Short Communications

Evidence Against the Proposed Interaction of Thionitrobenzoate with Protein Disulphide Bonds

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Thionitrobenzoate was prepared from 5,5'-dithiobis-(2-nitrobenzoic acid), and its reaction with several disulphide-containing proteins was examined under various conditions. No evidence for any cleavage of disulphide bonds was obtained. The claim by Robyt *et al.* [Arch. Biochem. Biophys. (1971) 147, 262–269] that thionitrobenzoate allows the quantitative determination of disulphide bonds in proteins is not substantiated.

Since its introduction by Ellman (1959) the reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) has been widely used for the quantitative determination of thiol groups. The primary reaction between Nbs₂ and a thiol proceeds at neutral or alkaline pH according to eqn. (1):

where $\phi =$

The electron-attracting substituents on the aromatic rings of Nbs₂ facilitate the cleavage of its disulphide bond by the nucleophilic thiol anion. The equilibrium position of reaction (1) consequently lies far to the right, and thiols may be determined by the stoichiometric formation of the yellow thionitrobenzoate ion. The other reaction product is the mixed disulphide formed between thionitrobenzoate and the attacking thiol.

Robyt *et al.* (1971) claimed to have observed attack by thionitrobenzoate on the disulphide bonds of proteins. They studied the reaction of Nbs₂ with proteins containing both disulphide and thiol groups and found that the modified proteins appeared to contain more $\phi-S-S-$ groups than would be expected solely from the reaction of Nbs₂ with the thiol groups. They therefore concluded that disulphide groups also entered into the reaction and postulated the following scheme to account for their findings:

In this scheme, the thionitrobenzoate ($\phi-S^-$) formed initially by reaction of Nbs₂ ($\phi-S-S-\phi$) with the thiol groups attacks the disulphide bonds of the protein to form the mixed disulphide and further thiol; the latter then reacts with more Nbs₂. Such a scheme of reactions would ultimately result in the cleavage and modification of each disulphide bond of the protein. Robyt *et al.* (1971) subjected the modified proteins to a brief treatment at pH 10.5, which they claimed brought about reaction (5):

Measurement of the absorbance increase at 412 nm thereby produced permitted the determination of the number of disulphide bonds originally present in the protein. Robyt *et al.* (1971) proposed that this could form the basis of a general method for determining disulphide bonds in proteins. In the case of proteins containing no thiol groups it would be necessary first to generate some thionitrobenzoate from Nbs₂ by introducing a small quantity of mercaptoethanol.

Were such a method to be generally applicable it would indeed constitute the simplest way of determining the disulphide content of proteins. However, as pointed out by Brocklehurst *et al.* (1972), it is unlikely that thionitrobenzoate would attack cystinyl disulphide bonds (CyS-SCy) to produce CyS-S- ϕ since the electron-attracting properties of the ϕ ring should result in the stability of disulphide bonds being in the order CyS-SCy > CyS-S- ϕ > $\phi-S-S-\phi$. The equilibrium position of reaction (3) would thus be expected to lie far to the left. This is supported by the interchange reaction between the mixed disulphide protein-S-S- ϕ and another thiol group within the same protein, resulting in the formation of an intramolecular disulphide bond and the release of thionitrobenzoate. Such an interchange has

been reported for lactate dehydrogenase (Holbrook & Jeckel, 1967), glyceraldehyde phosphate dehydrogenase (Boross, 1969), transglutaminase (Connellan & Folk, 1969) and α -amylase (Telegdi & Straub, 1973).

The claims of Robyt *et al.* (1971) have been challenged by Brocklehurst *et al.* (1972), who showed that the disulphide bonds of papain are not cleaved in its reaction with Nbs_2 ; similar comments have been made by investigators studying other proteins (Ando & Steiner, 1973; Telegdi & Straub, 1973). These results therefore cast serious doubt on the operation of reaction (3).

It seemed that this controversy might be resolved by a more direct examination of the interaction of thionitrobenzoate with protein disulphide bonds. For this purpose it would be desirable to use a preparation of thionitrobenzoate containing no Nbs_2 , thiol or mixed disulphide. This has been done by converting Nbs_2 into thionitrobenzoate quantitatively by electrolytic reduction and then examining its interaction with several disulphide-containing proteins. The results presented here do not support the reaction scheme proposed by Robyt *et al.* (1971).

Experimental

Nbs_2 , insulin (bovine pancreas), lysozyme (hen egg-white) and trypsin (bovine pancreas) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Electrolytic reduction of Nbs_2 was performed by the same procedure as that described for the reduction of lipoamide (Weitzman *et al.*, 1974). A 10 ml portion of 0.05 mM- Nbs_2 in 0.1 M-Tris-HCl buffer, pH 8.0, was electrolytically reduced by applying 1.5 V across the electrodes, and the progress of the reduction was followed by withdrawing samples and measuring the extinction at 412 nm. Quantitative reduction of 0.05 mM- Nbs_2 to thionitrobenzoate should result in the concentration of the latter being 0.1 mM and therefore having an extinction at 412 nm of 1.36 (Ellman, 1959). In practice I obtained a maximum value of 1.3 after about 20 min. Addition of cysteine to this solution resulted in no further increase in extinction, thereby demonstrating the absence of any unreduced Nbs_2 .

Results and Discussion

The preparation of thionitrobenzoate from Nbs_2 by electrolytic reduction has the clear advantages over alternative methods that no other products are formed, that the reduction proceeds to completion and that no separation or purification is required. The extinction coefficient of the fully reduced Nbs_2 is in close agreement with the accepted value of 13 600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Ellman, 1959) and does not support

the value of 11 400 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ proposed by Robyt *et al.* (1971). Other investigators have also produced evidence against this low value (Collier, 1973; Degani & Patchornik, 1971; Gething & Davidson, 1972), and examination of the cleavage of Nbs_2 by sulphite (Weitzman, 1975) also supports the higher value.

The solution of thionitrobenzoate was mixed with several proteins containing disulphide bonds, and evidence for the occurrence of reaction (3) was sought by following the absorbance at 412 nm; this would be expected to decrease with any consumption of thionitrobenzoate. Equal volumes of 0.1 mM-thionitrobenzoate and 1 mM-insulin, -lysozyme or -trypsin (each made up in 0.1 M-Tris-HCl buffer, pH 8.0) were mixed at room temperature and the absorbance at 412 nm was followed for 2 h against a blank containing the same concentration of thionitrobenzoate but without protein. With none of the proteins was any decrease in absorbance observed. Similar experiments were done with solutions of these proteins containing 8 M-urea, i.e. giving a final concentration of 4 M-urea in the reaction with thionitrobenzoate; again, no decrease in absorbance was observed. The absence of any reaction of these proteins with thionitrobenzoate was further confirmed by dialysing mixtures of thionitrobenzoate and protein (after 2 h interaction) against 0.1 M-Tris-HCl buffer, pH 8.0, to remove the thionitrobenzoate. Addition of mercaptoethanol or dithiothreitol to the dialysed proteins produced no yellow colour, thereby demonstrating the absence of any ϕ -S-S- groups (see eqn. 3). It is conceivable that reaction (3) would be pulled to the right by reaction (4). This possibility was tested by repeating the above experiments in the additional presence of 0.1 mM- Nbs_2 , removing both thionitrobenzoate and Nbs_2 by dialysis, and treating the dialysed protein with dithiothreitol. Again, no evidence for the formation of ϕ -S-S- groups was obtained.

Insulin, lysozyme and trypsin contain, respectively, 3, 4 and 6 disulphide bonds per molecule, and other experimental evidence (see, e.g., Cecil & Wake, 1962; Cecil & Weitzman, 1964) suggests that at least some of these bonds are readily accessible and sensitive to cleavage reagents. Their non-reactivity to thionitrobenzoate, even in 4 M-urea, is therefore unlikely to be due to unusual stability.

The present results clearly demonstrate that thionitrobenzoate does not attack and cleave disulphide bonds in proteins and do not support the claim by Robyt *et al.* (1971) that such a reaction permits the quantitative determination of these bonds.

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