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Chemical Modification of Proteins. VI.[1] The Reaction of N-(*p*-(2-Benzimidazolyl)phenyl)maleimide (BIPM) with the Thiol Groups of Egg Albumin[2]

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The reaction of N-(*p*-(2-benzimidazolyl)phenyl)maleimide (BIPM) (1) with egg albumin was examined and detailed chemical and spectroscopic analyses of the modified protein were performed. BIPM reacted specifically with thiol groups in egg albumin to give fluorescent adducts in agreement with the empirical rule previously proposed by the authors. The incorporated BIPM exists as a succinamic acid form 3 in the protein. These results are of significance in the use of BIPM as a fluorescent thiol reagent for proteins.

In view of the important biochemical and physiological roles of thiol groups, an extensive methodology for analysis of thiols in a variety of forms of preparations is needed.[4] During our systematic study on the fluorescent thiol reagents,[5] we have found that N-(*p*-(2-benzimidazolyl)phenyl)maleimide (BIPM) (**1**), nonfluorescent by itself, reacts readily with various thiol compounds to form fluorescent addition products (**2**)[6] (Chart 1), and that BIPM is useful for microanalysis of *in vitro* thiol samples[7] as well as for elucidating biochemical functions of thiols in biological system.[8] These results together with a number of our related findings[5–10] have led us to an empirical rule that N-substituted maleimide derivatives are generally nonfluorescent, while their addition products with thiols are fluorescent (Chart 1, R=fluorophores in general).

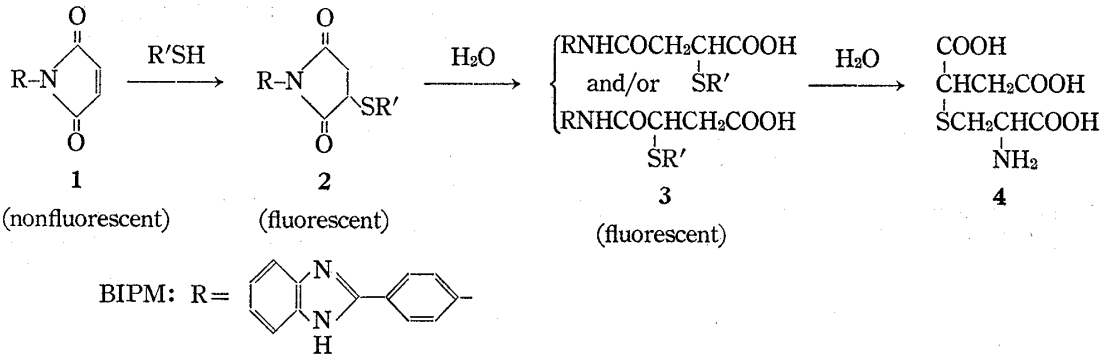

Chart 1

1) Part V: Y. Kanaoka, M. Machida, M.I. Machida, and T. Sekine, *Biochim. Biophys. Acta*, **317**, 563 (1973).
2) Fluorescent Thiol Reagents, VIII. For Part VII see: M. Machida, M. Bando, Y. Migita, and Y. Kanaoka, "submitted."
3) Location: *a) Kita-12, Nishi-6, Sapporo, 060, Japan; b) Hongo-2 chome, Bunkyo-ku, Tokyo, 113, Japan.*
4) A.F. Boyne and G.L. Ellman, *Anal. Biochem.*, **46**, 639 (1972).
5) Y. Kanaoka, M. Machida, H. Kokubun, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **16**, 1747 (1968); and the preceding papers cited therein.
6) Y. Kanaoka, M. Machida, K. Ando, and T. Sekine, *Biochim. Biophys. Acta*, **207**, 269 (1970).
7) T. Sekine, K. Ando, M. Machida, and Y. Kanaoka, *Anal. Biochem.*, **48**, 557 (1972).
8) K. Kimura, A. Watanabe, M. Machida, and Y. Kanaoka, *Biochem. Biophys. Res. Comm.*, **43**, 882 (1971).
9) M. Machida, M. Bando, M.I. Machida, Y. Migita, and Y. Kanaoka, "in preparation."
10) Y. Kanaoka and M. Machida, to be published.

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Apparently BIPM combines with thiols in proteins such as Takaamylase[6] and ribosomal proteins[8] on the basis of the absorption and fluorescence spectra and other properties of the reaction products. However, direct chemical evidence for the introduction of BIPM into protein has been incomplete. In the present paper, the incorporation of BIPM into protein is comfirmatively illustrated with egg albumin as a typical substrate of a thiol-protein. In addition, fluorescent characteristics of the BIPM-protein conjugate are described in detail.

Experimental

Materials——Five times crystallized egg albumin was a commercial product of the Nutritional Biochemical Company. Concentrations of egg albumin solutions were determined based on the relation: mg of protein $ml^{-1}=1.36\times$O.D. at 280 nm.[11] N-Ethylmaleimide and *p*-chloromercuribenzoic acid (PCMB) were purchased from Nakarai Chemicals, Ltd. S-(N-Ethylsuccinimido)-L-cysteine was prepared according to the method described by Lee and Samuels.[12] Sodium lauryl sulfate was obtained from Koso Chemical Co., Ltd. Sephadex G-25 was a product of Pharmacia. N-(*p*-(2-Benzimidazolyl)phenyl)maleimide (BIPM)[6] was a product of Teika Seiyaku Co. N-(*p*-(2-Benzimidazolyl)phenyl)succinamic acid (BIPSA) (3) was synthesized as described previously.[6]

Titration of the Thiol Groups of Egg Albumin with PCMB——The content of the thiol groups of egg albumin was estimated spectrophotometrically by adaptation of the PCMB method of Boyer.[13] The technique used in the present work was the following: solutions of PCMB were made by first preparing a stock solution of 2.5×10^{-3} M in 10 ml of deionized water containing 0.2 ml of 1 N NaOH and 3 ml of 0.15 M sodium pyrophosphate, and 1 ml of this stock solution was diluted to 25 ml with 0.33 M acetate buffer (pH 4.6). A solution of PCMB as added in increasing amounts (up to 0.3 ml) to a series of tubes each containing 0.5 ml of a protein solution (approx. 10^{-5} M) in 0.33 M acetate buffer and the total volume was made to 1 ml with 0.33 M acetate buffer. The reaction mixture was allowed to stand for 4 hr at room temperature (pH 4.6) and optical densities were then determined at 255 nm. The content of thiol groups per mole of protein was calculated according to the graphical method.

Spectroscopic Measurements——Fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer MPF-2A which is equipped with a R-106 photomultiplier and coupled to a QPD-33 recorder of the same manufacturer. Calibration of the fluorometer and determination of quantum yield were carried out by the previously described method.[5] Fluorescence was measured in 0.1 M phosphate buffer at pH 7.0. To avoid inner filter effects, optical densities of the solutions were kept below 0.2 at the excitation wavelength.

Absorption spectra were recorded on a Hitachi-ESP-3T spectrophotometer. Absorbance was measured with a Hitachi-Perkin Elmer-139 spectrophotometer. Protein concentration and the degree of labeling were estimated, as well as by fluorometry, based on the absorption of the protein-reagent conjugates at 280 and 320 nm: the extinction coefficients used were $E^{1\%}_{cm}$ 7.35 for egg albumin and ε^{M}_{cm} 32850 for BISPA (at 320 nm) (Table I). For determining the concentration, the absorption at 280 nm was corrected for turbidity and contribution of the absorption of BIPM-derivative at this wavelength.

Reaction of BIPM with Egg Albumin——Sample A: 45 mg of egg albumin were dissolved in 20 ml of 0.1 M phosphate buffer (pH 7.0) and mixed with 2 ml of a stock solution of BIPM (14.5 mg/10 ml) in monoglyme (Tokyo Kasei, G.R. grade). The solution was stood in a cold room (4°) for 19—24 hr. Excess of BIPM was removed in either of the following two ways: (1) The reaction mixture was applied to a Sephadex G-25 column and eluted with water. Fractions containing materials which absorb at 280 nm were combined and lyophilized. (2) The reaction mixture was dialyzed for 3 days at 4° against five changes of 5 liters of deionized water then lyophilized.

Sample B: Egg albumin was reacted in the presence of 0.5% lauryl sulfate by the same procedure as that of sample A and the modified protein was dialyzed and lyophilized.

Sample C: 11.5 mg of egg albumin were dissolved in 5 ml of 0.1 M phosphate buffer (pH 7.0) and incubated with 0.5 ml of a monoglyme solution of BIPM (5×10^{-3} M) for 1 hr at room temperature. The unreacted BIPM was removed through a Sephadex G-25 column using 0.1 M phosphate buffer (pH 7.0) as an eluent. The fractions were monitored by observing the absorption at 280 and 320 nm. These samples were used for measurement of fluorescence spectra.

Sample D: The reaction was performed as in the case of sample A except that the incubation period was 14 hr. This sample was dissolved in 0.1 M phosphate buffer and the solution was adjusted to pH 10.6

11) L.W. Cunningham and B.J. Nuenke, *J. Biol. Chem.*, **234**, 1447 (1959).
12) C.C. Lee and E.R. Samuels, *Can. J. Chem.*, **39**, 1152 (1961).
13) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

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with 0.1 N NaOH then stood overnight. The solution was dialyzed for 3 days at 4° against five changes of 5 liters of deionized water then lyophilized.

Amino Acid Analysis——Protein samples were hydrolyzed in 6 N HCl at 110° for 72 hr in evacuated sealed glass tubes and the hydrolysates were subjected to quantitative amino acid analysis. Analyses were performed with a Hitachi KLA-3B amino acid analyzer by the method of Spackmann, *et al.*,[14] expect that the operational temperature was 55°.

S-(1,2-Dicarboxyethyl)-L-cysteine (S-succinylcysteine) **4** was prepared by the acid hydrolysis of S-(N-ethylsuccinimido)-L-cysteine.[15] This sample has an elution pattern in the chromatography on the amino acid analyzer identical with an authentic specimen which was prepared by reaction of maleic anhydride with L-cysteine.[16]

Results

Stoichiometry of the Reaction of BIPM with Thiols of Egg Albumin

The thiol content of native egg albumin was determined by the Boyer's PCMB method.[13] Fig. 1 shows that 3.2 moles of thiol groups are titrated in this manner. When the protein was incubated with BIPM as described in the sample A procedure, the titrable thiol groups remaining were now 2.5 moles as shown in Fig. 2. This decrease in the amount of thiols which react with PCMB in the sample after the treatment with BIPM, 0.7 mole per mole of egg

Table I. Extinction Coefficients (ε) of BIPS and BIPSA

nm	280	310	315	320
BIPS	13600	26000	22150	17500
BIPSA	13500	35550	35500	32850

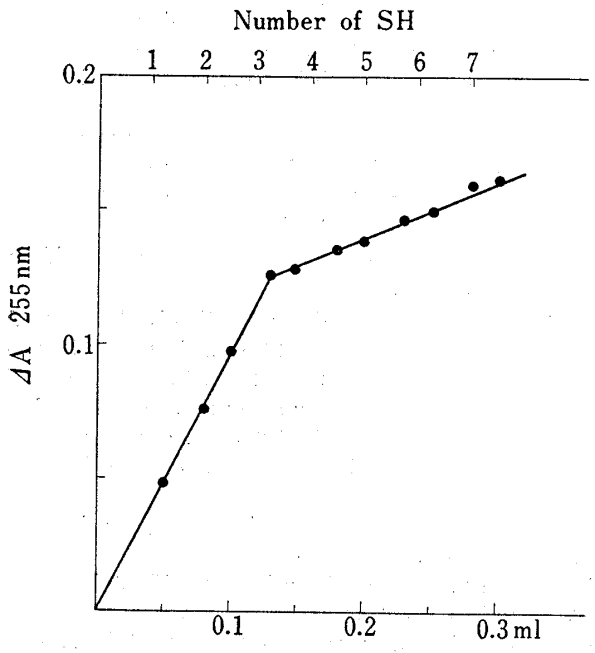

Fig. 1. Titration of Thiol Groups in Native Egg Albumin (4.09×10^{-6} M) with PCMB

The concentration of the added PCMB solution was 1.02×10^{-4} M.

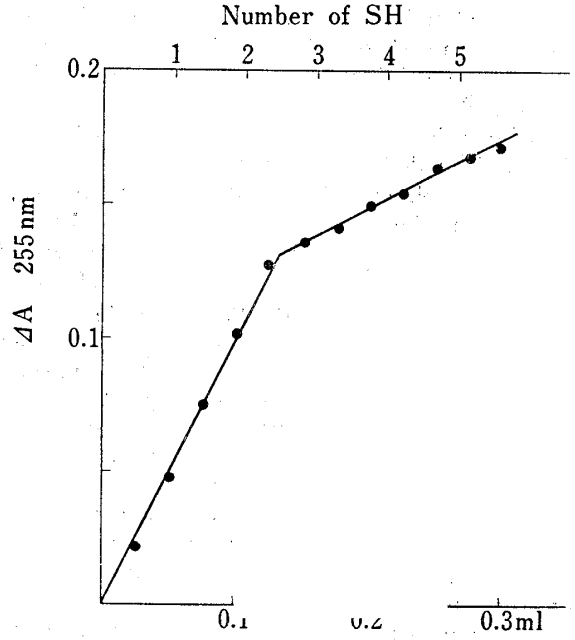

Fig. 2. Titration of Thiol Groups in the Lyophilized Sample A (2.80×10^{-6} M) with PCMB

The concentration of the added PCMB solution was 1.044×10^{-4} M.

14) D.H. Spackmann, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
15) D.G. Smyth, O.O. Blumenfeld, and W. Konigsberg, *Biochem. J.*, **91**, 589 (1964).
16) D.H. Calam and S.G. Waley, *Biochem. J.*, **86**, 226 (1963).

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albumin, nearly agreed with the value of the incorporation of BIPM, 0.63 mole, obtained from the absorption at 320 nm of the modified protein assuming that the incorporated BIPM exists in the ring-opened form (BIPSA). Extinction coefficients of BIPS and BIPSA used in the present work are listed in Table I.

The product of the reaction of cysteine with N-ethylmaleimide, a well-known thiol reagent, is S-(N-ethylsuccinimido)cysteine **2** (R=Et; R'SH=cysteine), which yields equal amounts of S-succinylcysteine **4** and ethylamine after hydrolysis in 6N HCl at 110° for 72 hr.[15] Fig. 3 shows the chromatographic separation of a new amino acid derivative (arrowed) in the amino acid analysis of the hydrolysate obtained in the similar manner from the BIPM-egg albumin conjugate (sample B). The compound was identified to be **4** by co-chromatography on an amino acid analyzer with the authentic specimen of **4**. The extent of labeling calculated from the amino acid analysis was 0.6 mole, in excellent agreement with the value obtained from the absorption measurement. Table II presents the comparison of the amino acid composition of the BIPM-egg albumin with that of the native protein. There is no significant change in the quantity of any of amino acids before and after the treatment of the protein with BIPM. On the basis of the amino acid analysis of **4**, the extents of labeling were 0.6 and 4.0—4.2, for sample A and sample B, respectively.

Table II. Amino Acid Composition of BIPM-labeled Egg Albumin

Amino acid	Reported values[a] egg albumin	Native egg albumin	Sample A[b]	Sample B[b]	Sample B[c]
Lys	20	20.9	20.8	21.1	20.8
His	7	6.7	6.9	6.8	6.6
Arg	15	15.1	15.1	15.4	15.5
S-Succinylcysteine			0.64	4.2	4.0
Asp	32	31.5	31.5	31.6	31.7
Thr	16	13.3	13.8	13.9	14.0
Ser	36	27.0	27.5	27.0	27.8
Glu	52	52.0	52.6	52.8	52.9
Pro	14	18.2	16.2	16.2	15.4
Gly	19	19.4	19.9	20.2	20.2
Ala	35	35.2	35.6	35.2	35.3
Val	28	30.3	30.4	30.0	30.0
Met	16	15.5	15.4	15.5	15.6
Ileu	25	23.6	23.7	23.7	23.7
Leu	32	32.0	32.0	32.0	32.0
Tyr	9	8.5	9.8	9.6	9.3
Phe	21	19.4	19.6	19.8	19.8

The amino acid compositions are expressed in terms of the molar ratios of the consituent amino acids. Each values based on 32 leucine per mole of protein are the result of a single analysis of protein hydrolyzed for 72 hr and not corrected for decomposition during hydrolysis.
a) G.R. Tristram, *Adv. in Protein Chem.*, 5, 83 (1949)
b) 10 moles of the reagent per molecule of protein
c) 20 moles of the reagent per molecule of protein

Absorption Spectra of the Modified Protein

A question is raised whether the initially formed succinimide ring **2** on reaction of BIPM with thiol groups in a protein is retained or further hydrolyzed to the more stable succinamic acid form **3** (Chart 1). Generally it has been known that succinimide derivatives are susceptible to hydrolytic ring-opening under relatively mild conditions.[17] N-(*p*-(2-Benzimidazolyl)-phenyl)succinimide (BIPS) (**5**) was selected as a model compound of the adduct **2**[6] since

17) *a*) M.K. Hargreaves, J.G. Pritchard, and H.R. Dave, *Chem. Rev.*, **70**, 439 (1970); *b*) M. Machida, M.I. Machida, and Y. Kanaoka, "in preparation."

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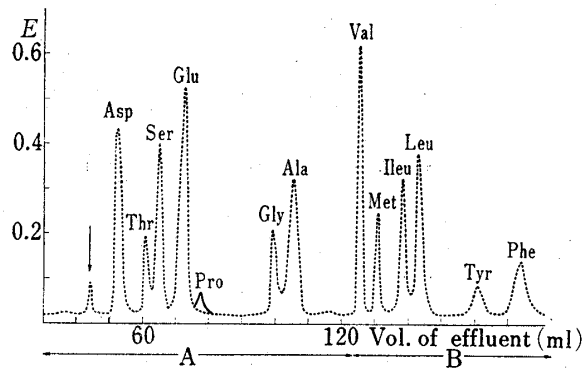

Fig. 3. Amino Acid Analysis of a 72 hr Acid Hydrolysate of Sample B by the Method of Spackman, *et al.*[14]

A, eluted with 0.2 M sodium citrate buffer, pH 3.25; B, eluted with 0.2 M sodium citrate buffer, pH 4.25

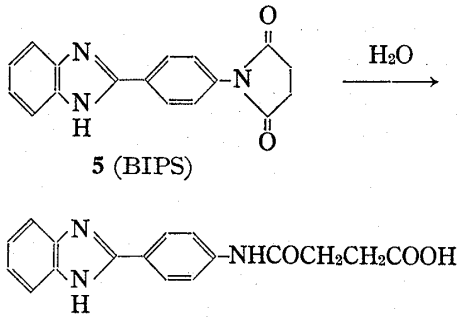

Chart 2

the sulfide moiety may, but not significantly affect the spectroscopic properties of the system **2**. Thus the related model compound of the ring-opened product **3** in the spectroscopic studies is the corresponding succinamic acid (BIPSA) (**6**) (Chart 2).

The ultraviolet absorption spectra of BIPS (**5**), BIPSA (**6**) and mixtures of native egg albumin and BIPS, and BIPSA are shown in Fig. 4. In going from a succinimide (BIPS) **5** to a succinamic acid (BIPSA) by the ring-opening the absorption undergoes hyperchromic and bathochromic changes and, it can be expected that the absorption spectra may well serve as criteria whether the incorporated BIPM exists in a form of a succinimide **2** or a succinamic acid **3** in the individual microenvironments of the protein. In Fig. 5 are shown the absorption spectra of sample A and D, which are nearly identical and are very close to the pattern of the spectrum of the mixture of egg albumin and BIPSA (Fig. 4), indicating that the introduced reagent takes the form of the succinamic acid under these conditions as a result of hydrolysis in the course of the labeling treatment.

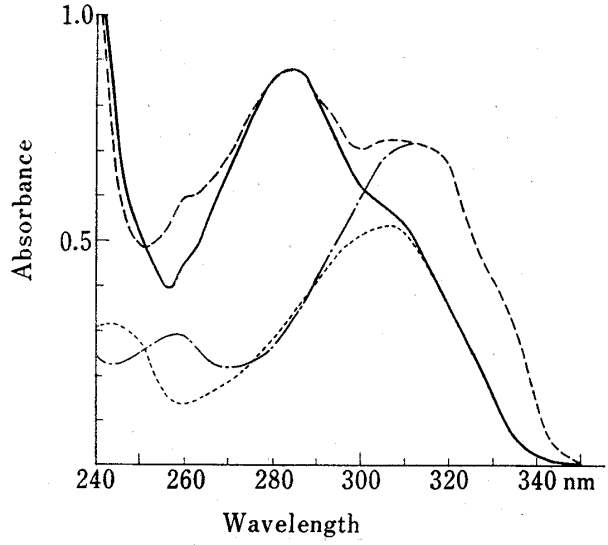

Fig. 4. Ultraviolet Absorption Spectra of BIPS and BIPSA in the Absence and Presence of Egg Albumin

concentration 2×10^{-5}M in 0.1M phosphate -------: BIPS, —·—·: BIPSA, ——: a mixture of BIPS and native egg albumin (1:1), ----: a mixture of BIPSA and native egg albumin (1:1)

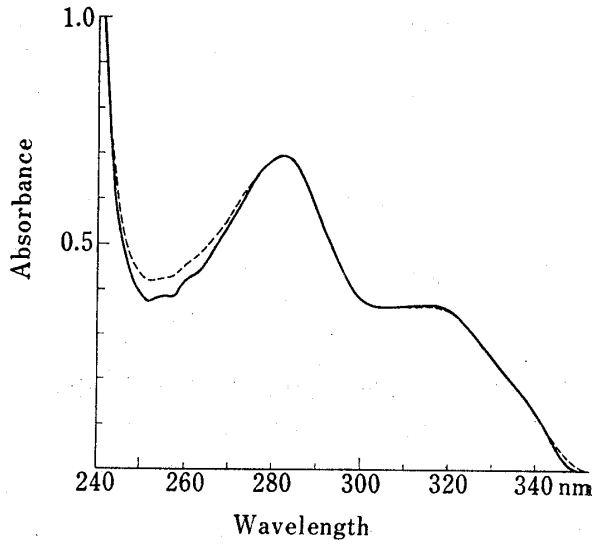

Fig. 5. Ultraviolet Absorption Spectra of BIPM-treated Egg Albumin in 0.1M Phosphate Buffer (pH 7.0)

Concentration of protein was approximately 10^{-5}M. ——: sample A, ----: sample D

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Fluorescence Spectra of the Modified Protein

The fluorescence excitation and emission spectra of BIPSA and BIPM-egg albumin (sample D) are compared in Fig. 6. The excitation spectrum of BIPSA, measured at 360 nm, causes a small red-shift when BIPSA is covalently bound to the protein in sample D. In addition, in the emission spectra a slight but clear difference between BIPSA alone and the modified protein is observed at around 345 nm and 375 nm. To see the solvent effects on the emission of the fluorophore, fluorescent spectra of BIPSA in dioxane containing various amounts of water were shown in Fig. 7. While the fluorescence intensity increases with increase of the dioxane fraction of aqueous dioxane accompanied by appearance of vibrational structures (340 and 375 nm), the emission maximum was nearly unaltered.

The extent of labeling can also affect the fluorescence spectra of the conjugated proteins. The amounts of the reagent introduced into thiol groups in sample C and sample D are estimated to be 0.1 mole and 0.7 mole, respectively. As shown in Fig, 8, the fluorescence spectra of sample A and D are superimposable. However, the excitation spectrum measured at 360 nm of sample C, which is labeled to a less extent than A and D, has a strong maximum at 294 nm

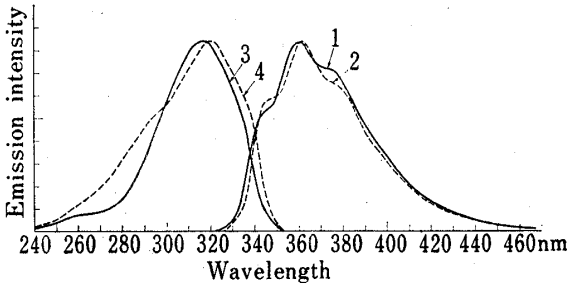

Fig. 6. Fluorescence Excitation and Emission Spectra of BIPSA and BIPM-Egg Albumin in 0.1M Phosphate Buffer (pH 7.0)

The emission spectra (curve 1 and 2) were obtained with excitation at 310 nm and the excitation spectra (3 and 4) were measured on the emission monochrometor set at 360 nm. ——: BIPSA, ----: BIPM-egg alubmin (sample D)

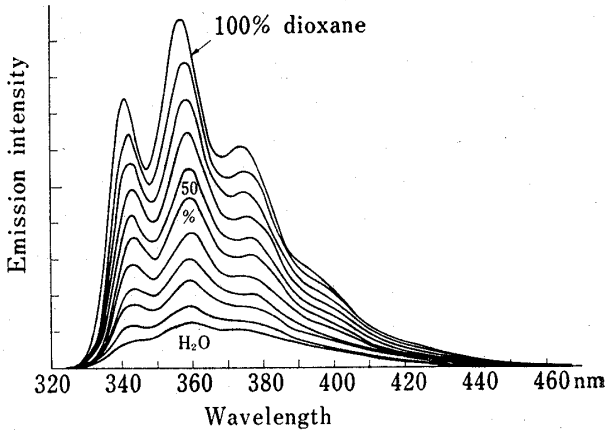

Fig. 7. Fluorescence Emission Spectra of BIPSA in Dioxane-Water Mixtures with Excitation at 320 nm Obtained by Changing a Dioxane Content at 10% Intervals.

Stock solution was prepared as follows: BIPSA (6.9 mg) was dissolved in 0.2 ml of dimethylformamide and diluted to 25 ml with ethanol. 0.15 ml of the stock solution was diluted to 25 ml with various aqueous dioxane solutions, and used for measurement.

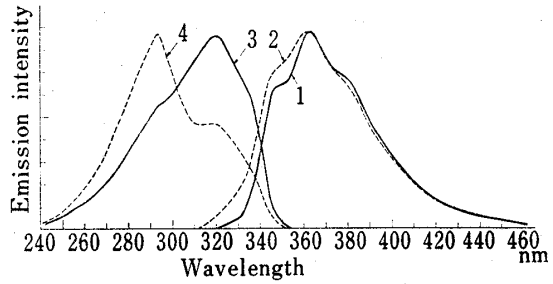

Fig. 8. Fluorescence Spectra of BIPM-treated Egg Albumin in 0.1M Phosphate Buffer (pH 7.0)

The emission spectra (curve 1 and 2) were obtained with excitation at 310 nm and the excitation spectra (3 and 4) were obtained on the emission monochrometor set at 360 nm. ——: sample A and D, ——: sample C

Table III. Fluorescence Quantum Yields of the BIPM-Proteins

Excitation (nm)	310	320	330
Sample A	0.17	0.17	
Sample C		0.18	0.18
Sample D	0.11	0.12	
BIPSA	0.13	0.13	

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due to "intrinsic" fluorescence of aromatic amino acid residues of egg albumin as well as a weaker peak at around 320 nm due to the introduced BIPM. In consistent with this relatively less importance of the "Extrinsic" chromophore in the excitation spectrum, the fluorescence emission spectrum of sample C shows a different pattern from that of sample A and D to indicate relatively important contribution of an intrinsic fluorescence particularly at around 350 nm. Fluorescence quantum yields of these spectra are listed in Table III.

Discussion

Egg albumin was selected as a standard thiol-protein in the present work in order to evaluate BIPM as a fluorescent thiol reagent for protein, because the reactions of the thiols of this protein have been well studied using several conventional reagents. Boyer found that the thiol content per mole of egg albumin titrable with PCMB are 3.2 at pH 7.0 in 0.05 M phosphate buffer and 4.0 at pH 4.6 in 0.33 M acetate buffer, respectively,[13] while Alexander reported that 2.2 equivalents of thiols react with N-ethylmaleimide in pretreated egg albumin with Duponol.[18] In the case of sample A, for example, on the basis of both the PCMB titration and the absorption measurement, approximately 0.6—0.7 mole of BIPM was incorporated into one mole of egg albumin.

Although the reagent has been apparently introduced into the thiol group on the basis of the above ultraviolet absorption analysis as well as the concomitant development of fluorescence, the convincing chemical evidence is now available from the amino acid analysis of the BIPM-protein, which shows the existence of 0.6 mole of S-succinylcysteine **4** that must arise from the addition product **2** of BIPM with the cysteine residue in the protein. It has been known that N-ethylmaleimide could react with functional groups in protein other than thiol, such as amino and imidazole, under certain conditions.[19-21] A comparison between the amounts of the introduced BIPM into egg albumin and of **4** in an acid hydrolysate of the BIPM-treated protein provides a quantitative measurement of the specificity of the reagent toward thiol groups. Thus, since BIPM reacts specifically with thiols, the amounts of the incorporated reagent and the yield of **4** are nearly equal. Consistently, the results of amino acid analysis of the hydrolysate in Table II showed no indication that any other amino acid residues including lysine and histidine were modified. These basic amino acid residues were intact even in the case of extensively modified sample B, for which 20 equivalents of BIPM were applied in the presence of lauryl sulfate to react 4 thiols.

On the basis of the absorption spectra of the modified proteins, it is evident that the introduced BIPM in sample A exists in the form of the succinamic acid **3**. In fact, the spectra of sample A coincide with those of sample D which has been incubated in an alkaline solution in an attempt to complete the hydrolysis. These results are worthy to note in view of our observation that the rate of the hydrolysis of a succinimide ring is generally much slower than that of a maleimide ring.[22] The microenvironments of the thiols in this protein would facilitate the hydrolytic ring opening of the incorporated BIPM molecule.

The shapes of the emission spectra of BIPSA and BIPM-egg albumin are slightly different. In the spectra of the modified proteins, the shoulder at 375 nm in that of BIPSA alone has disappeared and conversely, the shoulder at 345 nm has been intensified (Fig. 6). Apparently this change in the spectra of the modified proteins is ascribed to contribution of intrinsic fluorescence of tryptophan (and tyrosine) residues in the protein. The influence of the in-

18) N.M. Alexander, *Anal. Chem.*, **30**, 1292 (1958).
19) C.F. Brewer and J.P. Riehm, *Anal. Biochem.*, **18**, 248 (1967).
20) G. Guidotti and W. Konisberg, *J. Biol. Chem.*, **239**, 1474 (1964).
21) D.G. Smyth, A. Nagamatsu, and J.S. Fruton, *J. Am. Chem. Soc.*, **82**, 4600 (1960).
22) M.I. Machida, M. Machida, and Y. Kanaoka, "in preparation."

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trinsic fluorescence is clearer in the spectra of sample C, a less extensively modified example (Fig. 8). In fact, when excited with the light of wavelength above 320 nm, the fluorescence spectra of sample C stayed unchanged, while with the light below 320 nm, small change in their shapes was observed, the peak at 345 nm being more sensitively influenced than the one at 375 nm. As a whole, however, quantum yields of BIPSA alone and the incorporated fluorophore are of the same order (Table III).

Fluorescence characteristics of BIPSA showed dependence on solvent-polarity in the intensity (Fig. 7) but practically not in the maxima. Usually fluorescence quantum yield is much more variable than the maximum and the polarity-dependent nature of microenvironments of thiols in protein will be best described in relation to the fluorescence maximum wave number.[1] Therefore BIPM is rather unsuitable for a hydrophobic probe[23] of thiols. Design of such an appropriate probe has been described elsewhere.[1]

Primarily, the question of specificity of a reagent should be treated as a separate problem with each protein under investigation. However, the above studies now provide a basis for the consideration of general reactions to be expected between BIPM and proteins. Under these conditions described in the present work, BIPM behaves in principle as a fluorescent reagent with a high degree of specificity for thiol groups in protein.

Acknowledgement This work was supported in part by grants from Matsunaga Science Foundation (to M.M.) and the Naito Foundation (to Y.K.).

23) W.O. McClure and G.M. Edelman, *Biochemistry*, 5, 1908 (1966).

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