ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 110, 205–209 (1965)

Enrichment of Proteins with Amino Acid Residues, Employing p, p′-Difluoro-m, m′-Dinitrodiphenyl Sulfone[1, 2]

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Received December 7, 1964

The bifunctional reagent, *p,p′*-difluoro-m,m′-dinitrodiphenyl sulfone, was employed to conjugate a variety of amino acids to the protein, gelatin. Among the amino acids tried, tyrosine conjugated most readily. The tyrosyl derivative of gelatin was characterized by electrophoretic, ultracentrifugal, and spectral studies. The conjugation of tyrosine to gelatin appeared to take place through the phenolic groups, although it was also shown to occur through the amino groups when the phenolic groups were blocked.

In previous studies, the bifunctional reagent, *p,p′*-difluoro-*m,m′*-dinitrodiphenyl sulfone (FF-sulfone, FNPS) (1), was shown to be useful for preparing protein-protein conjugates (2–4) and certain polyamino acid derivatives (5). The intermolecular linkages in these derivatives were apparently established across the amino, phenolic, sulfhydryl, and imidazolyl groups by means of dinitro diphenylsulfone (NPS) bridges. Since proteins enriched with amino acid residues are of potential interest for a variety of immunological, nutritional, and analytical studies (6), the possibility of employing FNPS for conjugating amino acids to proteins was examined. A derivative of gelatin enriched with tyrosine was characterized.

EXPERIMENTAL

The commercial sources of the proteins, amino acids, and other reagents employed in these studies were: gelatin, Allied Chemical Corporation; bovine serum albumin (BSA), Pentex; unlabeled amino acids, General Biochemicals; lysine-C^{14}, Volk; histidine-C^{14}, Schwartz; aspartic acid-C^{14}, alanine-C^{14}, and serine-C^{14}, Calbiochem; tyrosine-C^{14}, Nuclear Chicago; *N*-acetyl tyrosine and *O*-benzyl tyrosine, Cyclochemical; and fluorescein amine, Nutritional Biochemicals. FNPS (FF-sulfone) was prepared as described by Zahn and Zuber (1) or purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

Conjugation of C^{14}-amino acids to gelatin. Forty mg of gelatin (0.8 μmole) and 28–30 μmoles of each carrier amino acid, mixed with sufficient C^{14}-amino acid to give a radioactivity of 0.25 μc, was dissolved in 4 ml of 1% sodium carbonate; to this was added with stirring 1 ml acetone containing 10 mg FNPS. The reaction mixture, which gradually turned yellow, was stirred at room temperature for 24 hours, filtered if necessary, and dialyzed exhaustively against water. In control experiments, gelatin was treated with either FNPS alone (the product is hereafter referred to as NPS-gelatin), or with the amino acid alone, under the above reaction conditions. Radioactivity of the solutions was measured before and after dialysis. For this, the solutions were diluted tenfold and 0.1 ml of the diluted solution was placed on planchets and counted in duplicate in a Nuclear model C 115 gas-flow counter.

Conjugation of tyrosine, N-acetyl tyrosine, and O-benzyl tyrosine to gelatin. Two hundred mg of gelatin, mixed with 40 mg or equivalent of tyrosine, *N*-acetyl tyrosine, or *O*-benzyl tyrosine, was dissolved in 20 ml of 1% sodium carbonate. To this was added with stirring 10 ml acetone containing 40 mg FNPS. The reaction was allowed to proceed,

[1] For a preliminary communications, see *Federation Proc.* **23**, 140 (1964).

[2] Supported by grants from Horace H. Rackham School of Graduate Studies (R-635) and the Michigan Memorial Phoenix Project, 284.

[3] Student Medical Research Fellow.

TABLE I

INCORPORATION OF C^{14}-AMINO ACIDS INTO GELATIN BY MEANS OF FNPS[a]

Amino acid reacted	% Radioactivity incorporated into gelatin	Moles of amino acid incorporated per 40,00 gm gelatin
Tyrosine	80–90	20–25
Lysine	44	16
Aspartic acid	22	8
Histidine	20	7
Serine	9	3
Alanine	10	3

[a] Reaction: 0.8 μmole gelatin and 30 μmoles amino acid dissolved in 4 ml 1% sodium carbonate + 1 ml acetone containing 10 mg FNPS. Controls: gelatin + amino acid; NPS-gelatin + amino acid, in identical reaction conditions.

with stirring, for 24 hours, and the products were dialyzed and lyophilized. These products are designated, respectively, as Tyr-gelatin, *N*-acetyl tyr-gelatin, and *O*-benzyl tyr-gelatin.

Analytical procedures. Amino groups were estimated by colorimetric ninhydrin procedure (7). The Folin-Ciocalteu method was employed for the determination of phenolic groups (8). Nitrogen determinations were made by the micro-Kjeldahl procedure (9).

Electrophoresis of the various protein derivatives was carried out in barbital buffer, pH 8.6, ionic strength 0.05, on cellulose acetate paper. Absorption spectra of aqueous solutions of selected gelatin derivatives were determined in a Cary automatic spectrophotometer. Sedimentation patterns of the proteins were obtained on 1% solutions in phosphate buffered saline, (pH 7.0) at 20°, 59,780 rpm, employing a Spinco model E ultracentrifuge.

RESULTS AND DISCUSSION

Studies on the conjugation of various amino acids to gelatin employing FNPS are shown in Table I. It can be seen that all the amino acids tested were incorporated into the protein. Controls, which included gelatin treated with the C^{14}-amino acid in the absence of FNPS but under otherwise identical conditions, and gelatin pretreated with FNPS prior to the addition of the amino acid, showed no radioactivity, suggesting that the attachment of the amino acid to gelatin was mediated by FNPS and was not due to nonspecific adsorption. The reasons for the wide differences in the extent of incorporation of the different amino acids, ranging from 90% for tyrosine to 10% for serine, are not clear. Fluorescein amine was also shown to conjugate to gelatin under similar reaction conditions, indicating the general usefulness of FNPS for such conjugations.

In the following studies, the conjugation of tyrosine to gelatin was investigated in greater detail, since, in addition to being the most reactive amino acid for the FNPS-mediated conjugation to protein, tyrosine has been implicated as essential to the immunogenicity of proteins, thus becoming an attractive candidate for use as a "hapten" in studies of the chemical basis for the antigenicity of proteins (6).

Electrophoretic patterns at pH 8.6 of the various enriched gelatin derivatives were compared with those of gelatin and NPS-gelatin. The cationic mobility of gelatin was greatly reduced when reacted with FNPS in accordance with the expected loss of some of its amino groups by modification. Tyr-gel, interestingly, exhibited mobility intermediate to those of gelatin and of NPS-gel, indicating a gain of net positive charge over NPS-gelatin. *N*-Acetyl tyr-gel and *O*-benzyl tyr-gel exhibited mobility indistinguishable from that of NPS-gel, as did the gelatin derivatives of other amino acids. Control studies, which included electrophoresis of mixtures such as gelatin plus poly NPS-tyrosine (5), gelatin plus NPS-gelatin, and NPS-gelatin plus poly NPS-tryosine, confirmed that the observed mobility of tyr-gel was due to the formation of a covalent compound and not from an artifactual adsorption compound of one

TABLE II

ESTIMATION OF AMINO AND PHENOLIC GROUPS IN GELATIN DERIVATIVES[a]

Gelatin derivative	% Amino groups (ninhydrin)	% Phenolic groups (Folin-Ciocalteu)
Gelatin	100	100
NPS-Gelatin	49	94
Tyr-Gelatin	70	84

[a] The groups present in modified gelatins are calculated as a percentage of those present in gelatin.

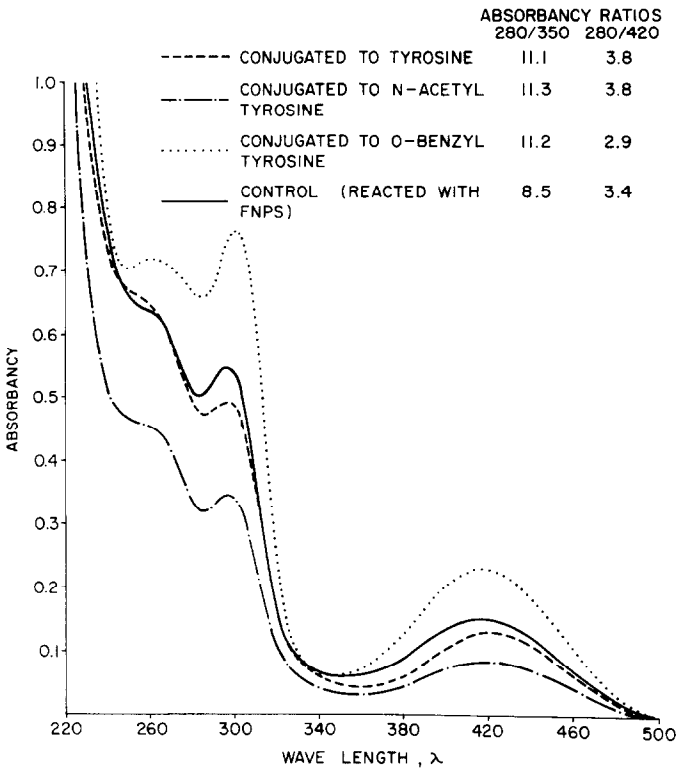

FIG. 1. Absorption spectra of gelatin derivatives. Protein concentration, 0.1 mg per milliliter.

of the above mixtures. Similarly prepared analogous derivatives of BSA showed electrophoretic behavior parelleling that of the gelatin derivatives.

Data from the colorimetric determination of the amino and phenolic groups of gelatin, NPS-gelatin, and tyr-gelatin are shown in Table II. Treatment of gelatin with FNPS results in a loss of both amino and phenolic groups in agreement with the previous studies on collagen (10–12) and BSA (4). Tyr-gel, on the other hand, had more amino groups and fewer phenolic groups than NPS-gelatin. This indicates that the conjugation of tyrosine to gelatin might involve primarily the phenolic groups rather than the amino groups. Such a reaction would also explain the observed electrophoretic behavior of tyr-gelatin. This linkage appears to be relatively stable since treatment with acid (pH 2.5) or alkali (pH 11.5) for 60 minutes at room temperature did not result in the liberation of free tyrosine as determined by the Folin-Ciocalteau method (8).

Further insight into the nature of linkage between tyrosine and gelatin was sought from the absorption spectra of various gelatin derivatives (Fig. 1). Gelatin which exhibits a weak absorption in the ultraviolet due to its low content of aromatic amino acids is not included. Introduction of NPS groups into the protein results in absorption in the regions of 260, 300, and 420 mμ (13), while the added tyrosine contributes in the region of 280 mμ. In analogy with dinitrophenyl (DNP) derivatives of tyrosine and other amino acids, only the *N*-derivatives are yellow while the *O*-derivative is colorless (14). Accordingly, the *O*-benzyl-tyr derivative of gelatin is intensely yellow, since in this case the conjugation takes place exclusively through the amino groups. High absorption at 420 mμ and in the ultraviolet region (due to the *O*-benzyl groups) are the characteristic

features of this derivative. Comparison of the absorption ratios, 280/350 and 280/420 (Fig. 1), gave some clues as to the nature of linkages in the various derivatives. The increase in 280/350 absorption in the three tyrosine derivatives relative to NPS-gelatin is in accordance with expected contribution of tyrosine, and it confirms that tyrosine residues were incorporated into the protein in all the three cases. The identity of the ratio 280/420 for tyr-gelatin and the *N*-acetyl tyr-gelatin suggests that conjugation has occurred in an identical manner in these derivatives. Since in the latter derivative only the phenolic groups are free for reaction with FNPS, it follows that in free tyrosine also the phenolic groups were the primary site of reaction with FNPS. The low value of this ratio for the *O*-benzyl tyrosine derivative probably arises from the fact that the 420 mμ (yellow) absorption of this derivative was the highest since it is an exclusively N-NPS derivative. These findings suggest that, when free tyrosine was used, the conjugation takes place through its phenolic group; when the phenolic group is blocked, then conjugation occurs through the amino group, as it does in the case of other amino acids such as lysine or alanine.

Sedimentation patterns of gelatin, NPS-gelatin and tyr-gel are compared in Fig. 2.

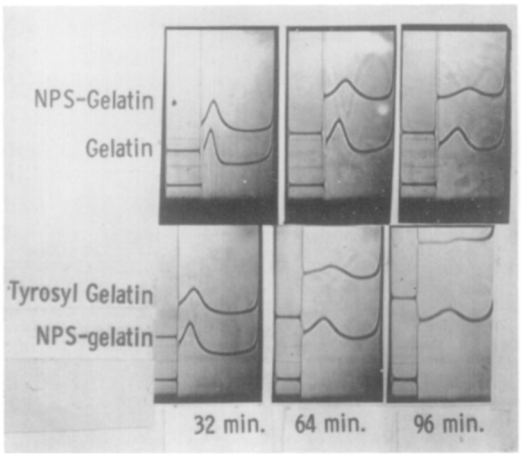

FIG. 2. Sedimentation patterns of gelatin derivatives. Speed, 59,780 rpm; temperature, 20°; solvent, phosphate buffered normal saline, pH 7.0.

The sedimentation constant of gelatin was 1.8, compared with 2.7 of NPS-gelatin. Parallel changes which occured in the sedimentation properties of bovine serum albumin after reaction with FNPS were shown to be due to the dimerization of the protein (4). Tyr-gelatin had a still higher sedimentation constant of 3.4, which is probably a reflection of an increase in molecular weight from dimerization as well as the addition of about 25 NPS-tyrosine moieties, and/or structural changes occurring during the conjugation reaction.

COMMENTS

When one uses *N*-carboxy amino acid anhydrides (6) for attaching amino acids to proteins, polymers of amino acids (polypeptides) rather than single residues are incorporated into the protein. With bifunctional reagents such as FNPS, the incorporation can take place in two ways: (a) amino acids such as alanine or valine will attach to the protein as single NPS-residues; or (b) bifunctional amino acids, such as tyrosine or lysine, which have been shown to form poly-NPS-amino acids (5), may, on the other hand, attach to the protein as single residues or as units of $(\text{NPS-amino acid})_n$ or both.

Some preliminary immunochemical studies with various gelatin derivatives suggest that the aromatic and bulky character of the NPS-residues may make such derivatives unsuitable for certain biological studies. NPS-gelatin and tyr-gelatin cross reacted with rabbit antibodies obtained against gelatin in agar diffusion studies. In reciprocal studies, gelatin did not react with either anti-NPS-gelatin or anti-tyr-gelatin. Both NPS-gelatin and tyr-gelatin were better antigens than gelatin and exhibited complete reciprocity in cross reactions. The data indicated that the NPS-residues were the major determinants in antibody formation, and the role of tyrosine in antibody formation could not be evaluated. The need for simple nonaromatic bifunctional reagents for use in such studies is thus indicated.

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