[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Preparation of C^{14}-Polypeptidyl-proteins[1]

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Bovine plasma albumin, egg albumin, chymotrypsin, chymotrypsinogen and lysozyme were modified by reaction with either N-carboxy-glycine-1-C^{14} anhydride or N-carboxy-L-phenylalanine-1-C^{14} anhydride. The effect of *p*H upon the number of sites acylated and the average length of the added peptides indicated an increase in both cases with increasing *p*H between 6.3 and 9. Quantitative end group studies indicate that the reaction favors the acylation of the reactive amino groups. The absence of significant amounts of bound peptides and amino acids was demonstrated by reaction of the modified proteins with ninhydrin and carboxypeptidase.

Introduction

The preparation of a number of polypeptidyl-proteins by reaction of α-amino acid-N-carboxy-anhydrides (NCAs) with proteins in buffered aqueous solutions has been reported.[3-8] The amino groups of the protein appear to be the preferred sites of acylation by the anhydride.[4,7] Additional evidence is provided by the isolation of polypeptidyl-ε-lysine peptides from several modified proteins.[9] However, other groups of the protein could initiate polymerization and thus become sites of acylation. Quantitative studies of the N-terminal groups of the modified proteins could give information regarding this possibility. Further, it is known that the *p*H of the reaction medium influences the course and extent of acylation reactions. This paper is concerned with the preparation of several polypeptidyl-proteins, the specificity of the reaction of proteins with NCAs and the effect of *p*H on the nature and size of the products. Preliminary results were reported previously.[10]

Results and Discussion

N-Carboxyamino acid anhydrides prepared from carboxyl-labeled amino acids were used in these experiments in order to facilitate the assay for the number of amino acid residues added to the proteins. In addition, the reaction of the C^{14}-modified proteins with ninhydrin, or with carboxypeptidase followed by ninhydrin, provided a convenient test for the presence of bound amino acids and peptides. A series of proteins were modified by reaction with glycine-1-C^{14} NCA, and in one case with L-phenylalanine-1-C^{14} NCA, in phosphate buffer at *p*H 7.3 and a molar ratio of NCA to protein of 350:1. The polypeptidyl-proteins were assayed for the amount of added amino acid residues and for the number of ε-amino groups of lysine remaining after the reaction. The results are summarized in Table I.

TABLE I

POLYPEPTIDYL-PROTEINS

Protein	Amino groups per mole[a]	Moles amino acid added per mole[b]	Amino groups acylated per mole[c]	Av. chain length
Bovine plasma albumin[d]	62	39	20	2
Bovine plasma albumin	62	41	21	2
Polyglycyl-BPA	62	172	26	7
Egg albumin	22	41	8	5
Chymotrypsin	16[e]	55	10	5
Chymotrypsinogen	14[f]	15	8	2
Pepsin	3	10	..	..
Lysozyme	6	35	5.5	6

[a] H. D. Springall, "The Structural Chemistry of Proteins," Academic Press, Inc., New York, N. Y., 1954, pp. 307, 309. [b] Determined by radioactivity assay of modified protein. [c] Determined by dinitrophenyl method. [d] L-Phenylalanine-1-C^{14} NCA was used. In all other cases listed glycine-1-C^{14} NCA was used. [e] Based upon changes involved in activation of chymotrypsinogen. See H. Neurath, *Advances in Protein Chem.*, 12, 319 (1957). [f] P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1957).

It will be noted that similar results were obtained when bovine plasma albumin was treated with N-carboxyglycine anhydride and N-carboxy-L-phenylalanine anhydride under the same conditions. Of interest also is the large increase in the amount of glycine added when polyglycyl-bovine plasma albumin was further modified by repeating the reaction with anhydride. The increase is apparently due to the replacement in the first treatment of ε-amino groups by α-amino groups, which are more efficient initiators at *p*H 7.3 than the ε-amino groups of lysine. The larger amount of glycine added to chymotrypsin as compared to chymotrypsinogen may be due in part to the fact that the former contains three α-amino groups compared to only one for the latter. Furthermore, the availability of the initiating sites to the anhydride is apparently of considerable importance.

The effect of *p*H on the amount of amino acid added and on the number of lysine residues acylated was studied by reaction of glycine-1-C^{14} NCA and bovine plasma albumin. The number of unreacted ε-amino groups was determined by the dinitrophenyl (DNP) procedure, and the total glycine units added was determined by radioactivity assay of the proteins. The results are shown in Fig. 1. A gradual increase in the number of glycyl residues added occurs between *p*H 6.2 and 7.6

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(2) Taken in part from the Ph.D. dissertation of W. H. Konigsberg, presented to the Faculty of Pure Science, Columbia University.

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(8) M. Sela, *Bull. Res. Council Israel*, **4**, 109 (1954).

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(10) W. H. Konigsberg and R. R. Becker, Am. Chem. Soc., New York Meeting, September 1954, p. 88c.

and between *p*H 8.1 and 9. The relatively large increase in the amount of added glycine observed between *p*H 7.6 and 8.1 is probably related to the *pK* of the α-amino group. The increase from *p*H 6 to 9 in the number of ε-amino groups acylated would be expected since as the *p*H is increased, the effective concentration of free ε-amino groups available for reaction increases. From the data of Fig. 1, the average chain length of the added peptides can be calculated by dividing the number of sites acylated into the total moles added, assuming complete acylation of the N-terminal aspartic acid group of bovine plasma albumin. Below *p*H 7.6, the average chain length of the glycine peptides is about 4.6, and above *p*H 8.1, a value of about 7.5 is obtained. The rather sharp increase between *p*H 7.6 and 8.1 may be due to the fact that many more α-amino groups become uncharged in this range and are thus able to compete more effectively with hydroxyl ions for the consumption of anhydride. The constant value of about 7.5 for the average length of the glycine chains in the higher *p*H range could be caused by the insolubilization of the derivative by the further addition of glycine residues. Thus at *p*H 7.3 the yield of soluble polyglycyl-bovine plasma albumin was 85%, at *p*H 8.3, 50% and at *p*H 9.1 only 20%.

A marked effect of buffer type on the extent of polymerization of N-carboxyglycine anhydride has been shown.[11] These findings apparently apply also to polymerizations initiated by amino groups of proteins[4,6] and may be involved in the observed variation of chain length with *p*H noted in Fig. 1, since a phosphate buffer was used for the *p*H range between 6.2 and 7.8 and a borate buffer for the higher values. However, the increase noted up to *p*H 7.8 cannot be explained on the basis of a buffer effect.

Experiments using a bicarbonate–CO_2 buffer and a phosphate buffer under analogous conditions confirmed the results of Tsuyuki, Van Kley and Stahmann[6] which showed that bicarbonate buffer is superior to phosphate buffer for the modification reaction. Using bovine plasma albumin and glycine-1-C^{14} NCA at *p*H 7.3, the results show that under the same conditions, 41 moles of glycine residues per mole of protein was added in phosphate buffer compared with 72 moles in bicarbonate buffer. An increase in the number of ε-amino groups of lysine acylated from 20 in phosphate buffer to 35 in bicarbonate buffer was found.

In addition to the effect of *p*H upon the reaction, it would be expected that the concentrations of both the anhydride and the protein would influence the extent of acylation. It was found that reaction of bovine plasma albumin at *p*H 7.3 with a large excess of glycine NCA (mole ratio anhydride to protein 840:1) resulted in complete precipitation of the protein. However, a stepwise treatment in which polyglycyl-bovine plasma albumin (mol. wt. 77,200) was retreated two times with a 70:1 ratio of glycine NCA to protein gave a soluble derivative in 10% yield. When this product was analyzed, it was found that 200 moles of glycine

(11) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).

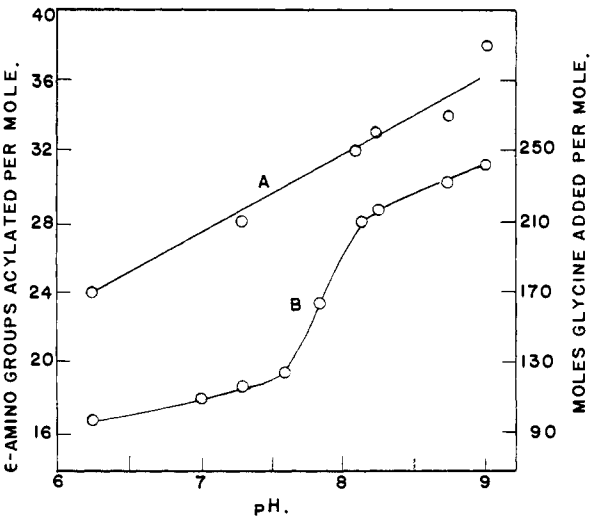

Fig. 1.—Effect of *p*H on the reaction of glycine NCA with bovine plasma albumin: A, moles ε-amino groups acylated per mole protein; B, moles glycine added per mole protein.

was added per mole of protein, and about thirty amino groups were acylated, compared to the starting material which contained 172 moles of added glycine with 26 sites acylated. This product appears to represent a degree of substitution close to the limit of solubility of the derivative prepared in this way, although a more highly substituted product can be prepared at higher *p*H values (Fig. 1). A marked effect of protein concentration is indicated by the data on preparations which were carried out at bovine plasma albumin concentrations of 10 and 20 mg./ml., with other conditions identical. When the lower concentration was used, 41 moles of glycine was added per mole of protein (Table I). When the higher concentration was used, 116 moles of glycine was added per mole of protein (Fig. 1).

It is well known that proteins are capable of binding a variety of compounds, including amino acids and peptides. To test for contamination by free amino acids, proteins modified with radioactive glycine and phenylalanine were treated with ninhydrin and the resulting CO_2 assayed for C^{14}. From the specific activities obtained, it was calculated that 0.3% of the total radioactive glycine was present as the free amino acid in polyglycyl-bovine plasma albumin. The corresponding value for poly-L-phenylalanyl-1-C^{14} bovine plasma albumin was 0.7%. The presence of absorbed glycyl or phenylalanyl peptides was determined by incubation with carboxypeptidase[12] followed by use of the ninhydrin procedure.[13] The amount of bound glycyl or phenylalanyl peptides was negligible.

To determine the acylation sites, the native and the modified proteins were both quantitatively analyzed for N-terminal groups and for unreacted lysine residues by the dinitrophenyl procedure.[12] The results are listed in Table II. Extensive

(12) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy in "Methods of Biochemical Analysis," Vol. II, Ed. D. Glick, Interscience Publishers, Inc., New York, N. Y., 1955, p. 359.

(13) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen and P. Hamilton, *J. Biol. Chem.*, **141**, 627 (1941).

TABLE II

STOICHIOMETRY OF THE REACTION OF PROTEINS WITH N-CARBOXYAMINO ACID ANHYDRIDES

	Mol. wt.	Moles N-terminal per mole	N-terminal group	Moles ε-DNP-lysine per mole	Total accounted for
Bovine plasma albumin (BPA)	69,000	0.7	DNP-Asp	57	57.7
Polyglycyl-BPA	71,300[a]	18.3	DNP-Gly	37	55.3
Polyglycyl-BPA	77,200[a]	23.4	DNP-Gly	31	54.5
Poly-L-phenylalanyl-BPA	75,400[a]	18.4	DNP-Phe	37.5	55.9
Poly-DL-phenylalanyl-BPA	76,000[a]	19.3	DNP-Phe	38	57.3
Egg albumin	44,000[b]	..	.....	19.0	19.0
Polyglycyl-egg albumin	47,300[a]	6.2	DNP-Gly	13.9	20.1
Chymotrypsin	24,500[c]	1.5	DNP-Ileu, DNP-Ala	10.4	11.9
Polyglycyl-chymotrypsin	25,700[a]	8.2	DNP-Gly	3.5	11.7

[a] Calculated on basis of amino acid residues added. [b] K. Linderstrøm-Lang and M. Otteson, *Compt. rend. trav. lab. Carlsberg*, **26**, 16 (1949). [c] Based on molecular weight of 25,000 for chymotrypsinogen reported by P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. Biophys. Acta*, **24**, 72 (1957), and the molecular weights of the peptides released in activation process. See Table I, footnote *e*.

destruction of DNP-glycine under the hydrolytic conditions employed requires large corrections for the polyglycyl-protein data. However, in the case of the polyphenylalanyl-bovine plasma albumins this difficulty is avoided, and the total number of amino groups accounted for agrees quite well with the number determined in the native protein. These results indicate that the amino groups are the main sites of acylation. In addition, it appears that no significant amounts of ureido acids were formed[14,15] and the reaction of the NCAs with proteins appears to follow a normal course of amine-initiated polymerization.[16]

To confirm the N-terminal studies and provide additional information, the phenyl isocyanate degradation was applied to a few polyglycyl-proteins. Two techniques were employed, paper strip[17] and solution.[18] The results are given in Table III. In all cases the phenylthiohydantoin of glycine was obtained as the N-terminal group, in agreement with the results of the DNP method. The recoveries in these experiments are low, and the data indicate fewer N-terminal groups in all cases than those reported in Table II. Of interest is the fact that in the polyglycyl-bovine plasma albumins where the average chain length of the added peptides is 2, the determination shows a decrease from 14 in the first stage to 8 in the second stage, whereas in the protein where the average chain length is about 7, the second stage shows no decrease. Since no phenylthiohydantoin of glycine would be released from sites in which a single glycine residue is attached to the ε-amino group of lysine, the results of the second stage indicate that in the case where the average chain length of the glycine peptides is 2, there are sites which have more than two glycine residues attached. Undoubtedly there are also sites containing only one glycine residue. However, when the average chain length is about 7, the data indicate that each site acylated must have attached a peptide containing at least three glycine residues.

(14) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).
(15) K. D. Kopple, *ibid.*, **79**, 662 (1957).
(16) D. H. G. Ballard and C. H. Bamford, *Proc. Roy. Soc. (London)*, **223A**, 495 (1954).
(17) H. Fraenkel-Conrat, THIS JOURNAL, **76**, 3606 (1954).
(18) M. B. Williamson and J. M. Passmann, *J. Biol. Chem.*, **222**, 151 (1956).

TABLE III

RESULTS USING THE EDMAN TECHNIQUE ON MODIFIED PROTEINS

Modified protein	Mol. wt.	Technique employed	Stage	Moles of PTH[a] per mole of protein
Poly-glycyl-BPA[b]	71,300	Paper strip	1	14
Poly-glycyl-BPA	71,300	Paper strip	2	8
Poly-glycyl-BPA	71,300	Solution	1	11
Poly-glycyl-BPA	71,300	Solution	2	7
Poly-glycyl-BPA	77,200	Solution	1	18
Poly-glycyl-BPA	77,200	Solution	2	18
Poly-glycyl-	25,700	Paper strip	1	8
chymo-	25,700	Paper strip	2	4
trypsin	25,700	Solution	1	5

[a] Identified as the phenylthiohydantoin (PTH) of glycine in each case. [b] BPA = bovine plasma albumin.

Within the limits of the errors inherent in the experimental methods used, it appears that the only sites of acylation are the amino groups of the proteins. Further evidence was afforded by testing directly for other groups of the protein that might react with the anhydride. If the hydroxyl group of tyrosine were acylated in the modification reaction, it would be expected that the magnitude of the phenol–phenoxide shift[19] would be altered in the modified proteins. When spectra of the native protein and of the modified protein were recorded in both acidic and basic solution, no differences between the two proteins were observed in the region from 230 to 320 mμ, indicating that acylation of the tyrosine hydroxyl groups had not occurred. The reactivity of the sulfhydryl group under the conditions employed here was determined by treating papain with the NCA of glycine. It was found that the nitroprusside color value[20] diminished, but returned to its original value after 24 hours. The increase in nitroprusside value to the initial value could be obtained in a few minutes upon addition of aqueous ammonia. Bartlett and Dittmer[21] have reported similar results in their studies of the reaction of DL-phenylalanine

(19) G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, **7**, 323 (1952).
(20) R. R. Grunert and P. H. Phillips, *Arch. Biochem. Biophys.*, **217**, 30 (1951).
(21) P. D. Bartlett and D. C. Dittmer, THIS JOURNAL, **79**, 2159 (1957).

NCA with cysteine and glutathione. It appears that in the reaction of NCAs with sulfhydryl-containing proteins, an initial attack on the sulfhydryl group occurs also, followed by transfer of the acyl group to an amino group,[22] or by hydrolysis of the thioester. In any event, the experimental conditions used in the preparation of the polypeptidyl-proteins result in a product in which no peptide chains are attached to the protein by thioester linkages. Any initial attack of the NCAs on the imidazole group of histidine would be expected to form an unstable intermediate,[23] and transfer of the acyl group in a related fashion would be expected.

Experimental

Glycine-1-C^{14} NCA.—Carbobenzoxyglycine-1-C^{14} was prepared as described in the literature[24,25] and converted to the anhydride by cyclization of the acid chloride.[11] The anhydride had a specific activity of 2.2×10^5 c.p.m./mg.

L-Phenylalanine-1-C^{14} NCA.—L-Phenylalanine-1-C^{14} was converted to the NCA according to the method of Sela and Berger[14]; m.p. 94–95°; specific activity, 5.8×10^3 c.p.m./mg.

Reaction of NCAs with Proteins.—The reactions were carried out with only slight modification of the method of Becker and Stahmann.[4] In a typical experiment, 200 mg. of glycine-1-C^{14} NCA (2.2×10^5 c.p.m./mg.) was added to 20 ml. of a solution of 400 mg. of crystalline bovine plasma albumin (Armour) in 0.067 *M* phosphate buffer, *p*H 7.3. The reaction was allowed to proceed at 0° for 24 hr. The mixture was then centrifuged at $17,000 \times g$ for 1 hr. at 5°. The supernatant fraction was transferred into a Visking dialysis sac and dialyzed against distilled water at 0–5° for 4 days. The protein solution was lyophilized, giving a yield of 350 mg. of modified protein. The specific activity of the product was 3.8×10^4 c.p.m./mg.

The reactions in which the effect of *p*H were studied were carried out under the same conditions using 0.067 *M* phosphate buffer for the *p*H range from 6.2 to 7.8, and 0.1 *M* borate buffer for the *p*H range 8.2 to 9.1. For the reaction of the NCAs with the series of proteins listed in Table I, phosphate buffer at *p*H 7.3 was used throughout, with protein concentrations of 10 mg./ml. Bicarbonate–CO_2 buffers were prepared by diluting a solution saturated with $NaHCO_3$ at 5° five, ten, fifteen and twenty times. Each solution was then saturated with 95% O_2–5% CO_2 gas mixture, giving *p*H values of 7.6, 7.3, 7.1 and 6.9, respectively. Egg albumin, 5× crystallized, lysozyme, 3× crystallized, and pepsin, 3× crystallized, were obtained from Pentex, Inc., Kankakee, Ill.; chymotrypsinogen, 3× crystallized, and chymotrypsin, 3× crystallized, were obtained from Worthington Biochemical Corp., Freehold, N. J.

Assay for Added Amino Acids.—The proteins were combusted to CO_2 using the reagents of Van Slyke[26] and the barium carbonate samples counted according to standard procedures in a flow gas counter.

Test for Free Amino Acids.—A portion of the protein was treated according to the ninhydrin carboxyl CO_2 method[13] at *p*H 2.5 and the $C^{14}O_2$ formed was assayed as barium carbonate.

Test for Bound Peptides.—An incubation mixture of diisopropylfluorophosphate treated carboxypeptidase[12] and the protein was prepared as follows: 2 ml. modified BPA, 3% solution, 0.1 ml. of a 0.5% solution of carboxypeptidase, 3 ml. of 0.067 *M* phosphate buffer *p*H 7.6 and 96 mg. of KCl to give an ionic strength of 0.5. Aliquots were removed at various times and analyzed by the ninhydrin CO_2 technique.

Reaction of NCAs with Protein Sulfhydryl Groups.—To a solution of 200 mg. of crude papain in 0.067 *M* phosphate buffer at *p*H 7.3, was added 50 mg. of glycine NCA. Aliquots were removed at suitable times and tested for sulfhydryl groups by use of the nitroprusside reaction.[20] At zero time, an optical density of 0.350 was obtained. After 30 min., the optical density had dropped to 0.300. After 24 hr., an optical density of 0.350 was again obtained. Similar results were obtained when glycine NCA was reacted with cysteine or with thioglycolic acid, although the optical density increased to the initial values within 1 hr. No attempts at quantitative evaluation of these data were made.

Protein Spectra.—Spectra of bovine plasma albumin and polyglycylbovine plasma albumin (mol. wt. 77,200) were obtained in 0.1 *N* HCl and in 0.1 *N* sodium hydroxide, using a Cary Model 11 Recording Spectrophotometer.

End Group Studies.—Modified proteins were converted to their DNP derivatives and hydrolyzed in constant boiling HCl at 105° for 16 hr.[12] ε-DNP-lysine and other DNP amino acids were estimated by the method of Porter.[27] Portions of the bands from the column were identified by paper chromatography. The recovery in control experiments of DNP-glycine under these hydrolytic conditions was only about 20%. Hydrolysis with perchloric acid in acetic acid[28] resulted in recoveries of approximately 50%. For the determination of the protein content of the DNP-proteins, the use of radioactive amino acids in the modification reaction provided a check on other methods of determination, since the ratio of the specific activities of the DNP-modified protein to the modified protein itself × 100 yields directly the percentage of the protein in the DNP derivative. Good agreement among values obtained by amide determination,[29] by calculation assuming complete substitution of groups reacting with dinitrofluorobenzene and by radioactivity measurements were found in most cases studied. For DNP-polyglycylchymotrypsin, the amide value was 90%, the calculated value 90% and the value based on radioactivity, 91%. The corresponding value for DNP-polyglycyl-chymotrypsinogen were 91, 89 and 90%, respectively.

The paper strip method of Fraenkel-Conrat[17] and the solution technique of Williamson and Passmann[18] for the Edman method were used.

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