LIMITED REACTION OF LYSOZYME WITH A FLUORESCENT LABELING AGENT

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SUMMARY

Lysozyme reacts with dansyl chloride to give a conjugate having a maximum of about 1 dansyl group per mole of protein. The conjugate exhibits cold lability but is enzymically active. ε-Dansyl lysine was recovered from hydrolysates, and preliminary tryptic digests suggest that only 1 lysine is labeled. The dansyl group is lost from the conjugate over several days with an apparent increase in quantum yield and lifetime. The dansyl group seems to be in a very exposed region, as the emission is orange and peaks at 560 nm. The extinction coefficient of the bound dansyl group, 5270 $cm^{-1}M^{-1}$, was determined with C^{14}-dansyl chloride and is unusually high for proteins.

The labeling of proteins with dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) yields conjugates which are very useful in studies utilizing polarization of fluorescence (1,2). It was originally assumed that the dansyl groups attach rigidly to the protein in a random manner, thus justifying the assumption that the polarization of dye fluorescence will reflect the rotational motion of the protein as a whole (1,3). However, there are now so many cases where dye is attached neither randomly nor rigidly that specific labeling by the dansyl group may be the rule rather than the exception (4). In this communication, data supporting specific labeling of lysozyme by dansyl chloride is presented. The fluorescent conjugate appears to have a maximum of 1 dansyl group per mole of protein, and unlike the native protein is cold-labile. The presence of a single dye moiety on a protein of well-defined structure promises to allow close correlation of dye microenvironment with some of the unusual fluorescence and other physical properties. A fluorescent dye bound at a specific site may act as a "reporter group" (5) signalling changes in

conformation, environment, or rigidity of the attachment site.

MATERIALS AND METHODS

Crystalline hen egg lysozyme preparations were purchased from Sigma (Lot 88B-8050) and Worthington Biochemical Corp. (Lot LY 7GA). Bovine and human serum albumins were crystalline preparations from Armour and Pentex, respectively. β-Lactoglobulin, crystalline, was obtained from Sigma. Crystalline ovalbumin and chymotrypsinogen were purchased from Worthington, and bovine γ-globulin, fraction II, was obtained from Pentex. α-Lactalbumin was a gift of Dr. R. L. Hill. Dansyl chloride was purchased from Pierce Chemical Co. C^{14}-Dansyl chloride was obtained from Schwarz BioResearch and recrystallized as described previously (6). Fluorescence spectra were obtained with an Aminco-Bowman spectroflurometer and corrected as described (7). Fluorescence lifetimes were determined on a TRW decay time apparatus (8).

RESULTS

Reactivity of Lysozyme with Dansyl Chloride. Although it was possible to prepare conjugates of bovine serum albumin and γ-globulin with a wide range of dye/protein (D/P) ratios using dansyl chloride (9,10), all such attempts to insert various numbers of dansyl groups into lysozyme failed. Table I shows that lysozyme is, compared with other proteins, rather unreactive towards dansyl chloride. Under conditions where albumins incorporate about 18 dansyl groups, lysozyme and chymotrypsinogen incorporate less than 1. When an excess of labeling reagent was added to lysozyme solutions which were stirred in the cold, it was found that protein precipitates formed, the loss of protein being correlated with the amount of reagent added (Table II). It has not been possible to obtain conjugates of native lysozyme having D/P ratios greater than 1.1 or so in spite of large excesses of reagent. Interestingly, whenever dansyl lysozyme studies have been reported in the literature, the D/P ratio is always quite low (11-17).

The loss of protein which occurred under the conditions of Table II was

TABLE I

REACTIVITY OF NATIVE PROTEINS TOWARDS DANSYL CHLORIDE

Protein	Molecular Weight	D/P of conjugate*
Bovine serum albumin	67,000	17.4
β-Lactoglobulin	35,500	11.0
α-Lactalbumin	14,000	4.1
Lysozyme	14,300	0.87
Bovine γ-globulin	160,000	4.48
Ovalbumin	46,000	6.60
Chymotrypsinogen	21,000	0.71
Human serum albumin	69,000	18.3

After 30 mg of each protein was dissolved in 3 ml 0.1 M potassium phosphate buffer, pH 7.0, 0.4 ml of dansyl chloride (20 mg/ml in acetone) was added, and the solutions mixed at 23° for 3 hours. After centrifugation, the solutions were passed through Sephadex G-15 columns to remove free dye.

*Dye/Protein ratios were determined spectrophotometrically using the following extinction coefficients ($cm^{-1}M^{-1}$) 5,270 for lysozyme, 3,700 for chymotrypsinogen (6), 3,000 for ovalbumin (6), and 3,400 for all other proteins. Protein was determined by the Lowry method (25). Optical densities for D/P ratios were read at the absorption maxima, which ranged from 333-345 nm.

found to be due to cryoprecipitation. When the reaction is carried out at room temperature, precipitation does not occur; dansyl lysozyme is now routinely prepared in this laboratory at room temperature. Fig. 1 shows the rapid development of turbidity which occurs when a dansyl lysozyme preparation is placed in a spectrofluorometer cell kept at 8°.

Extinction Coefficient of the Bound Dansyl. A simple method for determining the extinction coefficient of bound dansyl groups using C^{14}-dansyl chloride has been reported previously (6). For dansyl lysozyme, ε^{333} was found to be 5,270 cm^{-1} M^{-1}, a value which is considerably higher than that found for other proteins (6).

TABLE II

LABELING OF LYSOZYME BY DANSYL CHLORIDE

Sample	Dansyl-Cl added /mole of lysozyme	Protein recovered	D/P of conjugate*
1	0	[100]	0
2	0.88	82	0.28
3	1.76	58	0.42
4	2.64	41	0.59
5	3.52	37	0.65
6	5.28	25	0.95
7	7.04	17	1.13
8	8.80	15	1.06

The conjugates were prepared by adding various amounts of dansyl chloride (10 mg/ml acetone) to solutions containing 0.1 M $NaHCO_3$ and 0.033 M NaCl and 10 mg lysozyme/ml. The mixtures were stirred in an ice bath for 3 hours, centrifuged, and the supernatants were put through Sephadex G-15 columns to remove free dye.

*Dye/Protein ratios were calculated from the OD_{333} assuming an extinction coefficient at this wavelength of 5,270 $cm^{-1}M^{-1}$ (see text), and from protein concentrations determined by the method of Lowry et al (25).

Site of Attachment of Dnasyl Group. When dansyl lysozyme was subjected to hydrolysis by 6N HCl and the product examined by thin-layer chromatography (18) and paper electrophoresis (19), the only fluorescent spot found corresponded to ε-dansyl lysine. In spite of the fact that the N-terminal amino acid is lysine (20), no spot corresponding to di-dansyl lysine was found.

Table III shows the results of quantitative amino acid analysis for native and dansylated lysozyme. The recovery of amino acids is good except for the drop in lysine content. The data are not sufficiently precise, however, to rule out some participation of histidine in the labeling process. On the other hand, recent preliminary experiments using "fingerprints" of tryptic digests of performic acid oxidized dansyl lysozyme, strongly indi-

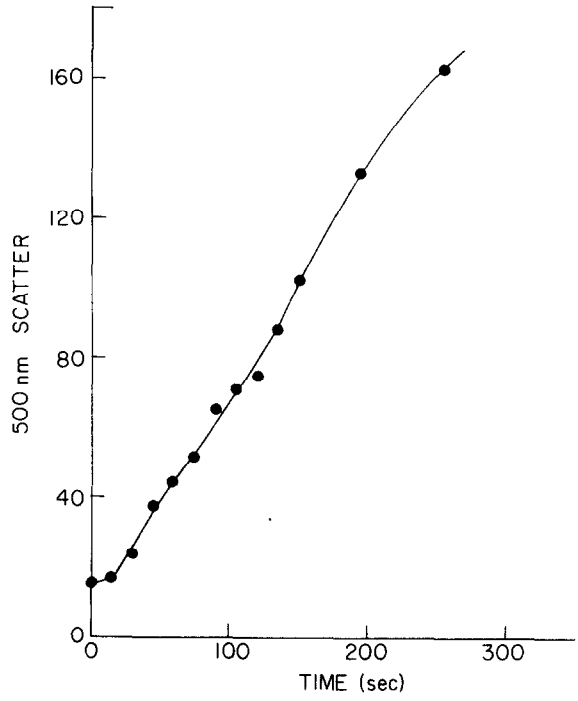

Fig. 1. Development of turbidity in a dansyl conjugate of lysozyme with a D/P ratio of 0.9, prepared at 25°. The protein, 7 mg/ml, in 0.1 M potassium phosphate buffer, pH 7.7, was monitored at 8° in a spectrofluorometer.

cate that only one site is dansylated. Experiments are under way to determine which of the six lysines is involved.

Stability of the Conjugate. The tendency of dansyl lysozyme to precipitate in the cold has already been mentioned. Also, it has been noted that fresh conjugates have a light orange fluorescence under a UV lamp, but the color changes to yellow and finally to green after a few days. Rechromatography of the solution on Sephadex reveals the presence of free dye. In one experiment a conjugate of lysozyme having an initial D/P ratio of 0.86 was found after 2 and 4 days to have D/P ratios of 0.67 and 0.57, respectively. In contrast, a dansyl bovine serum albumin conjugate with a D/P ratio of 0.70 showed no evidence of loss of dye for months.

Fluorescence of the Conjugate. The light orange fluorescence of dansyl lysozyme was found to peak at 560 nm and to extend beyond 750 nm. The

TABLE III

AMINO ACID COMPOSITIONS OF CARBOXYMETHYLATED LYSOZYMES

	Residues per Mole of Protein	
	Unlabeled Lysozyme	Dansyl Lysozyme
Aspartic Acid	20.3	20.3
S-Carboxymethylcysteine	7.2	6.9
Threonine	7.6	7.7
Serine	10.2	10.9
Glutamic Acid	5.0	5.0
Proline	2.2	2.5
Glycine	12.0	11.9
Alanine	11.9	12.2
Valine	5.1	5.0
Methionine	1.2	1.7
Isoleucine	5.6	5.7
Leucine	8.6	8.8
Tyrosine	2.9	2.9
Phenylalanine	3.0	3.2
Lysine	5.9	5.3
Histidine	1.2	0.9
Arginine	11.0	11.0

Native lysozyme and a dansyl lysozyme conjugate containing 0.9 dye group per mole of protein were carboxymethylated by the procedure of Canfield and Anfinsen (26). After acid hydrolsysi the samples were analyzed in a Beckman Spinco model 120B amino acid analyzer. (The analysis was obtained through the courtesy of Dr. Irwin M. Chaiken).

fluorescence is therefore unusual, because the emission peaks of other dansylated proteins such as serum albumin or gamma globulin are at wavelengths 20 to 60 nm shorter (9,10). The dansyl lysozyme spectrum is quite symmetrical, a finding which is consonant with a homogeneous fluorescence. The fluorescence lifetime of freshly prepared dansyl lysozyme in 0.1 M potassium phosphate buffer, pH 7.7 was 7.8 nsec. Curve-fitting (9) on the TRW lifetime apparatus was good, again suggesting that the solution fluorescence was homogeneous.

Other Properties of Dansyl Lysozyme. The conjugate was fully active in the M. lysodeikticus assay (21). Ageing of solutions resulted in evolution of free dye and apparent increases in quantum yield and lifetime. Bound dye fluorescence was strongly dependent on temperature and was found to be

markedly sensitive to solvent perturbants such as propylene glycol. Polarization of fluorescence studies suggest that the dye has considerable freedom of rotation independent of the protein rotation. In dansyl lysozyme, tryptophan fluorescence is partially quenched due to energy transfer. Details of these and other results will be reported elsewhere.

DISCUSSION

Under the usual labeling conditions, it is evident that only one dansyl group is incorporated onto one of the 6 lysines of lysozyme; and it appears that the lone dye moiety is labile in spite of the fact that dansyl amino acids normally are stable to hydrolysis by strong acid (19). The cause of the lability is obscure; possibly a neighboring group catalyzes the hydrolysis of the sulfonamide linkage.

The propensity of the conjugate to precipitate was also noted by Lagunoff and Ottolenghi (22). They said, "...there is a maximum level of labeling which, if exceeded, leads to the formation of an insoluble product." While they did not speculate on what the maximum level was, attempts in this laboratory to isolate insoluble conjugated lysozyme with more than 1 dansyl group have not been fruitful. It seems possible that the insoluble product is the mono-substituted lysozyme itself.

The dansyl group of the conjugate appears to be highly exposed. Not only is the fluorescence emission spectrum red-shifted, indicating that the emitting group is in a polar environment (23), but the extinction coefficient of the bound dansyl is higher than reported for other proteins (6) and similar to those of simple dansyl sulfonamides (1). Correlation of site topology with these anomalous properties should be possible once the attachment site of the dansyl group can be located in the 3-dimensional model of lysozyme obtained from crystallographic data (25).

The short lifetime of dansyl lysozyme fluorescence indicates that previous work on the polarization of fluorescence of this enzyme based on an

assumed decay time of 13-14 nsec (13,17) must be reexamined. A recent report (17) claimed that dansyl lysozyme emission was heterogeneous, with one component which made up 30% of the emission having a decay time of 15 nsec. Such heterogeneous fluorescence is unlikely from the evidence presented here that only one dansyl group is incorporated per mole of protein, that the emission band is symmetrical, and that a longer lifetime component was not found in our nanosecond lifetime apparatus with fresh conjugate.

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