FLUOROMETRIC ASSAY FOR DISCRIMINATING THE STATES OF AMINO GROUPS IN INSULIN, LYSOZYME AND ASPARAGINASE WITH FLUORESCAMINE

Y. TAMAURA, K. TODOKORO, M. IKEBE, H. MAKINO, S. HIROSE and Y. INADA
Laboratory of Biological Chemistry, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo, Japan

Received 25 November 1974

1. Introduction

Since Weigele et al. [1] synthesized 4-phenylspiro [furan-2 (3H), 1′-phthalan]-3,3′-dione, fluorescamine, which reacts specifically with primary amine to yield highly fluorescent product, Udenfriend et al. [2–4] applied this reagent to fluorometric assay of amino acids, peptides and proteins. In order to clarify the higher order structure of proteins and enzymes, a number of chemical modification reagents which discriminate the states of amino acid residues have been explored by many investigators [5,6]. The reaction of fluorescamine with a substance containing amino groups proceeds almost instantaneously at a room temperature and in aqueous medium. Fluorescamine might be, therefore, the most suitable reagent for discriminating the states of amino groups in the protein molecule.

In the present paper it is shown that the states of amino groups in insulin, lysozyme and asparaginase are discriminated by measuring the fluorescence intensity of the product formed by the reaction of fluorescamine with each protein.

2. Materials and methods

Bacitracin, bovine insulin, hen egg white lysozyme and asparaginase (*Escherichia coli* HAP) in crystal forms were used, and their concentrations except bacitracin were determined spectrophotometrically assuming $\epsilon_M = 5.7 \times 10^3$ M^{-1} cm^{-1} at 280 nm for insulin, $\epsilon_M = 3.9 \times 10^4$ M^{-1} cm^{-1} at 280 nm for lysozyme and $\epsilon_M = 8.83 \times 10^4$ M^{-1} cm^{-1} [7] at 278 nm for asparaginase. The reaction of each protein with fluorescamine was carried out as follows; to 3 ml of a protein solution ($1.3 \times 10^{-7} - 4.0 \times 10^{-5}$ M) in 0.2 M borate buffer (pH 9.0) was added 50 μl of fluorescamine (less than 63 mM) in acetone. The sample solution was diluted three times with the buffer solution and the fluorescence intensity, F, was measured with the excitation wavelength at 390 nm and emission at 478 nm, using a Shimazu spectrofluorometer RF-502 with corrected spectra attachment. Alkali denatured proteins were prepared by standing each protein solution at alkaline pH's for 3 hr in the case of lysozyme and asparaginase and for 3 days in the case of insulin. After that the pH of each sample solution was put back at neutral pH. The number of amino groups, n, reactive with fluorescamine was estimated from the value of $F/C\cdot\phi$, a fluorescence intensity (F) divided by a protein concentration (C) and by a quantum yield (ϕ), in reference to the value of $F/C\cdot\phi$, obtained for bacitracin containing one amino group in the molecule. The sample for determining the quantum yield, ϕ, was prepared as follows; the protein modified by 0.53 mM fluorescamine was isolated by passing through a Sephadex G-25 column. The ϕ value was determined by measuring the area of the fluorescence band of the modified proteins and the transmittance at 360 nm, using quinine sulfate as reference. The ϕ value of quinine sulfate (10 μM in 0.5 N H_2SO_4) was assumed to be 0.55(8). To make sure of the number of the modified amino groups in the protein molecule, an absorption spectrophotometric assay was performed, using the protein modified by 0.53 mM fluorescamine, which were isolated by passing through Sephadex G-25. The number of amino groups modified by fluorescamine was calculated from the absorbance at 380 nm (a peak position of the absorption band of fluorescamine which reacted with proteins) and the protein concentrations.

 North-Holland Publishing Company – Amsterdam

3. Results and discussion

Fig.1 shows plotting of the fluorescence intensity, F, of the product formed by the reaction of fluorescamine with bacitracin (curve A), insulin (curve B), lysozyme (curve C) and asparaginase (curve D) against their protein concentrations. The fluorescence intensity increases linearly as the protein concentration increases. Fig.2 represents the reaction curves obtained for bacitracin, insulin and lysozyme against various fluorescamine concentrations. The vertical line in the figure indicates the fluorescence intensity divided by each protein concentration and by quantum yield, $F/C\cdot\phi$. The reaction curve obtained for bacitracin (curve A) rises sharply at lower concentrations of the reagent and keeps a constant level at higher concentrations. Similar reaction curves were obtained for insulin (curve B), denatured insulin (curve B′), lysozyme (curve C) and denatured lysozyme (curve C′). The constant $F/C\cdot\phi$ value obtained for the reaction curve of bacitracin was taken as unit (n=1). In the case of insulin, 2 out of 3 amino groups in the molecule are reactive with fluorescamine and the remaining one becomes reactive after an alkali denaturation. In the case of lysozyme,

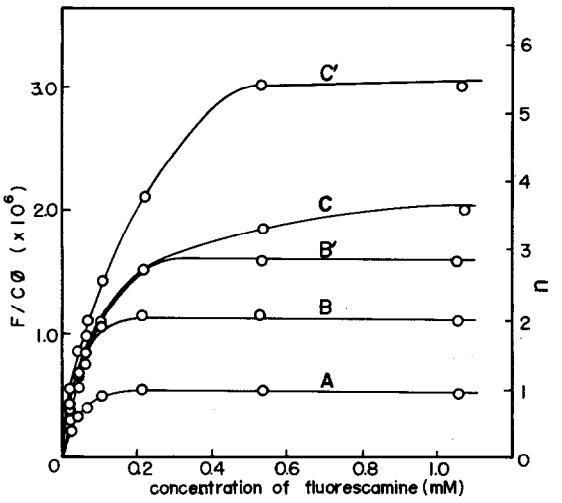

Fig.2. Plotting of $F/C\cdot\phi$ (fluorescence intensity/protein concentration, quantum yield) against fluorescamine concentration. Curve A; 28.2 μM bacitracin, Curves B and B′; 17.2 μM native insulin and 17.2 μM insulin denatured at pH 13.0, repectively. Curves C and C′; 8.73 μM native lysozyme and 7.80 μM lysozyme denatured at pH 12.2, respectively. The value of $F/C\cdot\varphi$ at a constant level obtained for the reaction curve of bacitracin was taken as n = 1.

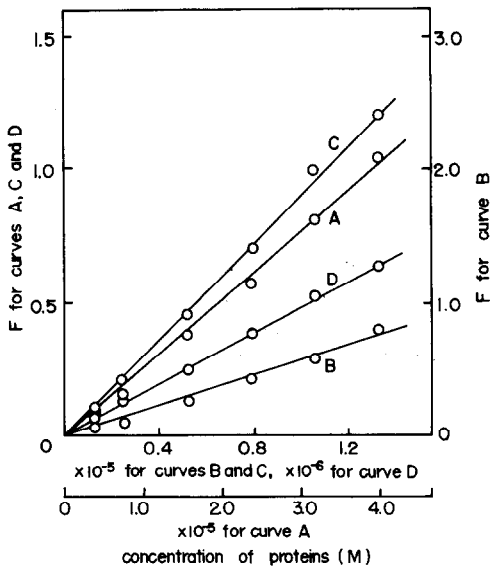

Fig.1. Relation between fluorescence intensity, F, of 0.53 mM fluorescamine reacted with bacitracin (curve A), insulin (curve B), lysozyme (curve C) or asparaginase (curve D) and protein concentration.

4 out of 7 amino groups in the molecule are reactive with fluorescamine (curve C) and 2 of the remaining groups become reactive with fluorescamine by an alkali denaturation. These results are compatible with those obtained for insulin and lysozyme using naphthoquinone disulfonic acid as a chemical modification reagent [9].

The quantum yield, ϕ, of fluorescamine which reacted with each protein was determined and the results are shown in table 1, together with the number of amino groups in each protein molecule modified with fluorescamine. The ϕ value of bacitracin, 0.047, is in good agreement with native insulin, 0.048, and alkali denatured insulin, 0.047, and also native lysozyme, 0.048. The ϕ value obtained for alkali denatured lysozyme, 0.029, which is smaller than that for native lysozyme, agrees with that obtained for native asparaginase, 0.030 or alkali denatured asparaginase, 0.030–0.031.

Quite recently, the primary structure of asparaginase from *Escherichia coli* HAP was determined by Matsuda et al. [10] and it was found that there exist 88 lysine residues and four terminal amino groups in the molecule. Fig.3 shows plotting of $F/C\cdot\phi$ of native asparaginase (curve A) and asparaginase denatured at pH

Table 1
Number of amino groups, n, reactive with fluorescamine in the bacitracin, insulin and asparaginase molecule.

Proteins	ϕ	$F/C\cdot\phi$ ($\times 10^5$)	n	n_t	absorption spectrophotometric assay n
Bacitracin	0.047	5.53	1.0	1	1.0
Insulin					
pH 7.0	0.048	12.0	2.2	3	2.1
pH 13.0	0.047	15.5	2.8		2.9
Lysozyme					
pH 7.0	0.048	18.2	3.3	7	3.4
pH 12.2	0.029	30.3	5.5		5.5
Asparaginase					
pH 7.0	0.030	155	28	92	26
pH 12.5	0.031	359	65		62
pH 13.5	0.030	431	78		80

ϕ; Quantum yield
n; Number of amino groups reacted with fluorescamine
n_t; Total number of amino groups in the protein molecule

12.5 (curve B) and at pH 13.5 (curve C) against fluorescamine concentration. Each curve keeps a constant level at higher concentration than 0.3 mM fluorescamine. Approximately 30 out of 92 amino groups in the molecule are reactive with the reagent, 35 of the remaining groups become reactive with the reagent by an alkali denaturation (pH 12.5) and the remaining 13 groups become reactive by a strong alkali denaturation (pH 13.5). The same result as above was obtained by an absorption spectrophotometric assay for insulin, lysozyme and asparaginase, as seen in table 1. Amino groups nonreactive with fluorescamine in the native protein molecule may be located in the interior of the protein molecule or be in bound state such as ionic linkage and hydrogen bonding in the molecule. Fluorescamine can be used for discriminating the states of amino groups in the protein molecule with the unique merit of the high sensitivity of detection and the moderate reactivity for discrimination.

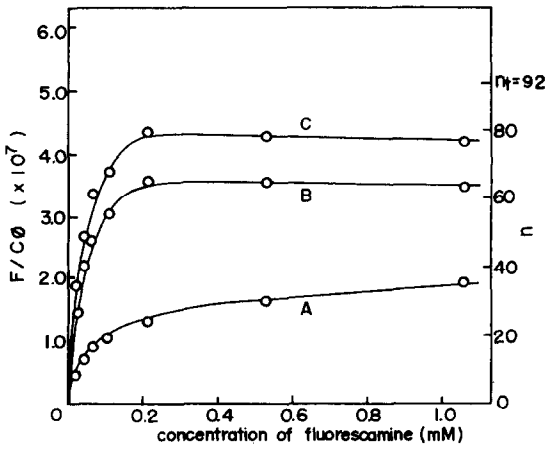

Fig.3. Plotting of $F/C\cdot\phi$ (fluorescence intensity/protein concentration, quantum yield) obtained for asparaginase against fluorescamine concentration. Curves A, B and C, 1.27 μM native asparaginase, 0.97 μM asparaginase denatured at pH 12.5 and 0.687 μM asparaginase denatured at pH 13.5, respectively.

References

[1] Weigele, M., DeBernardo, S. L., Tengi, J. P. and Leimgruber, W. (1972) J. Amer. Chem. Soc. 94, 5927–5928.
[2] Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972) Science, 178, 871–872.
[3] Stein, S., Böhlen, P., Stone, J., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 202–212.

[4] Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.
[5] Vallee, B. L. and Riordan, J. F. (1969) Annu. Rev. Biochem. 38, 733–793.
[6] Niederwieser, A. and Patake, G. (1971) New Techniques in Amino Acid, Peptide and Protein Analysis (1st Edn.) pp. 341–385, Ann Arbor Sci.
[7] Nishimura, Y., Makino, H. Takenaka, O. and Inada, Y. (1971) Biochim. Biophys. Acta. 227, 171–179.
[8] Melhuish, W. H. (1961) J. Phys. Chem. 65, 229–235.
[9] Matsushima, A. Sakurai. K., Nomoto, M., Inada, Y. and Shibata, K. (1968) J. Biochem. 64, 507–514.
[10] Maita, T., Morokuma, K. and Matsuda, G. (1974) J. Biochem. 76, No. 6.