4,4′-Difluoro-3,3′-dinitrophenylsulfone as a Cross-Linking Reagent for Lysozyme (34095)

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Several bifunctional reagents have been recently employed to introduce stable covalent cross-links into biologically active proteins without adversely affecting activity (1). Such cross-links generally confer additional stability toward such denaturing agents as heat, urea, alkali, or oxidants. Thus, 1, 5-difluoro-2, 4-dinitrobenzene (FDP) has been used to form dinitrophenylene bridges in ribonuclease (2). Stabilizing cross-links have been introduced into lysozyme using phenol-2,-4-disulfonyl chloride (3) or α, α'-dibromoxylenesulfonic acid (4). Bovine serum albumin is extensively cross-linked by treatment with 4, 4′-difluoro-3, 3′-dinitrophenylsulfone (FNPS) (5).

Current studies in this laboratory are directed toward the preparation of enzyme derivatives more stable toward ionizing radiation than the corresponding native enzyme. To accomplish this the use of bifunctional reagents presents an attractive possibility. Those used with lysozyme have the disadvantage of introducing no distinctive marking characteristic into the protein derivative. Derivatives formed with FDP or FNPS have markedly different spectra from their native counterparts; *i.e.*, two new absorption bands in their visible spectra. Such a property enhances greatly the possibility of eventually defining the location of bridge ends in the peptide chain sequence. Derivatives formed from several proteins with FDP have a tendency to aggregate at or near neutral pH values unless urea or detergents are present. Thus FNPS seems the more desirable reagent. Also, the two reactive fluorines in FNPS are equal and independent in reactivity.

The present paper presents studies of the reaction between FNPS and lysozyme and reports separation of a cross-linked preparations.

Materials and Methods. FNPS was purchased from the Pierce Chemical Co. and used without further purification. Egg-white lysozyme was obtained from the Worthington Biochemical Corporation (2× crystallized, LY636). Bio-Gel P-10, 100–200 mesh, was a product of Bio-Rad Laboratories. In other instances, reagent grade chemicals and glass-distilled water were employed.

The procedure of Wold (5) was used with some modification to effect cross-linking. 1.25 g. (87.4 μ) lysozyme was dissolved completely in 3.5 l. 1% sodium carbonate. FNPS 35.0 mg (100 μmoles) was dissolved in 2.0 ml acetone and 8.0 ml methanol. The FNPS solution was added to the lysozyme solution dropwise at room temperature (26°) over a 10-min period during which time the lysozyme solution was stirred continuously using a magnetic mixing bar. Stirring was continued for another 10 min. A yellow color appeared immediately in the solution and deepened with the passage of time indicating reaction of the reagent had proceeded with protein and solvent. The solution became cloudy in 4 hr indicating the formation of intermolecular aggregates. Twenty hours after reagent addition 60.0 ml concd HCl was added dropwise with continuous stirring to bring the pH from 11.10 to 6.0. The volume of the solution was decreased to 250 ml by lyophilization. A smaller amount of lysozyme was subjected to the above treatment omitting FNPS; no destruction of enzymic activity was noted.

The FNPS-lysozyme concentrate contained a precipitate which was removed by centrifugation and washed once with 50 ml of water and once with 30 ml of water. The precipitate was largely soluble in water. The combined water washes (NPS–Lys WW) and washed precipitate (NPS-Lys Res.) were lyophilized to obtain their dry weight.

Ten milliliters of glacial acetic acid were added to the supernate obtained from centrifugation of the FNPS-lysozyme concentrate and this solution was applied to a 5.5 × 70.0 cm column of Bio-Gel P-10 previously equilibrated with 0.1 *M* acetic acid. Fines had been removed by repeated suspension in and decantation of 0.1 *M* acetic acid. After the sample had passed into the column, elution was accomplished with 0.1 *M* acetic acid using a Technicon variable speed proportioning pump to deliver elutant at a rate of 21.0 ml in 20 min. Twenty-minute fractions were collected in a Buchler refrigerated bath (5°) model 3R-4002 fraction collector. Optical density measurements on the eluate were conducted in 1.00 cm quartz cells in a Beckman model DB spectrophotometer. Fractions were pooled as indicated in Fig. 1 and lyophilized to obtain their dry weight.

Ultraviolet and visible spectral scans were performed on selected eluate fractions and lyophilized pooled fractions using a Beckman model DK-2A ratio recording spectrophotometer. Peak absorption at 420 mμ was used to calculate the number of NPS groups per

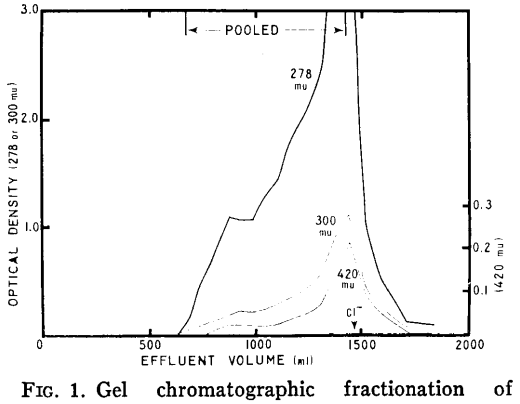

FIG. 1. Gel chromatographic fractionation of lysozyme after reaction with 4,4′-difluoro-3,3′-dinitrophenylsulfone. Chlorides were first detected in the eluate at the point indicated with an arrow.

mole weight of lysozyme using a molar extinction coefficient of 8000 for an NPS grouping (9).

Enzymic activity measurements employed lyophilized *Micrococcus lysodeikticus* cells (Mann Research Laboratories) as substrate at pH 6.24 essentially according to the procedure of Shugar (6), at a temperature of 30°. The decrease in turbidity as cells were lysed was assessed by following the optical density at 570 mμ for 3 min. Initial absorbance in our assay system was about 0.95 to 1.00. Initial rates of turbidity decrease were used to calculate enzymic activity in samples. Standard solutions with known concentrations of crystalline lysozyme were assayed on the same day as samples and these results used to construct a standard curve for calculating enzymic activity. Enzymic activity measurements were also conducted in a similar manner using a suspension of cells in 0.05 *M* glycine–sodium hydroxide buffer, pH 9.21 (7).

Drastic alterations in spectral properties during cross-linking with FNPS would invalidate any assay for protein based on direct spectral measurements at 280 mμ. Therefore, protein estimations were based on the ninhydrin color yields of samples submitted to complete acid hydrolysis (8). Hydrolysis was accomplished by placing 1.0 ml of the sample solution (0.1 – 4.0 mg protein) in a Pyrex tube with 1.0 ml concn. HCl; freezing, evacuating, and sealing the tube; and heating the sealed tube at 115° for 16 hr. Contents of the hydrolysis tube were neutralized with sodium hydroxide and an aliquot of the hydrolysate reacted with ninhydrin reagent. The ninhydrin reagent contained 20 g ninhydrin and 1.5 g hydrindantin dissolved in 650 ml of methyl cellosolve and 350 ml of 4.0 *N* sodium acetate buffer, pH 5.5. The latter buffer was added to other components dissolved in methyl cellosolve after bubbling nitrogen through the methyl cellosolve solution for 15 min. This reagent could be stored at 5° for 3 weeks without adversely affecting its quality for these assays. Color development was done by adding a neutral aliquot of sample hydrolyzate to 3.0 ml of 4.0 *N* sodium acetate buffer, pH 5.5, and 2.0 ml of

ninhydrin reagent, heating 15 min at 93°, cooling the reaction mixture, adding 5.0 ml ethanol:water (1:1) mixture, and reading the absorbance at 570 mμ. If necessary, dilutions were made of the reaction mixture with the ethanol:water mixture. Norleucine and crystalline lysozyme standards were carried through the entire procedure with each set of samples. Color yields from unknown samples were expressed as norleucine equivalents. An experimentally established value of 8.00–8.70 norleucine equivalents per milligram of crystalline lysozyme was then used to calculate protein content of samples.

Results and Discussion. From the elution profile in Fig. 1, two considerations are important. First, protein is cleanly separated from excess reagents and salts in the reaction mixture. Second, at this molar ratio of reagent: protein (1.14) and protein concentration practically all of the reagent is reacted with protein. This means that the water wash fraction contains very little unreacted or hydrolyzed reagent. Absorption spectra provided further support for the contention that almost quantitative reaction of the reagent had been obtained. The absorption maximum at 420 mμ was 13% lower in 0.1 *N* HCl than in 0.1 *N* NaOH for both F38-78, the fractions pooled from the gel column (Fig. 1), and NPS-Lys WW indicating that the majority of functional fluorine groupings had reacted with lysozyme. Free nitrophenol groupings whether attached to a reagent molecule whose other end had reacted with protein or existing free in solution would give rise to a pH-dependent shift and decrease in the visible spectrum. Similar results have been observed by Wold (5) and Tawbe *et al.* (9) upon reacting FNPS with bovine serum albumin.

The major product of the cross-linking reaction under these conditions is NPS-Lys WW accounting for about 40% of the original lysozyme. This material retains significant lytic activity at pH 6.24. The nonintegral number of NPS groups apparently present in this material and its lack of complete solubility in water after lyophilization indicate it is probably a mixture of molecules with a single cross-link and molecules with more than one cross-link. Intramolecular, rather than intermolecular cross-links are probably the dominant new feature in this material since it has been observed to behave as a monomer preparation during gel filtration on P-100 columns in 4.0 *M* urea. It has been observed in other laboratories (1) that incorporation of aryl groups into proteins can lead to extensive aggregation without cross-linking, particularly in neutral salt solutions, since the presence of additional nonpolar groups favors protein–protein interaction rather than protein–solvent interaction. Thus, solubility behavior must be considered with caution and molecular weight estimations made in the presence of agents such as urea which disrupt noncovalent forces prior to concluding that intermolecular cross-linking has occurred.

NPS-Lys WW is under further study with an aim toward resolving its components via ion-exchange chromatography. Then a better estimation of the number of cross-links can be made by subjecting components to complete acid hydrolysis prior to making spectral measurements at 420 mμ. Such a procedure would be feasible since NPS-bis-lysine derivatives are stable during acid hydrolysis and their spectral examination would not be invalidated by the possibility that the pH on or near the protein surface may be sufficiently different from that in solution to prevent hydrolysis of unreacted fluorobenzene groupings.

Because of its lack of complete solutibity in even concentrated urea (up to 10 *M*) quantitative spectral examination of NPS-Lys Res. has not been possible. However, the material is deep yellow in color and probably is an aggregate held together by intermolecular NPS cross-links. Addition of a large amount of this material (1000-fold above that level of lysozyme giving rapid lysis of *Micrococcus lysodeikticus* cells) to a suspension of *Micrococcus lysodeikticus* cells results in no decrease in turbidity at either pH 6.24 or pH 9.21 indicating complete loss of enzymic activity in this material. That this material accounts for less than 10% of the original lysozyme is a further indication that intra- rather than intermolecular cross-linking

TABLE I. Products of FNPS Cross-Linking in Egg White Lysozyme.

Material[a]	Dry weight (g)	Protein in fraction (%)	Original lysozyme protein (%)	Sp act[b] at pH 6.24	Sp act[b] at pH 9.21	NPS groups calcd/mole wt lysozyme
F38-78	0.53	60.0[c]	25.5	0.40	0.31	0.22
NPS-LYS Res.	0.14	70.7[d]	7.6	0	0	—
NPS-LYS WW	0.71	70.0[e]	39.6	0.21	0.07	1.68

[a] See Fig. 1 for designations.

[b] Specific activity of crystalline lysozyme was taken as 1.00.

[c] 100% soluble in water after lyophilization.

[d] 63.3% soluble in water but 100% soluble in 2.0 *M* urea, pH 8.0, after lyophilization.

[e] 44.4% soluble in 2.0 *M* urea, pH 8.0, after lyophilization.

is favored under our conditions.

Lytic activity was measured at pH 6.24 and at pH 9.21 on both soluble materials. The observation that cross-linking affects lytic activity differently depending on the assay pH used (Table I) is consistent with the suggestion of Neuberger and Wilson (7) that the proton donor at alkaline pH values is the epsilon-amino group of lysine residue 97 rather than the carboxyl group of glutamic acid residue 35. α,α'-Dibromo-*p*-xylenesulfonic acid has been studied extensively as a cross-linking agent for lysozyme and found to form links between lysine 96 and lysine 97 and between lysine 33 and lysine 116 (3). FNPS is believed to react preferentially with amino groups and tyrosine phenolic groups. Thus cross-linking with this reagent could be expected to depress lytic activity measured at pH 9.21 more so than that measured at pH 6.24.

The loss of enzymic activity at both assay pH values may be due to several things. Incorporation of the NPS residue (a bulky grouping) may occur at such a position that the active region of the enzyme is masked from interaction with substrate molecules. If this is true, cross-linking should abolish the difference spectra seen when glycol chitin forms an enzyme-substrate complex with lysozyme (10). A further possibility is that the cross-linking process *per se* may have caused extensive unfolding of the native structure so that groupings functional in the active site are not in required juxtaposition for maximal lytic activity (11). These possibilities will be subjected to closer scrutiny as more highly purified cross-linked derivatives become available.

Cross-linked derivatives of lysozyme should have great potential value as subjects for radiation study. We are examining these derivatives currently for stability towards radiation and other denaturing agents. Derivatives more stable than native lysozyme hopefully can be examined to determine the position of the stabilizing cross-linking leading to an inference as to the position of portions of the enzyme molecule essential to lytic activity. As for other enzymes, more stable derivatives of certain proteolytic enzymes such as trypsin could be quite useful experimental tools. The lability of trypsin towards heat and self-digestion is a drawback to widespread use of this enzyme in amino acid sequence studies where its narrow specificity would be a very desirable feature.

Summary. Active cross-linked derivatives of egg-white lysozyme have been prepared by reaction with 4,4′-difluoro-3,3′-dinitrophenylsulfone and isolated from excess reagents and salts by gel chromatography. Lytic activity of the enzyme at pH 9.21 is almost abolished in one cross-linked derivative which still retains 20% of its lytic activity at pH 6.24. The potential importance of such cross-linked enzymes is discussed in connection with stabilization of enzyme structure towards inactivation.

1. Wold, F., *in* "Methods in Enzymology" (C. H. W. Hirs, ed.) Vol. 11, p. 617. Academic Press, New York (1967).

2. Marfey, P. S., Nowak, H., Uziel, M., and Yphantic, D. A., J. Biol. Chem. **240**, 3264 (1967).

3. Herzig, D. J., Rees, A. W., and Day, R. A., Biopolymers **2**, 349 (1964).

4. Hiremath, C. B. and Day, R. A., J. Am. Chem. Soc. **86**, 5027 (1964).

5. Wold, F., J. Biol. Chem. **236**, 106 (1961).

6. Shugar, D., Biochim. Biophys. Acta **8**, 302 (1962).

7. Neuberger, A. and Wilson, B. M., Biochim. Biophys. Acta **147**, 473 (1967).

8. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. **30**, 1190 (1958).

9. Tawde, S. S., Ram, S. R., and Lyengar, M. R., Arch. Biochem. Biophys **100**, 270 (1963).

10. Hayashi, K., Imoto, T., and Funatsu, M., J. Biochem. Tokyo **54**, 381 (1963).

11. Moore, G. L. and Day, R. A., Science **158**, 210 (1968).

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