THE EFFECT OF DENATURING AGENTS ON MYOSIN*

I. SULFHYDRYL GROUPS AS ESTIMATED BY PORPHYRINDIN TITRATION

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Myosin is a protein distinguished by its very high viscosity and intense double refraction of flow. It is also one of the few proteins which possess titratable sulfhydryl groups even in the native state; in the presence of certain denaturing agents the number of such groups is very greatly increased. Study of the action of a large number of denaturing agents on myosin has revealed certain phenomena quite different from those accompanying denaturation of the "globular" proteins. The present paper deals with the effect of various agents on sulfhydryl groups, as determined by the porphyrindin titration; Paper II deals with the effects of the same agents on viscosity, double refraction of flow, and solubility. These studies have revealed that myosin is profoundly altered by many very mild reagents not ordinarily classed as denaturing agents for proteins. The effects of these reagents are particularly clearly revealed by measurements of viscosity and double refraction; but their effects on sulfhydryl groups are also significant, and have been reported in this paper.

EXPERIMENTAL

Preparation of Myosin

Rabbit Myosin—This was prepared from leg muscles of rabbits by a process somewhat modified from the earlier procedure of Edsall (5). The animals were killed by exsanguination under

* A preliminary account of part of the work reported in this paper and Paper II has already been given (6).

anesthesia. The blood from the hind legs was drained out and the muscles were rapidly chopped up and ground as fine as possible. The ground muscle was then immediately transferred into 10 to 15 parts of a solution containing potassium chloride (0.5 N) and sodium bicarbonate (0.03 N) at 4°. The mixture was gently stirred mechanically for 2 hours in the cold, strained through cheese-cloth, and filtered through paper pulp on a Buchner funnel.

The filtrate, which generally showed strong double refraction of flow in a layer 2 cm. in depth when tested by means of the apparatus described in Paper II, was now brought to a pH near 6.3 by the slow addition of dilute acetic acid, with constant stirring. At this pH the protein solution was appreciably more opalescent than before the addition of acid, but no precipitate formed.[1] It was now diluted with 5 to 10 volumes of cold distilled water; the flocculent precipitate which formed was allowed to settle out overnight in a cold room, and the supernatant fluid was siphoned off. The precipitate was centrifuged in the cold, washed once or twice by centrifuging with cold distilled water, and dissolved by the addition of finely ground potassium chloride crystals with constant stirring. Usually the final concentration of KCl needed for complete solution at this pH was 0.4 to 0.5 N. Dilution with 2 volumes of cold distilled water readily reprecipitated the protein. The precipitate was again centrifuged, washed with cold distilled water, and redissolved in KCl. The solutions so obtained generally showed strong double refraction of flow.[2]

The myosin solutions, dissolved in KCl at a pH of approximately

[1] On the acid side of pH 6.0 myosin is insoluble in salt solutions, and may rapidly become irreversibly denatured, at least in part. On the other hand, the precipitate formed on dilution with water does not settle well unless the pH is below 6.5 (unpublished observations of H. O. Singher in this laboratory). At pH near 6.3 the conditions are nearly optimal for the precipitation of undenatured myosin.

[2] Occasionally preparations were found in which double refraction had largely or completely disappeared. The reasons for this alteration were not discovered, but the observations recorded in Paper II indicate some of the many possible mechanisms which may have been involved. We wish to emphasize the importance of testing the protein solution for double refraction at every stage of the preparation, as this measurement apparently affords the most sensitive criterion of the undenatured state of the protein.

6.2, were treated with a few drops of toluene and held at 4°. If preserved free from bacterial contamination, they still showed intense double refraction after several weeks or even months, but the experiments described in this paper were in general carried out as soon as possible after the protein was prepared.

Protein concentration was determined by the micro-Kjeldahl procedure of Koch and McMeekin (8), the nitrogen content of the solution after digestion being determined by nesslerization. The protein content was calculated from the nitrogen content, Bailey's (3) figure of 16.6 for the percentage of nitrogen in myosin being used. Bailey's value was obtained on myosin preparations which had been precipitated, and then thoroughly washed with alcohol and ether before being dried. Under these conditions, the dried myosin is obtained as a fine white powder. On the other hand, some preparations made in this laboratory were prepared by washing myosin (precipitated from salt solution by dilution) with water, and then removing the water by evaporation *in vacuo* at low temperature over phosphorus pentoxide. Under these circumstances, the dry myosin is yellowish, glassy in appearance, and difficult to grind. The nitrogen content was found to be only 15.7 per cent. Probably dry myosin so prepared contains some lipids which are removed by Bailey's alcohol-ether treatment. The difference in nitrogen content between the two types of preparation is in harmony with this explanation, as is also the statement of Weber (14) that myosin contains approximately 10 per cent of lipid material. Todrick and Walker (13) have reported an even lower nitrogen content of 15.3 per cent for myosin. For the present, we consider Bailey's figure of 16.6 per cent as the most satisfactory basis for calculation of protein content. Sharp (12) has recently reported the figure of 16.8 per cent, in close agreement with Bailey.

Estimation of Sulfhydryl Groups in Proteins

These groups were estimated by titrating the protein with porphyrindin (9, 7). A fuller and more explicit description of this method than that given in the earlier papers appears desirable here.

The method rests upon two assumptions; namely, (1) that a positive nitroprusside reaction in a protein solution is given only by —SH groups, and (2) that porphyrindin is reduced in a neutral

protein solution, at room temperature, within 1 to 2 minutes, only by —SH groups. It is known that the carbonyl group will give a positive nitroprusside reaction, but the permanency and tint of the color so developed are in marked contrast to the rapidly fading color given by simple mercaptans and by proteins. It is known that tyrosine and phenols in general in alkaline solution will affect porphyrindin, but the rate of this reaction is much slower than the very rapid reduction by mercaptan groups. Moreover, the color developed by the dye in the presence of phenols is an orange-yellow, in contrast to the practically colorless condition of the reduced form of the dye brought about by simple mercaptans and by proteins which presumably contain —SH groups. The possibility cannot be excluded that there may exist in protein molecules other groups of an unknown nature which give a positive nitroprusside reaction and reduce porphyrindin to the colorless leuco form. However, in the absence of further evidence, we have chosen tentatively to regard as sulfhydryl the reactive groups in proteins which respond to these tests.

The reason for using both criteria is the general observation that proteins which do not give a positive nitroprusside reaction do not reduce porphyrindin, whereas those proteins which give the former reaction also reduce the dye. The parallel feature of the two types of reaction is further emphasized by the readily observable fact that as one adds porphyrindin progressively to a solution of an appropriate protein, the initial nitroprusside reaction becomes weaker and finally negative. At this point the dye has been completely reduced and the solution is colorless. Excess of dye beyond this point results in persistence of the blue color for a period of several minutes to half an hour, depending upon the protein. On the basis of these observations the conditions for the estimation of the sulfhydryl groups in proteins have been chosen. Briefly stated, the amount of added dye just necessary to cause a negative nitroprusside reaction in the protein was taken to be a measure of the sulfhydryl groups present.

The method is quite simple and rapid. In each of several test-tubes of 10 ml. capacity are placed 2.0 cc. of the protein solution. To the first is added a certain volume of the porphyrindin solution, and the tube is shaken for not more than 1 to 2 minutes. A drop of dilute ammonia is added, followed by about 0.5 ml. of dilute

sodium nitroprusside. If the reaction is positive, the second tube is treated with a larger quantity of porphyrindin. A positive nitroprusside reaction in this case is followed by the addition to the third tube of a still larger quantity of porphyrindin. The procedure is thus carried on by successive approximations until just the amount of dye is added which will bring about a loss in nitroprusside reaction. The end-point by this method is easily reproducible. Both before and after the titration of the protein the dye solution is standardized against a known solution of cysteine. The amount of dye consumed by the protein in order to bring it to the specified end-point is expressed in terms of its equivalent of cysteine for 100 gm. of protein.

The protein sulfhydryl groups estimated as above may properly be designated as "titratable." A few proteins in the *native* state contain such titratable groups.[3] Among them is myosin. When

[3] Balls and Lineweaver (4) have recently questioned the general applicability of the nitroprusside and porphyrindin reactions to the identification of sulfhydryl groups in proteins. Their objection to this method was based on the failure of crystalline native papain to give a positive nitroprusside reaction or to reduce porphyrindin, although the protein apparently reacted with iodoacetic acid. It is clear from these authors' description that native papain behaves toward nitroprusside and porphyrindin exactly as do native egg albumin, edestin, excelsin, and globin (7). These proteins, as well as papain, do not react with the above reagents in the *native* state, although the proteins do contain —SH groups which are revealed by denaturation. The recent experiments of Anson (1) illustrate this point further, for he states that ferricyanide, although it reacts with denatured egg albumin, does not react with native egg albumin. Iodine and iodoacetamide, on the other hand, do so. Anson's value for —SH groups in egg albumin, as determined by titration with dilute ferricyanide in the presence of Duponol PC, agrees well within the limits of experimental error with Greenstein's value (7) obtained by titration with porphyrindin in the presence of concentrated guanidine hydrochloride. (Their values are 1.21 and 1.24 per cent respectively, expressed as percentage of cysteine.) If a similar agreement is obtained for other proteins, it will go far to establish the validity of both methods for estimation of the maximal number of titratable sulfhydryl groups in proteins. The apparent conflict between the various points of view may be readily resolved by drawing a distinction between two types of sulfhydryl groups in proteins. Groups of the first type do not react with nitroprusside, porphyrindin, or ferricyanide, but do react with iodine and iodoacetic acid. Groups of the second type react with all the reagents mentioned. Many native proteins such as egg albumin, edestin, and papain apparently possess sulfhydryl groups of the first

urea, guanidine hydrochloride, or methylguanidine hydrochloride was added to a solution of myosin, an appreciably greater number of titratable sulfhydryl groups was revealed.

Results

Todrick and Walker (13) estimated the sulfhydryl groups in native myosin by determining the amount of phenol indo-2, 6-dichlorophenol dye reduced over a period of 12 hours. The result of these investigators, calculated as per cent cysteine, is 0.27. Mirsky (10) estimated the sulfhydryl groups in myosin by comparing the cysteine content of the protein hydrolysate before and after treatment of the protein with iodoacetate. His value for native rabbit myosin was 0.31 per cent cysteine. After treatment with trichloroacetic acid, the cysteine apparently rose to about 0.57 per cent.

Our first experiments were concerned with estimating the amount of sulfhydryl groups in native myosin dissolved in KCl and in myosin dissolved in urea and in guanidine hydrochloride (Table I). The values given for the protein in solutions of urea and guanidine hydrochloride include the cysteine found in the native protein. The proportion of cysteine is apparently independent of the protein concentration. The value in the native protein, 0.42 per cent cysteine, is somewhat higher than that given by Mirsky (10) and is appreciably higher than that given by Todrick and Walker (13).[4] In solutions of urea and of guanidine hydro-

type which on denaturation of the protein are transformed into groups of the second type. It is difficult to say at present whether this transformation is effected by a change in the spatial configuration of the protein (2) or whether denaturation causes a breaking of certain loose bonds involving sulfhydryl. In this connection the work of Schubert (11) on the compounds of aldehydes and ketones with mercaptans is suggestive. Schubert finds that the loosely bound hemimercaptals fail to give a nitroprusside reaction but do reduce iodine. It would be premature to postulate labile hemimercaptal linkages in the native protein molecule, but the parallelism is striking. The work of Balls and Lineweaver (4) indicates that the sulfhydryl group which is apparently necessary for specific activity of papain is of the first type.

[4] The —SH content of myosin solutions slowly diminishes on standing. In one preparation the cysteine equivalent of the —SH groups had fallen from 0.41 per cent to 0.30 per cent in 2 weeks at 4°.

TABLE I

Sulfhydryl Groups in Native Rabbit Myosin and in Myosin Treated with Urea and Guanidine Hydrochloride

2.0 cc. of protein solution were used in each determination. The dye was made up in 1.16×10^{-3} M concentration (0.0325 per cent) in 0.5 M KCl solution. Urea and guanidine hydrochloride were added to the protein solution so that the concentration in each case was 16.7 mM per gm. of H_2O.

Preparation No.	Protein concentration	Per cent cysteine found in		
		KCl, 0.5 M	Urea	Guanidine HCl
	per cent			
II	0.60	0.41	0.62	1.19
I	0.85	0.40	0.64	1.12
II	1.00	0.41	0.66	1.18
III	0.63	0.43	0.65	1.12
III	0.93	0.42	0.63	1.11
IV	2.30	0.39		1.12
IV	1.90	0.46	0.68	1.18
V	0.78	0.40	0.64	1.16

TABLE II

Effect of Varying Concentrations of Guanidine Hydrochloride on Myosin

2.0 cc. of a 0.85 per cent solution of protein were used in each determination. The dye concentration was 1.16×10^{-3} M (0.0325 per cent) in 0.5 M KCl.

Guanidine HCl added	Dye	Cysteine
mM per gm. H_2O	*mM $\times 10^4$*	*per cent*
0.0	2.96	0.40
0.5	2.96	0.40
1.0	4.22	0.57
2.1	6.07	0.82
4.2	7.48	1.01
6.3	8.06	1.09
8.4	8.12	1.12
10.4	8.12	1.12
12.5	8.12	1.12
14.6	8.12	1.12
16.7	8.12	1.12

chloride, the amount of sulfhydryl groups increases markedly, the increase being greater in guanidine hydrochloride.

Myosin was further investigated in solutions of varying concen-

tration of guanidine hydrochloride. The strong effect of this reagent is revealed in Table II, the results being comparable to those obtained with other proteins.

The data obtained on the different preparations of rabbit myosin were extremely consistent. In five different preparations the value found in potassium chloride solution was 0.42 ± 0.03 per cent cysteine and in concentrated guanidine hydrochloride (above 8 M) it was 1.15 ± 0.04 per cent cysteine. In one preparation (No. IV) double refraction and viscosity were unusually low and the solution could be concentrated to an extent quite unusual in myosin preparations. Nevertheless, the sulfhydryl content both in potassium chloride solution and in guanidine hydrochloride was found to be the same as that in the other preparations.

The effects of several derivatives of urea and guanidine hydrochloride and the effects of several inorganic salts on myosin are reported in Table III. Methylguanidine hydrochloride is as effective as the parent substance, whereas the methylated ureas produce a distinctly different effect.

Ammonium chloride, methylammonium chloride, dimethylammonium chloride, arginine monohydrochloride, O-methylisourea hydrochloride, hydrazine monohydrochloride, and acetamide reduce the content of titratable sulfhydryl groups to 0.[5] This effect was also produced by N-methylurea in high concentration and by glycine in a concentration as low as 0.01 M. None of these substances produced any such effect on cysteine or other simple —SH compounds.[6] The effect has been found repeatedly and invariably in all the myosin preparations tested. Addition of concentrated guanidine hydrochloride to the mixture of myosin with glycine or ammonium chloride or arginine monohydrochloride immediately restored the full content of titratable sulfhydryl groups found in the completely denatured protein (see Table I).

In connection with the studies reported in Table III, the experiment was tried of adding to the native protein solution just

[5] The protein solution under these conditions gives a negative nitroprusside reaction and fails to reduce porphyrindin.

[6] Ammonium salts are usually employed to enhance the color developed by the nitroprusside reagent with —SH groups. It is obvious that the use of these salts with various tissues calls for a certain amount of caution, particularly if it is desired to estimate the sulfhydryl content of such tissues.

sufficient porphyrindin to oxidize the free —SH groups completely. When urea, guanidine hydrochloride, or methylguanidine hydrochloride was added to this oxidized protein, the amount of free —SH groups which appeared exactly corresponded to the differences found between the native and denatured protein in any

TABLE III

Effect on Myosin of Various Substances

2.0 cc. of a 0.78 per cent solution of protein were used in each determination. The dye was used in 1.16×10^{-3} M (0.0325 per cent) concentration in 0.5 M KCl.

Reagent	Concentration of reagent	Cysteine
	mM per gm. H_2O	*per cent*
0.5 M KCl in H_2O		0.40
Urea	16.7	0.64
N-Methylurea	10 –16.7	0
	1.7	0.40
O-Methylisourea HCl	16.7	0
Guanidine HCl	16.7	1.16
" H_2SO_4	1.1	0.40
Methylguanidine HCl	16.7	1.16
Arginine monohydrochloride	0.5	0
Glycine	0.01– 1.0	0
Ammonium chloride	1.7	0
Methylamine HCl	1.7	0
Dimethylamine HCl	1.7	0
Hydrazine monohydrochloride	1.7	0
Acetamide	16.7	0
KI	1.0	0.40
KSCN	1.0	0.40
$MgCl_2$	1.0	0.40
$CaCl_2$	1.0	0.40
LiCl	1.0	0.40

one of these solvents. Addition of N-methylurea and O-methylisourea hydrochloride to the solution of oxidized native myosin produced no increase in —SH groups. Thus, if one titrates the free —SH groups of the native protein, then adds the denaturing reagent, and resumes the titration, the total amount of dye consumed is the same as if the denaturing agent had been added at the beginning.

A number of salts—KI, LiCl, $CaCl_2$, $MgCl_2$, KSCN—were tested for their effect on the sulfhydryl groups with negative results, the —SH content in all these solutions being the same as in potassium chloride.

DISCUSSION

The best analytical data on the sulfur of the muscle proteins are those of Bailey (3) who gives the total sulfur of rabbit myosin as 1.10 per cent and the methionine sulfur as 0.73 per cent.

In concentrated guanidine hydrochloride, the porphyrindin titration yields the equivalent of 1.14 per cent of cysteine, or 0.305 per cent cysteine sulfur in myosin. This is approximately five-sixths of the difference between total sulfur and methionine sulfur in myosin; 95 per cent of the total sulfur can thus be accounted for as cysteine and methionine sulfur.[7]

Myosin apparently contains a fairly high proportion of free and titratable sulfhydryl groups in the native state (Table I). The amount of these groups on the one hand is greatly increased by treatment of the protein with guanidine hydrochloride and, on the other hand, is reduced to 0 by treatment with certain ammonium derivatives. Is the protein "denatured" by either procedure or by both? Obviously, a definition of denaturation based on either procedure would be arbitrary.

We have no explanation to offer at present as to what becomes of the —SH groups of native myosin when the latter is treated with ammonium salts. They reappear, together with additional —SH groups, when the protein is treated with guanidine hydrochloride. Thus a mixture of myosin with glycine, like native egg albumin, shows no titratable —SH groups, but does so after treatment with guanidine. Myosin is remarkable in that the appearance or the disappearance of sulfhydryl groups may be caused by the use of reagents in themselves non-reactive toward mercaptans. The amount of reagent required to effect disappearance is very

[7] It may be pointed out that the value of the porphyrindin titration, expressed as cysteine sulfur, for all the proteins which have been investigated (7) has never been greater than the value for the total sulfur minus methionine sulfur. If the reagent reacted with other groups in addition to sulfhydryl groups, one would not expect such a relation to be generally found.

much less than that required to effect appearance of sulfhydryl groups.

The total —SH content of myosin is the sum of the sulfhydryl groups titrated when the protein is in the native state plus the additional sulfhydryl groups revealed when the protein is denatured in concentrated guanidine hydrochloride solution. The total amount of dye consumed is apparently the same whether one titrates the native protein first, and then adds the guanidine salt and resumes titration, or whether the titration is conducted from the start in the presence of guanidine salt. In the former case it is clear that porphyrindin has oxidized the free sulfhydryl groups characteristic of the native myosin to a form which is not affected by the subsequent addition of guanidine salt. That this oxidized form may be disulfide seems probable, but is not proved. Porphyrindin readily and quantitatively oxidizes cysteine to cystine—whether it oxidizes sulfhydryl in the protein to disulfide remains for further investigation.

SUMMARY

1. An improved method for the preparation of myosin is described.

2. The free sulfhydryl groups of rabbit myosin have been estimated by the use of porphyrindin and the results are expressed in terms of cysteine for 100 gm. of protein. A critical discussion of the significance of the porphyrindin titration is given.

3. The cysteine equivalent of the —SH groups in native rabbit myosin is 0.42 per cent. In concentrated urea this figure rises to 0.65 per cent, and in concentrated guanidine hydrochloride or methylguanidine hydrochloride to 1.15 per cent. Treatment of the native protein with various ammonium derivatives and amino acids reduces titratable —SH to 0. Addition, however, of guanidine hydrochloride to these latter solutions brings out all the sulfhydryl groups characteristic of the fully denatured protein.

4. If one titrates the free —SH groups of the native protein, and then adds guanidine hydrochloride and resumes the titration, the total amount of dye consumed is the same as if the denaturing agent had been added at the beginning.

5. 95 per cent of the total sulfur of myosin may be accounted for as methionine plus cysteine sulfur.

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