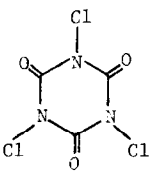
Tetrahedron Letters No. 49, pp 4893 - 4896, 1973. Pergamon Press. Printed in Great Britain.

REACTION OF AMINO ACIDS AND PROTEINS WITH TRICHLOROISOCYANURIC ACID

M.Z. Atassi
Department of Chemistry, Wayne State University, Detroit, Michigan 48202

(Received in USA 4 September 1973; received in UK for publication 22 October 1973)

The purpose of the present work was to study the applicability of trichloroisocyanuric acid (TCIC)[1] to oxidation of the thioether side-chain of methionine as a possible approach for modification of methionine residues in proteins. Reaction of TCIC with amino acids was studied under various conditions of temperature and pH to determine those under which the reagent acted with specificity. Hen egg white lysozyme and hen egg albumin were used as the protein models. TCIC was stored in the dark under anhydrous conditions at 0°.



TCIC

In a preliminary experiment, to a magnetically-stirred solution (2 ml) of methionine (40 mg) in water at pH 3.5 was added a solution (1 ml) of TCIC (1.8 g TCIC in 6 ml of acetone). The pH was kept constant by the addition of 2 N NaOH on a pH-stat. After 2 hr reaction at 0°, thin layer chromatography (Eastman chromatogram sheets; silica gel) in n-butanol-acetic acid-water (4:1:5 v/v) showed that no methionine (R_f, 0.42) was present and it converted entirely to a single new product (R_f, 0.123) which coincided with authentic methionine sulfone (R_f, 0.129).

To examine the selectivity of the reaction, a solution (2 ml) of equimolar quantities of 19 amino acids was reacted with an aliquot (300 µl) of TCIC (106 mg in 2.2 ml acetone) at pH 3.5 on the pH-stat. Aliquots (200 µl) were examined at intervals by amino acid analysis. Methionine, cystine, tyrosine and tryptophan disappeared completely (100%) within 4 min of reaction, while histidine suffered a very appreciable modification (0 time, 1.00; 4 min reaction, 0.26; 10 min, 0.17 µmole). The disappearance of methionine was accounted for partly by the appearance of methionine sulfone (0.43 µmole) and only a trace (less than 0.1 µmole) of methionine sulfoxide. Although there was some indication of cysteic acid formation after 4 min of reaction, the main reaction product of cystine appeared under glycine. In addition, at least five other new reaction products appeared on the analyzer. Under these conditions aspartic and glutamic acids reacted quite rapidly (Asp: 4 min, 0.42; 10 min, 0.32; 45 min, 0.24 µmole; Glu: 4 min, 0.73; 10 min, 0.51; 45 min, 0.29 µmole). In fact all other amino acids reacted more slowly at varying rates.

Reaction of the amino acid mixture with similar excess of TCIC at pH 7.0 and 0° resulted in com-

plete disappearance of cystine, methionine and tryptophan within 1.5 min of reaction. No other amino acids were modified even after reaction for 45 min. Methionine was accounted for partially by the appearance of methionine sulfone (0.2 μmole) and some sulfoxide (0.1 μmole). The reaction product of cystine eluted at a position which coincided with that of glycine. In addition, a reaction product appeared 6 min before aspartic and another product coincided with proline.

For reaction of proteins with TCIC, a solution (2 ml) of the protein (30 mg), at 0°, was adjusted to the appropriate pH (pH 3.5 or 7.0) with 2 N NaOH. An aliquot (100 μl) of TCIC solution (100 mg of TCIC in 2 ml acetone) was added very slowly to the magnetically-stirred protein solution and the pH was kept constant by the addition of 2 N NaOH on the pH-stat. After reactions for 2 hr at 0°, samples were centrifuged (5000 rpm, 0°, 20 min), dialyzed against distilled water and then freeze-dried. Separate hydrolyses in constant boiling HCl[2] and for tryptophan determination[3] were carried out. Reaction of lysozyme with TCIC at pH 3.5 and 0° was entirely non-specific and resulted in complete modification of methionine, cystine, tyrosine and tryptophan residues. Methionine and cystine were oxidized quantitatively to methionine sulfone and cysteic acid respectively. The product from reaction of lysozyme at pH 7.0 and 0° gave, after dialysis, an insoluble fraction which was freeze-dried, hydrolysed and analyzed separately. In the insoluble fraction (about 15 % of the total), methionine was completely oxidized to methionine sulfone and the six tryptophan residues suffered a great degree (90 %) of modification (i.e. 0.64 residues were unmodified). Partial oxidation of the cystine residues (2.52 residues out of 8) was also encountered. In the soluble fraction, the reaction was entirely confined to oxidation of the two methionines to the sulfone (Met, 0; $MetO_2$, 1.93 residues) and 90% of the six tryptophans (Trp, 0.63 residues). No other amino acids were modified.

Action of TCIC on egg albumin at pH 3.5 and 0° resulted in oxidation of all 16 methionine residues primarily to the sulfone. The five cysteine residues were quantitatively oxidized to cysteic acid. Also, the tryptophans were completely modified. In addition, drastic modification of the tyrosines (decreased from 9.57 residues in albumin to 1.88 after reaction with TCIC) and histidines (decreased from 7.16 to 2.32 residues) was obtained. On the other hand in reaction at pH 7.0, the modification was almost restricted to the methionines (decreased from 16.2 to 0 residues) and the tryptophans (decreased from 2.81 to 0 residues). Methionine was primarily oxidized to its sulfone (14.3 residues). Only a slight modification of cysteine (0.69 residue, about 14 %) was observed.

At acid pH, modification of aspartic and glutamic acids (and of other amino acids on long reaction periods) was presumably due to the formation of acid chlorides which may be favored close to the pK of the carboxyl group. This was completely avoided when reaction was carried out at pH 7.0. Also, at pH 7.0 modification of tyrosine and histidine did not take place. Therefore, action of TCIC

on free amino acids at pH 7.0, exhibited good selectivity for methionine, cystine and tryptophan.

Oxidation of ethers of formula $R-CH_2-O-R'$ to carboxylic acid esters $R-\overset{O}{\overset{\|}{C}}-O-R'$ has been achieved in good yield by reaction with TCIC in the presence of excess water[4]. In contrast, the present findings show that oxidation of the thioether side-chain to the corresponding thioester does not take place since, as expected, oxidation of the sulfur in thioethers is greatly favored. The formation of methionine sulfone was appreciably higher at pH 3.5 than at pH 7.0, and it was the only reaction product detected by thin layer chromatography. Most likely, the first reaction product of methionine will be an adduct between the thioether sulfur and a nitrogen in the heterocyclic ring of the reagent. This adduct is slowly hydrolyzed at pH 3.5 and more rapidly in the acidic solvent during thin layer chromatography to yield predominantly the methionine sulfone. In protein derivatives, strong acid hydrolysis converts this adduct quantitatively into methionine sulfone.

Reaction of cystine with TCIC at pH 3.5 or at pH 7.0 should give rise to the thiolsulfonate (i.e., cystine dioxide, $Cy.SO_2.S.Cy$), which eluted on the analyzer under glycine. The formation of cystine dioxide has been demonstrated by the action of other oxidizing agents on cystine[5-9]. The disulfide dioxides were in fact the first intermediate oxidation products to be prepared from disulfides[10,11]. The thiolsulfonate structure of disulfide dioxides has been very well confirmed[12-15]. Cystine dioxide is very difficult to obtain in the pure form since interconversion to the isomer (the disulfoxide, Cys.SO.SO.Cy) can take place during isolation[16]. At any rate, acid hydrolysis in 6 N HCl converts cystine dioxide to cysteic acid[17,18]. This explains why in the case of oxidized lysozyme only cysteic acid is detected after acid hydrolysis of the derivative. In the case of proteins containing thiol groups which cannot favorably form disulfides on oxidation (e.g., egg albumin), the thiol group will be oxidized to cysteic acid.

Reaction of TCIC with tryptophan will, most likely, lead to the formation of an oxindole derivative. The complete oxidation of tryptophan at pH 7.0 in proteins may be useful for cleavage at tryptophyl peptide bonds if oxidation is followed by mild acid treatment (e.g., 0.1 N HCl, room temperature). This will be analogous to cleavage of tryptophyl peptide bonds by very mild acid treatment after oxidation of tryptophan with periodate[19]. However, since with TCIC oxidation of tryptophan residues in proteins proceeds to completion, acid treatment should result in higher cleavage yields than those obtained with periodate oxidation. This is being investigated.

The remarkable narrowing down in specificity of TCIC reaction, observed with free amino acids on increasing pH from 3.5 to 7.0, reproduced quite well with proteins. The amino acids modified in the two proteins were those expected from the behavior of the mixture of free amino acids. Egg albumin was chosen because it has a high methionine and thiol group contents. Lysozyme on the other hand,

has a high tryptophan content and carries only disulfide bonds and no cysteine residues. Therefore, selectivity of the reaction at pH 7.0 for methionine and tryptophan which has been demonstrated here on two sufficiently different proteins will, most likely, be of general applicability to other proteins.

The author would like to thank Miss Noemi Heckenast for her technical assistance. The work was supported by a grant (AM-13389) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service; and in part by a grant (71-910) from the American Heart Association.

REFERENCES

1. Stenberg, V.I., Olson, R.D., Wang, C.T. and Kulevsky, N. (1967) J. Org. Chem. 32, 3227.
2. Atassi, M.Z. (1968) Biochemistry 7, 3078.
3. Liu, T.Y. and Chang, Y.H. (1971) J. Biol. Chem. 246, 2842.
4. Juenge, E.C. and Beal, D.A. (1968) Tetrahed. Lett. 5819.
5. Toennies, G. and Lavine, T.F. (1936) J. Biol. Chem. 113, 571.
6. Emiliozzi, R. and Pichat, L. (1959) Bull. Soc. Chim. Fr. 1887.
7. Nischwitz, E. (1963) Z. Anal. Chem. 193, 190.
8. Consden, R. and Gordon, A.H. (1950) Biochem. J. 46, 8.
9. Toennies, G. (1934) J. Amer. Chem. Soc. 56, 2198.
10. Gilman, H.H., Smith, L.H. and Parker, H.H. (1925) J. Amer. Chem. Soc. 47, 851.
11. Connor, R. (1943) in "Organic Chemistry" (Gilman, H., ed.), p. 905, Wiley, New York.
12. Cymerman, J. and Willis, J.B. (1951) J. Chem. Soc. 1332.
13. Allen, P., Berner, P.J. and Malinowski, E.R. (1963) Chem. and Ind. 208.
14. Crenshaw, R.R. and Owen, T.C. (1961) Proc. Chem. Soc. 250.
15. Sweetman, B.J. (1959) Nature 183, 744.
16. Savige, W.E. and Maclaren, J.A. (1966) in "The Chemistry of Organic Sulfur Compounds" (Kharasch, N. and Meyers, C.Y. eds.) Vol. 2, p. 367, Pergamon, London.
17. Maclaren, J.A., Leach, S.J. and Swan, J.M. (1960) J. Text. Inst. 51, T665.
18. Schirlé, C. and Meybeck, J. (1952) C.R. Acad. Sci, Paris 235, 298.
19. Atassi, M.Z. (1967) Arch. Biochem. Biophys. 120, 56.