REACTION OF SULFENYL HALIDES WITH CYTOCHROME *c*. A NOVEL METHOD FOR HEME CLEAVAGE

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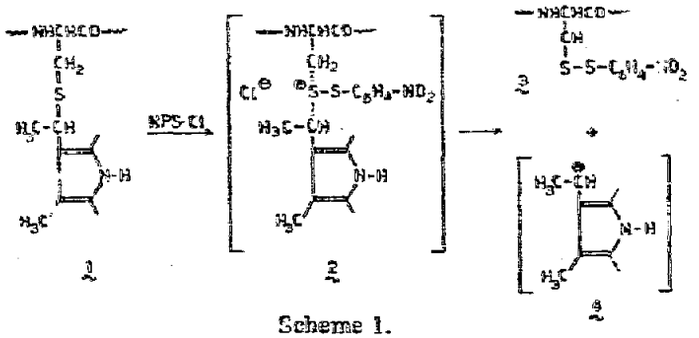
1. Introduction

The heme group of cytochrome *c* is linked to the polypeptide chain by thioether bridges [1]. Methods presently used for heme cleavage involve the use of heavy metals such as silver and mercury [2, 3], performic acid [4], or, as recently reported, iodine and cyanogen bromide [5].

Attempting to selectively modify the single tryptophan residue of horse heart cytochrome *c* by sulfenyl halides [6], we found that these reagents also allow a rapid and quantitative cleavage of the thioether bonds linking the heme to the protein.*

Sulfenyl halides are known to react with unsymmetrical thioethers giving rise to disulfides among other products depending upon the structure of the thioethers [7, 8]. These reagents have found an application in the removal of S-protecting groups in peptide synthesis [9].

The results herewith reported indicate that 2-nitrophenylsulfenyl chloride (NPS-Cl)** reacts (see scheme)



Scheme 1.

with the thioether function of cytochrome (1) to form a thiolsulfonium ion (2) which decomposes to a mixed disulfide, S-NPS-cysteine derivative (3). Cleavage is successfully effected due to the easy formation of the carbonium ion (4) stabilized by the conjugated porphyrin system.

* The results here reported were in part presented at the 12th Eur. Peptide Symposium, Schloss Reinhardsbrunn, September 1972.

Abbreviations:

NPS, 2-nitrophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; NCPS, 2-nitro-4-carboxyphenylsulfenyl; NPS_3-cytochrome *c*, derivative with two NPS groups linked to the two cysteine residues and one to the tryptophan residue; NPS_1-cytochrome *c*, derivative with the NPS group linked to the tryptophan residue.

2. Experimental

2.1. *Materials*

NPS-Cl and DNPS-Cl were obtained from Fluka AG (Basle, Switzerland). NCPS-Cl was synthesized according to the literature [10]. S-NPS-glutathione [11] and 2-NPS-tryptophan [12] were synthesized as described previously.

Horse heart cytochrome *c* (type III) was obtained from Sigma, Chem. Company (St. Louis, Mo., USA). The heme undecapeptide (sequence 11–21) was prepared by peptic digestion of cytochrome *c* following

the procedure described by Harbury and Loach [13]. The peptide was further purified on a Sephadex G-25-SF column (2.8 × 140 cm) equilibrated and eluted with 10% acetic acid. The heme peptide corresponding to the sequence 1–38 of horse heart cytochrome *c* was prepared by trypsin digestion of N_ϵ-maleyl-cytochrome *c* and subsequent deacylation in 30% acetic acid at 37° for 40 hr. The peptide was purified on a Sephadex G-50-SF column (2.8 × 140 cm) and then by ion exchange chromatography on a CM-cellulose (Serva, Heidelberg, Germany) column (1.6 × 12 cm) equilibrated with 0.01 M ammonium acetate buffer, pH 6.5. The peptide was eluted by an exponential gradient from 0.01 M to 0.5 M ammonium acetate buffer, pH 6.5. The peptides were homogeneous on thin-layer chromatography on cellulose plates (Merck AG, Darmstad, Germany) with butanol: pyridine: acetic acid: water (12:24:4:30) as eluent.

2.2. *Sulfenylation of cytochrome c and related heme peptides.*

The reaction was carried out by dissolving the protein or peptide (3–5 mg/ml) in 50% acetic acid and then adding with stirring 100 equivalents of the sulfenyl chloride dissolved in glacial acetic acid (final solvent conditions 66% acetic acid). The reaction was followed by thin-layer chromatography. After 10 min stirring at room temp., the reaction mixture was diluted three times with water and then extracted several times with ethyl acetate. The aqueous layer was concentrated *in vacuo* at 37° and then applied to a Sephadex G-25-SF column (1.6 × 20 cm) equilibrated and eluted with 10% acetic acid. The modified protein or peptides were located in the eluate via their absorption at 280 and 365 nm.

2.3. *Removal of the sulfenyl chromophore from the cysteine residues*

The sulfenylated protein or peptide (0.5–1 μmole) was dissolved in 1 ml of 8 M urea, pH 7.2, containing 0.1% β-mercaptoethanol. After 5 min at room temp. the solution was directly applied to a Sephadex G-25-SF column (1.6 × 20 cm) equilibrated and eluted with 10% acetic acid. The NPS_1-cytochrome was located in the effluent by spectrophotometric reading at 365 nm, and the peptides by the reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent) [14].

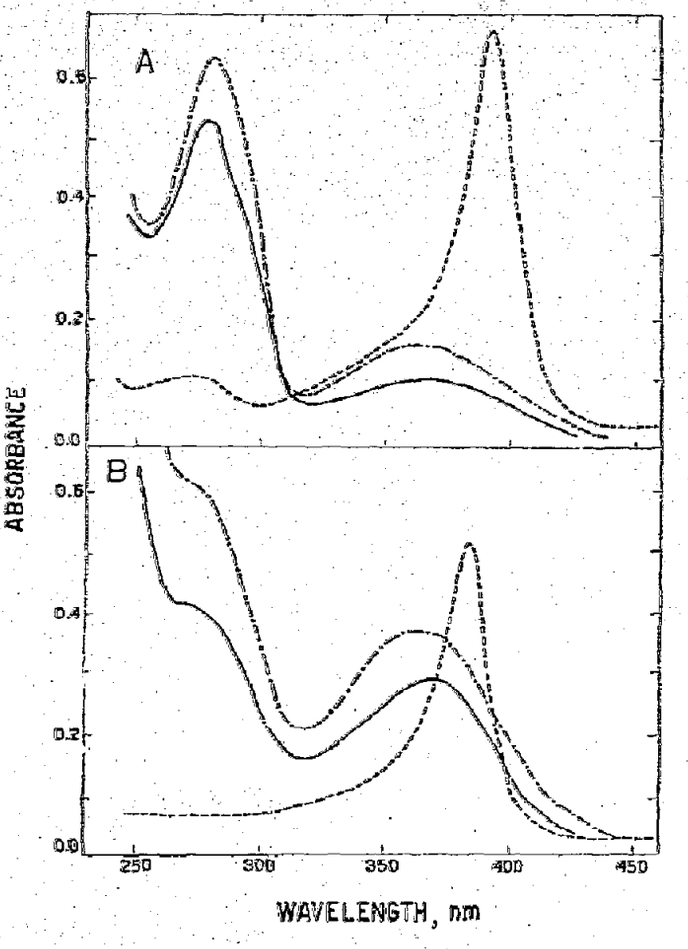


Fig. 1. A) Absorption spectra in 10% acetic acid of cytochrome (-----), NPS_1-cytochrome (——) (obtained by reduction of NPS_3-cytochrome; see Experimental) and of 2-NPS-tryptophan (-·-·-). B) Absorption spectra of the peptic heme undecapeptide (sequence 11–21) (-----), of its S-NPS-derivative (——) and of S-NPS-glutathione (-·-·-).

3. Results

Kinetic analysis of the reaction of sulfenyl halides with the thioether functions of cytochrome and heme peptides by thin-layer chromatography showed that the removal of the heme in 50% acetic acid with 50–100 equivalents of NPS-Cl occurs quantitatively in less than 1 min. The S-sulfenyl derivatives were easily separated from the heme, excess reagent and side products by gel filtration on Sephadex G-25.

The thiol function of cysteine is easily restored from the mixed disulfides (3) by the general procedure of reduction of disulfide bonds by thiols extensively used in protein chemistry [15]. The treatment

Table 1
Amino acid composition of cytochrome and heme peptides reacted with NPS-Cl and then reduced with β-mercaptoethanol in 8 M urea (theoretical values are in parentheses).*

Amino acid	NPS-cytochrome	1–38	11–21
Lysine	18.1 (19)	6.5 (7)	1.1 (1)
Histidine	3.1 (3)	2.5 (3)	1.0 (1)
Arginine	2.2 (2)	0.9 (1)	
Aspartic acid	8.3 (8)	1.9 (2)	
Threonine	10.2 (10)	1.9 (2)	0.9 (1)
Glutamic acid	12.6 (12)	4.0 (4)	2.8 (3)
Proline	4.3 (4)	1.0 (1)	
Glycine	12.2 (12)	5.9 (7)	
Alanine	6.2 (6)	1.2 (1)	1.1 (1)
Cysteine**	1.7 (2)	1.6 (2)	1.7 (2)
Valine	2.9 (3)	2.5 (3)	1.8 (2)
Methionine	1.7 (2)		
Isoleucine	4.8 (6)	1.1 (1)	
Leucine	6.0 (6)	1.9 (2)	
Tyrosine	3.8 (4)		
Phenylalanine	4.0 (4)	1.8 (2)	

* Composition was determined after 22 hr hydrolysis in sealed tubes under vacuum in 6 N HCl at 110°. Amino acid analyses were carried out with a C. Erba analyzator, Model 3A27. No corrections for destruction or low recovery of amino acid were made. The results are expressed in terms of residues per mole of protein or peptide.
** Determined by the Ellman's reagent [14].

Table 2
Properties of the sulfenylated cytochrome *c* and cytochrome *c* heme containing peptide sequences obtained by reaction of the corresponding protein or peptides with sulfenyl chlorides.

	Derivative	R_f*	λ_{max}** (nm)	ε_M** $\times 10^{-3}$
Cytochrome *c*	NPS_3***	–	363	11.0
	NPS_1****	–	368	4.1
1–38	NPS	0.31	358	7.6
11–21	NPS	0.57	360	8.1
	DNPS	0.60	316	17.5
	NCPS	0.44	353	4.4

* Thin-layer chromatography on cellulose plates (Merck) with butanol:acetic acid:pyridine:water (42:24:4:30). The plates were sprayed with ninhydrin.
** Values determined in 10% acetic acid.
*** Two NPS-groups bound to the two cysteine residues and one to the tryptophan residue.
**** One NPS-group bound at the tryptophan residue.

with sulfenyl halides also sulfenylated the single tryptophan-59 residue at the 2-position of the indole ring [6].

Fig. 1A shows the absorption spectrum in 10% acetic acid of NPS_1-cytochrome, with a single NPS-group on the tryptophan residue. This derivative was obtained by reduction of NPS_3-cytochrome with β-mercaptoethanol in 8M urea and subsequent gel filtration on Sephadex G-25. The spectrum closely resembles that of the model compound 2-NPS-tryptophan. In fig. 1B is shown the absorption spectrum of the NPS-derivative of the undecapeptide 11–21 (two NPS-groups bound at the two cysteine residues), which is similar to the spectrum of the model compound S-NPS-glutathione.

Reaction of sulfenyl halides with cytochrome or the heme peptides 1–38 and 11–21 and removal of the S-sulfenyl groups by reduction does not cause alteration of any other amino acid (table 1). This is in accordance with the known high specificity of the reaction of these reagents with proteins [6].

In addition to NPS-Cl, other arylsulfenyl chlorides were tested, such as DNPS-Cl and NCPS-Cl [6], with similar results in reactivity and efficiency of the reaction. In table 2 are reported the absorption maxima and molar extinction coefficients of the sulfenyl derivatives of cytochrome and heme peptides, as well as their chromatographic behaviour.

Azobenzene-2-sulfenyl bromide, a reagent which has been shown to react selectively with cysteine and not with tryptophan [16], was ineffective in removing the heme from cytochrome. The protein after exposure to 100 equivalents of reagent in 50% acetic acid for 1 hr was recovered unchanged.

Attempts to perform the sulfenylation reaction in the presence of 100 equivalents of indole as a scavenger for tryptophan sulfenylation failed. The heme was removed in low yields and tryptophan was partly modified.

4. Discussion

The reaction of sulfenyl halides with thioethers is a useful alternative to the available methods for heme

cleavage in cytochrome *c* and in heme peptides. Heavy metals are slow in cleaving off the heme and particularly with small heme peptides may not be quantitative [1,4].

Performic acid treatment is a severe procedure, destroying several amino acid side chains [17]. The iodine-cyanogen bromide reaction is not a quantitative procedure and cleaves methionine peptide bonds [5]. These disadvantages are not presented by sulfenyl halides, which react fast and quantitatively and, except for the modification of tryptophan, are specific.

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References

[1] E. Margoliash and A. Scheyter, Adv. Protein Chem. 21 (1966) 114.
[2] K.-G. Paul, Acta Chem. Scand. 5 (1951) 389.
[3] S. Sano and K. Tanaka, J. Biol. Chem. 239 (1964) PC 3109.
[4] E. Margoliash, N. Frohwirt and E. Wiener, Biochem. J. 71 (1959) 559.
[5] F. Lederer and J. Tarin, European J. Biochem. 20 (1971) 482.
[6] A. Fontana and E. Scoffone, in: Methods in enzymology, Vol. 25, eds. C.H.W. Hirs and S.N. Timasheff (Academic Press, New York, 1972) p. 482.
[7] C.G. Moore and M. Porter, Tetrahedron 9 (1960) 58.
[8] M. Oki and K. Kobayashi, Bull. Chem. Soc. Japan 43 (1970) 1223 and 1229.
[9] L. Moroder, G. Borin, F. Marchiori and E. Scoffone, presented at the 12th European Peptide Symposium, Schloss Reinhardsbrunn, September 1972.
[10] A.J. Havlik and N. Kharasch, J. Am. Chem. Soc. 77 (1955) 1150.
[11] A. Fontana, E. Scoffone and C.A. Benassi, Biochemistry 7 (1968) 980.
[12] A. Fontana, F. Marchiori, R. Rocchi, P. Pajetta, Gazz. Chim. Ital. 96 (1966) 1301.
[13] H.A. Harbury and P.A. Loach, J. Biol. Chem. 235 (1960) 3640.
[14] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[15] C.H.W. Hirs, in: Methods in enzymology, Vol. II, ed. C.H.W. Hirs, (Academic Press, New York, 1967) p. 199.
[16] A. Fontana, F.M. Veronese and E. Scoffone, Biochemistry 7 (1968) 3901.
[17] C.H.W. Hirs, in: Methods in Enzymology, Vol. II, ed. C.H.W. Hirs (Academic Press, New York, 1967) p. 197.