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The Use of Glycidol to Introduce Aldehyde Functions Into Proteins – Application to the Fluorescent Labelling of Bovine Serum Albumin and Avidin

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Keywords: Amination / Protein modifications / Lucifer Yellow CH / Fluorescent probes / Nucleophilic substitution

The reactions between 2,3-epoxypropan-1-ol (glycidol) and either the nonglycosylated protein bovine serum albumin (BSA) or the glycoprotein avidin (AV) allowed the successful introduction of diol groups through the addition of some of the proteins' nucleophilic residues to the epoxide ring. The extent of glycolation could be readily tuned by varying the reaction time, the pH of reaction and the concentration of glycidol. Treatment of the stable protein-diol intermediates with sodium periodate resulted in the generation of reactive aldehyde functionalities through the oxidation of the glycol moieties. At this step, the amount of generated aldehydes could also be modulated by changing the time and/or pH of oxidation. As an application, both formylated proteins were successfully labelled with the hydrazide-containing fluorescent dye Lucifer Yellow CH (LyCH), affording highly fluorescent conjugates. Mild oxidation of the avidin-diol left untouched glycol moieties that contributed to greatly enhance water solubility in the avidin-LyCH bioconjugates. In certain cases the avidin-LyCH conjugates retained a good affinity for biotin, as shown in a competitive binding assay. Glycidol thus appears to be an attractive low-cost reagent for protein chemical activation aimed at further coupling of amine-containing species.

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Introduction

Reductive amination is a very useful strategy for conjugation of a protein to another partner, this being, for example, another protein, a probe or a DNA fragment.[1] This strategy requires that one of the two species to be conjugated should carry aldehyde functions, while the other species should be an amine-containing compound (amine, hydrazine or oxime). Several chemical procedures to generate aldehyde groups in proteins are already available. Mild oxidation of glycoproteins[2] or of peptides carrying N-terminal serine or threonine residues (i.e., β-amino alcohol groups)[3] by sodium periodate allows generation of aldehyde functionalities, but this strategy is not universal although it generally allows site-selective conjugation. Alternatively, acylation of protein amino groups with bifunctional reagents such as *N*-succinimidyl *p*-formylbenzoate (SFB) and *N*-succinimidyl *p*-(formylphenoxy)acetate (SFPA) provides a more general means to introduce aldehydes.[4] Unfortunately, because of their hydrophobicities, coupling of these reagents may cause precipitation of the conjugates, especially when they are highly loaded.[1]

In a totally different area, conversion of terminal hydroxy groups of agarose into aldehyde groups has been described. Its principle is based on the addition of the low-cost glycidol to the hydroxy groups of the matrix under basic conditions to afford 1,2-diols that can readily be oxidized by $NaIO_4$ to provide aldehyde functions.[5] This pioneering work was later extended to other matrices.[6] Additionally, glycidol treatment was shown to induce surface hydrophilization,[7] which is an interesting feature when the hydrophobic character of SFB and SFPA is borne in mind. We felt this strategy might be transposable to protein functionalization, as these macromolecules also contain a number of nucleophilic sites such as the ε-amino group of lysine, the β-sulfhydryl group of cysteine and to a lesser extent the β-hydroxy group of serine. To the best of our knowledge, the use of glycidol to introduce aldehyde functions into proteins had never previously been attempted. The reaction behaviour of glycidol with the proteins bovine serum albumin (BSA) and avidin (AV) under various reaction conditions was therefore studied. Further treatment of the protein-diol species with $NaIO_4$ yielded the formylated proteins, as qualitatively evidenced by the Purpald colorimetric test. As an application, BSA and AV were labelled with the hydrazide-containing fluorescent dye Lucifer Yellow CH (LyCH). This is a preliminary work, as our final objective is to label AV with metallocarbonyl PAMAM dendrimers, as part of our ongoing development of Carbonyl Metallo Immuno Assay.[8]

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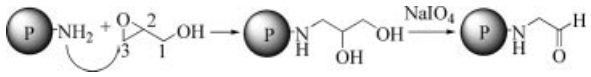
Results and Discussion

The introduction of aldehyde groups into BSA and AV was performed in two steps as shown in the general Scheme 1.



Scheme 1. Preparation of formylated proteins (P = BSA or AV).

Reaction of Glycidol with BSA

Optimization of the reactional conditions was performed on the model protein BSA. Under the chosen reaction conditions, the nucleophilic residues of BSA, especially the ε-amino groups of its lysine side-chains, attack the less hindered epoxide carbon (carbon C3) of glycidol.[9] The addition reaction is probably accompanied by a competitive ring-opening reaction of glycidol by hydroxide anions present at basic pH, to afford glycerol. The reaction between BSA and a large excess of glycidol was studied as a function of glycidol concentration and reaction time at pH 9.2. After dialysis of the protein, the degree of amine conversion was indirectly determined by the colorimetric TNBS assay.[10] This assay is known to be specific for primary amines, as secondary amines are known to react at best two orders of magnitude more slowly than primary amines.[11] Experimental results are displayed as bar charts in Figure 1.

The TNBS assay on the BSA-diol samples showed that the extent of amine conversion increased both with the concentration of glycidol (Figure 1, A) and with the incubation time (Figure 1, B). Nearly quantitative modification of BSA's primary amines was achieved on treatment with glycidol (1.11 M) at pH 9.2 for 72 h. Treatment with glycidol at various concentrations performed at pH 7.2 instead of 9.2 resulted in an amine conversion rate lower by a factor of two (data not shown). Let us note, however, that the formation of diglycol species and reaction of other nucleophilic groups of BSA under the reaction conditions (i.e., large excess of glycidol over amine) cannot be ruled out.

Oxidation of BSA-diol by $NaIO_4$

Oxidation of BSA and BSA-diol conjugates was performed under the conditions described by Wolfe et al.[12] for IgG-type antibodies, typically at neutral or acidic pH and room temperature. The sodium periodate concentration in all reactions was set to 10 mM. The oxidized samples were purified by size exclusion chromatography in a slightly acidic buffer (pH 6) to avoid self-condensation reactions. The presence of aldehyde groups in the protein samples was qualitatively determined by the Purpald colorimetric test. It was not possible to calibrate the assay with a simple aldehyde such as acetaldehyde, since absorption maxima and molar extinction coefficients of the blue-violet tetrazine derivatives vary a lot as a function of the aldehyde substituent,[13] so the concentration of aldehyde measured for a given concentration of protein was expressed as OD_{550}.

In a first series of oxidation experiments, several BSA-diol conjugates resulting from treatment of native BSA with glycidol at various concentrations for 48 h at pH 9.2 were treated with $NaIO_4$ at pH 6. A significant amount of aldehydes was observed for the BSA-diol samples (see part A of Figure 1, grey bars), while, as expected, no aldehyde was detected for the BSA sample (data not shown). This two-step approach therefore provides an efficient and low-cost means to generate aldehyde functionalities on the nonglycosylated protein BSA. Incidentally, under these oxidation conditions, the concentration of generated aldehydes was not related to the number of available glycol groups. In a second series of oxidation experiments, a BSA-diol sample with an amine conversion rate of 68% was oxidized at different pH. Oxidation performed at pH 5 and 4 yielded 2.5 and 6 times more aldehydes, respectively, than oxidation performed at pH 6, as determined by the Purpald test. This is in good agreement with the trend relating to the oxidation of IgGs reported in literature data.[12] Consequently,

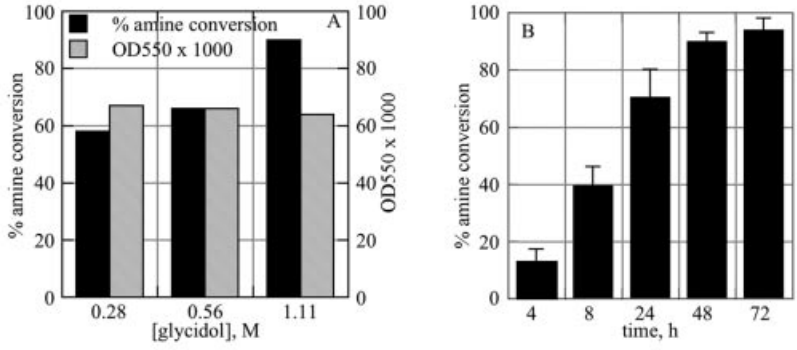


Figure 1. Reaction between glycidol and BSA (10 mg mL^{-1}; 150 μM; 9 mM in amines) at pH 9.2. A) Extent of amine conversion as a function of glycidol concentration after 48 h of reaction. Number of aldehydes (expressed as OD_{550}) generated by oxidation by 10 mM $NaIO_4$ at pH 6 for 1 h. B) Extent of amine conversion as a function of glycidol reaction time ([glycidol] = 1.11 M).

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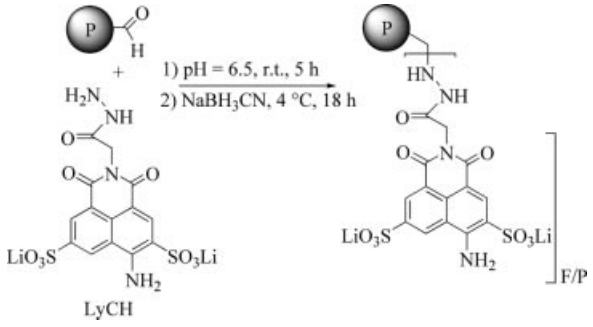
when oxidation is carried out under weakly acidic conditions as in the first series of experiments, it is to be expected that a fraction of the diol groups will remain untouched.

Reaction between Glycidol and Avidin and Oxidation by $NaIO_4$

Avidin-diol samples were prepared by treatment of native avidin with glycidol (1.11 M) at pH 9.2 for 24 h and were oxidized with $NaIO_4$ at pH 4 for 1 h. The aldehyde group contents of both samples were qualitatively evaluated by the Purpald test. Avidin being a glycoprotein, the number of generated aldehydes expressed as OD_{550} was this time not equal to zero. However, it was 5.6 times higher for the avidin-diol sample, which clearly shows the benefit of treating avidin with glycidol before its oxidation.

Labelling of BSA and Avidin with Lucifer Yellow CH Fluorescent Dye

As an application, BSA and AV were each labelled with the hydrazide-containing fluorescent dye Lucifer Yellow CH (LyCH).[14] Firstly, BSA and four BSA-aldehyde samples resulting from treatment with glycidol (1.11 M) at pH 9.2 for various times and oxidized at pH 4 for 1 h were treated with large excesses of LyCH under the conditions previously described for periodate-oxidized antibodies.[12,15] The hydrazone intermediate was then reduced by treatment with $NaBH_3CN$ (Scheme 2).



Scheme 2. Reaction of formylated proteins with LyCH (P = BSA or AV).

Excess LyCH was separated by preparative size exclusion chromatography. The BSA concentration and the fluorophore-to-protein (F/P) ratios were calculated from optical density measurements at 280 nm and 428 nm and the extinction coefficients of BSA and LyCH at these wavelengths (Table 1).

Conjugation of LyCH to the oxidized BSA-diol species yielded labelled proteins with $(F/P)_{uv}$ values ranging from 2 to 23, appearing to depend on the glycidol incubation time (i.e., on the number of diol groups borne by the protein; see Figure 1, B). As a control, no coupling between oxidized BSA and LyCH was observed when the glycidol addition step was omitted (Entry 1), thus confirming that the labelling reaction occurred exclusively through addition of the hydrazide of LyCH to the previously generated aldehyde functions.

Table 1. Conjugation of LyCH to BSA and BSA-diol ([glycidol] = 1.1 M, pH = 9.2) oxidized at pH 4 for 1 h. Fluorophore-to-protein ratios calculated by UV/Vis spectrometry [$(F/P)_{uv}$] and fluorimetry [$(F/P)_{fl}$].

Entry	Glycidol incubation time, h	$(F/P)_{uv}$	$(F/P)_{fl}$
1	0	0	0
2	4	2	2
3	8	5.5	5.5
4	24	11	13.4
5	32	23	11.5

BSA-LyCH samples were also analysed by fluorescence spectroscopy. The concentrations of LyCH in the BSA-LyCH samples were measured from a calibration curve established with standard solutions of LyCH, and the F/P ratio was again calculated (Table 1). It appears that the F/P ratios calculated by both methods were close to each other for the BSA conjugates with low to medium F/P ratios. Conversely, for highly loaded bioconjugates the two sets of values differed considerably. This discrepancy is probably due to self-quenching, which would be expected to occur for proteins labelled with high loads of fluorescent probes.[16]

Secondly, avidin-diol samples resulting from treatment of native avidin with glycidol (1.1 M) at pH 9.2 for 8, 24 and 32 h were treated with $NaIO_4$ at pH 4 for 1 h and allowed to react with LyCH. Here, immediate precipitation was observed upon addition of LyCH for all the samples. Nonetheless, the filtrates were subjected to size exclusion chromatography. No peak corresponding to the protein was observed on the chromatograms, indicating that all conjugates, including the one resulting from the oxidation of native AV, were fully insoluble. We had previously experienced precipitation of avidin when conjugated to more than 10 dicobalt hexacarbonyl IR probes and had attributed it to the additional presence of the hydrophobic $Co_2(CO)_6$ moieties on the protein surface.[17] We inferred that such a phenomenon could also be occurring for the AV-LyCH conjugates, as LyCH features a hydrophobic core despite the presence of two sulfonate groups. Thus, to reduce the number of reactive aldehyde functions on AV-diol and AV and therefore to keep unreacted more diol groups, oxidation was carried out at pH 4 for 15 min (instead of 1 h) and coupling of LyCH was performed as above. Precipitation was again observed for AV and for two of the AV-diol samples (Table 2, Entries 1–3).

Filtrates were again subjected to size exclusion chromatography. A very small peak corresponding to the AV-LyCH conjugate was observed for Entries 1 (native AV) and 2 (AV treated with glycidol for 8 h), indicating that most of the protein had again precipitated during LyCH conjugation. The two other AV-diol species gave partially to fully soluble AV-LyCH conjugates, which were analysed as above to determine their F/P ratios. Both soluble/partly soluble AV-LyCH conjugates displayed high levels of fluo-

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Table 2. Conjugation of LyCH to AV and AV-diol ([glycidol] = 1.1 M, pH = 9.2) oxidized at pH 4 for 15 min. Fluorophore-to-protein (F/P) ratio and IC_{50}.

Entry	Glycidol incubation time, h	Solubility	$(F/P)_{uv}$	$(F/P)_{fl}$	IC_{50}, μM
1	0	insoluble	–	–	–
2	8	almost insoluble	–	–	–
3	24	partly soluble	14	13.2	0.5
4	32	fully soluble	16	16.3	0.7

roprobes without self-quenching effects as observed when the $(F/P)_{uv}$ and $(F/P)_{fl}$ data were compared. It seems that the presence of more remaining diol moieties resulting from longer glycidol incubation times (from Entry 2 to Entry 4) made the AV-LyCH conjugates more soluble, in a similar manner as grafting of PEG chains.[8b,18] The extent of fluorescent labelling was independent of the number of diol groups, because under the short-time oxidation conditions, only a proportion of the available diols were converted into aldehydes. These experiments, carried out with AV that displays lower solubility after chemical modification, clearly show the benefits of using glycidol. Indeed, it brings two advantages: easy access to aldehyde functions after mild oxidation, as well as increased solubility of the modified protein when oxidation is carried out under the conditions in which a proportion of the grafted diol functions are involved.

To assess the usefulness of AV-LyCH as fluorescent tracer, we performed a competitive binding enzymatic assay using commercially available streptavidin-coated microtiter plates and HRP-biotin. The assay was based on the competition between AV or AV-LyCH conjugate and immobilized streptavidin for the binding of HRP-biotin. The amount of surface-bound enzymatic tracer was quantified with OPD/H_2O_2 as substrates, and percent bound tracer was plotted as a function of AV or AV-LyCH concentration in the log/logit format (Figure 2). Under the conditions of the assay, the IC_{50} value of AV was 0.1 μM while the IC_{50} values of the two soluble AV-LyCH conjugates was equal to 0.5 and 0.7 μM (Table 2). Thus, both AV-LyCH conjugates retained satisfactory affinities for biotin relative to AV.

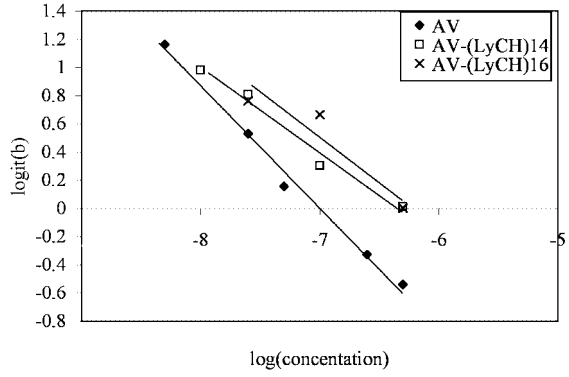

Figure 2. Competitive binding assay of AV and AV-LyCH conjugates.

Conclusions

In conclusion, treatment of the nonglycosylated protein BSA or the glycoprotein AV with glycidol, followed by mild oxidation of the intermediate glycol moieties, afforded reactive aldehyde functionalities, the number of which could be modulated by varying the reaction conditions in the first and/or second steps. As an application, the functionalized proteins were derivatized with the fluorescent dye Lucifer Yellow CH, affording highly fluorescent bioprobes without altering their recognition properties in the case of avidin. In addition, glycidol derivatization greatly improved the water solubility of the final avidin-LyCH bioconjugates. Glycidol thus appears to be a low-cost and attractive reagent for protein chemical activation aimed at further coupling of amine-containing species such as enzymes, dendrimers and so on. It could also be interesting to apply this route to the preparation of a new generation of macromolecular metallocarbonyl markers for Carbonyl Metallo Immunoassay.

Experimental Section

BSA fraction V and horseradish peroxidase-biotin (HRP-biotin) were purchased from Sigma. Avidin and the dilithium salt of LyCH were purchased from Fluoroprobes. Glycidol (racemic form) and Purpald™ (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) were purchased from Aldrich. All other reagents are commercially available and were used as received. Dialysis was performed using tubular 6–8 kDa cutoff Spectra/Por® (Spectrum) membranes at 4 °C for 48 h. "NaPB buffer" refers to 10 mM sodium phosphate, 150 mm NaCl, pH 7.2. UV/Vis spectra were recorded on a Uv/mc2 spectrometer (Safas). Fluorimetric measurements were performed on a F-2000 fluorimeter (Hitachi).

General Procedure for the Synthesis of Protein-Diol Conjugates: Glycidol (final concentration ranging from 0.26 to 1.11 M) was added to a protein solution (10 mg mL^{-1} in 0.1 M carbonate buffer pH 9.2 or phosphate buffer pH 7.2) and the solution was stirred at room temp. for 4 to 72 h. The modified protein was purified by extensive dialysis in NaPB buffer. The concentration of the purified protein-diol was determined by UV/Vis analysis at 280 nm [ε_{280}(BSA) = 44000 M^{-1} cm^{-1};[19] ε_{280}(AV) = 102300 M^{-1} cm^{-1}[20]].

Determination of the Extent of Modification of Amines in Protein-Diol Samples: Solutions of the BSA and BSA-diol conjugates (0.4 mg mL^{-1}; 1 mL in 0.1 M borate buffer, 0.15 M NaCl, pH 8.6) were prepared in test tubes in duplicate. An aqueous solution of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 12 μL; 1% solution w/v) was added to each tube. After 40 min incubation in the dark at room temp., the absorbance of the yellow solutions was measured at 420 nm.

General Procedure for the Preparation of Formylated Proteins: A sodium periodate solution (0.1 M in citrate-phosphate buffer solution at pH 4, 5 or 6; final concentration 10 mM) was added to a solution of protein-diol (2–10 mg mL^{-1} in the same buffer solution as above), and the solution was stirred at room temp. in the dark. The solutions were typically oxidized for 15 or 60 min. Excess oxidant was quenched by adding ethylene glycol (0.25 mL mL^{-1} protein solution). After 10 min, the formylated protein was purified by size exclusion chromatography using a commercial dextran column (D-Salt®, Pierce, 5 mL bed volume, eluent: 10 mM citrate buffer pH 6, fraction size = 0.5 mL). The elution was monitored at

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280 nm and the concentration of the pooled formylated protein fractions was determined by UV/Vis analysis as above.

Qualitative Determination of Aldehyde Functions in Formylated Proteins: Solutions of formylated protein (0.5 mg mL^{-1} in citrate-phosphate buffer, pH 6) were prepared in test tubes in duplicate. A freshly prepared solution of Purpald (300 μL; 10 mg mL^{-1} in 1 M NaOH) was added to each tube. After 30 min of incubation at room temp., a freshly prepared solution of sodium borohydride (500 μL; 2 mg mL^{-1} in 1 M NaOH) was added. The absorbance of the solutions was measured at 550 nm.

Labelling of Formylated Proteins with LyCH: An aqueous solution of LyCH (22 mM; 250 molar equiv.) was added to a solution of formylated protein (0.2–2 mg mL^{-1} in 0.1 M phosphate buffer, 0.15 M NaCl, pH 6.5), and the solution was carefully stirred for 5 h at room temp. in the dark. Sodium cyanoborohydride (10 μL mL^{-1}; 5 M in the same buffer as above) was added, and the solution was kept for 18 h in the dark at 4 °C. Ethanolamine (20 μL mL^{-1}; 3 M in the same buffer) was added to block unreacted aldehydes. After 10 min, the solutions were filtered and subjected to size exclusion chromatography using a commercial HPLC column (300 × 7.8 mm Biosilect SEC250–5, Biorad). The labelled protein was separated from excess LyCH using NaPB as eluent at a flow rate of 0.7 mL min^{-1} and monitoring at 280 nm. The fractions containing the labelled protein were pooled and the fluorophore to protein (F/P) ratio was calculated as follows. Firstly, the concentration of LyCH was measured from the OD_{428} [ε_{428}(LyCH) = 11300 M^{-1} cm^{-1}]. Alternatively, it was determined fluorimetrically (λ_{ex} = 428 nm, λ_{em} = 540 nm) from a calibration with standard solutions of LyCH in NaPB in the 50 to 150 nM range. Secondly, the protein concentration was calculated from OD_{280} after subtraction of LyCH contribution [ε_{280}(LyCH) = 24170 M^{-1} cm^{-1}].[21]

Competitive Binding Assay of AV-LyCH Conjugates: Standard solutions of AV or AV-LyCH conjugate (50 μL; range 0–2 μM) prepared in PBS – 0.1% BSA were applied in duplicate to a streptavidin-coated microtiter plate (Labsystems), followed by application of HRP-biotin solution (50 μL; 200 ng mL^{-1} solution in PBS – 0.1% BSA). The plate was incubated for 60 min at room temp., and washed four times with PBS – 0.05% Tween 20. A solution of OPD (100 μL; 0.7 mg mL^{-1} in citrate-phosphate buffer pH 5 containing 0.04% H_2O_2) was added to the wells. The enzymatic reaction was stopped by addition of H_2SO_4 (2.5 M, 50 μL) and the OD_{490} was read with a microtiter plate reader (model 680, Biorad).

Acknowledgments

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