THIO-MICHLER'S KETONE, A NEW REAGENT FOR SULFENYL IODIDE AND THIOL PROTEINS

Ludek Jirousek and Morris Soodak
Graduate Department of Biochemistry, Brandeis University
Waltham, Massachusetts 02154

Received June 19,1974

SUMMARY

Halogens oxidize thio-Michler's ketone (I) to an intensely blue disulfide (II), but sulfenyl iodides react with the thioketone (I) to give turquoise green mixed disulfides. The same turquoise colored products are formed from a thiol protein and the blue disulfide of thio-Michler's ketone (II). Both reactions can find use in studies of protein sulfenyl iodide derivatives and of thiol proteins. The high values of molar extinction of the colored compounds result in high sensitivity of the reactions.

Feigl et al. (1) described a sensitive reaction of thio-Michler's ketone (4,4'-bis (dimethylamino) thiobenzophenone) with traces of halogens (eqn.1) and suggested the quinoid formula (II) for the unstable intensely blue product. The formula is used here for the sake of simplicity.* Other positive iodine compounds, e.g. N-iodosuccinimide, ICN etc., also oxidize the thioketone (I) to the blue Feigl's disulfide (II).

On the other hand, sulfenyl iodides do not oxidize (I) to (II), but produce turquoise-green mixed disulfides (III), (eqn.2). The high ϵ_M values of (III) permits sensitive testing for protein sulfenyl iodide derivatives which have been previously (3-6) suggested as possible intermediates in biological iodinations. Evidence has accumulated indicating that sulfenyl iodides are obligatory intermediates of thiol oxidations by iodine (7). The formation of sulfenyl iodide groups was also found in model experiments with peroxidases (8). However, until now methods for the estimation of sulfenyl iodide groups were not quite satisfactory (4,5, 9-11).

The reaction of sulfenyl iodides with thio-Michler's ketone (I) is described. The use of the reagent can be extended to studies of protein thiol

*The blue color is likely to come from resonating forms of the aromatic system, in which the participation of quinone forms is negligible. Comp. ref. (2).

groups, either via the sulfenyl iodide derivative, or directly, using the blue Feigl's disulfide (II) see eqn. 3. Feigl's disulfide reacts with thiol groups in proteins to give also the turquoise colored mixed disulfides (III) by a thiol-disulfide interchange. The analytical applications of these reactions are currently under investigation.

MATERIALS AND METHODS

Thio-Michler's ketone was obtained from Eastman Kodak Co., Rochester, N.Y. Methanolic or dimethylsulfoxide solutions were used and were kept in darkness. The solutions are moderately light sensitive.

The proteins used were commericially available preparations of highest purity and were not further purified.

The solid sulfenyl iodide, N-p-chlorophenyl-2-(benzyloxycarbonamido)-3-iodothio-3-methylbutanamide (12) was obtained by courtesy of Drs. Jerry E. White and Lamar Field, Vanderbilt University, Nashville, Tenn.

Spectral measurements were performed on a Beckman DB Spectrophotometer. The experiments with proteins were run at 0-2°. Water maintained at 0° was circulated through the cuvette assembly. It has since been found that sulfenyl iodide derivatives of β-lactoglobulin and bovine serum albumin are quite stable at room temperature.

The disulfide from thio-Michler's ketone (Feigl's disulfide) (II) was prepared in solution by oxidizing 2.84 mg (0.01 mmole) of thio-Michler's ketone by 0.005 mmole of I_2 in 10 ml CCl_4. The mixture was extracted with thorough shaking 2x with 4 ml of water and the combined extracts were washed 2x with 1 ml portions of CCl_4. The resulting intensely blue solution was used directly.

On titration of thio-Michler's ketone in 33% methanol by I_2 the disulfide is formed quantitatively at a ratio of 2 moles of the thioketone : 1 mole of I_2. The ϵ_M of Feigl's disulfide was estimated as ca. 68.000.

Mixed disulfide of β-lactoglobulin and thio-Michler's ketone via the protein sulfenyl iodide. β-Lactoglobulin (38 mg, 1 μmole, 2 μequiv. of -SH) in 1 ml of 0.1 M acetate buffer, pH 5.1, at 0° was mixed with 2 μmoles of I_2 in water solution and kept for 30 min. (A saturated I_2 solution in water is approximately 1 mM at 10°). Thio-Michler's ketone (2,84 mg., 10 μmoles) in 0.2 ml of dimethylsulfoxide was added into the solution of the protein sulfenyl iodide derivative. After 30 min at 0° the mixture was passed through a Sephadex G-25 column (3x30cm) in the cold room. The green colored protein fraction separated from the unreacted orange colored thioketone and from a small quantity of the blue Feigl's disulfide which had also formed. Yield, 2.036 μmoles of the mixed disulfide = 101.8% of theoretical, was calculated with the use of the ϵ_M value of 70,720.

Mixed disulfides from protein sulfenyl iodides and thio-Michler's ketone at a 1:1 ratio. (Table 1). Protein (0.25 μmoles) in 0.5 ml of 0.1 M acetate buffer, pH 5.1 and an equimolar amount of I_2 in water solution were mixed at 0°. Thioketone (0.25 μmoles in 0.1 ml of dimethylsulfoxide was added as soon as the yellow color of iodine disappeared from the solution. After 5 min, an

aliquot was passed through a Sephadex G-25 column (1x30 cm) equilibrated with the same buffer. Absorbancies of the eluted fractions were measured at 630 and 280 nm. The absorption curves of the most concentrated protein fractions were recorded from 740 to 240 nm. The yields were calculated with the use of the ϵ_M value for the mixed disulfide of β-lactoglobulin and thio-Michler's ketone, since the values for the other proteins are not known yet.

Mixed disulfide of β-lactoglobulin and thio-Michler's ketone from the protein and Feigl's disulfide. β-Lactoglobulin (0.25 μmoles) was dissoved in 0.1 ml of 0.1 M acetate buffer, pH 5.1 and treated at 0° with 1 ml of the blue solution of Feigl's disulfide for 30 min. Then an aliquot was passed through a Sephadex column as above and the green protein fraction was collected for measurements.

Other proteins were used in a similar way.

The dependence of the yield of the mixed disulfide on the ratio of β-lactoglobulin sulfenyl iodide : thioketone. β-Lactoglobulin sulfenyl iodide in 0.1 M acetate buffer, pH 5.1 was prepared from the protein and the aqueous I_2 solution. The use of only 0.2 equivalents of I_2 per 1 equivalent of -SH ensured a complete conversion of the added iodine to sulfenyl iodide groups. The procedure was performed at 0°. In control experiments the absence of free iodine in the solution of the protein sulfenyl iodide derivative was checked spectrophotometrically or with N,N-dimethyl-p-phenylenediamine, which gives a strong purple red color with iodine, but no reaction with the sulfenyl iodide groups.

Then, to a series of aliquots of the solution containing 0.014 μequiv. of the protein sulfenyl iodide and the excess of β-lactoglobulin, amounts of the thioketone as shown in Table 2 were added to make the final volume of each sample 2 ml. $A_{630\ nm}$ values were measured against water. The values of blanks, containing the same amounts of β-lactoglobulin of which no part was converted into the sulfenyl iodide derivative, and of the thioketone, were subtracted. The ϵ_M values for the β-lactoglobulin mixed disulfide were determined as an assymptote for an infinite excess of the thioketone.

Cleavage of the mixed disulfide of β-lactoglobulin and the thioketone with nucleophilic reagents. A solution of β-lactoglobulin-thioketone mixed disulfide prepared as above contained 1.83 mg of the mixed disulfide per 1 ml and had an $A_{630\ nm} = 7.2$. Aliquots of this solution (0.2 ml) were added to a series of 1.8 ml portions of reagent solutions in which the concentrations of the reagent ranged from 10^{-7} to 10^{-1} M. The reagents used were: sodium azide, sodium sulfite, sodium thiosulfate and potassium cyanide. The mixtures were kept at 0° for 30 min and the resulting absorbancies at 630 nm were recorded. The concentrations of the nucleophilic reagent required to cleave 50% of the mixed disulfide were determined from a graphical plot of the experimental data.

RESULTS AND DISCUSSION

On oxidation of thio-Michler's ketone with I_2 the orange maximum at 470 nm disappears and a composite blue band of Feigl's disulfide is formed (Fig.1), with overlapping maxima at 630, 590 and 530 nm. In comparison, the spectra of the protein mixed disulfides (III), (Fig.2), lack the maximum at 530 nm and display a new band at about 450 nm, making the mixed disulfide visibly turquoise-green, whereas the Feigl's disulfide is ultramarine blue. The 450 nm band of the mixed disulfide does not come from the thioketone adsorbed on

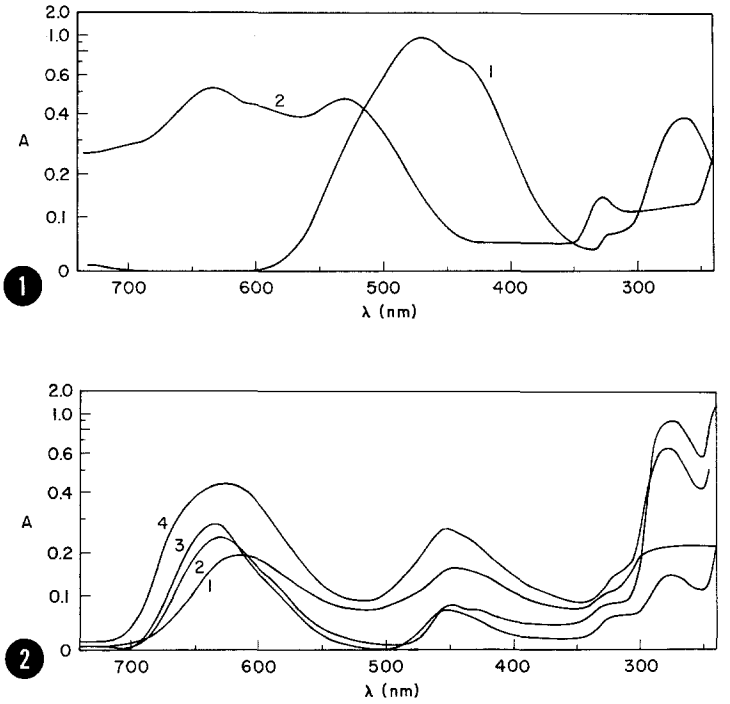
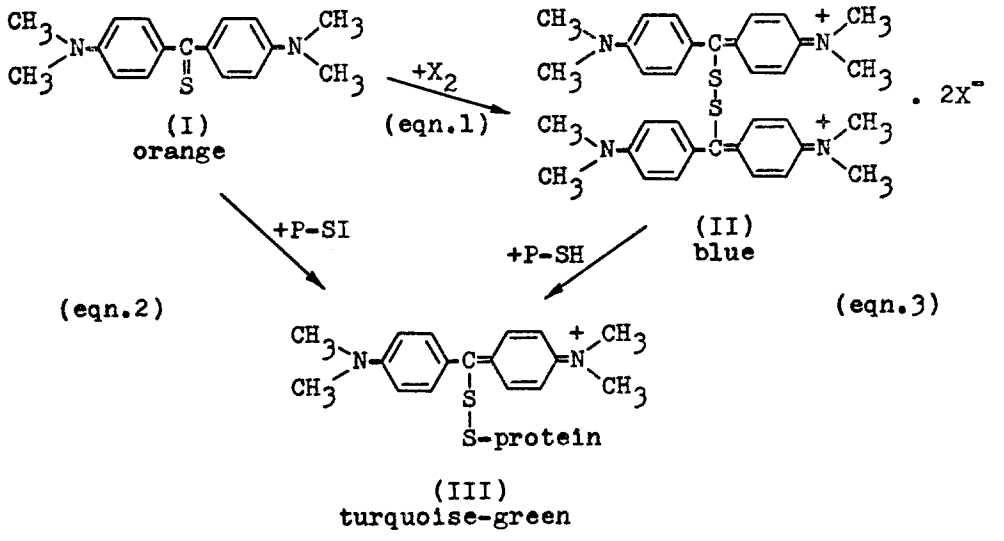


Fig. 1. Absorption spectra of 0.05 mM thio-Michler's ketone in 33% methanol (1) and of approximately 7 μmolar aqueous solution of its blue oxidation product, the Feigl's disulfide, which was extracted from CCl_4 solution of the thioketone and iodine into water.

Fig. 2. Absorption spectra of protein fractions containing the turquoise-green mixed disulfides. The fractions were obtained by gel-chromatography on Sephadex G-25 of the following reaction mixtures: (1), glyceraldehyde-3-phosphate dehydrogenase + Feigl's disulfide; (2), bovine serum albumin + Feigl's disulfide; (3) β-lactoglobulin sulfenyl iodide + thio-Michler's ketone; (4), ovalbumin sulfenyl iodide + thio-Michler's ketone.

protein: the mixed disulfides of β-lactoglobulin or bovine serum albumin could be precipitated by alcohols, acetone or ammonium sulfate with no loss of the 450 nm band, and further attempts to remove it by extractions with organic solvents failed. For comparison, the thioketone adsorbs on a number of proteins to give orange colored products displaying single absorption maxima at 450-455 nm; the adsorbed dye is easily removed by the above procedures.

The A_{630nm} values of the mixed disulfide of β-lactoglobulin slightly decrease with an increasing pH at the same time the A_{450nm} value slightly increases. Above neutrality the colors of both the Feigl's and the mixed



disulfides fade with different velocities. In a refrigerator at pH 5.1 the solution of β-lactoglobulin mixed disulfide gradually faded in a course of several weeks and deposited some rusty colored substance.

The disulfide nature of the above products is suggested by the methods of their preparation. The solid sulfenyl iodide of Field and White (12) also produced a turquoise product with the thioketone. The disulfide nature is further supported by the results of nucleophilic cleavage experiments. A 50% cleavage of the β-lactoglobulin-thioketone mixed disulfide occured at 0° after 30 min. in $10^{-5.7}$M $S_2O_3^=$, $10^{-4.8}$M $SO_3^=$, $10^{-4.5}$M CN^- and $10^{-3.2}$M N_3^-. These strong nucleophilic activities of the above reagents are similar to data obtained with the mixed disulfide of β-lactoglobulin and ^{14}C-thiouracil (9). Potassium iodide also displayed a significant nucleophilic activity toward the Feigl's and the mixed disulfides, which is also similar to the cleavage of β-lactoglobulin-^{14}C-uracil disulfide by iodide. This is in contrast to the data on the low nucleophilic activity of iodide toward more simple disulfides (13). Mercaptoethanol, cysteine or thioureylene compounds also destroyed the colored disulfides; interchange reactions leading to turquoise-green mixed disulfides of these agents with the thioketone were observed

Table 1
Relative yields of the mixed disulfides of proteins with thio-Michler's ketone, as obtained at pH 5.1 and a 1:1:1 ratio of protein: I_2:thioketone

Protein	Groups/mole Protein		Protein used,	Yield	
	-SH	-S-S-	n moles	n moles	%
	Thiol proteins				
β-Lactoglobulin	1	2	100	44	44
Bovine serum albumin	0.7	16	118	72	61
Papain	1	3	129	34	26
Ovalbumin	4	1	100	32	32
Lactate dehydrogenase	7		100	17	17
Glyceraldehyde-3-phosphate dehydrogenase	14		132	9	7
	Disulfide proteins				
Insulin	0	3	250	0	0
Ribonuclease	0	4	144	0	0
Chymotrypsinogen A	0	5	129	0	0
α-Chymotrypsin	0	5	129	0	0

Protein: I_2 ratios of 1:1 for the preparation of the sulfenyl iodide derivative permitted only the reactionof iodine with an equivalent of one thiol group. The yields were calculated from A_{630nm} values of the pooled fractions obtained by gel-chromatography of the reaction mixtures. The ϵ_M value for the mixed disulfide of β-lactoglobulin was used for the calculations, since the values for the other protein derivatives are not yet known.

(comp. ref. 14). Furthermore, the reduction by $NaBH_4$ is also consistent with the disulfide nature of these products.

Data in Table 1 show that on iodine treatment each of the tested thiol proteins reacted with the thioketone. The thioketone did not react with any of the native thiol proteins and the treatment with I_2 (with the avoidance of its excess) was required to elicit the formation of these green products. None of the disulfide proteins, free of thiol groups, reacted with the thioketone, either with or without I_2 treatment. These data demonstrate the specificity of the reaction for the sulphenyl iodide group and strongly support Danehy's view (7) that sulfenyl iodides are obligatory intermediates in iodine oxidation of thiols. Due to their high reactivity such derivatives were difficult

Table 2
The dependence of yields of the mixed disulfide of β-lactoglobulin and thio-Michler's ketone on the ratio of the reagent to the protein sulfenyl iodide derivative

thioketone μmoles	Ratio $\frac{\text{thioketone}}{\text{LG-SI}}$	A_{630nm}	Yield %
0.005	0.35	0.137	27
0.01	0.70	0.204	41
0.02	1.4	0.425	85
0.03	2.1	0.438	87.5
0.05	3.5	0.457	91.3
0.1	7.0	0.484	96.7
0.15	10.6	0.493	98.5
∞	∞	0.500*	100.0

*determined as an assymptote.
LG-SI = β-lactoglobulin sulfenyl iodide derivative.
0.014 μequivalents of β-lactoglobulin sulfenyl iodide were treated with the quantities of thio-Michler's ketone shown, in a final volume of 2 ml. Blank values of A_{630nm} (β-lactoglobulin + thioketone) were subtracted.

to detect until now. Thio-Michler's ketone appears therefore to be a valuable reagent for such a purpose.

Experiments in Table 2 show that the formation of the mixed disulfide from β-lactoglobulin sulfenyl iodide becomes practically complete with a relatively small excess of the thioketone. β-Lactoglobulin sulfenyl iodide was used for several reasons: it is the most stable protein sulfenyl iodide derivative yet known; during its preparation, at pH 5.1, the loss of I_2 due to iodination of tyrosyls is negligible (11,15), and at pH 5.1 the mixed disulfide is relatively stable and rapidly formed.

This paper describes a new sensitive reaction by which one may determine protein sulfenyl iodide groups.

Feigl's disulfide represents a new potential tool for the determination of thiol groups in proteins. It has been generalized (14) that exchange reactions of thiols with Ellman's reagent or pyridine disulfide are likely

to represent interesting properties of aromatic sulfhydryl disulfide reagents. With Feigl's disulfide, a new reagent in this group, highly but differently colored disulfides can be prepared.

ACKNOWLEDGEMENTS

We thank Mr. Arnold Freedman for valuable technical assistance and Dr. Farahe Maloof for effective support. The work was supported in part by funds from the Rosenstiel Medical Science Grant 1973.

REFERENCES

1. Feigl, F., Goldstein, D. and Rosell, R. A. (1957) Z. analyt. Chem. 158, 421-427; Feigl, F. (1958) Spot Tests in Inorganic Analysis, pp. 368-369, Elsevier, New York.

2. Brooker, L.G.S. and Sprague, R.H. (1941) J. Amer. Chem. Soc. 63, 3203-3213.

3. Maloof, F. and Soodak, M. (1963) Pharmacol. Rev. 15, 43-95.

4. Cunningham, L.W. (1964) Biochemistry 3, 1629-1634.

5. Jirousek, L. and Cunningham, L.W. (1968) Biochim. Biophys. Acta 170, 160-171.

6. Fawcett, D. (1968) Canad. J. Biochem. 46, 1433-1441.

7. Danehy, J. P. (1971) in Sulfur in Organic and Inorganic Chemistry (Senning, A., ed.), pp. 327-339, Marcel Dekker, New York.

8. Soodak, M. and Jirousek, L., to be published.

9. Jirousek, L. (1968) Biochim. Biophys. Acta 170, 152-159.

10. Jirousek, L. (1974) Analyt. Biochemistry, in press.

11. Jirousek, L. and Pritchard, E.T. (1971) Biochim. Biophys. Acta 229, 618-630.

12. Field, L. and White, J. E. (1973) Proc. Nat. Acad. Sci. USA 70, 328-330.

13. Parker, A. J. and Kharasch, N. (1959) Chem. Rev. 59, 583-628; (1960) J. Amer. Chem. Soc. 82, 3071-3075.

14. Glazer, A. N. (1970) Ann. Rev. Biochem. 39, 101-130.

15. Cunningham, L. W. and Nuenke, J. B. (1960) J. Biol. Chem. 235, 1711-1715; (1959) J. Biol. Chem. 234, 1447-1451.