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CONVERSION OF EXPOSED ASPARTYL AND GLUTAMYL RESIDUES IN PROTEINS TO ASPARAGINYL AND GLUTAMINYL RESIDUES

S. D. LEWIS AND J. A. SHAFER

Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

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SUMMARY

1. The feasibility of converting exposed carboxyl groups in proteins to carboxamido groups using 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) as a condensing agent was demonstrated.

2. The effectiveness of ammonia as a nucleophile in the EDC-mediated condensation with carboxyl groups in proteins was determined. In 5.5 M NH_4Cl at pH 4.75, 11.5, 14.7, 9.0, 5.0 and 5.1 carboxyl groups are converted to carboxamido groups in chymotrypsinogen A, α-chymotrypsin (EC 3.4.4.5), lysozyme (EC 3.2.1.17), ribonuclease (EC 2.7.7.16) and trypsin (EC 3.4.4.4), respectively.

3. The number of carboxamido groups found was determined from the amount of extra ammonia appearing in acid-hydrolyzed protein which was reacted with EDC and NH_4Cl. In one case, the number of β- and γ-carboxamido groups formed was also determined enzymically by digesting treated protein with proleolytic enzymes and then enzymically assaying the digest for free asparagine and glutamine. Both acid hydrolysis and enzymatic assays gave essentially the same value for the yield of carboxamido groups.

4. 5.5 M NH_4Cl, 1 M glycine methylester, and 1 M glycinamide were all equally effective in modifying carboxyl groups. Since previous studies showed that the glycine derivatives quantitatively modify exposed carboxyl groups in proteins, it was concluded that NH_4Cl may be used to quantitatively convert exposed carboxyl groups in proteins to carboxamido groups. Now that it is possible to convert aspartyl and glutamyl residues in proteins to asparaginyl and glutaminyl residues, it should be possible to establish with greater certainty the role of carboxyl groups in the function and structure of proteins.

INTRODUCTION

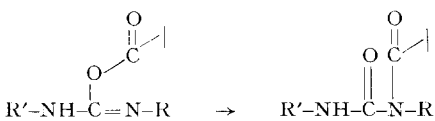
The carbodiimide-mediated condensation of carboxyl groups in proteins with

Abbreviation: EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide.

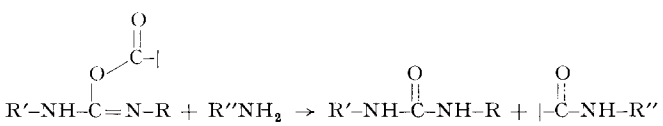
nucleophiles appears useful as a tool for assessing the function of carboxyl groups in proteins. This reaction was initially developed by Hoare and Koshland[1,2] as the basis of a procedure for the quantitative determination of carboxyl groups in proteins. The procedure involved condensing carboxyl groups in proteins with glycine methyl ester or glycinamide using a water soluble carbodiimide such as 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) as the condensing agent. For proteins containing no free thiol groups, side reactions do not normally complicate this condensation. Phenolic hydroxyl groups of tyrosyl residues can add to the cumulative double bond of EDC to form *O*-aryl isoureas[3]. However, treatment of the protein with hydroxylamine decomposes the *O*-aryl isourea and regenerates unsubstituted tyrosyl residues[3].

By condensing carboxyl groups in ribonuclease with phthalimidomethyl esters of glycine and alanylglycine and then removing the phthalimidomethyl group, Wilchek *et al.*[4] were able to study the effect of moving the carboxyl side chains in ribonuclease further away from the protein backbone. Loss of enzymic activity accompanying the EDC-mediated condensation of proteins with glycine derivatives has been taken as evidence for the involvement of carboxyl groups in binding of substrate by trypsin[5], and in the maintenance of the tertiary structure of chymotrypsin[6,7].

The role of a carboxyl (or carboxylate) group in the activity of a protein could be better assessed through this reaction if the group condensing with the carboxyl group were nearly the same size as the OH (or O^-) being replaced. From this point of view, ammonia is preferred as the nucleophile over glycine methyl ester or glycinamide. Hoare and Koshland[2] showed, however, that the ability to obtain quantitative condensation depended on the nucleophile. For example, norleucine methyl ester was not quantitatively incorporated into protein carboxyl groups. It is likely that rearrangement of the *O*-acylisourea to an *N*-acylisourea (Eqn 1) and other reactions with amino acid side chains compete with intermolecular nucleophilic attack (Eqn 2),



(1)



(2)

and become significant when nucleophilic attack is impeded. This work demonstrates that ammonia can be made as effective as glycine methyl ester or glycinamide in the EDC-mediated condensation with carboxyl groups in proteins. Thus, it is possible to convert exposed carboxyl groups in a protein to carboxamido groups.

MATERIALS AND METHODS

Ammonia and amino acids

These were determined by the method of Spackman *et al.*[8] except that after development of the color the absorbance at 570 nm was measured continuously on a Gilford Model 2000 multiple sample absorbance recorder with 0.5- and 1-cm flow through cells. Areas under the peaks were measured with a compensating polar

planimeter sold by Gelman Instruments Co., Ann Arbor, Mich. The amino acid analyzer was calibrated with an 18 amino acid standard solution from Calbiochem. All ammonia determinations were corrected for ammonia in the diluting buffer used to prepare samples and standards, and for the extra ammonia introduced during the hydrolysis procedure. These ammonia blanks were determined several times and found to be constant. The 0.5-ml samples put on the analyzer contained 12 nmoles ammonia from the diluting buffer and 13 nmoles introduced during the hydrolysis procedure, and at least 250 nmoles of ammonia arising from the protein. In order to keep the ammonia blanks to a minimum constant value, all glassware used in ammonia analysis was rinsed with distilled water and dried immediately before use. The distilled water was prepared by running the distilled water supplied to the laboratory through a demineralizer and redistilling it in an all glass still. All solutions were prepared with this water.

Routine procedure for condensation of proteins with ammonia

Protein (25–50 mg was used for most of these runs) was added to two 10-ml portions of 5.5 M NH_4Cl. Both solutions were adjusted to pH 4.75. To one solution were added three 1 mmole portions of solid EDC (from Ott Chemical Co., Muskegon, Mich.) at 1-h intervals. The pH of this solution was maintained at 4.75 by addition of 0.5 M HCl with a Radiometer TTT-1 pH-Stat. An hour after the last addition of EDC (3 h total reaction time), 3 ml of 1 M acetate buffer, pH 4.75, was added to both the reacting solution containing EDC and the no EDC control. After stirring for an additional 15 min, insoluble material was removed from the samples by centrifugation and both solutions dialyzed together against three 2 l portions of 0.001 M HCl. The acetate was added to decompose the EDC. This step could result in acetylation of the protein. Addition of acetate should be omitted when it is necessary to isolate a protein modified only at carboxamido groups. This step was omitted in isolating the trypsin derivative. For ribonuclease, gel filtration on Sephadex G-25 replaced dialysis. The last traces of non-covalently bound ammonia were removed by passing 10–15 ml of the dialyzates through a 2.5 cm × 38 cm column of Sephadex G-25, using 0.001 M HCl to elute the protein. The fractions rich in protein were combined and lyophilized.

Determination of carboxamido groups by ammonia liberated on acid hydrolysis

Lyophilized protein (4–5 mg) was hydrolyzed in 1 ml of constant boiling HCl in an evacuated sealed tube for 22 h at 107 °C. The HCl, packaged in 1-ml sealed ampules, was obtained from Pierce Chemical Co., Rockford, Ill. After hydrolysis, the sample was taken to dryness on a rotary evaporator, 1 ml of water added and taken to dryness again. The residue was taken up in 5 ml of pH 2.2 diluting buffer[8] and 0.5 ml of this solution put on the amino acid analyzer. The ammonia recovered (after correction for the blank) was compared with the recovery of arginine and lysine. Ammonia contents based on the known lysine and arginine contents of the protein were determined. The number of ammonia residues in the no EDC control was substracted from that in the sample to give ammonia residues covalently incorporated on reaction with EDC. The amounts of ammonia incorporated, using as a basis of calculation the known lysine contents and the known arginine contents, were averaged.

Determination of asparagine and glutamine by enzymatic digestion followed by treatment with asparaginase

Trypsin treated with 5.5 M NH_4Cl in the presence and absence of EDC was isolated as described above and then digested with pepsin, Viokase and asparaginase in the manner described below. Lyophilized trypsin 30 mg was incubated at 37 °C with 0.1 mg of pepsin in 6 ml of 0.073 M NaCl at pH 3. After 3 h, the solution was treated with Viokase by a procedure similar to that described by Tower *et al.*[9]. 1 ml of 0.1 M NaOH was added to the pepsin-digest and the pH of the resulting solution adjusted to pH 8.2–8.3 with 0.5 M HCl. A suspension of Viokase (2 ml) was then added and the pH of the mixture readjusted to 8.2–8.3. A drop of toluene was added and the mixture was stirred for 24 h at 37 °C. The suspension of Viokase was freshly prepared prior to use by adding 400 mg Viokase to 10 ml of 0.11 M NaCl and dialyzing the resulting suspension against 3 changes of 1 l of 0.11 M NaCl for 24 h. After incubation, insoluble material was removed from the incubation mixture by centrifugation and the resulting supernatant was subjected to ultrafiltration on an Amicon Corp. Diaflow equipped with a PM-10 membrane. Of the approx. 9 ml put into the apparatus approx. 6 ml was passed through the membrane. The pH of the ultrafiltrate was adjusted to 10–10.5 with 1.0 M NaOH and 5.0 ml of this solution was lyophilized. The residue produced on lyophilization was dissolved in 5 ml of water. A sample of this solution was diluted 1 to 6 with pH 2.2 diluting buffer and subjected to amino acid analysis. Other samples (3 ml) of the solution of lyophilized digest in water were incubated with 0.2 ml of asparaginase (1 mg/ml in 0.85% NaCl) and 2 ml of borate buffer (0.135 M, borate buffer, pH 8.5). The final pH of this incubation mixture was 8.6. After 4 h, 1-ml samples were removed and added to 0.1 ml of 1.5 M trichloroacetic acid. The solution was subjected to centrifugation and 0.5 ml of the supernatant added to 3.0 ml of pH 2.2 diluting buffer for amino acid analysis. A blank containing no trypsin was carried through the entire procedure. The yields of amino acids obtained from the blanks before and after treatment with asparaginase were subtracted from the yields of amino acids obtained from the samples containing trypsin.

Source of proteins

Pepsin (EC 3.4.4.1) swine stomach, two times crystallized; trypsin (EC 3.4.4.4) bovine pancreas, two times crystallized; α-chymotrypsin (EC 3.4.4.5) bovine pancreas, three times crystallized; chymotrypsinogen A bovine pancreas, five times crystallized; ribonuclease (EC 2.7.7.16) beef pancreas, lyophilized salt free; lysozyme (EC 3.2.1.17) egg white, two times crystallized; and asparaginase (EC 3.5.1.1) *Escherichia coli*, purified lyophilized powder, were all obtained from Worthington Biochemical Corp., Freehold, N.J. Viokase (4 N.F. Pancreatin) was obtained from Viobin Corp., Monticello, Ill.

RESULTS

Table I illustrates variables influencing formation of carboxamido groups in the EDC-mediated condensation of chymotrypsinogen and chymotrypsin with ammonia. At pH 4.75 in 5.5 M NH_4Cl, 11.5 $\pm$ 0.9 and 14.7 $\pm$ 1.6 carboxyl groups are converted to carboxamido groups in chymotrypsinogen and chymotrypsin, respec-

TABLE I

CONVERSION OF CARBOXYL GROUPS TO CARBOXAMIDO GROUPS

Protein	*pH*	*[NH_3] in EDC reaction (M)*	*Residues NH_3 incorporated**	*No. of free carboxyl groups***
Chymotrypsinogen	4.75	1.0	6.9 ± 1.7	15
Chymotrypsinogen	4.75	3.0	8.6 ± 0.4	
Chymotrypsinogen	4.75	5.5	11.3 ± 1.3, 11.8 ± 0.5	
Chymotrypsinogen	4.75***	5.5	11.0 ± 0.1	
Chymotrypsinogen	5.75	5.5	8.0 ± 0.5	
Chymotrypsin	4.75	5.5	14.7 ± 1.6	17
Lysozyme	4.75	5.5	9.0 ± 1.0	11
Ribonuclease	4.75	5.5	5.0 ± 0.2	11
Trypsin	4.75	5.5	5.1 ± 0.4	7

* Variance represents difference from average using as a basis of calculation 14 lysine and 4 arginine residues for chymotrypsinogen, 14 lysine and 3 arginine residues for chymotrypsin, 6 lysine and 11 arginine residues for lysozyme, 10 lysine and 4 arginine residues for ribonuclease, and 14 lysine and 2 arginine residues for trypsin.

** Sum of aspartic acid, glutamic acid and α-carboxyl groups.

*** Three 0.5-mmole portions of EDC added instead of three 1-mmole portions of EDC (see Material and Methods).

tively. Ammonia analysis of native chymotrypsinogen passed through a Sephadex G-25 column yielded 27 residues of ammonia. Chymotrypsinogen controls incubated with 1.0–5.5 M NH_4Cl also had an average ammonia content of 27 residues. Chymotrypsinogen incubated with EDC in the absence of ammonia yielded 26 ammonia residues. These results eliminate the possibility that the ammonia incorporated into protein (Table I) arises from a reaction involving only EDC or from non-covalently bound ammonia. There are 24 carboxamido groups in chymotrypsinogen. Undoubtedly, partial degradation of serine, threonine, cystine, and tryptophan which accompanies acid hydrolysis of protein is responsible for the slightly high yield of ammonia. This deviation from the expected value for the amide ammonia was greatest for chymotrypsinogen. Other procedures for liberating amide ammonia which minimize the yield of excess ammonia have been discussed by Wilcox[10]. It was not necessary to use these procedures in this work. The small reproducible extra ammonia did not effect the determination of the number of carboxamido groups formed by the EDC treatment, since the carboxamido groups formed were determined from the difference between the ammonia content of protein treated with ammonia in presence of EDC and protein treated with ammonia in the absence of EDC.

With 1.0 M glycine methyl ester or glycinamide, 10.6 and 12.7 carboxyl groups are modified in native chymotrypsinogen and chymotrypsin, respectively[6]. Thus, 5.5 M ammonia and 1.0 M glycine methyl ester appear equally effective in the EDC-mediated condensation of exposed carboxyl groups in proteins. Although there are 15 carboxyl groups in chymotrypsinogen and 17 carboxyl groups in chymotrypsin[11–13] not all of them are accessible to EDC unless the proteins are denatured[6]. In 8 M urea EDC mediates condensation of all the carboxyl groups of these proteins with glycinamide or glycine methyl ester. X-ray crystallographic analysis of native chymotrypsin indicates two aspartyl carboxyl groups are inaccessible to water[14].

Examination of Table I reveals the expected decrease in yield of carboxamido

groups with decreasing concentration of ammonia. Raising the pH of the reaction to 5.75 also causes a decreased yield of carboxamido groups. Interestingly, halving the amount of EDC added causes only a barely detectable decrease in the yield of carboxamido groups.

The data in Table I illustrate the conversion of 9 of the 11 carboxyl groups in lysozyme to carboxamido groups. Under similar conditions using 1 M glycine methyl ester Hoare and Koshland[2] were also only able to modify only 7–8 of the 11 carboxyl groups in lysozyme. However, denaturation of the lysozyme with 5 M guanidinium·HCl resulted in the modification of all 11 carboxyl groups in lysozyme by 1 M glycine methyl ester.

As shown in Table I, 5 out of 11, and 5 out of 7 carboxyl groups in ribonuclease and trypsin, respectively, are modified by treatment with EDC in 5.5 M NH_4Cl.

In an effort to demonstrate that aspargine and glutamine are formed in the EDC-mediated condensation, trypsin treated with ammonia in the presence and absence of EDC was digested with pepsin and then with Viokase (a mixture of proteolytic enzymes). The resulting mixture of amino acids was treated with asparaginase for 4 h. This treatment was sufficient to hydrolyze all the free asparagine and glutamine in standard samples of asparagine and glutamine. The asparaginase used in this work was apparently contaminated with glutaminase so that addition of glutaminase was unnecessary. The results summarized in Table II lead to the con-

TABLE II

CHANGES IN AMINO ACIDS ON TREATING ENZYMATIC DIGESTS OF TRYPSIN WITH ASPARAGINASE*

Digest	*ΔAsp*	*ΔGlu*	*ΔNH_3*	*ΔGln + ΔAsn*
Trypsin treated with 5.5 M NH_4Cl and EDC	14.6	8.2	21.0	−22
Trypsin treated with only 5.5 M NH_4Cl	10.3	6.3	16.3	−17.4

* Yields normalized to 14 lysine residues. The asparaginase was probably contaminated with glutaminase since this treatment with asparaginase was sufficient to hydrolyze standard solutions of asparagine and glutamine.

clusion that the extra ammonia incorporated into trypsin exists as aspargine and glutamine. It should be noted, however, that this conclusion rests on the assumption that the same degree of completion of proteolysis is obtained with trypsin and trypsin treated with EDC, since proteolysis was not complete. For example assuming 100% recovery of the 14 lysines only 17 of the 29 glutamines and asparagines in untreated trypsin were recovered by this hydrolytic procedure.

DISCUSSION

The carbodiimide mediated condensation of amines with carboxyl groups probably ceases being quantitative when the rate of nucleophilic attack on the *O*-acylisourea is impeded so that the rate of this reaction becomes comparable to the rate of intramolecular side reactions such as rearrangement of the *O*-acylisourea (Eqn 1). The rate of nucleophilic attack should decrease when: (a) The steric bulk

around the attacking nitrogen atom is increased. (b) The total concentration of the nucleophile is decreased. (c) The pK of the nucleophile is increased. The reactivity of similar nucleophiles toward carbonyl carbon should be proportional to less than the first power of their basicities, whereas the fraction of the nucleophile in the deprotonated form at pH 4.75 (the pH for the condensation) will be inversely proportional to the first power of their basicities (when the pK values of the nucleophiles are > 4.75).

An idea of the relative effectiveness expected for ammonia and glycine ethyl ester as nucleophiles in this reaction can be obtained by comparing their known relative reactivities toward another active ester, *p*-nitrophenyl acetate. Ammonia is four times more reactive toward *p*-nitrophenyl acetate than glycine ethyl ester[15,16]. Ammonia is also about 33 times more basic than glycine ethyl ester (pK 9.25 for ammonia *vs* pK 7.73 for glycine ethyl ester)[17]. Thus at pH 4.75, a solution 8.25 M in NH_4^+ will displace *p*-nitrophenol from *p*-nitrophenyl acetate at about the same rate as a 1 M solution of glycine ethyl ester hydrochloride.

Condensation of exposed carboxyl groups in proteins with glycine derivatives is quantitative in 1 M solutions of the glycine derivative. Assuming that the relative reactivities of NH_3 and glycine ethyl ester toward *p*-nitrophenyl acetate and the *O*-acylisourea intermediate are similar, quantitative condensations of exposed protein carboxyl groups should be possible in concentrated solutions NH_4^+. The results reported here show that 5.5 M NH_4Cl is indeed as effective in modifying exposed carboxyl groups in proteins as 1 M glycine methyl ester.

It is therefore possible to convert exposed carboxyl groups in proteins to carboxamido groups. It should be pointed out, however, that care must be taken in correlating changes in properties of proteins with formation of carboxamido groups. Effects of possible side reactions such as reaction with hydroxyl groups of tyrosine[3] and serine[18] and reaction with thiol groups of cysteine[19] must be excluded in order to assess the effects of converting carboxyl groups to carboxamido groups. Fortunately, as Carraway and Koshland[20] pointed out in their review of these reactions that the effects of these side reactions can be evaluated by observing the effect of carbodiimide in the absence of added nucleophile. Also these side reactions can be prevented or reversed. As already mentioned, reaction with tyrosine can be reversed with hydroxylamine. Reaction with thiol groups can be prevented by reagents which reversibly block these groups[20]. The addition of serine to the cumulative double bond of a carbodiimide appears to be rare. It has been reported only for the addition of the active serine in α-chymotrypsin to a carbodiimide having an apolar group which directs the carbodiimide to the active site[18]. However, this reaction is also reversed by the addition of nucleophiles such as hydroxylamine[18].

Since complications due to side reactions can be avoided, the conversion of exposed carboxyl groups to carboxamido groups demonstrated in this work should prove a useful tool in evaluating the function of carboxyl groups in the activity of proteins.

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