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SULFUR-CONTAINING AMINO ACIDS OF THE POLYHEDRAL PROTEIN OF THE SILKWORM YELLOWS VIRUS

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Some time ago, Wellington [1] showed that the proton of the inclusion bodies of the virus of nuclear polyhedrosis of the silkworm contained cystine residues. He found that there was 0.6 ± 0.08 g of cystine and (or) cysteine per 100 g of protein. Tarasevich [3] established that the inclusion bodies contained sulfhydryl groups.

Sulfhydryl Groups in Polyhedral Protein

Polyhedral protein*	Conditions of determination	SH groups in the protein, μmole/mg	
		titration with CH_3HgNO_3	determination by Ellman's method
Unreduced	Without denaturant	0	—
	8 M urea	0	—
	8 M guanidine hydrochloride	0	0
	0.2 M sodium dodecyl sulfate	0	—
Reduced in 8 M area solution	Without denaturant	Insoluble	
	7 M urea	0.026**	0.033
	7 M guanidine hydrochloride	0.030	0.029
	0.2 M sodium dodecyl sulfate	—	0.030
Reduced in 7 M guanidine hydrochloride solution	7 M guanidine hydrochloride 0.2 M sodium dodecyl sulfate	—	0.030
Reduced in 0.2 M sodium dodecyl sulfate solution		—	0.028

* All solutions of protein 0.01 M with respect to sodium ethylenediaminetetraacetate.

** The sample of protein was reduced for 4 hr.

In the present paper we report the results of the reduction and carboxymethylation of the polyhedral protein. Analyses of the sulfhydryl groups in this protein before and after reduction and data on the determination of the cysteic acid in oxidized polyhedral protein are also given.

It can be seen from the table that the polyhedral protein isolated under alkaline conditions contains no sulfhydryl groups. In the protein reduced in 8 M urea solution, 0.029–0.033 μmole of SH groups per mg of protein was found. Since the polyhedra contain sulfhydryl groups, it is quite likely that during the isolation of the protein at pH 10.5–11.0 its sulfhydryl groups could be completely oxidized.

The amount of S-carboxymethylcysteine found in acid hydrolysates of reduced and carboxymethylated polyhedral protein (0.031 μmole per mg of protein) agrees very well with the amount of sulfhydryl groups in reduced polyhedral protein. However, oxidized polyhedral protein contains far more cysteic acid (0.069 μmole/mg).

Since an 8 M solution of urea is not always a sufficiently powerful agent for the complete unfolding of the polypeptide chain [4], it was assumed that under the conditions described the disulfide bonds in the polyhedral protein were not all reduced.

To check this assumption, the reduction of the polyhedral protein was also carried out in a 7 M solution of guanidine hydrochloride and in a 0.2 M solution of sodium dodecyl sulfate. The protein reduced in the guanidine hydrochloride and sodium dodecyl sulfate solutions contained just the same amount of sulfhydryl groups as the protein reduced in urea (see table).

The considerable difference in the content of SH groups in the reduced protein and of S-carboxymethylcysteine in the reduced and carboxymethylated protein, on the one hand, and the content of cysteic acid in the oxidized protein, on the other hand, is possibly explained by the partial conversion of the cysteine or cystine residues into residues of sulfur-containing amino acids which are not reduced under the conditions used. This assumption is confirmed to some extent by the presence of three additional peaks in the chromatograms of acid hydrolysates of the reduced and carboxymethylated protein obtained on an amino acid analyzer.

The first peak issues at about 40 ml, which corresponds to the position of cysteic acid. The second peak, issuing at 60 ml, must probably be assigned to cysteine sulfinic acid, which is fairly stable under the hydrolysis conditions used [5]. This peak was not present in chromatograms of an acid hydrolysate of the oxidized protein. The third extremely small peak issues at about 70 ml, which corresponds to the elution of taurine [6].

The possibility of the conversion of cysteine residues in alkaline solutions of proteins and peptides into products of deeper oxidation than cysteine has been reported by several authors [7, 8].

The question of whether the conversion of cysteine into the products mentioned is the only cause for the difference in the content of SH groups in the reduced protein and of S-carboxymethylcysteine in the reduced and carboxymethylated protein and the content of cysteic acid in the oxidized protein requires further investigation.

Experimental

We have studied the polyhedral protein of Borrelinavirus bombycis isolated by the alkali method [9], purified by gel filtration through Sephadex G-25, and equilibrated with 0.03 N aqueous ammonia. The content of nitrogen in the protein was 15.74% (15.5% according to Bergold and Wellington [2]).

Dissolution of the protein without the access of atmospheric oxygen. A weighed sample of the protein was transferred to a vessel permitting it to be dissolved with the mass being stirred by a magnetic stirrer in the absence of atmospheric oxygen in a current of nitrogen.

To prepare the solutions of protein in the presence of urea or guanidine hydrochloride, the protein was first dissolved in a 2 M solution of one of these substances at a pH of about 10.0, and then a concentrated solution was added in drops to give the desired concentration, the pH being carefully brought to the desired value. This expedient made it possible gradually to raise the concentration of urea or guanidine hydrochloride and to exclude the formation of a gel. A gel can be formed when certain proteins are dissolved directly in solutions with a high concentration of urea or guanidine salt [10]. Before use, the urea and guanidine hydrochloride were crystallized twice from ethanol.

Reduction of the protein. This process was carried out by the method of Anfinsen and Haber [11] in one of the solvents given in the table (the final concentration of protein was about 2%). After 16 hr, the protein was separated from the excess of reagents by gel filtration through Sephadex G-25 with subsequent freeze-drying [11] or by precipitation and repeated washing of the protein with a mixture of acetone and hydrochloric acid [12] with subsequent freeze-drying of a suspension of the protein in acidified water.

Carboxymethylation of the sulfhydryl groups of the reduced protein. After the elimination of the excess of mercaptoethanol [12], the reduced protein was dissolved without the access of air in an 8 M solution of urea with pH about 10.0 and with the addition of the disodium salt of ethylenediaminetetraacetic acid (0.01 M with respect to this acid), and then the pH was lowered to 8–9 and, with stirring by a magnetic stirrer, a tenfold excess (with respect to sulfhydryl groups) of iodoacetic acid, twice crystallized from petroleum ether, was added in drops. The pH of the medium was brought to 8–9 with a 5% solution of methylamine. The solution was stirred for 15 min, a tenfold excess of β-mercaptoethanol (with respect to the iodoacetic acid) was added, and the mixture was left for 1 hr. The S-carboxymethyl protein was separated from the excess of reactants by gel filtration through Sephadex G-25, equilibrated with 0.03 M aeueous ammonia solution, and freeze-dried.

The polyhedral protein was oxidized with performic acid by Moore's method [13].

Acid hydrolysis. The samples of proteins for acid hydrolysis were prepared exactly as recommended by Moore and Stein [14]. Hydrolysis was carried out at 110 ± 1° C for 20 hr and the hydrolysate was evaporated in a rotary evaporator at 45° C for 15–20 min. Twice-redistilled constant-boiling hydrochloric acid was used for the hydrolysis.

The amounts of cysteic acid, methionine sulfone, and S-carboxymethylcysteine were obtained on an amino acid analyzer.

S-Carboxymethylcysteine [15], cysteic acid [16], and methionine sulfone [17] were used for preparing the reference samples. These materials were purified by repeated crystallization from water.

Determination of the sulfhydryl groups in the protein. Two methods were used: titration with methylmercury nitrate [10] and with Ellman's reagent [18].

Titration with methylmercury nitrate. To prepare the methylmercury nitrate, 3.7 g of methylmercury iodide, purified by repeated crystallization from methanol [19], was suspended in 2 ml of distilled water. With stirring by means of a magnetic stirrer, a solution of 1.7 g of silver nitrate in the minimum amount of water was added dropwise to the suspension. The reaction was carried out at 35–40° C for 2 hr. The silver iodide was filtered off and the solution of methylmercury nitrate was evaporated to dryness and kept in a vacuum desiccator over phosphorus pentoxide. Yield

2.63 g (95%). The material was purified by dissolution in the minimum volume of ethanol with subsequent cooling to −30–40° C. The colorless crystals were collected and washed with cold ethanol, mp 58–59° C [20].

For titration, a 10^{-3} M solution was prepared and its titer with respect to cysteine was determined. We found the content of cysteine in the samples by means of Ellman's reagent. The working solution of methylmercury nitrate can be stored in a dark flask in the refrigerator for at least 4 months without change.

Titration. A weighed sample of protein (about 10 mg) was dissolved in 1 ml of one of the solvents (see table) at a pH of about 10.0 in a current of nitrogen as described above. A drop of freshly-prepared 10% sodium nitroprusside solution was added and the vessel containing the protein solution was placed in an ice bath and rapidly titrated with the methylmercury nitrate solution until the characteristic red-violet coloration disappeared. In this way the approximate volume of methylmercury nitrate solution necessary to bind the sulfhydryl groups was determined. Accurate results were obtained by adding about 90% of the approximate volume directly to the protein solution and then titrating rapidly until the color of the sodium nitroprusside disappeared. A correction was made to the final result for the threshhold concentration of sulfhydryl groups at which a coloration is still given with sodium nitroprusside.

Determination of the sulfhydryl groups with 5, 5'-dithiobis (2-nitrobenzoic acid) (Ellman's method [19]). A weighed sample (5–10 mg) of the protein was dissolved in 1 ml of 0.05 M sodium carbonate (pH about 11.0) in a current of nitrogen in a 10-ml measuring flask, stirred with a magnetic stirrer (the flask was calibrated with the magnet in position) and was made up to the mark with 0.1 M phosphate buffer at pH 7.9 (the buffer contained the disodium salt of ethylenediaminetetraacetic acid to a concentration of 0.01 M). To obtain a solution of protein in the presence of urea or other denaturing agent, dilution was carried out with 0.1 M buffer containing the denaturing agent (see table). To 3 ml of protein solution were added 0.04 ml of Ellman's reagent [39.67 mg of 5, 5'-dithiobis (2-nitrobenzoic acid) in 10 ml of pH 7.0 phosphate buffer], the mixture was stirred, and after the maximum development of the color the optical density was determined at 412 mμ against a control solution containing all the reagents apart from the 5, 5'-dithiobis (2-nitrobenzoic acid). The time of development of the maximum coloration is different for different proteins and different methods of denaturing them.

Since Ellman's reagent undergoes partial hydrolysis in an alkaline medium, the absorption was measured at 412 mμ of a solution containing all the components except the protein. The absorption proved to be very slight, amounting to 0.04–0.06 optical density units (SF-4 spectrophotometer). This correction was taken into account in the calculations. It must also be noted that Ellman carried out the determination using 0.02 ml of the reagent. We chose twice the amount of the reagent in order to decrease the time for the maximum development of color.

For the calculation we assumed $\varepsilon = 13603 \pm 163$. It has been shown [21] that this value is constant for a spectral slit width less than 6 mμ. Measurements on SF-4 and SF-4A spectrophotometers gave similar results.

In working according to Ellman's method, the determination must not be carried out in media with pH values greater than 8.5, since the rate of hydrolysis of 5, 5'-dithiobis (2-nitrobenzoic acid) then rises considerably, which leads to a falsification of the results.

Conclusions

1. The polyhedral protein of silkworm virus yellows isolated by the alkali method does not contain sulfhydryl groups.

2. The content of sulfhydryl groups in the reduced polyhedral protein has been determined by titration with methylmercury nitrate and by means of Ellman's reagent (0.029–0.033 μmole of SH groups per mg of protein). The content of S-carboxymethylcysteine groups in acid hydrolysates of reduced and carboxymethylated polyhedral protein has been determined with an amino acid analyzer (0.03 μmole/mg).

3. The content of cysteic acid in acid hydrolysates of oxidized polyhedral protein is 0.069 μmole/mg.

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