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Generation of a Free α-Amino Group by Raney Nickel after 2-Nitro-5-thiocyanobenzoic Acid Cleavage at Cysteine Residues: Application to Automated Sequencing†

Samuel Otieno

ABSTRACT: The selective reaction of SH containing proteins and peptides with NTCB (2-nitro-5-thiocyanobenzoic acid) has been reported (Degani, Y., & Patchornick, A. (1974) *Biochemistry 13,* 1; Jacobson, G. A., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248,* 6583). With this reagent, cysteinyl peptide bonds are selectively cyanylated and subsequently cleaved under alkaline conditions. In the present study we have successfully cleaved the β-chains of guinea pig hemoglobin at the single cysteine and the peptides thus obtained were separated. However, the C-terminal peptide was blocked at its N terminal by a thiazolidine ring and hence could not be used for Edman degradation sequence analysis. Deblocking of this peptide was successfully done by Raney nickel in the buffer medium of pH 7.0, and also in water, at 50 °C for 6 to 10 h. The Raney nickel reagent is used in large excess by weight (at least ten times the weight of sulfur compound) over the compound to be desulfurized. Under these conditions, control experiments on cysteine, methionine, and some other amino acids showed that only the sulfur containing amino acids are degraded by Ni(H). Cysteine and methionine were rapidly converted to alanine and β-aminobutyric acid, respectively. Gel electrophoresis of the iminothiazolidine peptide after exposure to Ni(H) showed no breakage of the chain.

The desulfurization of an organic compound by Raney nickel was first reported by Bougault (1940). Since that time, the reaction has been used with much success both for synthesis and the determination of structure of organic compounds. The desulfurization of thiophen derivatives and thiazoles with Raney nickel has been extensively investigated (Badger & Sasse, 1956; Badger & Kowanko, 1951). A Raney nickel desulfurization involves the breaking of a carbon–sulfur bond in an organic substance and, usually, the formation of at least one new carbon–hydrogen bond.

The reagent NTCB has been shown (Degani et al., 1970; Vanaman & Stark, 1970) specifically to cyanylate free thiol groups in proteins and to be of value in distinguishing truly essential thiols in various SH-dependent enzymes (Degani & Patchornik, 1974). It has been shown that cyanylated poly-

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peptides can undergo an intramolecular cleavage reaction at the amino peptide bond adjacent to the SCN-cysteinyl residue, upon incubation at 37 °C in alkaline medium (Jacobson et al., 1973; Degani & Patchornik, 1974). We have successfully used this reagent to specifically cleave guinea pig β-globin chains with a 95% yield. However, the cleaved peptide from the C-terminal end of the chain was blocked at its amino terminus and, hence, could not be subjected to sequence determination by Edman degradation. Thus, an attempt was made to unmask the blocked N-terminal group with Raney nickel. Thiazoles are reported to be more difficult to desulfurize because the lone electron pair of the heteronitrogen atom might compete for the active centers in much the same way that pyridine and similar heteroaromatic compounds act as catalytic "poisons" in hydrogenation reactions (Lindlar, 1952; Elsner & Paul, 1953; Badger & Sasse, 1956). Therefore, all of the present work was carried out with the very active (neutral) W-6 catalysts (Billica & Adkins, 1967). In the experiment reported here, water and methanol were routinely used since primary amines had been reported to be ethylated in the presence of Raney nickel, but not alkylated by methanol (Rice & Kohn, 1955; Kao et al., 1955).

When treated with W-6 catalysts which were made in this laboratory in methanol or in water at 50 °C, the acyliminothiazolide peptide was smoothly desulfurized in excellent yield (90%) to the expected alanine at the N terminus. The commercially available catalyst was less effective, however, as only a 55% yield was obtained.

In this report, experiments are presented in which the effects of Ni(H)[1] on sulfur containing proteins (i.e., hexokinase and phosphoglycerol kinase) were studied. Raney nickel was then used on cyanylated and subsequently cleaved β chains of guinea pig hemoglobin.

Materials and Methods

Yeast hexokinase B was kindly provided by Dr. E. A. Barnard. Yeast phosphoglycerol kinase was purchased from Sigma. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Pierce. 2-Nitro-5-thiocyanobenzoic acid was prepared by the method of Degani & Patchornik (1971). It was characterized by its melting point and by spectrophotometric titration of the thionitrobenzoate produced by reaction with glutathione at pH 7.0. Glutathione was obtained from Sigma. The commercially activated Raney nickel was obtained from Apache Chemicals Inc., Seward, Ill. Chelex-100 chelating resin was obtained from Bio-Rad laboratories. Dansyl chloride and dansyl amino acids were obtained from Pierce. Amino acids were obtained from Sigma Chemical Co. Polyamide layer sheets were obtained from Gallard-Schlesinger Chemical Mfg. Corp. Urea was obtained from Baker Chemicals and guanidine hydrochloride was from Sigma. RG 501-X8 resin was obtained from Bio-Rad Laboratories.

Amino acid analyses were carried out on a Beckman Model 120C amino acid analyzer. Oxidation of protein samples was carried out by the method of Moore (1963). Reduction of hexokinase and carboxymethylation with iodoacetic acid and iodoacetamide were done according to Jones et al. (1975). Protein concentrations were determined by the method of Lowry et al. (1951). $NaDodSO_4$ gel electrophoresis was done according to the method of de Haën & Gertler (1974) and the discontinuous Tris glycine buffer system of Laemmli (1970) was also used.

Preparation of W-6 Raney Nickel Catalyst. This was prepared by the addition of NaOH to Raney nickel–aluminum alloy (Adkins & Billica, 1948; Billica & Adkins, 1967). The procedures for preparation and the apparatus employed for washing the catalyst were similar to those described by Billica & Adkins (1967). After extensive washing to neutrality, the catalyst was transferred to the reaction flask of a hydrogenation apparatus and shaken for 15 min with 100 mL of ion-exchange resin (Amberlite IR120) and 100 mL of distilled water, under a hydrogen pressure of 3 atm. The contents were then transferred to a nylon gauze, suspended in water, then the catalyst was removed from the resin and was allowed to settle as indicated by Van Driel et al. (1971).

Globin Chain Preparations. Globin was prepared according to the method of Garrick et al. (1973). The sample (50 mg/mL) was added dropwise and with continuous stirring to 20 volumes of 2.5% (w/v) oxalic acid acetone at room temperature. Globin precipitate was collected by centrifugation at 18 000*g* at 4 °C for 10 min and the supernatant discarded. A column chromatographic method, developed by Dintzis (1961) to separate the two apoprotein globin chains, was routinely employed to separate the chains.

Pooled, freeze-dried α and β chains were further fractionated by the method of Clegg et al. (1966). The chains were denatured in 8 M urea containing 0.05 M sodium phosphate and 0.05 M 2-mercaptoethanol (pH 7.1) and placed on a column (2.5 × 18 cm) of CM-cellulose (Whatman CM-52) equilibrated with the same buffer. Chains were eluted with a 1200-mL linear gradient of increasing ion concentration (0.015–0.059 N). Fractions of 10 mL were collected. Pooled fractions were passed through a column (3.5 × 112 cm) of Sephadex G-25 coarse equilibrated with 0.5% formic acid, and freeze dried.

Reaction with NTCB. Guinea pig β chain (20 mg/mL) was reacted with 5 mM NTCB in 0.05 M Tris-HCl, pH 8.0, at 35 °C for about 6 h. The cyanylated protein was then subjected to cleavage. The reaction was conducted in 8 M urea or 4 M guanidinium chloride.

Cleavage of Cyanylated Protein and Separation of Peptides. The cyanylated protein (20 mg/mL) in 8 M urea,[2] 0.2 M Tris–acetate buffer, pH 9.0, was incubated for 72 h at 37 °C. The urea was then removed by gel filtration of a sample (1 mL) on a Sephadex G-25 column (2 × 60 cm) run in 0.05 M ammonium acetate, pH 7.0. The material absorbing at 280 nm, which was found in a sharp peak at the void volume, was collected in 1.5-mL fractions, pooled, and freeze dried. The peptide was dissolved in the minimum of buffer (0.05 M ammonium acetate, pH 7.0), containing 1% 2-mercaptoethanol. The sample was then applied onto an SP-Sephadex C-25 column (1.2 × 60 cm) equilibrated in the same buffer. The peptides were eluted with the same buffer (linear gradient of increasing buffer concentration of 0.005 M and 0.8 M), and fractions of 1 mL were collected. The separated peptides were lyophilized and were further purified on Sephadex G-75 Superfine and Bio-Gel P10 (1 × 75 cm) columns.

The N-Terminal Determinations. In principle, the technique of Hartley (1970) with a minor modification was followed. Peptide to be analyzed (1–2.5 nmol) is placed in a 6 × 50 mm test tube and 5 μL of 0.1 M $NaHCO_3$ is added and made to cover the base of the tube by vibrating it on a Vortex mixer. Five microliters of dansyl chloride solution (2 mg/mL) in acetone is then added, and after a brief mixing the mixture is allowed to react for 12 h at 20 °C. The mixture is hydrolyzed

[1] Abbreviations used: Ni(H), Raney nickel; NTCB, 2-nitro-5-thiocyanobenzoic acid; Pth, phenylthiohydantoin; CM, carboxymethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

[2] Urea was purified before use by stirring overnight with Bio-Rex RG Grade mixed bed resin-RG 501-X8.

TABLE I: Modifications Resulting from the Treatment of a Mixture of Amino Acids with Raney Nickel W-6 at 25 °C at Various pHs.[a]

duration of reaction (h)	amount amino acid remaining (μmol)							
	pH 5.0		pH 6.0		pH 7.0		pH 8.5	
	Cys	Met	Cys	Met	Cys	Met	Cys	Met
0	1	1	1	1	1	1	1	1
1	0.23	0.94	0.02	0.87	0.00	0.84	0.00	0.86
6	0.01	0.67	0.00	0.57	0.00	0.50	0.00	0.53

[a] The reaction was carried out with the W-6 Raney nickel preparation on a mixture containing 1 μmol of each amino acid except alanine. Reaction was determined by a decrease of cystine and methionine and by the appearance of alanine and α-amino-*n*-butyric acid. All other amino acids remained unchanged and therefore are not given in the table. For experimental details, see text.

with 6 N HCl, the residue obtained after evaporation dried in vacuo and the dansylamino acids and side products are then extracted by vibrating the test tube with 25 μL of ethyl acetate saturated with water. The extract is spotted on two polyamide layers (2 μL each) such that the spots on the starting line are less than 2 mm in diameter. Simultaneously, a standard mixture of dansyl amino acids is run in parallel, thus providing an internal control for accurate quantitation, as it eliminates differences in TLC plate batch behavior, humidity, solvents, and minor discrepancies in the chromatographic process itself. The solvent systems of Woods & Wang (1967), i.e., benzene–acetic acid (9:1), formic acid–water (1.5:98.5) and, later, ethyl acetate–methanol–acetic acid (20:1:1) were used.

Reaction of Amino Acids with Raney Nickel. Reactions were carried out with the W-6 Raney nickel preparation. Amino acid mixtures were dissolved in 2 mL of water and the solutions were adjusted to the appropriate pH (5.0, 6.0, 7.0, or 8.0) with 3 N NaOH. An aliquot (200 μL) was removed and mixed with 4 mL of 0.2 M citrate buffer (pH 2.25). The solution was frozen and kept refrigerated until it was used for standard runs. Raney nickel (300–600 mg) was added to the magnetically stirred amino acid solution and the pH was maintained at the desired value by addition of 6 M HCl using a pH stat. Portions (200 μL) were removed at intervals, cooled at 4 °C, then centrifuged at 4 °C (5000 rev/min, 20 min). The supernatant was passed through a Chelex-100 chelating column (0.6 × 5 cm). The column was then washed with 10 mL of 1 N ammonium hydroxide. The solution was freeze-dried. Reactions in water, methanol and at pH 7.0 and 8.0 were carried out at 50 and at 25 °C, respectively. All reactions were carried out under N_2 and H_2.

Reactions of Proteins with Raney Nickel. Desulfurization with W-6 Raney nickel was carried out on a solution of protein (6 mg) in water (2 mL); and/or methanol (2 mL), and also at pH 7.0 and 8.0 in 0.05 M Tris-HCl, at 50 and 25 °C. Reactions were carried out under an atmosphere of H_2 and N_2. The procedure employed was similar to that described for the amino acids. Aliquots were withdrawn at intervals and centrifuged at 4 °C (5000 rev/min, 20 min), and the supernatant was passed through a column (0.6 × 5 cm) of Chelex-100 chelating resin. The protein was eluted with 1 N ammonium hydroxide and then freeze-dried.

Extent of desulfurization for each sample was determined by all of the following criteria: (a) increase of alanine content in the acid hydrolyzates of each sample; (b) the cysteic acid content of the acid hydrolysate of the native, reduced, and carboxymethylated proteins before and after desulfurization; (c) the amount of CM-cysteine produced before and after desulfurization.

Desulfurization of Acyliminothiazolide Peptide. Desulfurization of the peptide was carried out at 50 °C, in 0.05 M Tris-HCl, pH 7. A mixture of 20 mg of the peptide and 200 mg of Ni(H)W-6 in 50 mL buffer was refluxed for 7 h, under an atmosphere of N_2. The solution was cooled to 4 °C and then centrifuged at 4 °C (5000 rev/min, 20 min), and the supernatant was treated as discussed before. The peptide was then subjected to Edman degradation using the the automatic sequencer.

Amino Acid Sequence Analysis. The peptide was subjected to 14 steps of degradation by the automated Edman procedure using a Beckman 890B protein sequenator employing the standard 0.1 M Quadrol program. Samples were dried under nitrogen and converted to the phenylthiohydantoin (Pth)-amino acids by treatment with 1 N HCl–0.001 N ethanethiol for 10 min at 80 °C. The converted Pth-amino acids were identified by three independent methods: thin-layer chromatography (Jeppsson & Sjoquist, 1967), gas chromatography, and each sample was regenerated by hydrolysis in 0.01 N NaOH according to Mendez & Lai, (1975), or in hydroiodic acid for 18 h at 120 °C (Smithies et al., 1971) and analyzed on a Beckman Model 120C amino acid analyzer. Yields of approximately 90% of the theoretical values were noted at step 1 and the repetitive yields averaged 95% for the peptide.

The solvent systems used for Pth derivations were 1,2-dichloroethane:propionic acid:heptane (25:17:58, v/v) (Jeppsson & Sjoquist, 1967) and chloroform:methanol (9:1, v/v) (Pataki, 1966). Aqueous phases were dried and tested for Pth-arginine by the phenanthraquinone spot test (Sanger & Tuppy, 1951). To distinguish between Pth-leucine and Pth-isoleucine, acid hydrolysis was carried out with (a) 0.6 mL of 6 N HCl; (b) 0.5 mL of 47% HI (Van Orden et al., 1964); and (c) 0.1 mL of 4 N methanesulfonic acid containing 0.2% tryptamine (Smithies et al., 1971), at 150 °C for 22 h, in evacuated and sealed tubes. The HCl and HI hydrolysates were evaporated to dryness in an Evapo-Mix. The methanesulfonic acid hydrolysates were diluted with water and/or buffer and applied directly to an amino acid analyzer.

Amino Acid Composition. The amino acid composition of the proteins and the acyliminothiazolide peptide was determined on a Beckman Model 120C. Duplicate protein samples were hydrolyzed in evacuated tubes with 6 N HCl for 24 h, at 100 °C, and also in 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu & Chang, 1971) for determination of tryptophan.

Results

Effect of pH on Desulfurization of Amino Acids at 25 °C. Desulfurization reactions were performed with the W-6 Raney nickel preparations. Desulfurization of amino acids at these conditions showed a decrease in the contents of cystine and methionine only (Table I). All the other amino acids remained unchanged. Desulfurization of cystine proceeded much more rapidly than that of methionine at lower pHs. At pH 7.0, methionine was 50% desulfurized after 6 h as compared with about 33% at pH 5, and 43% at pH 6. Cystine on the other hand was 98% desulfurized after 6 h of reaction at pH 5.0, and

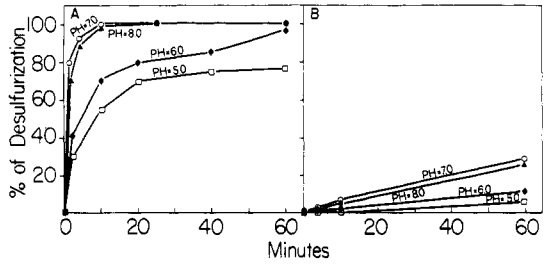

FIGURE 1: Rate of desulfurization at 50 °C of cystine (A) and methionine (B) at pH 5.0 (□—□), 6.0 (♦—♦), 7.0 (○—○), and 8.0 (▲—▲).

completely desulfurized at the higher pHs. The product of desulfurization of cystine was alanine. The reaction product of methionine was α-amino-*n*-butyric acid, in agreement with Ivanov et al. (1961, 1964), and was confirmed by analysis of a sample of α-amino-*n*-butyric acid which was obtained commercially.[3] Cysteine behaves in a manner similar to cystine.

At 50 °C, and especially at pH 7.0 and 8, desulfurization of cystine was extremely rapid and was virtually complete within 4 min. Reaction of methionine was much slower again under these conditions. Other amino acids, including cysteic acid, remained unmodified. Cystine was again quantitatively converted to alanine and the decrease in methionine was entirely accounted for by the appearance of α-amino-*n*-butyric acid.

Figures 1A and 1B summarize these findings. The diagram shows the rates of desulfurization of cystine and methionine at 50 °C and pH 5.0, 6.0, 7.0, and 8.0. From these results it can be concluded that pHs 7.0 and 8.0 were the most satisfactory conditions for desulfurization reactions.

When desulfurization was carried out in distilled–deionized water at pH 5.5, the desulfurization rates observed were between those at pH 5 and pH 6 as shown in Table I. The same results were obtained when the desulfurization reaction was carried out in methanol.

Desulfurization of Cystine Residues in Yeast Hexokinase and Phosphoglycerol Kinase. Reactions were carried out with these proteins because both contain free sulfhydryl groups which are accessible to reactions such as alkylations with haloacetates (Lazarus et al., 1968; Roustan et al., 1976). The results are summarized in Table IIA and B. The conditions used, pH 7.0, 50 °C were those under which the reaction with W-6 Raney nickel was the most rapid. Reaction for 6 h resulted in complete desulfurization of the 3.5 cysteine residues per subunit of hexokinase and 1 cysteine residue in phosphoglycerol kinase. It is important to note that the reaction of simple thiol containing amino acids (cysteine and cystine) with Raney nickel was virtually complete during the first 30 min of the reaction; whereas in intact proteins, it took at least 6 h to completely desulfurize cysteine and cystine to the corresponding alanine residues (Table IIA and B). The reaction was entirely specific for cysteine and methionine whose only reaction products were alanine and α-amino-*n*-butyric acid,

[3] A known quantity of commerically obtained α-amino-*n*-butyric acid was added as an internal standard to a known quantity of a protein solution before hydrolysis. A parallel experiment was done with the mixture of amino acids (except methionine, cystine, and cysteine) containing α-amino-*n*-butyric acid. The hydrolyzates and the mixture of amino acids were subjected to an amino acid analyzer, and the percent recovery of the internal standard in the hydrolyzate provided a direct measure of the percent recovery of the amino acid derivatized by Raney nickel in the protein. In determining the elution profile of α-amino-*n*-butyric acid, we employed the Pico buffer system IV for physiological amino acid analysis obtained from Pierce Chemical Co. In this system, α-amino-*n*-butyric acid eluted from the analyzer quantitatively between citrulline and valine.

TABLE II: The Effect of Raney Nickel on The Sulfur-Containing Proteins, the Native and Carboxymethylated Forms of Yeast Hexokinase and Phosphoglycerol Kinase, at 50 °C, pH 7.0.

(A) hexokinase[a]

amino acid composition	untreated hexokinase	Raney nickel treated hexokinase	Raney nickel treated CM-hexokinase
CM-cystine	3.7	0.0	0.0
Met	10.8	0.0	0.0
Val	20.4	19.8	21.0
Ala	32.6	38.5	39.2
Tyr	15.1	14.9	15.2
Trp[b]	3.5	4.0	3.2
Asp	53.1	53.0	52.1

(B) phosphoglycerol kinase[c]

amino acid composition	untreated phosphoglycerol kinase	Raney nickel treated phosphoglycerol kinase	Raney nickel treated CM-phosphoglycerol kinase
Cys	0.9[d]	0.0	0.0
Met	2.7	0.0	0.0
Val	33.4	32.9	33.5
Ala	40.0	41.1	42.0
Tyr	6.9	6.8	6.7
Phe	18.0	17.9	18.1
Trp	2.3[e]	2.4	2.2

[a] Reduced and carboxymethylated hexokinase from Schmidt & Colowick (1973). [b] Tryptophan recovery from alkaline hydrolysates. Hydrolysis was by 4.2 N NaOH (0.6 mL containing 25 mg of starch) at 110 °C for 16 h (Hugli & Moore, 1972). Hydrolysis with 4 N methanesulfonic acid was also performed on the protein (Liu & Chang, 1971). The desulfurization reaction was carried out for 6 h. [c] Roustan et al. (1976). [d] Determined by DTNB (Ellman, 1959), and also by carboxymethylation of the protein as described by Crestfield et al. (1963). [e] Determined after alkaline hydrolysis and also by hydrolysis with *p*-toluenesulfonic acid (Liu & Chang, 1971). The values were obtained after 6 h of desulfurization reaction.

respectively. Methionine reactivity in this system was greater than that of the free methionine residue. The same results were obtained with yeast hexokinase when the reaction was carried out in water at 50 °C (Table III). Again, alanine and α-amino-*n*-butyric acids were the only products of the reactions of cysteine and methionine. All other amino acids were not affected as judged by amino acid analysis of the protein before and after desulfurization. To identify the modified residues during desulfurization of the model proteins, desulfurized hexokinase and phosphoglycerol kinase were subjected to complete acid and alkaline hydrolysis as indicated in Materials and Methods. Amino acid analysis of both acid and alkaline hydrolyzates clearly showed that aromatic amino acids, i.e., tryptophan, phenylalanine, and tyrosine were recovered undamaged after desulfurization of these proteins (Table IIA and B). The ratio of valine to tryptophan remains constant throughout the course of desulfurization, while that of alanine to tryptophan increases progressively. On the other hand, the methionine to tryptophan ratio was also affected, but the increase was not as high as that of alanine to tryptophan. All other amino acid residues are unaffected. Cysteine residues were completely desulfurized to corresponding alanine residues as indicated in Table IIA and B. These observations confirm our finding that desulfurization of proteins with W-6 Raney nickel is specific for sulfhydryl and methionine groups in proteins. Damage to peptide bonds or to other amino acids did not occur under our experimental conditions.

Characterization of NTCB Cleaved Peptides. β chains of

TABLE III: Desulfurization of Yeast Hexokinase with Raney Nickel in Water at 50 °C.[a]

treatment (h)	amino acid composition (mol/mol) native hexokinase none Cys	native hexokinase none Met	native hexokinase Raney nickel Cys	native hexokinase Raney nickel Met	reduced, carboxymethylated hexokinase none CM-Cys	reduced, carboxymethylated hexokinase none Met	reduced, carboxymethylated hexokinase Raney nickel CM-Cys	reduced, carboxymethylated hexokinase Raney nickel Met
1	3.5	10.8	2.5	8.0	3.5	10.8	2.4	8.8
3			1.2	7.5			1.25	7.0
6			0.0	5.5			0.00	4.8
12			0.0	3.7			0.00	3.5
24			0.0	1.2			0.00	1.0

[a] Reactions were carried out with the W-6 Raney nickel preparation as indicated. Values were obtained from duplicate acid hydrolyses, both before and after carboxymethylation and performic acid oxidation. Cystine was determined as *S*-CM-cysteine and cysteic acid after performic acid oxidation. Thiol groups were determined by titration of the intact protein according to the method of Lazarus et al. (1968). Values of all other amino acids remained unchanged; hence, they are not included in the table.

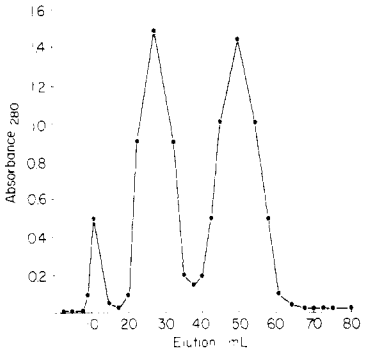

FIGURE 2: Chromatography of the products of the reaction of NTCB with guinea pig β-globin chains on SP-Sephadex C-25 (1.2 × 60 cm) equilibrated in 0.05 M ammonium acetate, pH 7.0. Experimental conditions are described in Materials and Methods. The peaks were pooled separately, freeze-dried, and then were subjected to N-terminal analysis.

TABLE IV: Raney Nickel Treatment of the Acyliminothiazolide Peptide.[a]

amino acid composition	untreated peptide	Ni(H)-treated peptide
Ala	7.0	8.2
Asp	5.0	5.0
Lys	3.0	3.1
Leu	6.0	5.9
His	4.9	5.0
Val	8.0	7.9
Pro	3.0	3.0
Glu	5.0	4.9
Phe	3.0	3.1
Arg	2.0	2.1
Tyr	2.0	2.2
Thr	1.0	0.9

[a] The β-globin chain from guinea pig was reacted with NTCB as discussed in the methods. The purity of the isolated acyliminothiazolide peptide was checked by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970). The peptide (30 mg) was treated with 300 mg of Ni(H) for 6 h at 50 °C, pH 7.0. The solution was cooled to 4 °C and then centrifuged at 4 °C (5000 rev/min, 20 min). The supernatant was passed through a Chelex-100 chelating column. After washing the column with 1 N ammonium hydroxide, the solution was freeze-dried.

guinea pig hemoglobin were cyanylated at pH 8.0 (see Materials and Methods) and then cleavage was achieved by incubation in 8 M urea, pH 9.0 at 37 °C for 72 h. The cleaved products were separated on a SP-Sephadex C-25 column (1.2 × 60 cm; Figure 2). The first peak to emerge corresponded to the intact protein (β-globin chain) as judged by its amino acid composition. The second peak is the original N-terminal portion of the protein since its N-terminal residue was valine as obtained by the dansylation method of Hartley (1970) and also by the Edman degradation technique. The first 25 residues of this peptide corresponded to those obtained with the intact chain (data to be published elsewhere).

The third peak contained a blocked N-terminal residue since no amino acid residue was liberated by the dansyl-Edman procedure according to Gray & Hartley (1963), Hartley (1970), and Bruton & Hartley (1970). This peptide was further purified by gel filtration on Sephadex G-75 and Bio-Gel P-10. Its purity was checked by $NaDodSO_4$ gel electrophoresis and by determination of the amino acid composition.

Unlike other peptides and sulfur-containing proteins, the acyliminothiazolide peptide has a thiazolidine ring at its N terminal and, hence, is useless for sequence determinations. When this peptide was treated with W-6 Raney nickel at 50 °C, pH 7.0, under an atmosphere of N_2 and H_2, for 6 h, desulfurization proceeded to about 90% as judged by sequence analysis in the automatic Edman degradation sequenator. Desulfurization was also affected in water at 50 °C for 10 h. It was found that the recovery of alanine was rather low (≃60%) when the reaction was run in the presence of air. Since cysteic acid was not desulfurized under our experimental conditions, it was concluded that this low yield was due to air oxidation of the thiazolidine ring.

Amino Acid Composition of the Acyliminothiazolide Peptide. The amino acid compositions of the blocked and unblocked thiazolide peptide are summarized in Table IV. The blocked peptide liberated approximately 7 residues of alanine upon hydrolysis in 6 N HCl (Table IV), and no cysteic acid peak was observed. After deblocking the thiazolide ring with W-6 Raney nickel, an extra residue of alanine was released, thus accounting for the thiazolide ring at the N terminus of the peptide. The absence of tryptophan was inferred from the lack of any fluorescent spot in any of the peptide maps, and also by alkaline hydrolysis of the peptide. The reliability of detection of Trp by fluorescence under long wavelength UV excitation has been previously established during sequence studies of Trp-containing proteins (Brosius & Chen, 1976), where the presence or absence of Trp was confirmed by the use of Erlich's reagent and direct identification of Pth-Trp. Spraying the tryptic peptide maps of the acyliminothiazolide peptide with 1% dimethylbenzaldehyde in 2 N HCl according to Spies & Chambers (1949) was also employed in this study.

Sequence Analyses. Fourteen cycles of automated Edman

degradation of the desulfurized peptide gave the sequence shown in Table V.

This sequence included the blocked N-terminal amino acid residue which was unblocked by desulfurization as discussed in Materials and Methods. The homology of this sequence to that of the mouse (Garrick et al., 1975) is very obvious. The only difference in the sequences so far determined is at residue 12 of the peptide (see Table V). In the guinea pig β-globin chain this residue is lysine, whereas in the mouse it is arginine. The N-terminal amino acid of the desulfurized peptide is alanine, thus suggesting that desulfurization of an acyliminothiazolidine ring yields alanine as the new N-terminal amino acid residue.

TABLE V.

Cycle Residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Guinea pig peptide (desulfurized)	Ala	Asp	Lys	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Lys	Leu	Leu
Mouse (Garrick et al., 1975)	Cys	Asp	Lys	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg	Leu	Leu

Discussion

The present work, together with preceding results, confirms the observation of Degani et al. (1970) that NTCB can be a highly efficient, specific reagent for cleavage of proteins and peptides at cysteinyl residues. However, the blocked peptides obtained from the C terminal are not susceptible to Edman degradation procedures. A method to unmask these peptides is reported here.

Although some other preparations of Raney nickel were tried in search of a better catalyst (personal observation), the W-6 Raney nickel preparation was appreciably more efficient. Studies done at different pHs indicate that desulfurization at pH 7.0 and 8.0 were relatively fast and gave a single reaction product from each cystine (or cysteine) and methionine as shown in Table IIA and B. Also reaction at pH 7.0 or 8.0 proved more efficient for desulfurization of cystine at all temperatures tested than lower pH values, while the rate of desulfurization of free methionine remained unchanged. The modification of methionine residues in hexokinase and phosphoglycerol kinase had different kinetics. In this case, methionine was completely desulfurized in 6 h suggesting a discrete desulfurization mechanism. However, it should be noted that at lower pH values and in distilled water or methanol, the rate of desulfurization of methionine residues in proteins was not significantly different from that obtained at high pH and temperature for simple methionine residues (Tables I and III).

Desulfurization in methanol in this study was the same as that obtained with distilled deionized water at pH 5.5. The influence of temperature on the rate of desulfurization was observed at all pHs studied. Desulfurization at pH 7.0 and at 50 °C was fast and entirely specific for cystine (or cysteine) and methionine. As expected, cysteic acid could not be desulfurized under any conditions used here.

Work with amino acid mixtures and also with hexokinase and phosphoglycerol kinase as model proteins showed that all other amino acids remained unchanged. Tryptophan, tyrosine, and phenylalanine were recovered undamaged as indicated in Tables IIA and B. This supports the findings by Gassman & Amick (1975) that the desulfurization of benzofurans did not affect the ring structure.

Since cysteic acid was not desulfurized in this study, we concluded that the low yield of desulfurization of an acyliminothiazolide peptide in air was due to air oxidation of the thiazolidine ring. When oxygen was flushed out with nitrogen, and the reaction carried out under this atmosphere, there was a great improvement in yield of the desulfurized peptide (90%) as observed by sequence analysis (Smithies et al., 1971).

The results of the present study taken together with those of Degani et al. (1970) clearly demonstrate that NTCB specifically cleaves proteins and peptides at cysteinyl residues and that the peptides produced with blocked N-terminal residues can be successfully unblocked by Raney nickel. On the basis of this finding, we believe that this technique can routinely be used to obtain specific cleavage of peptides and proteins at cysteinyl residues for sequence analysis. Since cysteine is a relatively rare amino acid, this procedure should be fully as useful as cyanogen bromide cleavage at methionine residues for the generation of large peptides. In addition, the desulfurization of proteins by Raney nickel may potentially be a powerful diagnostic test for enzymes believed to be sulfur-dependent in their enzymic activity.

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Acetylcholine Receptor and Ionic Channel of *Torpedo* Electroplax: Binding of Perhydrohistrionicotoxin to Membrane and Solubilized Preparations†

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ABSTRACT: The electric organ of the ray, *Torpedo ocellata,* can serve as a source for both the acetylcholine (ACh) receptor and its ionic channel. The two entities were identified by their specific binding of [^{3}H]ACh and [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX), respectively. Binding of [^{3}H]H_{12}-HTX was inhibited by certain drugs and toxins, e.g., histrionicotoxin (HTX), amantadine, and tetraethylammonium (TEA) ions at concentrations that did not inhibit [^{3}H]ACh binding. However, the specific carbamoylcholine-induced ^{22}Na efflux from microsacs from the electric organ membranes was blocked by inhibitors of either the receptor or its ionic channel. The ionic channel had the properties of a protein as judged by heat sensitivity and the inhibition of [^{3}H]H_{12}-HTX binding, after incubation of the electric organ membranes with protein reagents such as *p*-chloromercuribenzenesulfonic acid (PCMBS) or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The "binding" of [^{3}H]H_{12}-HTX at 4×10^{-8} M to lipids in the microsacs was 12% of the total binding to intact microsacs and was nonsaturable and insensitive to heat or specific drugs. After solubilization with cholate, the [^{3}H]H_{12}-HTX binding subunits retained the same affinities for toxins and drugs. The K_d for [^{3}H]H_{12}-HTX was 3×10^{-7} M. The majority of the ionic channel could be separated from the ACh receptors in the cholate extract by incubation with ACh-receptor affinity gel and ACh-receptor antibodies. The ACh receptor purified by this affinity gel contained only a few active ionic channel units as judged by low levels of high affinity binding of [^{3}H]H_{12}-HTX. On the other hand, after solubilization with Triton X-100, all the ionic channel molecules were either separated or denatured so that the purified ACh receptor did not exhibit high affinity binding for [^{3}H]H_{12}-HTX.

A basic assumption in nicotinic neuromuscular transmission is that binding of acetylcholine (ACh)[1] to receptor sites induces a change in conformation of the receptor, which in turn causes a channel to open for an average duration of a millisecond (Katz & Miledi, 1972; Neher & Stevens, 1977). It is suggested that at the endplate, Na^+ and K^+ currents are simultaneously "gated", so that there is only one channel type for both cations (Dionne & Ruff, 1977). The terms "ionophore" (Bon &

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[1] Abbreviations used: ACh, acetylcholine; HTX, histrionicotoxin; H_2-HTX, dihydroisohistrionicotoxin; H_8-HTX, octahydrohistrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; TEA, tetraethylammonium; DTT, 1,4-dithiothreitol; PCMBS, *p*-chloromercuribenzenesulfonic acid; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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