SULFHYDRYL GROUPS

Sulfhydryl groups are very reactive with many reagents. They react readily with most alkylating and arylating agents, combine with many heavy-metal ions, and are easily oxidized by even very mild oxidizing agents. They react readily with most acylating agents, but the resulting thiol esters are quite unstable in an aqueous environment and usually hydrolyze. Four of the more useful reagents for the modification of protein sulfhydryl groups and procedures for their use are given below.

**p-Mercuribenzoate.** Solutions of p-mercuribenzoate can be prepared by adjusting an aqueous suspension of the reagent (p-chlor- or p-hydroxymercuribenzoate) to pH 10 or 11 to achieve solubilization and then readjusting to a desired lower pH. Insoluble material, if present, can be removed by filtration or centrifugation. The reagent concentration can be determined by measuring the absorption at 232 mµ employing an extinction coefficient of 1.69 x 10^4 M^-1 cm^-1 (Boyer, 1954).

For qualitatively determining the effect of p-mercuribenzoate substitution, the desired amount of reagent can be added to the protein in water or a buffer compatible with the protein. The amount used is usually based on moles of sulfhydryl groups present, varying from a stoichiometric equivalence to a several-hundredfold excess. Sufficient time should be allowed for completion of the reaction because of large variation in reaction rates. For most proteins, maximum reaction rates are obtained at pH 4.5 to 5. To determine the kinetics of the reaction of a reagent with sulfhydryl groups, the desired amount of reagent is added and the rate of mercaptide formation determined from the rate of increase in absorbance at 250 to 255 mµ (see Figure 10-4). The increment of absorbance per mercaptide group can be estimated, if one knows the number of sulfhydryl groups, by determining the total increase in the presence of excess reagent. This value varies for different proteins and for the same protein under different conditions (Swenson and Boyer, 1957). Typically, values from 5 to 7.5 x 10^3 M^-1 cm^-1 are found. At pH values near neutrality, the change in absorbance is greatest at 250 mµ, while at pH 4.5 it is greatest near 255 mµ.

For quantitative determination of sulfhydryl groups with p-mercuribenzoate, a procedure such as that described by Boyer (1954) is recommended. This involves adding the protein in question to a solution of p-mercuribenzoate as in a titration and noting the endpoint as that amount of protein no longer giving a linear increment of increased absorbance (see Figure 10-5). Because both the reagent and the protein absorb strongly at the wavelengths employed, care must be exercised to employ proper blanks. Under such circumstances, for example, when the protein is available only in small amounts or has a very low content of sulfhydryl groups, it is more desirable to reverse the sequence of additions (i.e., adding the reagent to the protein and observing the change in absorbance and the endpoint in an analogous way).
**Iodoacetate.** The reactivity of sulfhydryl groups with haloacetates far exceeds that of other protein groups under almost all conditions (Section 6-1). It is therefore possible to employ an extremely wide range of conditions, usually designed primarily to satisfy requirements of the protein being modified, and still obtain selective reaction with sulfhydryl groups. While a pH between approximately 6 and 8.5 is optimal, reaction specificity continues high for a considerable distance on either side of this range. Specific modification can usually be enhanced by using just the theoretical amount of reagent required for reaction with sulfhydryl groups. For quantitative reaction, urea, SDS, or another denaturant is frequently needed. Solutions of haloacetates should be made up just prior to their use, since they are subject to slow hydrolysis (—CH$_2$—I $\rightarrow$ —CH$_2$—OH + I$^-$) under alkaline conditions. The procedure of Crestfield et al. (1963) for the reduction and S-carboxymethylation of proteins is:

To 5 to 100 mg of protein in a 12-ml screw-cap vial maintained under a nitrogen barrier, add 3.61 g of deionized, crystalline urea, 0.30 ml of EDTA solution (50 mg of disodium EDTA per ml), 3.0 ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 N HCl diluted to 30 ml with water), and finally 0.10 ml of mercaptoethanol. The solution is made up to a 7.5-ml mark with water, and a solution 8 M in urea and 0.2% in EDTA is used to fill the vial completely. A disk of polyethylene is slid over the top in order to exclude air and is held in place with the screw cap. After 4 hours at room temperature (22º-25º), the contents of the vial are transferred to a 25-ml beaker under the nitrogen barrier. A freshly prepared solution of 0.268 g of iodoacetate added is slightly less on a molar basis than the amount of mercaptoethanol. The —SH groups of cysteine residues react most rapidly, and since the excess iodoacetate reacts faster with mercaptoethanol above pH 8 than with thioether sulfur, alkylation of methionine is kept to a minimum. The three equivalents of Tris per equivalent of iodoacetate keep the solution more alkaline than pH 8.3.

Exclusion of light while working up the solution is important in order to prevent the formation of iodine which may react with tyrosine and histidine residues. Fifteen minutes after addition of the iodoacetate, the reaction is stopped by passing the sample through a column of Sephadex G-75 to separate the protein from other components of the reaction solution.

When haloacetates are used to determine the effects of sulfhydryl group substitution on a protein’s properties, conditions should be chosen under which the protein is stable. Temperature, pH, and the concentration of reagent can be adjusted to attain the desired extent or rate of reaction. The procedure of Battell et al. (1968) for the carboxymethylation of muscle phosphorylase $b$ is more or less typical:

The reaction as carried out in 0.13 M KCl, 0.033 M sodium glycerophosphate, 1.5 mM EDTA at pH 7.5 with a protein concentration of ~10 mg/ml, and 10 mM iodoacetamide. Aliquots are taken and the reaction stopped at various times by addition of a 50-fold excess of mercaptoethanol over iodoacetamide. After 8 hours incubation at 30ºC approximately 85% of the enzyme’s catalytic activity was lost. At this high excess of iodoacetamide (approximately 30-fold) inactivation was pseudo-first order to beyond 95% completion.

**N-Ethylmaleimide.** N-Ethylmaleimide shows high selectivity for reaction with more exposed protein sulfhydryl groups. At pH values near neutrality its reaction with sulfhydryl groups is sufficiently specific to allow considerable variation in other reaction parameters such as temperature and reagent concentration. It can be used to quantitatively determine sulfhydryl groups (see Section 6-2). However, its more
important use is for substitution of sulfhydryl groups to determine the effects of modification on various properties. The reaction of N-ethylmaleimide with isocitrate dehydrogenase (Colman and Chu, 1970) was done, for example, at pH 7.7 in 0.1 M triethanolamine buffer containing 0.3 M Na$_2$SO$_4$, 1 mM EDTA, and 10% glycerol. Enzyme (0.37 mg/ml) was incubated at 30°C with 0.09 mM reagent and the reaction was followed spectrophotometrically from the decrease in absorbance at 302 mµ using $\Delta \varepsilon = 620$ (see Section 6-2). After 60 minutes more than 80% of the dehydrogenase activity and almost all reductive carboxylation activity were lost. In the presence of the enzyme’s substrate, isocitrate, and the activating cation, Mn++, negligible activity was lost during this period. Under these conditions of reagent excess (approximately sevenfold based on two sulfhydryl groups per 58,000 g) the changes in catalytic properties appeared pseudo-first order for 60 minutes. Hydrolysis of the reagent was shown to have negligible influence on the observed rates under these conditions (i.e., half-life = 318 minutes).

5,5’-Dithiobis (2-nitrobenzoic acid) (DTNB). DTNB can be used to effect the substitution of sulfhydryl groups in proteins and to quantitatively determine them spectrophotometrically. Variations of the procedure first described by Ellman (1959) can be used for quantitation of sulfhydryl groups. A stock solution of DTNB (39.6 mg in 10 ml of 0.1 M phosphate buffer, pH 7.0) can be prepared and used for several weeks or longer until blank values become too high. To each 3.0-ml sample of protein in 0.1 M phosphate buffer (pH 8.0), 0.02 ml of stock DTNB solution is added; after 2 to 3 minutes, the optical density at 412 mµ is determined against a blank lacking protein. An extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ can be used to calculate the concentration of sulfhydryl groups reacting. Denaturants like sodium dodecyl sulfate, urea, or guanidine hydrochloride can be used to increase the reactivity of slow-reacting groups.

To determine the effect of substitution on the properties of a protein, reaction conditions should be chosen to be compatible with the protein. As an example, the procedure of Kastenschmidt et al. (1968) for reaction of DTNB with muscle phosphorylase b follows:

To 10 mg of phosphorylase b in 0.9 ml of 50 mM sodium glycerophosphate and 2 mM EDTA (pH 6.8) was added 0.1 ml of 10 mM DTNB in the same buffer. The molar concentrations of enzyme and DTNB in the solution were 5.4 x 10$^{-5}$ M and 1 x 10$^{-3}$ M, respectively, giving an 18.5-fold excess of DTNB. The reaction solution was incubated for as long as 7 hours at room temperature or until the desired extent of reaction was indicated by the increased absorbance at 412 mµ. To stop the reaction and separate the modified protein from excess reagent and reaction products, the solution was passed through a column packed with Sephadex G-25 preequilibrated with 50 mM glycerophosphate and 2 mM EDTA at pH 6.8. Under these conditions the reaction was biphasic: two sulfhydryl groups per dimmer of phosphorylase b react rapidly and two others more slowly. Reaction of the latter two effects changes in quaternary structure and a decrease in catalytic activity. Following a 6-hour incubation period a mixture of products was obtained consisting of catalytically active dimers with one modified sulfhydryl per monomer, an inactive monomer with two modified sulfhydryl groups, and partially active, polydisperse, high-molecular-weight aggregates with between one and two modified groups per monomer.
REFERENCES