THE CONVERSION OF TRYPTOPHAN TO 2-THIOLTRYPTOPHAN IN PEPTIDES AND PROTEINS

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SUMMARY

A method is described for the quantitative removal of 2,4-dinitrophenyl groups from 2,4-dinitrophenylsulfenyl-tryptophan residues in model compounds and proteins. The cleavage of 2,4-dinitrophenyl from modified tryptophan is carried out using mercaptoethanol as a thiolysis reagent. This method can be used for the introduction of additional sulfhydryl groups into proteins after modification of the tryptophan residues with 2,4-dinitrophenylsulfenyl-chloride.

Dinitrophenylsulfenyl-chloride (DNPS-Cl) has been widely used in protein chemistry for the selective modification of tryptophan to form the 2-thioether derivative (1,2,3). It was recently shown that the dinitrophenyl group of S-DNP-cystein, O-DNP-tyrosine and Im-DNP-histidine can be displaced by thiols under very mild reaction conditions (4,5). This study describes a method for the quantitative removal (thiolysis) of the dinitrophenyl group from DNPS-tryptophan in peptides and proteins giving 2-thioltryptophan derivatives.

MATERIALS AND METHODS

Subtilisin Carlsberg, human serum albumin (HSA), and horse heart cytochrome C were purchased from Sigma Chemical Co. DNPS-Cl was obtained from Fluka AG. S-DNP-2-mercaptoethanol was a gift of Dr. S. Shaltiel. DNPS derivatives of HSA, subtilisin and tryptophan were prepared according to Scoffone et al. (1) The pentapeptide Gly-Ilu-Thr-Trp(DNPS)-Lys was isolated from DNPS-cytochrome C (6)

abbreviations: DNPS-Cl, 2,4-dinitrophenylsulfenyl-chloride; Trp(DNPS), 2,4-dinitrophenylsulfenyl-tryptophan; DNP, 2,4-dinitrophenyl; HSA, human serum albumin.

on an anti DNP-Sepharose column (7). 2-Thioltryptophan was prepared according to Wieland et al. (8). Di-tryptophan-2-disulfide were prepared from tryptophan by the method of Wieland et al. (8) or by the oxidation with air of 2-thioltryptophan. Amino acid analyses were performed essentially as described by Moore and Stein (9) using a one column system. Amino acids and peptides were located by thin-layer chromatography (TLC) on silica gel (from Riedel-De Haen AG) using the following solvent systems. 1-butanol-acetic acid-water [4:1:1(v/v)] and 1-butanol-acetic acid-pyridine-water (30:6:24:20) by staining with 0.25% ninhydrin or Erlich reagent. Absorbance was read at appropriate wavelengths using a PMQ (Zeiss) or Cary 14 spectrophotometer.

RESULTS

DNPS-tryptophan was incubated with 100-fold molar excess of 2-mercaptoethanol at pH 8 for 4 hr at room temperature and applied on TLC. Only two compounds were observed. One of the compounds was yellow and moved similarly on the TLC to the synthetic S-DNP-2-mercaptoethanol. The other spot migrated identically to 2-thioltryptophan using a synthetic sample as a marker (8). The products of the reaction were also characterized spectrophotometrically. Extraction of the

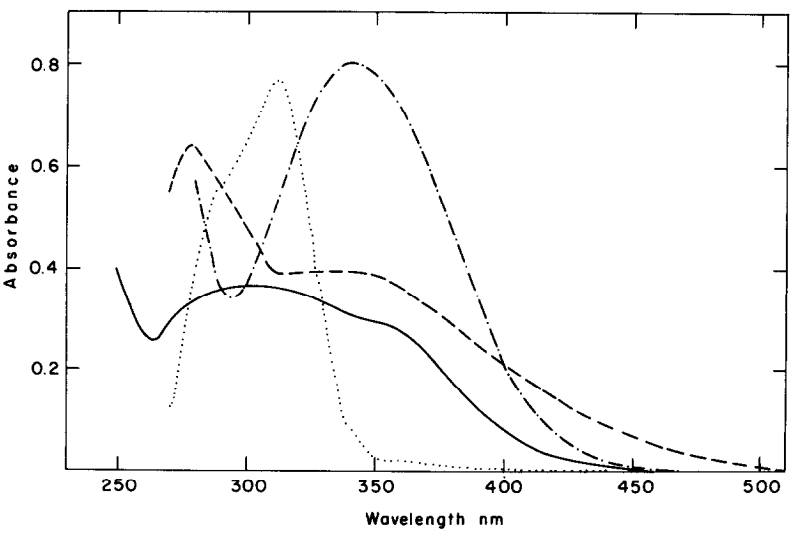


Fig. 1. Ultraviolet absorption spectrum of DNPS-tryptophan (– · –); 2-thioltryptophan (·····), Di-tryptophan-2-disulfide (——); and S-DNP-2-mercaptoethanol (– · –). All the samples were dissolved in 0.1 M NH_4HCO_3.

S-DNP-2-mercaptoethanol with ethyl acetate allowed an absorption scan of each of the reaction products. (Fig. 1). The 2-thioltryptophan has a characteristic absorption maximum at 313 nm, ε = 10.500.

Chromatography of the thiolysis mixture on a 16 cm column of the amino acid analyzer operated at 50° revealed a substance which eluted in the position of the synthetic 2-thioltryptophan. 2-thioltryptophan emerges between the hydrophobic amino acids and lysine at approximately 52 min. 2-thioltryptophan is not stable to conditions commonly used for hydrolysis of proteins, and a method for its detection from protein hydrolysates is not yet available. Finally, further chemical proof that the compound obtained was 2-thioltryptophan was that after reaction with 1-fluoro-2,4-dinitrobenzene at pH 5, DNPS-tryptophan was regenerated.

The pentapeptide Gly-Ilu-Thr-Try(DNPS)-Lys was reacted with 50-fold molar excess of 2-mercaptoethanol, and the thiolysis was followed spectrophotometrically Thiolysis of this peptide results in an increase of the u.v. absorption at 313 nm when scanned against DNPS-tryptophan as a blank, presumably due to the formation of thioltryptophan. (fig. 2A) After extraction of the S-DNP-2-mercaptoethanol with ethyl acetate, a pentapeptide containing thioltryptophan was isolated (Fig. 2B). This peptide had a spectrum different from both the DNPS-pentapeptide and the pentapeptide containing unmodified tryptophan.

In order to show that the thiolysis can be performed on more complex macromolecules, this reaction was checked on several proteins. The single tryptophan residue of HSA was modified with DNPS-Cl (6). The modified protein was treated with 100-fold excess of mercaptoethanol and the reaction was followed spectrophotometrically using DNPS-HSA as a blank. In contrast to the peptide models, the increase in the u.v. absorption was at 320 nm and not at 313 nm. This red shift may indicate that the sole tryptophan residue in HSA is buried. Gel filtration of the yellow mixture on Sephadex G-25 gave a colorless solution of protein and yellow S-DNP-2-mercaptoethanol.

The thiolysis was also performed on DNPS-subtilisin and the formation of

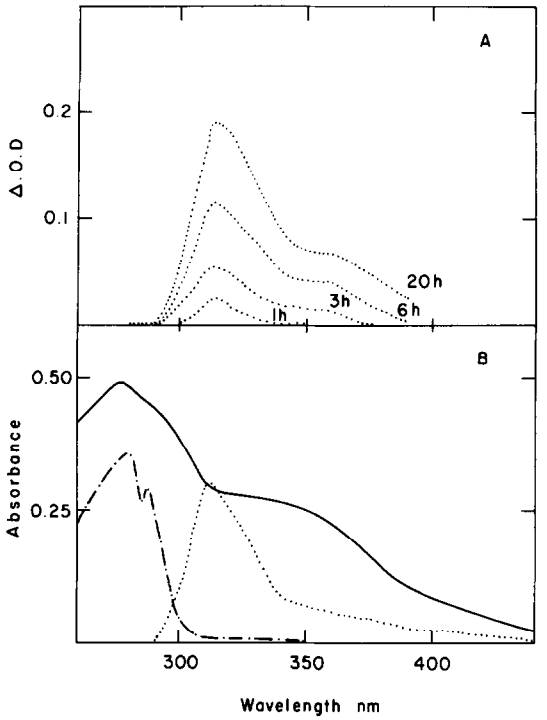


Fig. 2. (A) Changes of the u.v. spectrum with time of DNPS-pentapeptide (Gly-Ilu-Thr-Trp-Lys) upon thiolysis with 50-fold molar excess of mercaptoethanol (B) The ultraviolet absorption spectrum of pentapeptide (—·—); DNPS-pentapeptide (——); and 2-thioltryptophan pentapeptide (·····). All the samples were dissolved in 0.1 M NH_4HCO_3.

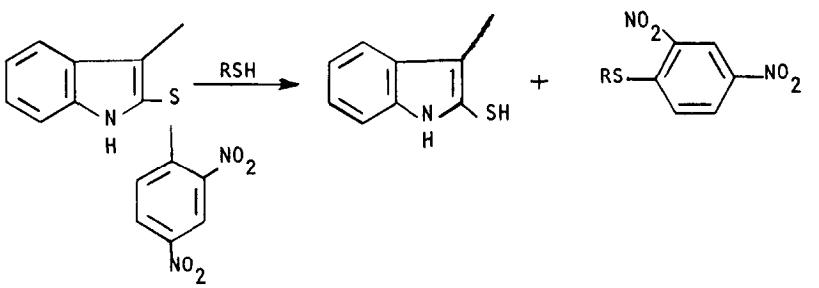
thioltryptophan could be followed at 313 nm. Since there is no red shift in the u.v. the thioltryptophan residue in subtilisin may be considered as "normal" and not buried. On extensive dialysis against 0.1 M NH_4HCO_3 the thioltryptophan-subtilisin precipitated. This precipitate was found by polyacrylamide gel electrophoresis to be approximately twice the molecular weight of subtilisin. Presumably the thioltryptophan was oxidized to the disulfide bridge resulting in the formation of dimers of the protein. The modified subtilisin had no enzymatic activity since the reaction with DNPS-Cl was carried out at acidic pH, under which condition subtilisin is irreversibly inactivated.

DISCUSSION

2-Nitro and 2,4-dinitrophenylsulfenyl-chloride are widely used in

modification of tryptophan residues in protein (1,2). The reaction can be easily quantitated by spectrophotometry since the nitrophenylsulfenyl group absorbs radiation in the visible part of the spectrum. The modification of tryptophan with these reagents introduces a bulky group into the protein. Conversion of the Trp(DNPS) to 2-thioltryptophan, even though it is not completely reversible, results in the removal of the bulky aromatic nitrophenyl group and consequently only a minor modification in tryptophan residues present in the binding or active sites of proteins is obtained. This method may enable the determination of whether the loss of activity induced by DNPS-Cl is due to the chemical modification of tryptophan residues or to steric blockage of the active site.

Schematically, the cleavage that occurs can be described as follows:



In view of the mild conditions under which the thiolysis proceeds, this chemical modification may find a variety of applications in the study of tryptophan residues in proteins. The introduction of thioltryptophan into the primary sequence of proteins provides the possibility for further modification. The thioltryptophan can react with heavy metals e.g. mercury. Such a modification gives another possible procedure to prepare derivatives for X-ray diffraction. Reaction with iodoacetic gives the carboxymethyl derivative of thioltryptophan while mild oxidation with air introduces disulfide bridges. In subtilisin, which has only one tryptophan, we were able to isolate the oxidized dimer. The reaction of proteins with bifunctional reagents leading to cross-linking may provide an important tool for the determination of the topography and inter-residue distances in the folded protein. The selective modification of the

thiol group of thioltryptophan can be achieved at low pH, then the pH of the reaction mixture can be raised allowing reaction with other active side chains.

The ready formation of thioltryptophan by thiolysis of Trp(DNPS) derivatives described here increases the number of procedures available for the modification of tryptophyl residues in proteins providing new permutations in the study of structure-function relationships of tryptophan containing enzymes. The effect of such modification on different proteins is under investigation.

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