Single Step Oxidative Binding of Antibodies to Hydrazide-Modified Eupergit C

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ABSTRACT

A single step periodate oxidative binding of glycoproteins to synthetic polymeric matrices is proposed. By this procedure, the glycoprotein to be immobilized is simultaneously mixed with sodium periodate and a hydrazide-containing matrix. The carbohydrate moieties of the protein are oxidized and react with matrix. The single step reaction is faster than the commonly-used multistep procedure and it allows conjugation of minute amounts of glycoproteins to the matrix.

Index Entries: Monoclonal antibodies; Eupergit C; carbohydrates; periodate oxidation.

Abbreviations: ADH, adipic dihydrazide; CPA, carboxypeptidase A; HRP, horseradish peroxidase; mAb, monoclonal antibody; PBS, phosphate buffered saline, pH 7.4; TNBS, Trinitrobenzene sulfonic acid.

INTRODUCTION

In a previous communication, we have described the binding of periodate-oxidized antibodies to adipic dihydrazide-modified Eupergit C (*1*). Highly active antibody preparations were obtained, possessing about twofold higher antigen binding activity than the same antibodies immo-

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bilized on intact Eupergit C via their amino groups. This effect was attributed to (a) binding of the antibodies via their carbohydrate-moieties which are remote from the antigen binding sites and are not involved in the antigen binding activity of the antibody, and (b) elimination of multipoint attachment of the antibodies to the matrix which may lead to inhibitory effects on their activity.

Binding of antibodies to a hydrazide-modified carrier is usually carried out by a multistep procedure (*1,2*). First, the antibodies are oxidized by sodium periodate, the excess of the periodate is removed from the reaction mixture by gel filtration, and the oxidized antibodies are allowed to react with the matrix. The removal of excess periodate is necessary to prevent excessive damage to the antibodies by periodate oxidation of amino acid residues in the antigen binding site, as well as damage to a periodate-sensitive matrix periodate (e.g., agarose). However, when a matrix does not carry carbohydrate groups that are sensitive to periodate oxidation, it may be of advantage to attempt simultaneous oxidation and binding of the antibodies to the matrix.

In the following communication, we describe a procedure for the one-step oxidative binding of antibodies to a hydrazide-modified Eupergit C when the oxidation of the carbohydrate moieties of the antibody by periodate is carried out in the presence of the matrix, in such manner that the oxidized antibody immediately binds to the matrix.

MATERIALS AND METHODS

Phycocyanine (from Anacystis R-2) was kindly received from Prof. Elisha Tal-Or, of the Faculty of Agriculture, The Hebrew University, Jerusalem, Israel. Hemoglobin, carboxypeptidase A (CPA), and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO).

The preparations of adipic dihydrazide-modified Eupergit C (Eupergit C-ADH) and of anti-CPA and anti-HRP monoclonal antibodies (mAbs), the determination of enzymatic activities of CPA and HRP, and the periodate oxidation of antibodies by the multistep procedure, were described previously (*1*).

Protein Binding to ADH-Eupergit C by the Single Step Procedure

In a typical experiment, 0.1–0.5 mg of protein were incubated with 100 mg of Eupergit C in 1 mL of 0.1 *M* sodium acetate buffer, pH 5.5, containing 1–10 m*M* sodium periodate. After 60 min of slow agitation at 4°C in the dark, the beads were thoroughly washed with 0.1 *M* sodium acetate buffer, pH 5.5. The degree of binding of antibodies to Eupergit C-ADH

was determined from the residual antigen binding activity in the reaction mixture supernatant by the ELISA method (3). Binding of horseradish peroxidase, phycocyanine and hemoglobin, were determined by following the decrease in absorbance at 390, 630, and 415 nm, respectively, in the reaction mixture supernatant. In order to distinguish between protein covalently-bound to the matrix and protein adsorbed to it, the beads were incubated with 8 *M* urea in 10% SDS for 10 min, and the amount of protein released to the solution was determined as described above.

The activities of the matrix-coupled anti-CPA and anti-HRP antibodies were determined by the enzymatic activities of the respective bound antigens, as previously described (*1*).

Sensitivity of Adipic Dihydrazide to Periodate Oxidation

In order to determine the sensitivity of the hydrazide groups to oxidation by sodium periodate, a solution of adipic dihydrazide (1 m*M* in 0.1 *M* sodium acetate buffer, pH 5.5) was incubated with various concentrations (0–30 m*M*) of sodium periodate at room temperature for 10 min. The total vol of the reaction mixture was 200 μL. Ethylene glycol (10 μL) was then added to titrate excess of periodate. The pH of the solution was raised to 10 by the addition of NaOH, and 50 μL of 1 mg/mL trinitrobenzene sulfonic acid (TNBS) were added. The reaction was allowed to proceed for 20 min at 37°C, and the intensity of the color developed was measured at 495 nm using a SLT-210 ELISA reader (Grodig, Austria).

In parallel, the sensitivity of ADH groups of Eupergit C-ADH to oxidation by sodium periodate was determined by titration of residual hydrazide groups on the matrix with TNBS. The beads (100 mg) were treated with sodium periodate (0–30 m*M*) in 0.1 *M* sodium acetate buffer, pH 5.5, for 10 min (total reaction vol was 1 mL). The beads were then extensively washed with PBS, the pH of the solution (1 mL) was raised to 10 by the addition of NaOH, and the beads were reacted with 80 μL of 3 mg/mL TNBS as described above. The intensity of the brown color developed on the beads was estimated visually.

RESULTS

The applicability of the single step approach to the coupling of glycoproteins to Eupergit C-ADH was demonstrated with two monoclonal antibodies: anti-CPA $mAb_{CPA}9$ and anti-HRP $mAb_{HRP}2$. Incubation of 1 mg of $mAb_{CPA}9$ with 1–10 m*M* of sodium periodate and 100 mg of the Eupergit C beads, resulted in an efficient binding of the antibodies to the matrix. As shown in Fig. 1, in the presence of 10 m*M* periodate the reaction required

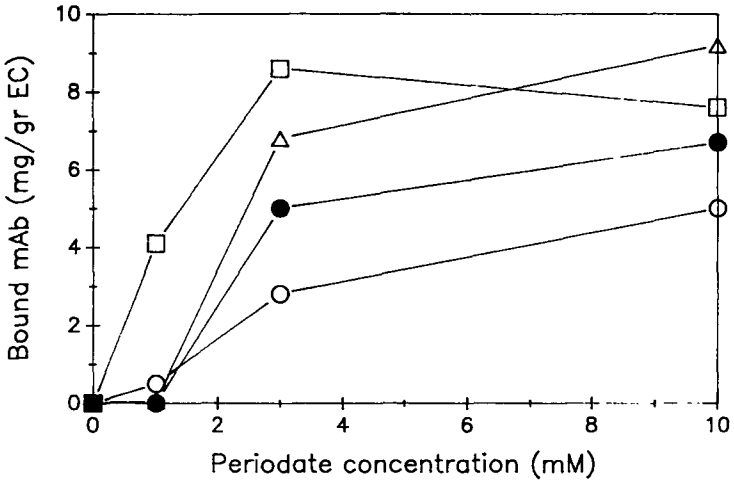

Fig. 1. Binding of anti-CPA $mAb_{CPA}9$ to Eupergit C-ADH. Fifty μg of affinity purified antibody was incubated with 5 mg of Eupergit C-ADH in 0.1 mL of 0.1 *M* sodium acetate buffer, pH 5.5, and various concentrations of sodium periodate. The binding of the antibody was followed by determination of the unbound antibody using a direct ELISA test. Binding was determined after 10 (○---○), 20 (●---●), 60 (△---△), and 120 min (□---□).

less than 60 min for completion, within this time period over 90% of the antibody applied (5 mg/r of matrix) were coupled to the matrix. It is pertinent to note that under similar conditions, binding by the multistep procedure was lower (about 60% of the antibody applied to the matrix). As shown in Table 1, the specific antigen binding activity of the conjugated antibodies was high, close to the theoretical value of 2 mol of antigen bound per mol of antibody, as long as the reaction time did not exceed 1 h. When the antibody was allowed to react with sodium periodate for 2 h, the degree of binding was not affected but the specific antigen binding activity decreased, apparently as a result of some damage caused to the antibodies by the presence of periodate (*1*).

When anti-HRP antibodies were coupled to Eupergit C-ADH by the single step procedure, a different binding-kinetics was observed. As shown in Fig. 2, higher concentrations of periodate and a longer reaction time were required to achieve maximal binding of this antibody to Eupergit C-ADH than those required to bind the anti-CPA antibodies. For comparison, when 50 μg of antibody were allowed to react for 20 min with 5 mg of the matrix in the presence of 3 m*M* periodate, 35 μg of anti-CPA vs 4 μg of anti-HRP were coupled to the matrix. When the reaction was allowed to proceed for 2 h, binding of anti-CPA was 43 μg and of anti-HRP 12 μg. In contrast, in the presence of 30 m*M* of periodate anti-HRP, mAb was efficiently coupled to the matrix within 2 h. The specific antigen

Table 1
Specific Activity of Bound Anti-CPA $mAb_{CPA}9$[a]

$[IO_4]$ (m*M*)	Time (min)	Specific activity[b] (mol CPA/mol Ab)
3	20	1.7
	60	1.9
	120	0.7
10	20	1.4
	60	1.8
	120	1.0

[a] The various antibody-matrix conjugates (described in Fig. 1) were incubated with 50 μg of CPA in 0.1 mL of PBS for 1 h. Excess of the antigen was washed off with PBS and the activity of the bound enzyme was determined with hippuryl-L-phenylalanine as previously described (*1*).

[b] The specific antigen binding activity was calculated using the data of antibody binding to the matrix presented in Fig. 1.

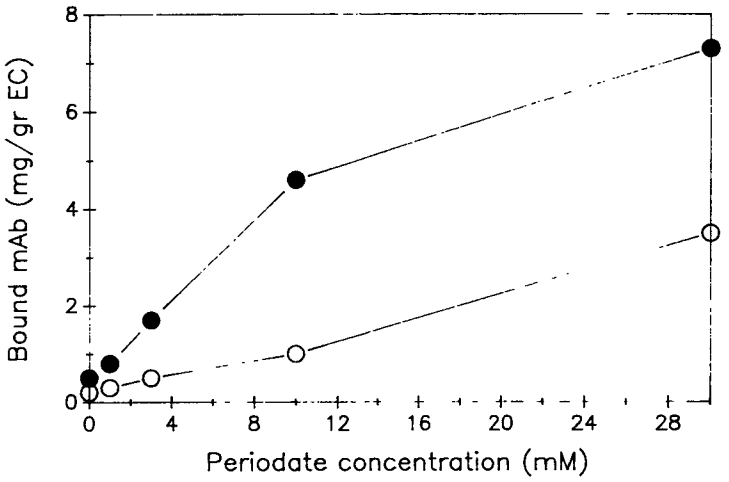

Fig. 2. Binding of anti-HRP $mAb_{HRP}2$ to Eupergit C-ADH. Conditions of binding were as described in legend to Fig. 1. Binding was determined after 20 (○---○) and 120 min (●---●).

binding activity of the antibody was similar to that of the same antibody bound by the multi-step procedure (about 0.8 mol antigen bound/mol of immobilized antibody).

Single step oxidative binding of glycoproteins to Eupergit C-ADH, when the hydrazide-modified carrier is mixed with periodate, may be interrupted by a side reaction in which the hydrazide group is oxidized by

periodate to form an activated carbonyl group (*4,5*) that may then react with amino groups of the protein(s) to be coupled to the matrix. This may lead to a mixed reaction: coupling of glycoproteins via their oxidized carbohydrate moieties to hydrazide groups of the matrix, as well as coupling via their amino residues to the carbonyl groups of the modified matrix. In our experiments, we indeed observed the release of nitrogen bubbles from the reaction mixture, especially when high concentrations (> 10 m*M*) of periodate were introduced, indicative for such side reaction.

Even though the extent of such side reaction should be limited at the low pH value (pH 5.5) used in our experiments, we decided to examine the possibility that such side reaction indeed interferes with the coupling of protein via oxidized carbohydrate moieties under the conditions used in our experiments. The sensitivity of ADH to periodate at pH 5.5 was first tested by reacting constant amounts of ADH with increasing concentrations of periodate and determining the residual ADH by reaction with TNBS. ADH was found to be stable at periodate concentrations of up to 3 m*M* (data not shown) but was destroyed in the presence of periodate concentrations exceeding 10 m*M*. Eupergit C-bound ADH was less sensitive to periodate oxidation. After reaction with 10 m*M* of periodate the beads were still stained brown by TNBS, to the same extent as the control beads that had not been treated with periodate.

The formation of active carbonyl groups on the surface of the matrix following periodate oxidation of the hydrazides should result in the binding to Eupergit C-ADH of nonglycosylated proteins (otherwise not capable of binding to the matrix). The binding to Eupergit C-ADH of two such proteins, hemoglobin and phycocyanine, was tested at various concentrations of periodate. As shown in Table 2, when each of the two proteins was incubated with Eupergit C-ADH (5 mg protein/g of matrix) in the presence of periodate at concentrations not exceeding 3 m*M*, protein binding was limited to about 15% of the protein input. With phycocyanine, about the same amount of protein was also bound to Eupergit C-ADH in the absence of periodate and may therefore represent binding of the protein amino groups to residual epoxy groups on the surface of the matrix. At 30 m*M* of periodate, binding of hemoglobin and phycocyanine increased to 28 and 70% of the input protein, respectively, apparently via reaction of active carbonyl groups formed with their amino groups. In a parallel experiment, the binding of HRP to Eupergit C-ADH in presence of various concentrations of periodate was examined. The extent of binding of HRP directly to Eupergit C via reaction of its amino groups with the matrix was found to be very low, apparently as a result of the low contents of lysyl residues (6 out of 308 amino acid residues) and its blocked *N*-terminus (*6*). In contrast, the degree of binding of HRP to the matrix in the presence of 1 and 3 m*M* of periodate was significantly higher than binding of the nonglycosilated proteins indicating binding via oxidized carbohydrate residues of HRP to the hydrazide groups of the matrix.

Table 2
Binding of Various Proteins to Eupergit C-ADH by the Single Step Procedure[a]

$[IO_4]$ (m*M*)	% of loaded protein bound[b] HRP	Phycocyanine	Hemoglobin
0	8	15	3
0.3	38	15	13
3	55	13	14
30	68	28	70
EC[c]	5	95	72

[a]The various proteins (0.5 mg each) were incubated with 100 mg of Eupergit C-ADH in 2 mL of 0.1 *M* sodium acetate buffer, pH 5.5, at 4°C in the dark for 60 min. Sodium periodate at the indicated final concentration was added to each tube.

[b]The amount of protein bound was determined by comparing the absorbance at 390, 630, and 415 nm of the reaction mixture supernatant of the three proteins, respectively, before, and after binding.

[c]Binding of the same proteins to intact Eupergit C as determined in a parallel experiment.

These experiments indicate that although the side reaction of ADH with periodate occurs at medium and high concentrations of periodate, at periodate concentration of 3 m*M* or lower the main coupling route of mAbs is via their oxidized carbohydrate moieties.

DISCUSSION

In this communication we have presented a procedure for a single step oxidative binding of glycoproteins to Eupergit C-ADH. This procedure, which involves the oxidation of glycoproteins with sodium periodate followed by an immediate coupling to the hydrazide-modified matrix, may, in principle, be applied to any synthetic polymeric matrix which does not contain carbohydrates. The single step procedure has some advantages over the more conventional multistep procedure: The overall reaction is much faster, requiring about one hour, rather than 5–6 h, for completion; the recovery of protein is higher since a gel filtration step to separate the periodate from the protein is eliminated; and finally, the procedure may be applied to coupling of minute amounts of protein to small quantities of matrix. This is especially important during a research and development stage when many analyses under different conditions are required and precious material may be saved. Obviously, in principle, the single-step procedure may be applied not only to Eupergit C but to any matrix which does not contain carbohydrates (e.g., Silica, TSK, and so on).

It has previously been suggested that incubation of a hydrazide-containing matrix with periodate may destroy the hydrazide groups by their oxidation (*5*). In such case, active carbonyl groups may be formed that

will bind glycoproteins via their amino groups rather than via the carbohydrate moieties. We have shown here that though this reaction may take place, the amount of protein bound via the amino groups is small, provided that the periodate concentration does not exceed 3 m*M*. This was also confirmed by titration of ADH with TNBS, that revealed no substantial damage to the hydrazide groups by periodate at concentrations of 3 m*M* or less.

The different response of the two mAbs used in our study, anti-CPA and anti-HRP, may reflect the two binding mechanisms. Apparently, binding to the hydrazide groups of the matrix via oxidized carbohydrate groups that takes place at low periodate concentration is faster than the side reaction occurring at higher periodate concentrations when active carbonyl groups are assumed to attack amino groups of the protein. Nevertheless, it is obvious that the single step periodate oxidative binding may not be applicable to all glycoproteins, just as the multi-step procedure is not applicable to all glycoproteins. However, whenever possible, we recommend the application of the single step procedure that is less material-consuming and easier to perform.

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