The synthesis of fluorescent chlorotriazinylaminofluorescein–concanavalin A and its use as a glycoprotein stain on sodium dodecyl sulphate/polyacrylamide gels

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Concanavalin A, a specific glycoprotein probe, was optimally labelled to a maximum stoichiometry of 0.4 mol of chlorotriazinylaminofluorescein (CTAF)/mol of concanavalin A monomer under mild reaction conditions (pH 8.0, 6 h), and under these conditions the CTAF–concanavalin A preparation retains its carbohydrate-binding ability and is able to penetrate SDS/7.5–15%–polyacrylamide gradient gels. CTAF–concanavalin A gives fluorescent bands for the glycoproteins transferrin, fetuin and deoxyribonuclease and shows no fluorescent response for the non-glycoproteins bovine serum albumin and soya-bean trypsin inhibitor. The detection limit of sensitivity for CTAF–concanavalin A, which is similar to that of fluorescein isothiocyanate–concanavalin A, is in the range 5–25 μ g of glycoprotein. CTAF–concanavalin A is a suitable probe for the detection of glycoproteins in higher-percentage ($\geq 10\%$) SDS/polyacrylamide gels, and will probably have other applications in, for example, fluorescent energy transfer and other structure-function studies.

INTRODUCTION

Fluorescent lectins have been shown to be very useful and specific probes of glycoprotein structure, function and location [1–7]. Fluorescein isothiocyanate- (FITC)-labelled lectins have been widely used and are commercially available from several manufacturers. However, FITC has a number of undesirable properties, such as variability in quality between manufacturers and batches, variable protein-labelling efficiency and multiple fluorescent and coloured impurities that can be a problem in obtaining consistently and maximally labelled fluorescent lectins [8,9]. In addition, FITC that efficiently labels lectin can decompose in only a few months of storage.

Conjugation of FITC to con A requires alkaline pH (pH 9.5) for extended times (e.g. 20 h) [6], and yet under these conditions con A forms a precipitate and in some cases is inactivated [10]. In using the procedure of Furland *et al.* [6] to conjugate FITC to con A (pH 9.5, 20 h), although a fluorescently labelled FITC–con A that binds to glycoproteins was obtained, it was not able to penetrate SDS/7.5–15%–polyacrylamide gradient gels for the staining of glycoproteins (C. W. Mahoney, unpublished work). The failure of commercial FITC–con A preparations to penetrate SDS/polyacrylamide gels of $\geq 10\%$ acrylamide has recently been documented by Cotrufo *et al.* [5]. Because of the precipitation of con A in coupling it to FITC at alkaline pH, it would be advantageous to employ a fluorescent labelling agent that can be covalently linked to con A under less alkaline reaction conditions.

Dichlorotriazinylaminofluorescein (DTAF), a fluorescent fluorescein analogue, reacts with free amino

groups under less alkaline conditions and more rapidly than FITC. DTAF has similar excitation and emission properties to those of FITC, and, in addition, can be synthesized economically, rapidly and in high yield and purity. DTAF also has the advantage that it is very stable in the solid state (98% purity for at least 15 months). These properties suggest that DTAF should be a more suitable fluorescent reagent for labelling con A. We report here the synthesis of DTAF, its solution stability and stoichiometric incorporation into con A, and the use of CTAF–con A as a glycoprotein probe.

MATERIALS AND METHODS

Materials

Cyanuric chloride, 4-aminofluorescein, and α -methyl D-mannoside were from Fluka Chemical (Buchs, Switzerland). Con A was from Fluka or Boehringer Mannheim (Rotkreuz, Switzerland). Hepes and Sephadex G-25 were from Sigma Chemical (Deisenhofen, Germany), and all other chemicals were of reagent-grade purity.

DTAF synthesis

DTAF was synthesized by the procedure of Blakeslee [8,9]. Organic solvents were dried over molecular sieves, and reactants with P_2O_5 *in vacuo*. A 50 mg (144 μ mol) portion of 4-aminofluorescein in 1.5 ml of anhydrous methanol was added dropwise to a stirred solution of 35 mg (190 μ mol) of cyanuric chloride in 250 μ l of anhydrous chloroform (at 4 °C). The reaction mixture was stirred for 2 h (at 4 °C), then quenched by the dropwise addition of 50 μ l of 10.3 M-HCl and stirred for an additional 1 h. The hydrochloride salt was then

Abbreviations used: con A, concanavalin A; DTAF, dichlorotriazinylaminofluorescein; CTAF, chlorotriazinylaminofluorescein; FITC, fluorescein isothiocyanate.

collected by vacuum filtration, washed with 14% (v/v) anhydrous chloroform in dry methanol and briefly dried. DTAF was obtained in 85–90% yield, and purity may be checked by t.l.c. on silica gel with methanol/chloroform (9:1, v/v) (R_F 0.95) or (1:9, v/v) (R_F 0.51). The purified product stored desiccated at 4 °C showed $\geq 98\%$ purity for at least 15 months.

Optimization of DTAF conjugation to con A

The final reaction mixture contained 2.1 mg of con A/ml (78 μM), 0.15 M-Hepes/NaOH buffer, pH 7.0 or 8.0, and a 1- or 3-fold molar excess of DTAF over con A, and the reaction was conducted in the presence or in the absence of 0.2 M- α -methyl D-mannoside (Scheme 1). At various times 100 μl samples were taken and applied to 1 ml of Sephadex G-25 in 0.15 M-Hepes/NaOH buffer, pH 7.0 or 8.0, containing 0.2 M- α -methyl D-mannoside in a 1 ml column and eluted with the same. Absorption spectra and protein assays were done on the fraction containing con A. Stoichiometries (mol of dye/mol of protein) were determined from the absorbance at 492 nm, the molar absorption coefficient of DTAF at 492 nm ($63400 \text{ M}^{-1}\cdot\text{cm}^{-1}$) [9] and the protein concentration.

Optimal DTAF conjugation to con A

The final reaction mixture contained 2.1 mg of con A/ml (78 μM), 0.15 M-Hepes/NaOH buffer, pH 8.0, and a 3-fold molar excess of DTAF over con A. The reaction was carried out for 6 h at room temperature (approx. 21 °C), was then quenched by making the solution 10 mM with respect to ethanolamine (by adding 1 M stock solution, pH 8.0) and 4 mM with respect to 2-mercaptoethanol, and the mixture was left to react for a further 6 h. The solution was then dialysed extensively against 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl, 1 mM-MnCl₂, 1 mM-CaCl₂ and 4 mM-2-mercaptoethanol. The dialysed solution was then diluted to 0.5 mg/ml with respect to con A, and a 3-fold molar excess of bovine serum albumin over con A was added. Further quenching under these conditions was carried out overnight (at room temperature). The solution was then adjusted to pH 5.5 with acetic acid and stored at 4 °C. Alternatively, quenching was carried out as follows. The solution was made 10 mM with respect to ethanolamine, left to react for 6 h (at room temperature) and then made 3 mM with respect to NaN₃ and 15.7 mg/ml with respect to bovine serum albumin and dialysed against 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl, 1 mM-MnCl₂, 1 mM-CaCl₂, 100 mM-2-mercaptoethanol and 3 mM-NaN₃ for 1 week (at room temperature) with several changes of dialysis solution. The protein solution was then diluted 10-fold and homogenized, and the pH was adjusted to pH 5.5 with acetic acid.

FITC conjugation to con A

The final reaction mixture contained 2.1 mg of con A/ml (78 μM), 0.15 M-Hepes/NaOH buffer, pH 8.0, and a 3-fold molar excess of FITC over con A. At various times 100 μl samples were removed, purified on Sephadex G-25, and analysed for the stoichiometry of mol of bound FITC/mol of protein as described above under 'Optimization of DTAF conjugation to con A'. The molar absorption coefficient at 492 nm for FITC

($76600 \text{ M}^{-1}\cdot\text{cm}^{-1}$) [9] was used for the stoichiometric calculations.

DTAF stability study

Solutions of DTAF (4.68 mM) in 100 mM-sodium borate buffer, pH 9.0, were maintained at various temperatures (−80 °C, −20 °C, 4 °C and room temperature) in ambient light (at room temperature) or in the dark (at room temperature). At various times samples were diluted 1000-fold into 100 mM-sodium borate buffer, pH 9.0, and absorption spectra were taken immediately. Decomposition of DTAF was calculated from the absorption decline at 492 nm as a function of time.

SDS/polyacrylamide-gel electrophoresis

SDS/7.5–15% -polyacrylamide gradient gels were cast in the absence of EDTA by the procedure of Laemmli [11]. After electrophoresis the gels were fixed for 1 h or overnight in 40% (v/v) methanol/10% (v/v) acetic acid and then equilibrated (4 × 30 min) in 50 mM-acetic acid/NaOH buffer, pH 5.5, containing 100 mM-NaCl and 4 mM-2-mercaptoethanol. The gels were then overlaid with the quenched DTAF-con A solution (40 ml/gel) and gently agitated overnight or for 2–3 days. The gels were then destained in the above equilibration solution (4 × 30 min) and dehydrated in 94% (v/v) ethanol. Photographs of the fluorescent gels were taken with the aid of a u.v. transilluminator and a Polaroid MP-3 camera with u.v. cut-off and orange filters.

Protein assay

Protein concentration was determined by the procedure of Lowry *et al.* [12], with con A (molar absorption coefficient at 280 nm $30780 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [15]) as standard.

RESULTS AND DISCUSSION

DTAF synthesis and stability

The reaction scheme for the synthesis of DTAF is shown in Scheme 1 and its absorption and emission spectra in Fig. 1. DTAF was obtained from the reactants 4-aminofluorescein and cyanuric chloride at greater than 98% purity (as determined by t.l.c. analysis, fluorescence and I₂ staining) in an 85–95% yield. In addition, the solid product when stored desiccated (at 4 °C) showed $\geq 98\%$ purity for at least 15 months of storage. Although DTAF in the anhydrous solid state is very stable, the contrary was found for alkaline solutions at pH 9.0 (Fig. 2). DTAF in 100 mM-borate buffer, pH 9.0, has decomposition half-lives of 11.8, 6.5, 5.0, 4.4 and 4.1 h for solutions maintained at −80 °C, −20 °C, 4 °C, room temperature (dark) and room temperature (ambient light) respectively (Fig. 2). Despite the fact that DTAF in alkaline solution (pH 9.0) has a half-life of 4–5 h (at room temperature), conjugation to con A at pH 8.0 (at room temperature) proceeds rapidly and efficiently to yield a fluorescent derivative with a similar labelling stoichiometry to that in the case of FITC-con A (see below). The presence or absence of ambient light had little effect on the decomposition of DTAF (Fig. 2).

DTAF coupling to con A

Purified DTAF was coupled to con A under various conditions of pH, time and DTAF/con A molar ratio

Scheme 1. Reaction scheme for the synthesis of DTAF from 4-aminofluorescein and concanavalin A

Fig. 1. Absorption and fluorescence-emission spectra of DTAF
—, Absorption spectrum; ·····, fluorescence-emission spectrum. Spectra were performed on solutions of DTAF in 100 mM-borate buffer, pH 9.0.

Fig. 2. Solution stability of DTAF at alkaline pH

Solutions of DTAF (4.68 mM) were maintained at various temperatures [○, -80 °C; ●, -20 °C; □, 4 °C; ■, room temperature (dark); △, room temperature (ambient light)], and spectra were taken as a function of time.

and in the presence or absence of α -methyl D-mannoside in order to optimize the coupling efficiency (Fig. 3). The presence or absence of 0.2 M- α -methyl D-mannoside had no significant effect on the kinetics or efficiency of DTAF

Fig. 3. Optimization of DTAF coupling to con A

Con A (2.1 mg/ml) in 0.15 M-Hepes buffer, pH 7.0 (○, ●, □ and ■) or pH 8.0 (△, ▲, + and ×), was treated with a 1-fold (○, □, △ and +) or 3-fold (●, ■, ▲, and ×) molar excess of DTAF over con A and in the presence (□, ■, + and ×) or in the absence (○, ●, △ and ▲) of 0.2 M- α -methyl D-mannoside for various lengths of time. See the Materials and methods section for further details.

coupling to con A, suggesting that the DTAF labelling does not occur at the carbohydrate-binding site of con A (Fig. 3). Little difference was also seen in the saturation kinetics at pH 8.0 relative to pH 7.0, although at the higher pH the labelling stoichiometry was approximately 2-fold that at pH 7.0. Similarly, increasing the molar excess of DTAF over con A from 1 to 3 results in an approximately 2-fold increase in the labelling stoichiometry. A maximum incorporation of 0.35 mol of DTAF/mol of protein monomer was obtained in ≥ 6 h at pH 8.0 with a 3-fold molar excess of DTAF over con A. In attempts to increase the labelling efficiency further conjugations were carried out at higher pH values, higher molar excesses of DTAF over con A and at higher temperatures (results not shown). In all cases a maximum labelling stoichiometry of 0.5 mol of DTAF/mol of protein monomer was obtained.

Con A undergoes monomer–dimer, dimer–tetramer and tetramer–aggregate equilibrium reactions in a pH-dependent manner, and in many cases the final aggregation is irreversible. Dimer formation occurs at

Scheme 2. Alternative models for the DTAF modification of con A

Fig. 4. Kinetics and stoichiometry of DTAF and FITC labelling of con A

Con A (2.1 mg/ml) in 0.15 M-Hepes buffer, pH 8.0, was treated with a 3-fold molar excess of each fluorophore (○, DTAF; ●, FITC) over con A for various times. After 49 h a fresh solution of DTAF was added to an additional 3-fold molar excess over con A to the CTAF-con A mixture. See the Materials and methods section for further details.

$\leq$ pH 5.6 and tetramer and aggregate formation at $\geq$ pH 5.6 [13]. Under the experimental conditions of the experiment of Fig. 3 there is no appreciable precipitation of con A. The maximum labelling stoichiometry of 0.5 mol of CTAF/mol of con A monomer suggests that a single site per con A dimer is accessible for modification and that the tetramer at $\geq$ pH 5.6 contains 2 mol of CTAF/mol. This stoichiometry further suggests that a single DTAF-modifiable site on monomer₍₁₎ is made unreactive or inaccessible by the binding of

monomer₍₁₎ to monomer₍₂₎. Such an effect can be caused either by a steric block or by a distal conformational alteration of the DTAF-modifiable site on monomer₍₁₎ distal to the monomer₍₁₎/monomer₍₂₎ interface (Scheme 2).

To ascertain that the labelling of con A with DTAF can occur under less drastic conditions than with FITC, parallel experiments were carried out under identical conditions with DTAF in one case and FITC in the other (Fig. 4). As expected, the labelling is much more rapid in the case of DTAF, and the reactions are essentially complete in 6 h for DTAF and in 30 h for FITC. Maximum incorporations of 0.4–0.5 mol of fluorophore/mol of protein monomer were obtained in both cases. If one assumes that the same site on con A is modified by DTAF and FITC, then the identical stoichiometric incorporations of both into con A and the similar fluorescent spectra of the free fluorophores suggest that the CTAF-con A and FITC-con A should have similar fluorescent properties.

Because of the instability of DTAF in alkaline solution [$t_{0.5}$ 4.1 h (at room temperature)] a freshly made solution of DTAF (4.68 mM in 100 mM-borate buffer, pH 9.0) was added to a final additional 3-fold molar excess over con A to con A that had already been treated with DTAF for 47 h (Fig. 4). Despite the labile nature of alkaline DTAF solutions, no further incorporation of DTAF into CTAF/con A mixture was seen (Fig. 4), indicating maximal incorporation of 0.4–0.5 mol of CTAF/mol of protein monomer in the presence or in the absence of additional fresh DTAF solution.

SDS/polyacrylamide-gel-electrophoretic analysis (under reducing conditions) of quenched CTAF-con A demonstrated the presence of a single fluorescent band corresponding to the con A monomer for the mildly quenched CTAF-con A (6 h) and a lack of aggregates at the stacking-gel/separating-gel interface when stained with silver. In the case of the extensively quenched CTAF-con A (1 week), although only a single fluorescent band at the position of the con A monomer

Fig. 5. Specificity of CTAF-con A as a glycoprotein stain on SDS/polyacrylamide gels

Various proteins (transferrin, fetuin, DNAase, bovine serum albumin, soya-bean trypsin inhibitor; lanes 1–5 respectively; 25 μ g each) were electrophoresed on an SDS/7.5–15% polyacrylamide gradient gel. After fixation and staining with a CTAF-con A solution, the gel was dehydrated in 94% ethanol and photographed over a u.v. transilluminator (a). The gel was then stained with Coomassie Brilliant Blue G250 and destained (b). See the Materials and methods section for further details.

Fig. 6. Sensitivity of CTAF-con A as a glycoprotein stain on SDS/polyacrylamide gels

Transferrin (25, 5 and 1 μ g; lanes 1–3 respectively) was electrophoresed on an SDS/7.5–15% polyacrylamide gradient gel. The CTAF-con A staining and photography were by the same procedure as indicated in the legend to Fig. 5.

was detected, higher aggregates were seen at the stacking-gel/separating-gel interface when stained with silver (results not shown). These results are indicative of the low reactivity of the final chloro substituent on the triazinyl ring of CTAF-con A. The three chloro substituents in trichlorotriazine have high, intermediate and low reactivities respectively, with the third chloro group showing 700-fold lower reactivity than the second chloro substituent [14].

CTAF-con A as a glycoprotein stain

Quenched CTAF-con A (0.2–0.5 mg/ml) acts as a specific and sensitive fluorescent glycoprotein stain when overlaid on SDS/7.5–15% polyacrylamide gradient gels (Fig. 5 and 6). A fluorescent response is apparent for transferrin, fetuin and DNAase (Fig. 5, lanes 1–3 respectively), which are all glycoproteins, and negative controls bovine serum albumin and soya-bean trypsin inhibitor (Fig. 5, lanes 4 and 5 respectively) show no fluorescence. Several and many fluorescent bands are seen for fetuin and DNAase respectively, indicating the heterogeneity of these commercial preparations. In the case of soya-bean trypsin inhibitor some higher- M_r

fluorescent bands are seen above the former, indicative of lack of purity of the commercial preparation. To demonstrate the detection-sensitivity of the CTAF-con A for glycoproteins in SDS/polyacrylamide gels various loads of transferrin were stained on 7.5–15% polyacrylamide-gradient gels (Fig. 6). Transferrin, which showed the strongest fluorescence response, has a detection limit of sensitivity of between 5 and 25 μg of transferrin. Although the fluorescence intensity/ μg of protein is variable among different proteins, similar limits of sensitivity were seen for all (5–25 μg of glycoprotein; results not shown). This detection limit of sensitivity for CTAF-con A compares favourably with that of FITC-con A (low microgram amounts of glycoprotein) [5,6].

In initial experiments with CTAF-con A as a glycoprotein stain on SDS/polyacrylamide gels, covalent incorporation of the fluorescent con A into glyco- and non-glyco-proteins was seen as demonstrated by the inability to remove the fluorescent probe with increasingly stringent washes containing various concentrations and combinations of reducing agents, salt, sugar, detergents, non-polar reagents and denaturants (urea and guanidinium chloride) (results not shown). Therefore the extensive quenching of CTAF-con A as well as staining under non-reactive acid pH conditions is critical to obtaining specificity for glycoproteins.

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