THE REACTIVITY OF PORPHYRINDIN IN THE PRESENCE OF DENATURED PROTEINS

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The estimation of sulfhydryl groups in native and in denatured proteins by titration with porphyrindin, a method first used by Kuhn and Desnuelle (10) and later developed and studied by one of us (6–8), has yielded much information concerning the distribution of these groups in the proteins. The advantages in the use of this dye for the estimation of mercaptan groups include (*a*) the rapid and stoichiometric reaction involved, (*b*) the lack of any effect on the dye by the presence of protein-denaturing agents (guanidine salts and urea derivatives), and (*c*) the fact that the reaction takes place at neutral pH. The disadvantages include (*a*) a long and costly synthesis of the dye, (*b*) a difficult visual end-point, (*c*) a loss in titer of the dye on standing in aqueous solution, and (*d*) the fact that the dye is reducible not only by mercaptan but also by tyrosine groups (4).

The first disadvantage has been partly overcome by simplifications in the synthesis introduced by Porter and Hellerman (12). The second disadvantage has been overcome by employing nitroprusside as an outside indicator; *i.e.*, by conducting the titration to the point where the —SH reaction with nitroprusside becomes negative. This method has been described in detail by Greenstein and Edsall (7) and has also been successfully employed by Anson (1) with various oxidizing agents. The third disadvantage has been obviated by standardizing the porphyrindin solutions against cysteine throughout the course of the titration with protein (6). In order to avoid the fourth disadvantage, the titration method was so devised (7) as to provide a stepwise and quite rapid estimation of the protein mercaptan groups, since the reaction of

porphyrindin with hydroxyphenyl groups is very much slower than with mercaptan groups. Inasmuch as the possibility nevertheless persisted that the tyrosine radicals interfered with the titration of protein mercaptan groups, it was considered desirable to investigate this problem further. For this purpose cysteine was added to solutions of denatured and in some cases previously oxidized proteins, and the cysteine so added titrated in the customary manner (7) with porphyrindin. The amount of cysteine recovered indicated that little or no interference by the non-mercaptan reducing groups in the proteins studied was encountered.

Procedure

Several aliquots of a neutralized solution of cysteine hydrochloride were added to solutions of various proteins in either 8 M guanidine hydrochloride or in 1 M glycine.[1] The cysteine solutions were standardized against iodine. The added cysteine was estimated in the presence of the denatured proteins by the stepwise procedure with porphyrindin, the titration being conducted to a negative nitroprusside end-point (7). The pH of the cysteine-protein mixtures varied from 6.4 to 6.8. The temperature was 25°. The time which elapsed between the addition of the cysteine to the protein and the beginning of the titration varied from 5 to 10 minutes. Titrations of cysteine in the protein solutions and in water alone with porphyrindin were conducted simultaneously. The reaction of the dye with cysteine alone was nearly instantaneous. The mixture of cysteine and protein, however, reacted more slowly with porphyrindin, and this rate of reaction was almost identical with that observed in the titration of mercaptan groups in denatured proteins. The time required for the added porphyrindin to oxidize completely the mercaptan groups present in a cysteine-protein mixture, or in a solution of denatured protein, was found to be approximately 30 seconds. Invariably, however, a full minute was allowed for this purpose. It is possible that the difference in the rate of titer of the cysteine in water alone and in

[1] Anson and Stanley (2) have rightly emphasized the necessity of using a pure grade of guanidine hydrochloride. The most satisfactory method of purifying this salt has been to dissolve it repeatedly in dry methanol at room temperature, and after filtering clear, to precipitate with an equal volume of anhydrous ether. The guanidine hydrochloride so obtained is neutral in reaction and free from possible oxidizing impurities (8).

the presence of the protein is due to the fact that, in the latter case, the mercaptan groups which are being titrated belong not to the added cysteine but to —SH radicals in the protein produced by reduction of the protein disulfide groups through action of the added cysteine.

The proteins used were of three types (6); namely, (*a*) amandin, in which sulfhydryl groups do not appear either in the native or in the denatured states of the protein (6), (*b*) horse serum albumin, in which sulfhydryl groups appear only in the denatured state of

TABLE I

Titration of Cysteine with Porphyrindin in Presence of Denatured Proteins

Protein	Denaturing agent	Dye added to oxidize protein —SH groups	—SH in denatured protein as cysteine	Cysteine added to solutions of oxidized proteins	Dye added to cysteine-protein mixtures	Recovery of added cysteine
		mM × 10^3	*per cent*	*mM* × 10^3	*mM* × 10^3	*per cent*
Amandin, 1 cc. 5.0% solution in 5.0% NaCl	8 M guanidine HCl	0	0	8.0	3.9	97
				16.0	7.6	95
				32.0	15.8	98
Horse serum albumin, 1 cc. 4.9% solution	" "	0.16	0.08	8.0	4.0	100
				16.0	7.9	98
				32.0	15.2	95
Rabbit myosin, 1 cc. 1.0% solution in 0.5 M KCl	" "	0.49	1.19	8.0	3.7	92
				16.0	7.6	95
				32.0	15.4	96
" "	1 M glycine	0	0	8.0	4.0	100
				16.0	7.9	98
				32.0	16.0	100

the protein (6), and (*c*) rabbit myosin, in which sulfhydryl groups are present when the protein is native, and either increase in amount when guanidine hydrochloride is added, or vanish when glycine and other ammonium salts are added (7).[2] Before the cysteine solution was added to solutions of serum albumin or of myosin in guanidine hydrochloride, the free —SH groups of the denatured proteins were exactly oxidized by the addition of the

[2] Horse serum albumin contains 4.65 per cent tyrosine (5). Myosin contains 3.38 per cent tyrosine (3). No data on the tyrosine content of amandin are available, but the protein gives an intense Millon reaction.

appropriate amount of porphyrindin. Cysteine was also added to a solution of native myosin in which the free —SH groups had been caused to disappear by the addition of glycine (7). Neither guanidine hydrochloride nor glycine interferes with the porphyrindin titration or with the selected end-point (6, 1).

The data are given in Table I.

DISCUSSION

The data in Table I reveal that the cysteine added to a solution of denatured protein can be nearly completely recovered by subsequent titration with porphyrindin. It is immaterial, apparently, whether the mercaptan titrated belongs to the added cysteine or whether it arises from disulfide groups in the protein which have been reduced to sulfhydryl by the added cysteine. In any event, there appears to be little or no interference by the non-mercaptan reducing groups of the denatured proteins when the method described (7) is closely followed.

Since the earlier investigations on estimating sulfhydryl groups in proteins by the use of porphyrindin were reported (6–8, 14), a number of similar studies employing a wide variety of oxidizing agents for sulfhydryl titrations have appeared in the literature (1, 2, 9, 11, 13). The data have been obtained on two well defined proteins, egg albumin and the tobacco mosaic virus, and are given in Table II.

With few exceptions, the data in Table II for each of the proteins studied show substantially good agreement. It must be remembered that one is dealing here as a rule with the oxidative titration by a variety of agents of autoxidizable groups in colloidal materials. Nevertheless, the results of Anson, Hellerman, and of Greenstein on egg albumin in guanidine and in duponol are nearly the same (Table II). Using the same denaturing agents, Mirsky obtained values for this protein somewhat lower than those obtained by the above investigators. The values of Rosner are the lowest of those given for egg albumin in Table II, and it may be that this is due to the fact that a substituting and not an oxidizing agent was employed for the titrations.

It is clear that the sulfhydryl group content of denatured proteins can be estimated by a wide variety of oxidizing agents. In the case of the tobacco mosaic virus all of the protein sulfur is accounted for as sulfhydryl sulfur (2). In the case of egg albumin,

about two-thirds of the cystine-cysteine sulfur is accounted for as sulfhydryl (6) (Table II). The sulfhydryl sulfur content of the denatured proteins studied so far has always been either less than

TABLE II

Sulfhydryl Groups of Denatured Proteins

Protein	Bibliographic reference	Denaturing agent	Titrating agent	—SH as cysteine
				per cent
Tobacco mosaic virus	Stanley and Laufer (14)	Guanidine HCl	Porphyrindin	0.76
" "	Anson and Stanley (2)	" "	Ferricyanide, tetrathionate, or mercuric benzoate	0.71
" "	Stanley and Laufer (14)	Urea	Porphyrindin	0.70
Egg albumin	Greenstein (6)	Guanidine HCl	"	1.28
" "	Anson (1)	" "	Ferricyanide, tetrathionate, or mercuric benzoate	1.24
" "	"	Duponol	" "	1.24
" "	Hellerman *et al.* (9)	Guanidine HCl	*o*-Iodosobenzoate	1.32
" "	Mirsky (11)	" "	Ferricyanide	0.96
" "	"	Duponol	"	0.96
" "	"	Urea	"	0.96
" "	Greenstein (6)	"	Porphyrindin	1.00
" "	Rosner (13)	"	Iodoacetate	0.87

or equal to the total cystine-cysteine sulfur of the proteins (2, 6–8) (Table II).[3]

[3] Egg albumin and the tobacco mosaic virus protein are two proteins whose —SH contents are revealed to nearly the same extent in urea and in guanidine hydrochloride. This does not mean that the two denaturing agents are necessarily equal in effect, since in the case of egg albumin the maximum —SH value is revealed at 8 M guanidine and at 16 M urea. In the case of myosin (7) 8 M guanidine hydrochloride liberates nearly twice as many —SH groups as does 16 M urea, and the effect of these agents on the physical properties of the protein is also in this order. The guanidine halides are much more powerful denaturing agents than urea (6).

SUMMARY

1. Cysteine was added in varying amounts to denatured proteins, and was nearly quantitatively recovered by subsequent titration of the mixtures with porphyrindin. This indicated little if any interference in the titration by other reducing groups of the proteins. The latter react too slowly under the conditions employed.

2. The data on the sulfhydryl group content of denatured tobacco mosaic virus protein and of egg albumin, obtained by the use of various oxidants and soluble denaturing agents, have been compared and discussed. In general, the results obtained by different investigators using diverse methods have been in substantially good agreement.

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