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Protection of Immobilized Sulfhydryl Groups Against Autooxidation by Alterations in Their Microenvironment

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Abstract

Immobilized sulfhydryl groups were prepared by partial thiolation of NH_2–glass beads. The microenvironment of the immobilized SH groups was varied by different chemical modifications of neighboring NH_2 groups. Introduction of a strong charge in the surroundings of immobilized sulfhydryls results in their dramatic stabilization against autooxidation. This effect is due to the salting of O_2 from the surface microlayer of the thiolated beads.

Index Entries: Sulfhydryl reagent; immobilized reagent; porous glass; autooxidation, of immobilized sulflydryl groups; heavy metal removal, and sulfhydryl group autooxidation; mercury, and sulfhydryl group autooxidation.

Introduction

Removal of mercury ions from waste waters is an important environmental problem (*1*, *2*). According to Jones (*1*), a number of different processes can be employed to remove and recover mercury ions down to the 1 ppm level, but only ion exchange and precipitation can be used for ionic mercury removal from aqueous solutions at lower concentrations. The high cost of operating ion exchange systems and the inconvenience and costs of a sulfide precipitation severely restrict their use (*1*, *3*). Also, both methods lack specificity toward mercury ions.

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Recently, Michelsen and co-workers (*3*, *4*) have successfully used tannery hair for removal of mercury ions from water. The interaction between the hair and Hg^{2+} largely results from a reaction between the latter and protein sulfhydryl groups. Chambers et al. (*5*) have found that the same result can be obtained by using crosslinked polymeric resins containing covalently attached sulfhydryl groups. These authors have reported that such resins can be used both for waste water treatment and for protection of immobilized enzymes in enzyme reactors from inhibition by ionic mercury.

One of the major obstacles in the practical application of SH group containing polymers is instability of (immobilized) sulfhydryl groups under aerobic conditions. At neutral and alkaline pH they are readily oxidized by atmospheric oxygen. This process, called autooxidation, results in disappearance of the ability to bind mercury ions. In this work, we have shown that immobilized SH groups can be greatly stabilized against autooxidation by chemical modification of their microenvironment

Materials and Methods

Materials

Controlled-pore glass beads (mesh size 20–80, 36.4 m^2/g, mean pore diameter 654 Å) were purchased from Sigma. Chemicals used in this work were of highest purity commercially available.

Amination of Glass Beads

Covalent attachment of NH_2 groups to the surface of porous glass beads was carried out in accordance with the aqueous silanization method of Weetall and Filbert (*6*). The amount of immobilized amino groups was determined using the picrate technique of Lee and Loudon (*7*) and was found to be 61 μmol/g of glass beads.

Thiolation of NH_2-Beads

N-acetyl-DL-homocysteine thiolactone was used as the thiolating agent (*8*). To 1 g of aminated beads, 50 mL of deoxygenated solution of 25 m*M* *N*-acetyl-DL-homocysteine thiolactone in 0.1*M* phosphate buffer (pH 10.5) containing 5 m*M* EDTA was added. The modification was carried out for 2 h under argon. It was terminated by decanting the liquid, washing with 1 m*M* HCl and acidic acetone, and subsequently drying with a stream of argon. This procedure yielded about 7 μmol of immobilized SH groups/g of beads (determined spectrophotometrically with Ellman's reagent as described below).

Blocking and Deblocking of Immobilized SH Groups

Most acylating reagents interact not only with amino groups but also with sulfhydryl groups. Therefore, in order to selectively modify the former, the latter

should be protected. To this end, we treated thiolated beads with Ellman's reagent, [5,5′-dithiobis-(2-nitrobenzoic acid)] (0.2 m*M*, pH 8), which results in the formation of the corresponding mixed disulfide. The blocked SH groups can be preparatively deblocked by treatment with 1 m*M* mercaptoethanol (pH 8, 5 m*M* EDTA), following a desired chemical modification of neighboring amino groups.

Acylation of Immobilized NH_2 Groups

Two different methods were studied: (i) acetylation of protected beads (see above) with pure acetic anhydride and (ii) succinylation of protected beads with 25% succinic anhydride in refluxing absolute ethanol. The modified beads were then thoroughly washed and treated with mercaptoethanol to deblock sulfhydryl groups.

Time Course of Autooxidation

Kinetics of O_2 oxidation of SH groups immobilized on porous glass beads was investigated as follows: the beads were suspended in 0.1*M* phosphate buffer, pH 8.0 (unless otherwise indicated), in the presence of 0.1 μ*M* Cu^{2+} and gently agitated under air. At desired time intervals the solution was removed and the beads were washed with 1 m*M* HCl and acidic acetone, dried, and assayed for sulfhydryl groups using Ellman's reagent (*9*).

Results and Discussion

Sulfhydryl groups immobilized on the surface of porous glass beads were chosen as a model system in this study. Amination of the glass beads as described above resulted in attachment of 61 μmol NH_2 groups/g of beads, which corresponds to about 1 amino group per 100 $Å^2$ of the surface. We then thiolated a portion of these amino groups with *N*-acetyl-DL-homocysteine thiolactone. Under conditions indicated in the experimental section, about 11% of all amino groups were converted to SH groups, leaving 89% of NH_2 groups available for other chemical modifications.

Exposure of thiolated glass beads suspended in phosphate buffer (pH 8.0) to air results in rapid disappearance of immobilized sulfhydryl groups (Fig. 1, curve a). We have demonstrated that this phenomenon is due to autooxidation of SH groups since under argon there is no appreciable decrease of sulfhydryl group content within the same period of time.

It is well known that autooxidation of thiols does not occur at a substantial rate in the absence of metal ions (*10–14*). And indeed, the immobilized sulfhydryl groups were absolutely stable under air in the presence of a metal-chelating agent, 0.1*M* EDTA. Therefore, in order to avoid ambiguity owing to possible variations in metal ions content in water, we have carried out all our autooxidation experiments in the presence of added 0.1 μ*M* Cu^{2+} in distilled deionized water.

The rate of O_2 oxidation of immobilized SH groups strongly depends on pH (Fig. 1): reduction in pH from 9.0 to 6.0 leads to a significant inhibition of the process.

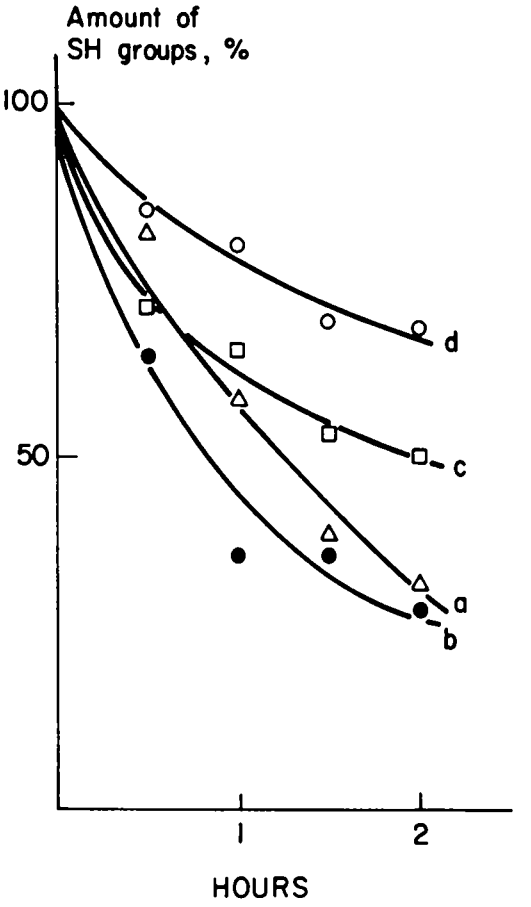

Fig. 1. pH dependence of the rate of autooxidation of immobilized SH groups: a, pH 8.0; b, pH 9.0; c, pH 7.0; d, pH 6.0. Conditions: 10 mg of thiolated glass beads in 5 mL of 0.1*M* phosphate buffer in the presence of 0.1 μM Cu^{2+}, gentle stirring under air, room temperature.

On the basis of both the results reported above and data from the literature, there are at least three different factors within the aminated microenvironment that may contribute to the stability of immobilized SH groups against autooxidation: (i) reduction in the local pH; (ii) electrostatic repulsion of metal ions from the proximity of SH groups; (iii) salting out of O_2 from the microenvironment of SH groups [this effect has been used by us to stabilize O_2-sensitive enzymes against oxygen inactivation (15)]. All these factors should be greatly affected by alterations in the electrostatic charge of the surroundings of the immobilized SH groups.

At neutral pH, the microenvironment of sulfhydryl groups immobilized on aminated glass beads is slightly positive due to partial protonation of NH_2 groups. This positive charge can be completely neutralized by, for example, acetylation of these amino groups. On the other hand, this positive charge can be replaced by a strong negative one if the aforementioned NH_2 groups are succinylated.

Figure 2 shows the effect of such modifications of NH_2 groups surrounding im-

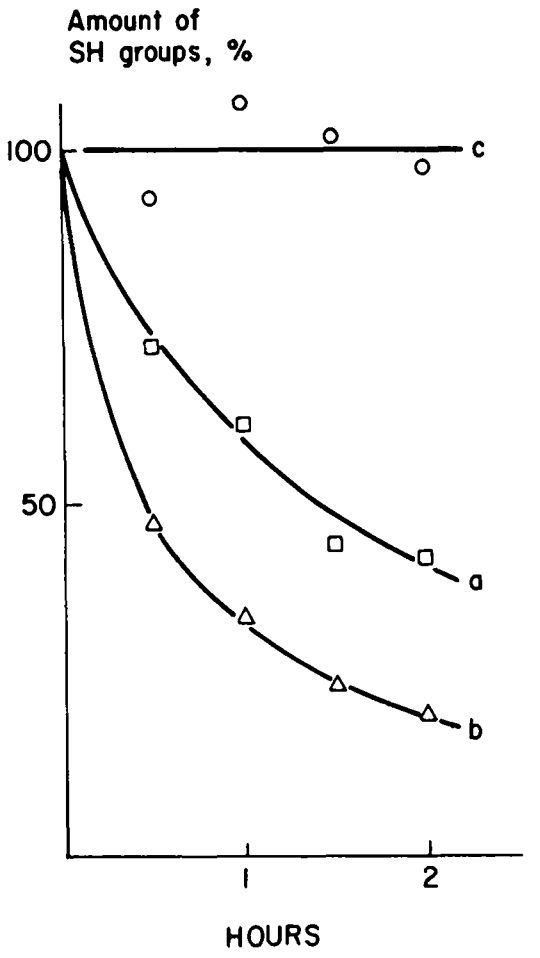

Fig. 2. Dependence of the rate of autooxidation of immobilized SH groups on chemical modification of the neighboring amino groups: a, nontreated HN_2 groups; b, acetylated NH_2 groups; c, succinylated NH_2 groups. Conditions: 10 mg of thiolated glass beads in 5 mL of 0.1*M* phosphate buffer (pH 8.0) in the presence of 0.1 μ*M* Cu^{2+}, gentle stirring under air, room temperature.

mobilized sulfhydryl groups on the O_2 stability of the latter. One can see that while acetylation destabilizes SH groups even further (Fig. 2, curve b), succinylation of NH_2 groups greatly protects immobilized sulfhydryl groups against autooxidation (Fig. 2, curve c). Whereas for nonacylated beads, the half-time of autooxidation is less than 1.5 h, even after 6 h succinylated beads retain over 90% of their SH groups.

It is clear that electrostatic partitioning of metal ions (e.g., Cu^{2+}) has nothing to do with the effects described, since it should have produced the opposite result (destabilization by succinylation owing to concentration of Me^{2+} near the resultant glass surface). The pH shift in the proximity of immobilized SH groups also cannot be responsible for the stabilization by succinylation; in this instance, acetylation should have resulted in a stability that is intermediate between non-

acylated and succinylated beads (because the ability to attract protons decreases as follows: nontreated amino groups < acetylated amino groups < succinylated amino groups), which is not the case (see Fig. 2). Hence it appears that the major cause of protection of SH groups by succinylation of the neighboring NH_2 groups is *salting out of* O_2 from the glass surface: since the solubility of oxygen in the charged microlayer near the surface is greatly reduced, immobilized SH groups are exposed to a much lower concentration of O_2 than exists in the bulk solution.

Acceptance of this concept completely explains the data in Fig. 2. Acetylation of amino groups reduces the micro-pH, but simultaneously removes a small but appreciable amount of existing positive charges from the glass surface (eliminating a partial salting out of oxygen) and therefore the overall result is destabilization. Succinylation both decreases the pH and introduces a strong negative electrostatic charge to the surface, which results in the dramatic protection of immobilized SH groups observed (Fig. 2).

Acknowledgments

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