and an absorption maximum at 590 nm with a shoulder at 560 nm. The Coomassie R solutions in DMSO showed similar spectra, but the absorption maximum shifted to 600 nm without a shoulder at 560 nm (data not shown). When water was added to the Coomassie R solution in methanol, the absorbance at 590 nm was decreased, and the color assumed a purple tint. The color change occurred gradually as more water was added and was characterized by a sharp fall in the absorption spectrum at 590 nm and the emergence of a peak at 560 nm. Addition of acetic acid had no effect on the absorption spectra.

When the proteins were added to the Coomassie R solution in the destaining solution (7.5% acetic acid and 5% methanol), the absorption spectra changed in various ways depending on the kind of protein added. The absorbance of the solution increased without a change in the spectrum when BSA was added. On the other hand, the solution turned pink-red and a new absorption shoulder emerged at 535 nm when collagen (SCL) was added. Its absorption spectrum was similar to the Coomassie R staining of collagen in the polyacrylamide gel (*1, 2*). The pink-red coloration was not observed just after the mixing of Coomassie R and collagen, but the absorption at 535 nm appeared 1 day after. Time-dependent metachromasia in the gel was also reported by McCormick *et al.* (*1*). They described that the pink-red coloration appeared after 24 h in the case of collagen staining in an acrylamide gel.

CD Spectra of Coomassie R in Protein Solution—Several reports have noted that the metachromasia of a dye on adding a polymer was accompanied by the induction of CD (*6-8*). The induction of metachromasia of the Coomassie R-collagen solution encouraged us to record the CD spectra in the visible region. A protein/dye ratio of 6 : 1 by weight was used. The metachromasia and CD were not detected 10 min after the mixing of the Coomassie R and protein. After 20 h, the induction of metachromasia and CD with a (+) band at 530 nm of Coomassie R was observed in the native collagen (SCL) solution. After one week, the CD band at 530 nm was increased 30% as compared with the CD band at 20 h, and the spectrum pattern was unchanged. Assuming that the CD became stable at this point, we measured the CD of Coomassie R in several protein solutions. The metachromatic stained proteins, which were native col-

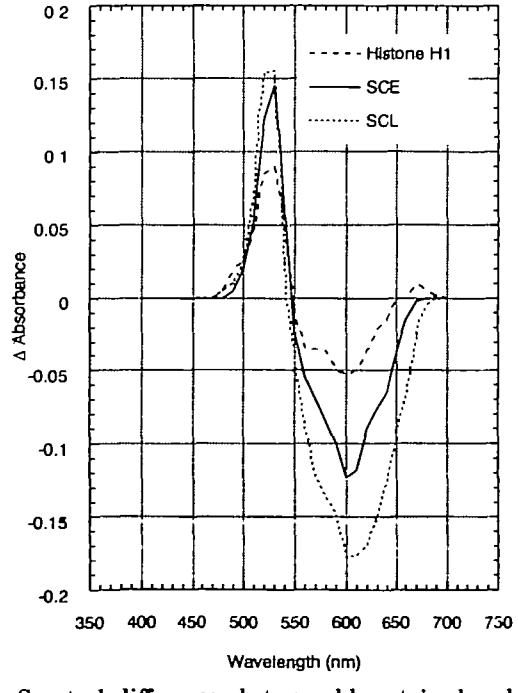

Fig. 1. **Spectral differences between blue-stained and pink-red-stained gels with Coomassie R.** Gel containing 0.01% BSA was used as the gauge against which we measured the differences in the absorption spectra of polyacrylamide gels containing proteins stained with Coomassie R. The concentrations of histone H1, collagen (SCE), and collagen (SCL) were 0.01, 0.02, and 0.02%, respectively.

TABLE I. **Amount of Coomassie R bound to various proteins.**

Protein	Coomassie R/protein[a] g/g	Coomassie R/protein[a] Molecules/100 amino acids	Number of[b] amino groups in the protein (mol %)	Reference
BSA	1.80	25.1	17.2	*19*
Histone H1 (bovine)[c]	1.77	21.4	30.9	SWISS
Ovalbumin (chicken)	1.57	21.2	11.2	SWISS
Cytochrome *c* (horse)	1.52	22.1	24.0	*19*
Chymotrypsinogen A (bovine)	1.03	13.1	8.6	*19*
κ casein (bovine)	0.99	11.7	10.7	*19*
Collagen type I (SCL)[d]	0.86	10.3	9.0	*9, 20*
Collagen type I (SCE)	0.84	10.0	9.5	*20*
β casein (bovine)	0.84	12.3	10.0	*19*
Trypsin (bovine)	0.76	9.4	8.7	*19*
Pepsin (porcine)	0.14	1.9	1.5	*19*

[a]Average of three experiments. Each measured value deviated by less than 5% from the average. [b]We counted the number of Arg, Lys, His, and N-terminals of the proteins. Data on amino acid compositions are quoted from the references as listed. SWISS means SWISSPROT database release 23. [c]As data for bovine histone H1 were not available, those for rabbit histone H1 were used. [d]As some arginine residues (25% of Arg) were converted to ornithine by the alkali treatment, the ornithine residues were counted as basic amino acids.

lagen (SCE, SCL), denatured collagen (SCL), and histone H1, induced CD (Fig. 3, a and b). On the other hand, BSA (Fig. 3a) and β casein (data not shown) which were stained orthochromatically, did not induce CD of Coomassie R in the visible region. The Coomassie R solution itself did not show CD in the visible region at the concentrations used (data not shown).

The induced CD spectra showed different patterns depending on the added proteins. The native collagens (SCE, SCL) induced a positive band at 530 nm that corresponded to the metachromasia-specific peak and a (+) band with low intensity at 490 nm. In contrast, the denatured collagen (SCL) induced CD with a (−) band at 530 nm and a (+) band of low intensity around 500 nm (Fig. 3b). Histone H1 induced a (+) band at 500 nm and a (−) band at 470 nm.

TABLE II. **Coomassie R-stained proteins: coloration and chemical properties.**

Protein[a]	Proline[b] residues (mol %)	Hydrophobic[c] residues (mol %)	Coloration[d]	Ratio of absorbance 535 nm/ 600 nm
Collagen type I SCE	22.1	6.9	P-R	2.39
Collagen type I SCE (native)[e]	22.1	6.9	P-R	2.42
Collagen type I SCL	21.9	7.1	P-R	2.50
Histone H1	11.2	10.8	P-R	1.59
BSA	4.8	24.9	B	1.04
κ casein	11.8	28.4	B	1.02
Chymotrypsinogen A	3.6	29.4	B	1.05
β casein	16.7	34.0	B	1.11
Ovalbumin	3.6	36.4	B	1.03
Rubrophilin[f]	11.1	2.0	P-R	ND[g]
Parotid saliva protein[f]	35.6	4.0	P-R	ND[g]

[a]The data on the amino acid composition and coloration are from the same references as in Table I. [b]Hydroxyproline is included in the proline content. [c]Trp, Ile, Leu, Phe, Tyr, Val, and Met were counted as hydrophobic amino acid residues (*13*). [d]P-R means pink-red-staining, B means normal blue-staining. [e]Collagen was trapped in the SDS-free polyacrylamide gel. [f]The data on rubrophilin and parotid saliva protein were obtained from Refs. *16* and *17*, respectively. [g]ND means no data.

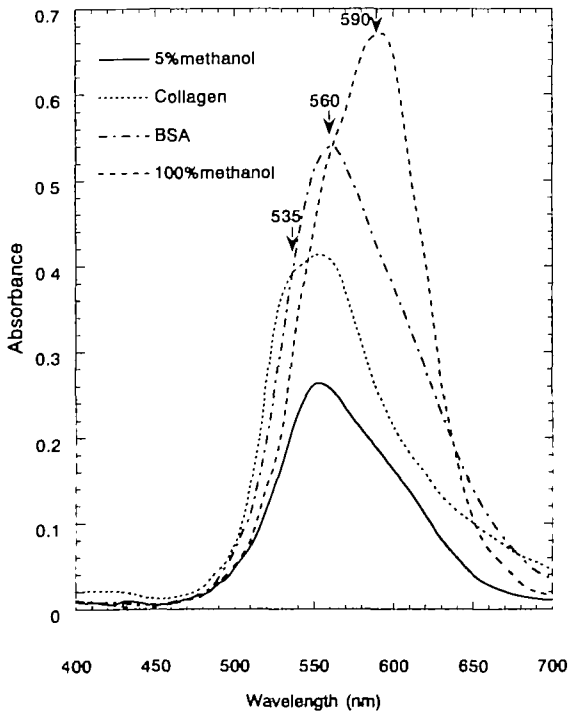

Fig. 2. **Absorption spectra of Coomassie R in various solvents.** Coomassie R was dissolved in 100% methanol or in 5% methanol/7.5% acetic acid at a concentration of 0.0015%. Collagen (SCL) or BSA (2 mg/ml in PBS) was added to 0.0015% Coomassie R in a 5% methanol/7.5% acetic acid solution thus forming a 0.02% protein solution. The absorption spectra of these Coomassie R solutions were scanned from 400 to 700 nm.

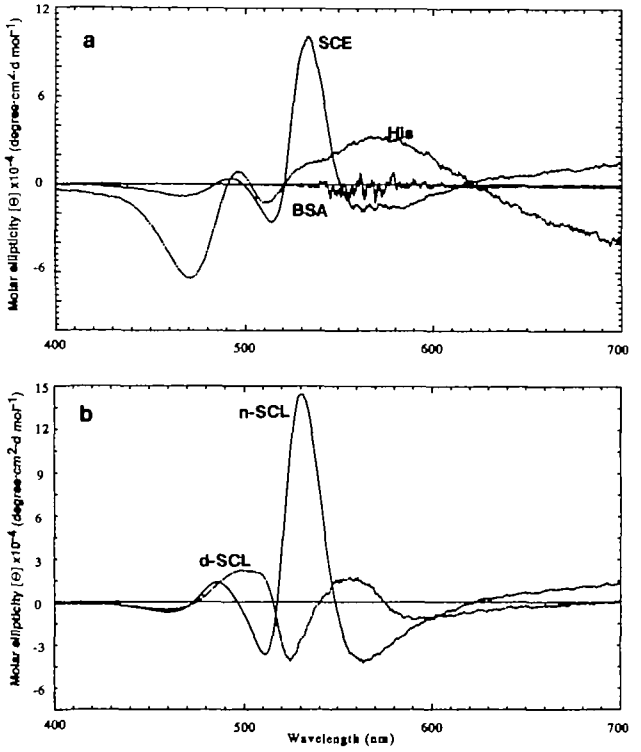

Fig. 3. **CD spectra of Coomassie R in different protein solutions.** The CD spectra of 6.05×10^{-5} M Coomassie R in 0.03% solutions of proteins. a: Native collagen SCE (SCE), histone H1 (His), and BSA (BSA). b: Native collagen SCL (n-SCL) and denatured collagen SCL (d-SCL).

TABLE III. **Coomassie R-staining of fluorescamine- or dansyl chloride-treated proteins: coloration and chemical properties.**

Protein	Treatment	Hydrophobic[a] residues (mol %)	The color[b] of staining	Ratio of absorbance 535 nm/ 600 nm
Collagen type I (SCE)	None	6.9	P-R	2.22
	Fluorescamine	13.5	P-R	1.66
Histone H1	None	10.8	P-R	1.59
	Fluorescamine	38.3	B	0.75
	Dansyl chloride	38.3	B	0.88
BSA	None	24.9	B	1.10
	Fluorescamine	38.5	B	0.77

[a]Basic amino acids modified by fluorescamine were regarded as hydrophobic amino acid residues. [b]P-R means pink-red-stained, B means normal blue-stained.

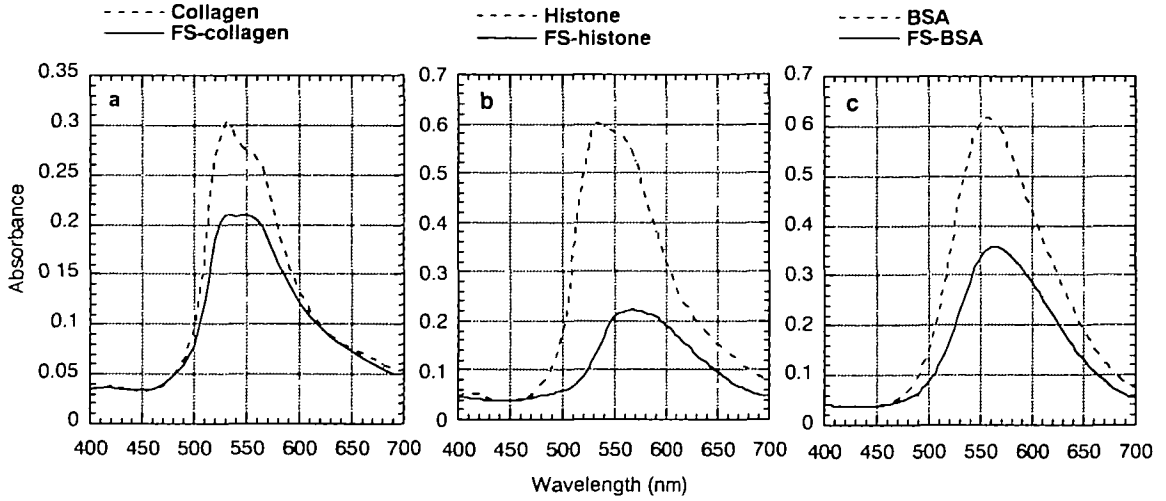

Fig. 4. **Absorption spectra of Coomassie R-stained proteins before and after fluorescamine treatment in polyacrylamide gel.** a: Collagen (SCE). b: Histone H1. c: BSA. FS denotes the fluorescamine-treated proteins.

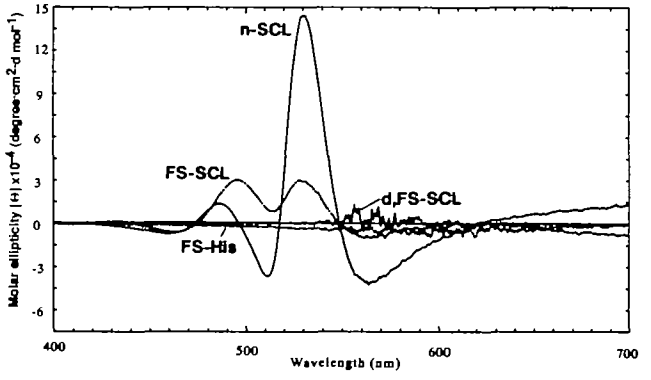

Fig. 5. **CD spectra of Coomassie R induced by fluorescamine-treated proteins.** The CD spectra of 6.05×10^{-6} M Coomassie R in 0.03% solution of native collagen SCL (n-SCL), native fluorescamine-treated collagen SCL (FS-SCL), denatured fluorescamine-treated collagen SCL (d FS-SCL), and fluorescamine-treated histone H1 (FS-His).

Coomassie R Metachromasia and CD Spectra in Proteins after Treatment with Fluorescamine or Dansyl Chloride— To test the correlation between the hydrophobic amino acid residue content of proteins and the metachromasia-CD induction of Coomassie R, hydrophobic residues were introduced into the amino groups of proteins by means of the fluorescamine treatment. The fluorescamine-treated proteins were colored faintly yellow but no absorbance was detected at wavelengths longer than 450 nm. The fluorescamine-treated histone H1 showed the largest increase in hydrophobic residue content, and its coloration changed to blue (Table III and Fig. 4b). Although the change was limited, collagen (SCE) and BSA also colored bluer after fluorescamine treatment (Fig. 4, a and c). After fluorescamine treatment, the content of hydrophobic amino acid residues in collagen was as low as 13.5%, which would account for the limited shift in color. The metachromatic indices of histone H1, collagen (SCE), and BSA, however, were lower after fluorescamine treatment (Table III and Fig. 4). When we used dansyl chloride instead of fluorescamine to convert the amino groups to hydrophobic groups, the dansyl chloride-treated histone H1 stained orthochromatically with Coomassie R and its metachromatic index became 0.88 (Table III). The staining of the fluorescamine- or dansyl chloride-treated collagen (SCL) showed the same color change as that of collagen (SCE).

The CD of Coomassie R in the presence of the fluorescamine-treated protein was measured one week after mixing (Fig. 5). The fluorescamine-treated histone H1 did not induce CD of Coomassie R. The induced CD band at 530 nm produced by the fluorescamine-treated native collagen (FS-SCL) was 5 times smaller than that induced by the untreated collagen. The CD of the native collagen (SCE) could not be measured because collagen (SCE) became insoluble during the fluorescamine treatment. The denatured fluorescamine-treated collagen (SCE, SCL) did not show the metachromasia in solution (data not shown) and also did not induce CD of Coomassie R (Fig. 5). The fluorescamine-treated collagen (SCL) was resistant to the trypsin digestion. This suggests that the collagen-specific helix of SCL was conserved after the fluorescamine treatment.

DISCUSSION

We agree with the current view that the differential staining of collagen-containing gel with Coomassie R is not due to the selective binding of reddish dye contaminants to the proteins but to metachromasia (*1, 3*) for the following two reasons. First, the pink-red coloration was observed in solution as well as in gel when collagen was added to the Coomassie R solution (Fig. 2). If a contaminant of Coomassie R were responsible for the pink-red-staining, we would not detect the color change in the solution system, which does not include the removal of unbound dye from the protein. Second, the pink-red-staining component of Coomassie R, which was extracted from the polyacrylamide containing collagen, stained the BSA its typical blue color (data not shown). The pink-red-staining was characterized by an absorption peak at 520–535 nm (*1, 2, 15*). In addition to the increase in the absorbance at 520–535 nm, our data

on the absorption differences revealed a prominent decrease in the absorption at 600 nm in the pink-red-stained collagen- and histone H1-containing gels (Fig. 1).

In the induction of Coomassie R metachromasia, the repeating glycine tripeptide structure of collagen was thought to facilitate the stacking of the Coomassie R and induce the spectral shift (*1, 2*). Duhamel *et al.* pointed out that this is not the case in histone H1, which stained pink-red but does not have a repeating glycine tripeptide (*3*). They proposed an alternative model in which closely-spaced proline residues in the sequence of the polypeptide serve as binding sites for dye stacking (*3*). To test this hypothesis, we compared the metachromatic indices of different proteins including proline-rich proteins (Table II). The pink-red-stained proteins that we examined contained more than 11% of proline residues; but the β casein-Coomassie R and κ casein-Coomassie R complexes stained blue, even though these proteins contained 16.7 and 11.8% of proline residues, respectively, and may have closely-spaced prolines in their amino acid sequences.

Next, we focused on the characteristically low content of hydrophobic amino acid residues in collagen, as described by Hayashi and Nagai (*14, 15*). The conversion of hydrophilic amino acid residues, which have amino groups, to hydrophobic residues by treatment with fluorescamine or dansyl chloride caused bluer staining and lowered the metachromatic indices of the proteins (Table III). Such treatment can not have affected the content or order of proline residues in the polypeptide sequence. The Coomassie R bound to collagen or histone H1 must be in a more hydrophilic environment than the Coomassie R bound to the blue-stained proteins. The nature of metachromatic stained protein, which has a low content of hydrophobic amino acid residues, may affect the electronic transition of Coomassie R and metachromasia is induced.

A specific interaction between the metachromatic stained proteins and Coomassie R was also observed as CD induction in the visible region. The pink-red-stained collagen (both native and denatured) and histone H1 induced the CD with different bands (Fig. 3, a and b). Similar to the metachromasia phenomenon, the CD induction of the collagen and histone H1 was decreased by the fluorescamine treatment. This suggests that both metachromasia and CD induction of Coomassie R are dependent on the same property of the protein bound to the dye.

The induction mechanism of metachromasia and CD appeared to involve the direct interaction of protein with Coomassie R rather than that of molecules of Coomassie R, because the estimated number of Coomassie R molecules bound to the proteins (from the data of Table I and the mixing ratio of protein and dye) was only 2.0 (in case of collagen and histone H1) to 2.3 (in case of BSA) per 100 amino acid residues in the polypeptides. It is known that the chiral conformation of the protein peptide induced the systematic aggregation of the dye along the backbone of the host peptide as described in case of dye-polyglutamic acid (*6*), dye-chitosan (*7*), or dye-glycosaminoglycan complexes (*8*). However, a helical protein does not always induce CD in the dyes it binds, as such induction depends on the ability of the ligand to bind with a twist in one sense (*7*). In the case of the Coomassie R-protein interaction, the binding of Coomassie R with a protein that contains a small number of hydrophobic amino acids may effect the alignment of Coomassie R. The binding of Coomassie R with the collagen or histone H1 conformation in a manner unallowing free rotation would induce the CD. The time dependency of the induction of CD and metachromasia may be understood by considering that a certain time is required for the dye molecule to settle in a stable position on the polypeptides.

This line of argument also explains why collagen in different conformations induced different CD bands (Fig. 3b) even though no difference was observed in the metachromasia. In the case of the CD induction of Coomassie R, not only the amino acid composition but also the conformation of the backbone of the polypeptide may effect the induction. This implies that the CD of Coomassie R could be used as a probe for the conformational change of collagen. The opposite sign of CD at 530 nm between the native collagen (SCL) and the denatured collagen (SCL) (Fig. 3b) may reflect the reverse helix chirality in collagen.

The lower intensity of the induced CD after the fluorescamine treatment of the proteins can also be explained in terms of the closely-spaced proline-Coomassie R interaction (*3*), as follows. The conformation was affected by the fluorescamine treatment, and this may change the proline-Coomassie R conformation that induced the metachromasia. Our results of trypsin digestion of the fluorescamine-treated collagen indicated that the collagen helical conformation was preserved even the fluorescamine treatment. This suggests that a protein containing a low number of hydrophobic amino acid residues is more likely to be responsible for the induction of Coomassie R metachromasia and CD.

The Bradford dye-binding protein assay using Coomassie Brilliant Blue G-250 (Coomassie G) is known to be insensitive to collagen unless modified by adding an appropriate amount of SDS (*18*). We found that pink-red-stained histone H1, showed no response in the Bradford protein assay unless the modified method of adding SDS (*18*) was used. However, the fluorescamine-treated collagen and histone H1 could be assayed by the normal method (data not shown). These results also show the specific interactions of collagen and histone H1 with Coomassie G, like those seen with Coomassie R.

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