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Reactivity of Protein Sulfhydryl Groups with Disulfides

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Abstract: The detection of sulfhydryl groups in proteins is limited by the inaccessibility of the sulfhydryls to the reagent, 5,5'-dithiobis(2-nitrobenzoic acid). The addition of other disulfides such as 2,2'-dithiobis(ethylamine) to the chromophoric disulfide increases both the rate and the extent of detection of "buried" sulfhydryl groups in proteins.

The relevance of sulfhydryl groups and disulfide bonds to the structure and function of proteins has led to the need for accurate means of quantifying the sulfhydryl groups present in the protein. Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) abbreviated as DTNB, reacts with sulfhydryl groups and releases a chromophoric product, the 2-nitro-5-thiobenzoic acid anion, which can be used to detect and quantify the number of sulfhydryl groups present in a protein sample. However, this technique is limited to the detection of unhindered, solvent-accessible sulfhydryl groups. Thus, in a protein molecule, even after its denaturation with chaotropic agents such as guanidinium chloride or urea, many sulfhydryl functions remain inaccessible to reaction with DTNB and are thus not detected[1,2].

The accuracy with which sulfhydryl groups can be detected, even in denatured proteins, needs to be improved. The use of rapidly reacting, more permeable disulfide groups such as cystamine, 2,2'-dithiobis(ethylamine), to carry out a primary reaction with protein sulfhydryl groups to release a readily-accessible solution-sulfhydryl for reaction with DTNB will be described.

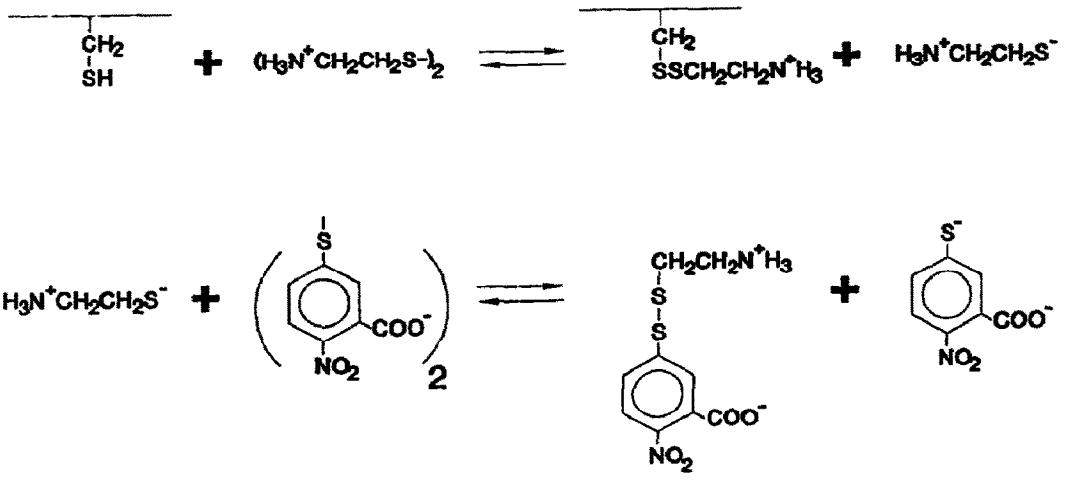
Free sulfhydryl groups were determined by adding either DTNB (0.5 mM) or the disulfide, cystamine (0.4 mM or 4.0 mM) or a mixture of both reagents using the procedure as described[3].

The number of sulfhydryl groups measured by DTNB increases as the protein is more fully denatured by the addition of urea or gdm-Cl. Although DTNB reacts quantitatively with free cysteine-HCl, the maximum number of sulfhydryl groups measured for each protein, even under denaturing conditions, is consistently below the number predicted by the primary peptide sequence. DTNB is known to react only with sulfhydryl groups accessible to the hydrophilic external environment[2,4]. Thus, a proportion of the sulfhydryl groups, even in denatured proteins, are still buried in sterically-hindered structures.

The chemical structure of DTNB containing both an aromatic ring and a negatively charged carboxylic acid may limit its reactivity in certain protein environments. A disulfide which is far more reactive than DTNB and is of sufficiently different chemical structure may react with the protein sulfhydryl and form the RS^- anion in solution which can then go on to react with DTNB to form the colored assayable nitrobenzoate anion. By this series of reactions, the second disulfide does not need to produce a colored anion, but merely needs to be much more reactive than the DTNB with the previously inaccessible protein sulfhydryl.

When cystamine is present in the reaction vessel with DTNB, the number of sulfhydryl residues which can be detected increases by as much as 10X for ovalbumin although the magnitude of the "cystamine-effect" varies between different proteins. This difference may be attributed to the different chemical environment of the sulfhydryl residues in different proteins. For example, ovalbumin contains four -SH groups[5] of which only 0.2 -SH is detected in the presence of DTNB while 2.0 -SH are detected in the presence of both DTNB and cystamine. In contrast, ß-lactoglobulin contains only one -SH group[5] which is detected equally well in the presence of DTNB or both DTNB and cystamine.

The utility of a second, more reactive disulfide binding to DTNB-inaccessible sulfhydryl groups is dependent on the slower rate of reaction of DTNB. Cystamine, a positively charged disulfide, is known to react fifteen times more rapidly than DTNB with BSA sulfhydryls[6]. Protein sulfhydryls which do not readily react with DTNB (either because of its negative charge or aromaticity) may be more reactive towards the positively charged cystamine.



Continuing studies will examine the "cystamine-effect" for sulfhydryls with known, chemical environments. At present, the addition of cystamine to aid the reaction of DTNB in native protein structures is recommended.

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