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Optimization of oxidation of glycoproteins: an assay for predicting coupling to hydrazide chromatographic supports

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ABSTRACT

A rapid, simple assay for aldehydes generated by oxidation of saccharide units in glycoproteins, using dyes containing hydrazide functionalities, is described. The assay is used, in conjunction with tests of biological activity, to predict oxidation conditions that will result in a maximum of active protein coupled to a hydrazide chromatographic support. Glycoproteins are labeled with Lucifer Yellow CH or Texas Red Hydrazide, and the extent of labeling is determined. Using the assay, it is shown that the efficiency of coupling to Affi-Prep Hydrazide is proportional to oxidation.

INTRODUCTION

Recently, several workers have reported the use of hydrazide-derivitized supports in the preparation of affinity chromatographic columns, where the coupled ligand is a glycoprotein (usually an antibody) [1–5] (for a recent review, see ref. 6). As these workers report, the primary attraction of the method is that the ligands are "oriented", with their active sites directed into solution, rather than randomly, as is the case in amine-directed coupling chemistries. Typically, the ligand is oxidized briefly under mild conditions with sodium periodate, to generate aldehydes from vicinal diols present on the saccharide units. The product is then made to react with the hydrazide functionalities present on the support, forming stable hydrazone linkages. The oxidation reaction is generally done "blind", that is, without analysis of the extent of oxidation in the product.

In our hands, the oxidation reaction has generally been reliable, yielding oxidized glycoproteins which couple to the support and produce affinity columns that behave as expected. Occasionally, however, a column exhibits a low coupling capacity. Under these circumstances, it has been difficult to determine whether this problem was the result of inadequate oxidation or an incomplete coupling reaction. Further, the optimum oxidation conditions for a new glycoprotein must be determined by trial and error, a process which can be expensive in terms of both time and material.

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An analysis of the extent of oxidation of the glycoprotein prior to coupling would overcome these problems. Avigad [7] reported a method based on analysis of formaldehyde released in the reaction, but it has the problems inherent in any indirect assay. Blotting techniques [8,9] have also been reported; however, they are cumbersome and are only semi-quantitative. O'Shannessy and Quarles [10] reported the use of immunoglobins labeled with fluorescent dyes in the direct immunofluorescence of cells. We report here the adaptation of this procedure to a simple, rapid, quantitative method for the determination of the extent of oxidation of glycoproteins.

EXPERIMENTAL

Reagents and chemicals

Human γ-globulins, horseradish peroxidase, bovine serum albumin, and [^{14}C]bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Sheep anti-bovine albumin was obtained from Bethyl Labs. (Montgomery, TX, USA). Immunoglobins were 3–4% carbohydrate; horseradish peroxidase had *ca.* 20% carbohydrate content. Lucifer Yellow CH and Texas Red Hydrazide were obtained from Molecular Probes (Eugene, OR, USA). Affi-Prep Hydrazide, Bio-Spin 6 desalting columns and the TMB peroxidase EIA substrate kit (containing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide) were obtained from Bio-Rad Labs. (Richmond, CA, USA). Other chemicals were obtained from commercial sources and were of analytical-reagent grade or better.

Oxidation of glycoproteins

Proteins were dissolved in, or dialyzed against, acetate buffer (0.02 *M* sodium acetate–0.15 *M* NaCl, pH 5.0 adjusted with glacial acetic acid). Protein concentrations varied from 3.8 to 10 mg/ml, and are given in the figure legends. To begin the oxidation reaction, 40 μl of 0.25 *M* sodium periodate in water were added per 1 ml of sample solution. After incubation for various times in the dark at room temperature, the reaction was quenched by adding 56 μl/ml of ethylene glycol and incubating for ⩾15 min. The reaction by-products were then removed and the protein was exchanged into fresh acetate buffer, either by passage over a 10DG desalting column (Bio-Rad Labs.) or dialysis (Spectrapore 2, Spectrum Medical), and used immediately or stored at 4°C until used (generally no longer than overnight). Oxidized proteins are designated o-IgG (oxidized human γ-globulins), o-αBSA (oxidized sheep anti-bovine albumin) and o-HRP (oxidized horseradish peroxidase).

Dye labeling of oxidized immunoglobins

A mixture of 10 μl of Lucifer Yellow solution (5 mg/ml of Lucifer Yellow CH in acetate buffer) and 100 μl of oxidized protein solution was incubated for 2 h at room temperature. To solubilize any precipitate, 10 μl of 1 *M* Tris (pH 8.0) were added. To remove the excess dye, a 75–100-μl aliquot was then desalted by centrifugation with a Bio-Spin 6 column (0.3 ml gel volume) equilibrated in 0.1 *M* Tris–0.15 *M* NaCl (pH 8.0 adjusted with HCl). The Bio-Spin column is designed for rapidly desalting 50–100-μl samples in a table-top centrifuge. The eluate was diluted to 0.5–1 ml, as required. Absorbances at 428 and 280 nm were measured with a Perkin-Elmer Lambda 3 dual-beam spectrophotometer, and the extent of labeling was calculated as the ratio of dye (determined by the absorbance at 428 nm) to protein (determined by the absorbance at 280 nm, after subtracting the absorbance at 280 nm due to dye).

Dye labeling of oxidized HRP

To 100 μl of o-HRP solution, exchanged into coupling buffer (0.2 *M* sodium acetate–0.5 *M* sodium sulfate, pH 4.5 adjusted with glacial acetic acid) by passage over a Bio-Spin 6 column, 10 μl of Texas Red Hydrazide solution (6 mg/ml Texas Red Hydrazide in pyridine) were added. The reaction was incubated for 2 h at room temperature. A 100-μl aliquot was then passed over a Bio-Spin 6 column, diluted, and absorbances were measured at 403 and 590 nm. The extent of labeling was calculated as the ratio of dye (determined by the absorbance at 590 nm) to protein (determined by the absorbance at 403 nm).

Coupling of oxidized proteins to Affi-Prep Hydrazide

Approximately 0.5–3.0 ml of Affi-Prep Hydrazide was equilibrated with coupling buffer. The volume of settled gel was determined, and an equal volume of coupling buffer was added to prepare a 50% slurry. To begin the coupling reaction, two volumes

of coupling buffer and one volume of oxidized protein solution (o-IgG or o-HRP) were added to one volume of the slurry.

Alternatively, the gel was equilibrated in 1 *M* sodium sulfate–0.1 *M* sodium acetate (pH 4.5), a 50% slurry prepared and an equal volume of oxidized immunoglobin solution (o-αBSA) added.

In either instance, the mixture was gently mixed overnight at 4°C. The mixture was then transferred to a Poly-Prep column (Bio-Rad Labs.) and washed with 3–5 bed volumes of 0.1 *M* Tris (pH 8.0). The concentration of protein in the eluate was determined by measuring the absorbance at 280 nm (403 nm for o-HRP), and the amount of coupled protein was determined by difference.

Assay of sheep anti-bovine albumin activity

Microtiter plates were incubated with 50 μl per well of BSA solution (1 μg/ml bovine albumin in 0.1 *M* sodium carbonate, pH 8.8) overnight at 4°C. Then 200 μl of blocking solution [1 mg/ml of ovalbumin (Calbiochem) in PTT buffer (10 m*M* sodium phosphate–0.15 *M* sodium chloride–0.05% Tween 20–0.01% thimersol, pH 7.4)] were added for 60 min at room temperature. The wells were then emptied and rinsed twice with 200 μl of PTT buffer.

The protein solution to be assayed was diluted in PTT buffer containing 0.01 mg/ml of ovalbumin and 100-μl aliquots were placed in duplicate wells. The plates were incubated for 60–120 min at room temperature. The wells were then emptied, rinsed three times with 100 μl of PTT and blotted dry.

Rabbit anti-sheep IgG–HRP conjugate (Bio-Rad Labs.) was diluted 800–1600-fold in PTT containing 0.01 mg/ml ovalbumin and 100 μl of the resulting solution were added to each well. The plates were then incubated for 60 min at room temperature, washed three times with PTT buffer and blotted dry. A 100-μl aliquot of TMB peroxidase EIA substrate was added and incubated for 5–15 min. Finally, 100 μl of 0.5 *M* H_2SO_4 were added to stop the reaction. The wells were read at 450 nm with a Bio-Rad Model 3550 microplate reader.

Assay of sheep anti-bovine albumin activity on Affi-Prep Hydrazide

[^{14}C]Bovine serum albumin was diluted *ca.* 50-fold with unlabeled bovine albumin in PBN buffer (10 m*M* sodium phosphate–0.5 *M* sodium chloride, pH 7.0) to give a 2.0 mg/ml solution with a specific activity of 79 000 cpm/nmol. Samples of Affi-Prep Hydrazide to which o-αBSA had been coupled were suspended in an equal volume of PBN buffer, and 50-μl aliquots were incubated with 100 μl of BSA solution (1.70 nmol BSA; $1.3 \cdot 10^5$ cpm) for 5 h at room temperature. The samples were then washed twice with 4.0 ml of PBN buffer. The samples were suspended in 10 ml of scintillation cocktail and the bound radioactivity was counted.

Assay of horseradish peroxidase activity

Samples were diluted to $2 \cdot 10^{-6}$, $1 \cdot 10^{-6}$ and $5 \cdot 10^{-7}$ of the initial concentration with acetate buffer. Volumes of 20 μl of the resulting solutions were placed in microtiter plate wells and 180 μl of TMB peroxidase EIA substrate were added. Absorbances were read at 655 nm, with data collection beginning when the absorbance of the $1 \cdot 10^{-6}$ dilution of the unoxidized sample reached 0.25. Five data points were then taken at 5-min intervals. Activity was calculated as the slope of the absorbance *vs.* time plot and averaged for the three dilutions.

RESULTS AND DISCUSSION

Our primary intent was to develop an assay that would predict coupling of a sample of oxidized protein to a hydrazide support without consuming a great deal of time and material. Our initial efforts focused on the use of Texas Red Hydrazide as a label, owing to its relatively high molar absorption coefficient (8×10^4 at 580 nm), and because it contains a hydrazide functionality which would mimic the coupling chemistry of Affi-Prep Hydrazide. However, it is only slightly soluble in water, and causes precipitation problems when coupled to immunoglobins (data not shown). Although Lucifer Yellow CH's molar absorption coefficient is lower by a factor of 6.7, it is soluble in water, and causes only slight precipitates in highly oxidized immunoglobin samples; these can be resolubilized by increasing the pH of the solution. For horseradish peroxidase, however, Texas Red Hydrazide was retained as the label of choice, as the protein absorbs in the same region of the visible spectrum as Lucifer Yellow CH and precipitation was not a problem.

In addition to its visible (428 nm) absorbance, Lucifer Yellow CH also has an absorption band at

280 nm, the wavelength used to determine protein concentration. To correct for this, it was therefore necessary to determine the relative absorbance at 280 nm of the dye coupled to IgG. Four samples of o-IgG, oxidized for various times, were labeled and assayed with the Bradford protein assay [11] (Bio-Rad Labs.), and UV–VIS spectra were obtained (data not shown). The molar absorption coefficient at 280 nm for the protein was determined for unlabeled samples and used to determine the absorbance at 280 nm due to protein. The ratio of excess absorbance at 280 nm (due to dye) to the absorbance at 428 nm was 2.5 ± 0.1. This factor was used in subsequent experiments to correct for dye absorbance at 280 nm.

To determine the length of time required for the labeling reaction, aliquots of a single preparation of o-IgG were incubated for various times with Lucifer Yellow CH solution and the extent of labeling was determined as described above. The reaction with dye is essentially complete after 1.5–2 h (data not shown).

The primary applications of the assay are to readily optimize oxidation conditions for immunoglobin molecules, and, once optimized, insure that subsequent oxidations are reproducible. This requires that the extent of dye labeling is a reliable predictor of coupling efficiency. Also, retention of biological activity is a major consideration in the optimization of oxidation conditions. One wishes to maximize the coupling of the protein while minimizing the loss of activity due to destruction of labile amino acid residues. Although these residues, primarily aromatic and sulfur-containing amino acids, react slowly with periodate [12], they often are critical for activity.

To determine whether the assay fulfils these requirements, a series of oxidation, dyelabeling, and coupling experiments were performed on human γ-globulins, sheep anti-BSA, rabbit anti-bovine IgG and horseradish peroxidase. The glycoproteins were oxidized with sodium periodate for various lengths of time and then assayed for dye labeling and for coupling to Affi-Prep Hydrazide. In some instances the biological activity of the oxidized glycoprotein was measured before and after coupling to the solid support.

The correlation between Lucifer Yellow labeling and coupling to Affi-Prep Hydrazide for human IgG, oxidized for varying lengths of time, is shown in Fig. 1. With increasing oxidation and generation of aldehydes, there is a very close parallel between the dye labeling and coupling to the support.

The effect of oxidation time on dye labeling, coupling and biological activity of o-αBSA is shown in Fig. 2. Biological activity was evaluated in solution (before coupling) by ELISA, and after coupling using ^{14}C-labeled BSA. In Fig. 2a, the extent of dye labeling is compared with the extent of coupling to the gel; the correlation is very close. Fig. 2b presents biological activity data; a steady decrease in the activity of the samples before coupling is observed. The net effect of this loss of biological activity, combined with increasing coupling, is that the two nearly cancel. The predicted activity on the support, calculated as the product of the fractional coupling efficiency and the fractional BSA binding activity, is nearly constant after 15 min of oxidation. The activity actually observed on the support is slightly lower than predicted, particularly at the 15- and 30-min time points, but the prediction that the coupled activity does not decrease is borne out.

A similar experiment was conducted with rabbit anti-bovine IgG (o-αbIgG in Fig. 3); in this instance, however, the activity on the support peaked at 60

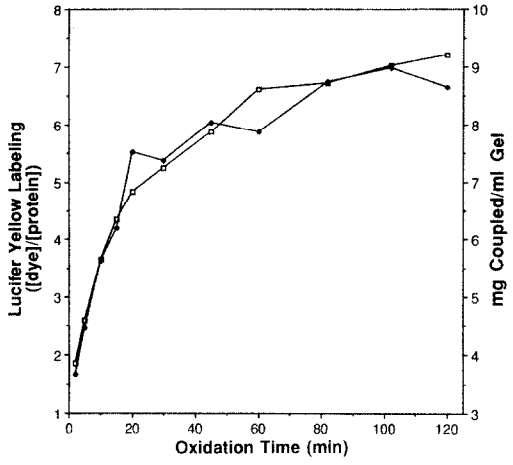

Fig. 1. Time course of oxidation of human γ-globulins. Human γ-globulins (10 mg/ml) were oxidized for various times and aliquots were labeled with Lucifer Yellow CH or coupled to Affi-Prep Hydrazide. The extent of labeling (□) and the amount of coupled o-IgG (◆) were determined for each time point.

min and declined thereafter (data not shown).

To determine if Lucifer Yellow CH labeling is a predictor of coupling to a hydrazide chromatographic support, the extent of protein oxidation was compared with the efficiency of coupling to a hydrazide support for a number of immunoglobins. Coupling is linearly correlated (r^2 = 0.91, using data from four experiments) with the number of dye molecules bound (Fig. 3). As different proteins have various numbers of carbohydrate moieties available, and couple to the support with various efficiencies, the values reported are relative to the respective maxima. The high correlation between relative oxidation and coupling is both gratifying and surprising, as the presumed mechanism of coupling requires only one aldehyde per molecule [13]. The assay, however, clearly gives a rapid indication of the relative amount of protein that one can expect to attach to the hydrazide support.

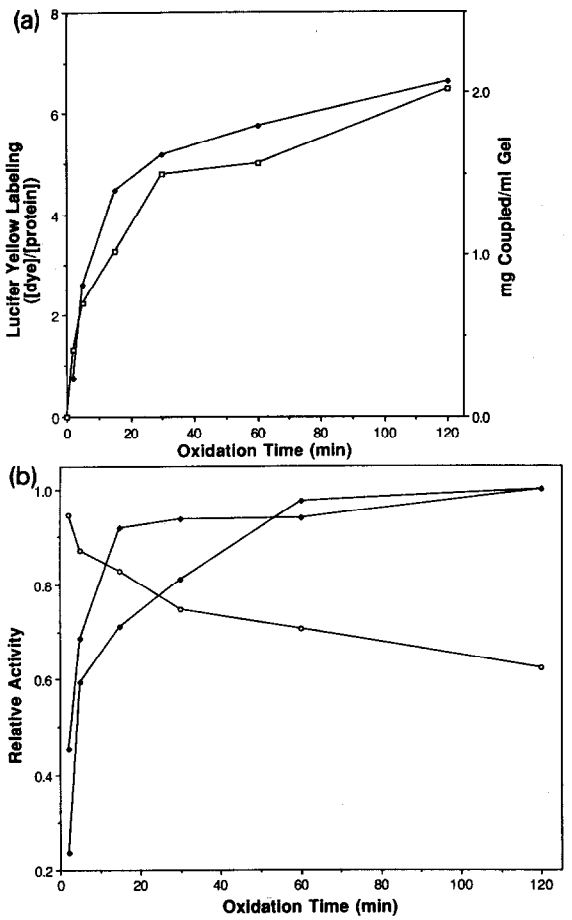

Fig. 2. Time course of oxidation of sheep anti-bovine albumin. (a) Sheep anti-bovine albumin (3.8 mg/ml) was oxidized for various times and aliquots were labeled with Lucifer Yellow CH or coupled to Affi-Prep Hydrazide. The extent of labeling (□) and the amount of coupled o-IgG (◆) were determined for each time point. (b) Activity of o-αBSA samples [(○) before and (◇) after coupling to Affi-Prep Hydrazide] were determined as described and normalized to the highest value (unoxidized material in the case of bulk). The predicted coupled activity (◆) was calculated as the product of the relative activity and the amount of protein coupled to the support.

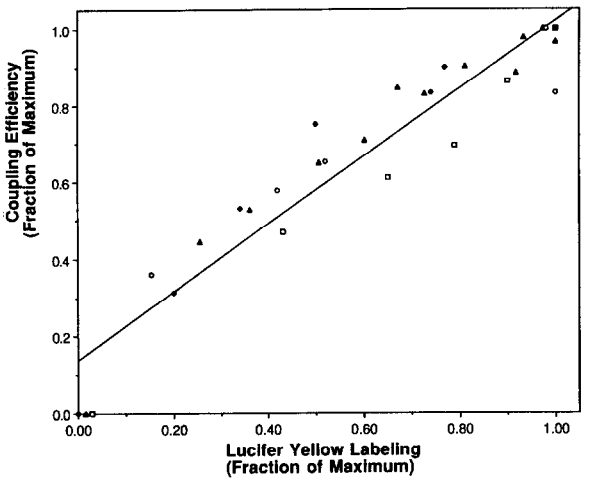

Fig. 3. Comparison of relative coupling efficiency and Lucifer Yellow labeling. Lucifer Yellow labeling and Affi-Prep Hydrazide coupling data from four immunoglobin oxidation time-course experiments were normalized, and a linear regression analysis performed on the resulting data. □, △ = o-IgG; ◇ = o-αBSA; ○ = o-αbIgG. y = 0.13725 + 0.87945x; r^2 = 0.906.

The assay is also useful for non-immunoglobin proteins; results for o-HRP are shown in Fig. 4. In this instance, the protein couples with high efficiency at relatively short oxidation times (86% of the input protein is coupled after only a 6.5-min oxidation time); the activity loss incurred from further oxidation therefore cannot be made up by increasing coupling, and the predicted and observed activities on the support agree well.

CONCLUSIONS

A simple assay, using hydrazide dyes to determine rapidly the extent of oxidation of glycoproteins, has been described. The results correlate well with the

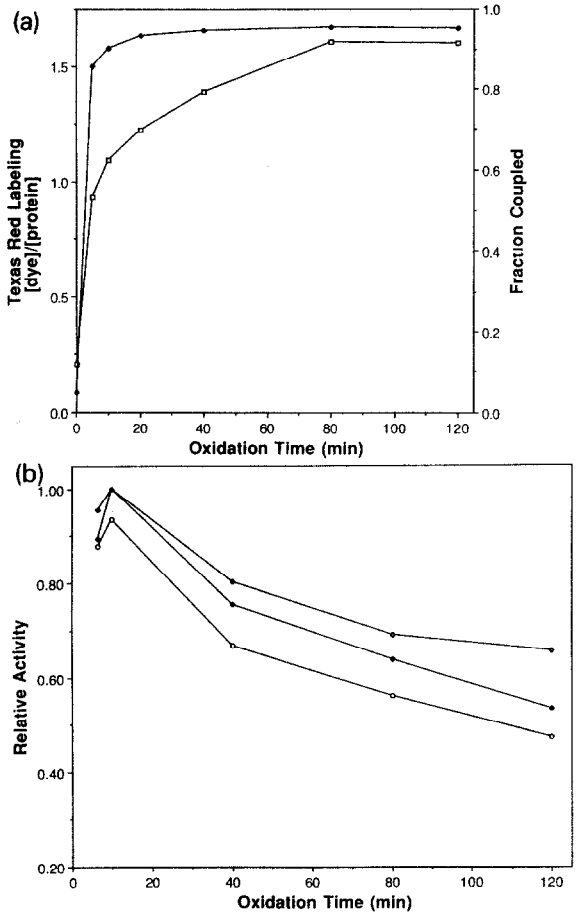

Fig. 4. Time course of oxidation of horseradish peroxidase. (a) Horseradish peroxidase (10 mg/ml) was oxidized for various times and aliquots were labeled with Texas Red Hydrazide or coupled to Affi-Prep Hydrazide. The extent of labeling (□) and the amount of coupled o-HRP (◆) were determined for each time point. (b) Activity of o-HRP samples [(○) before and (◇) after coupling to Affi-Prep Hydrazide] were determined as described and normalized to the highest value (unoxidized material in the case of bulk). The predicted coupled activity (◆) was calculated as the product of the relative activity and the amount of protein coupled to the support.

amount of protein coupled to a hydrazide chromatographic support.

Most important, the assay provides a convenient method of predicting the relative amount of protein that will couple to a hydrazide support under a given oxidation protocol. When combined with bioassay data, it can therefore be used to determine the optimum oxidation conditions, which will maximize the amount of biological activity on the column.

The assay simply involves the addition of a dye reagent to a small aliquot of the protein solution for a short period of time, rapid removal of the unbound dye with a Bio-Spin 6 column and reading the absorbance of the dyed protein. If the experimenter desires only a qualitative indication of the success of the oxidation reaction, the presence or absence of color in the Bio-Spin column eluate gives a simple yes/no answer.

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