Preparation and characterization of a recombinant DNA-derived ovine growth hormone variant internally labelled with sulphur-35

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ABSTRACT

$^{125}$I-Labelled polypeptide hormones have been extremely valuable for radioimmunoassays, receptor-binding studies and investigation of the processing and metabolism of hormones. However, such externally labelled material has the disadvantage that addition of one or more iodine atoms may alter the properties of the polypeptide. Furthermore, for studies on hormone metabolism and processing, the label may become separated from the hormone or its main breakdown products. Use of internally labelled polypeptides produced by biosynthesis can avoid such problems, but previously such material has usually been of low specific radioactivity, and unsuitable for many purposes. Here we describe the development of a procedure for the production of an internally labelled ovine GH analogue (oGH1) using a plasmid produced by recombinant DNA methods and expression in *Escherichia coli*.

Bacteria were grown in medium containing a low sulphate concentration, and then incubated in medium containing $^{35}$SO$_4^{2-}$ as the sole sulphur source. Under these conditions, the bacteria incorporated $^{35}$S into proteins including GH. Purification of such material required considerable modification of previously described methods, because of the need to handle very small amounts of highly radioactive material. The bacteria were lysed using lysozyme, and inclusion bodies were solubilized using 6 M guanidinium chloride. $^{35}$S-oGH1 was renatured and then purified by gel filtration on Sephacryl S-100, followed by immunoaffinity chromatography and a second gel filtration step. Material prepared in this way had a specific radioactivity of 6–27 $\mu$Ci/$\mu$g, and showed high 'bindability' to polyclonal and monoclonal antibodies and to receptors. $^{35}$S-Labelled material bound to receptors more effectively than $^{125}$I-labelled GH and showed improved stability. Such material appears to be well suited to receptor-binding studies and studies on the processing and metabolism of GH. The procedure developed should be applicable to other polypeptide hormones.

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INTRODUCTION

Polypeptide hormones labelled to a high specific radioactivity with $^{125}$I are widely used as labelled ligands in radioimmunoassays, radioreceptor assays, and to follow the internalization, processing and metabolism of such hormones. Externally labelled material of this type has the potential disadvantage that the chemical modification required to introduce the label may significantly alter the properties of the ligand. This is usually of little importance where the $^{125}$I-labelled ligand is used for a radioimmunoassay using a polyclonal antibody, presumably because the antibody can interact with several different epitopes on the polypeptide, not all of which will be affected by the iodination in any one molecule. When a polypeptide interacts with a monoclonal antibody or receptor, external labelling may cause problems due to modification of a unique binding site on the polypeptide in a fraction of the labelled molecules. Accordingly, it is frequently observed that $^{125}$I-labelled hormones show a 'bindability' (the fraction of labelled ligand which is able to bind to receptor etc.) of considerably less than 100% when interacting with receptors or monoclonal...
antibodies. Furthermore, modification of a receptor-binding site may lead to altered binding properties (e.g. lowered affinity) in a part of the labelled ligand, giving rise to a heterogeneity in binding properties which may be incorrectly interpreted in terms of heterogeneity of receptors. Such problems may be particularly important in the case of a hormone such as pituitary growth hormone (GH; somatotrophin) that has more than one binding site (de Vos et al. 1992). Use of iodinated material for studies on the metabolism and degradation of a hormone may cause particular problems, since the labelled hormone and fragments produced from it may behave quite differently from the endogenous hormone and fragments. As a consequence, the investigator may follow the fate of iodinated peptides or amino acids (or even free iodide), which bears little relation to that of unlabelled hormone.

In view of these limitations posed by the use of externally labelled material, there is a need for the production of internally labelled polypeptides of high specific activity. In some cases, semisynthesis can be used for this purpose; for example, Jones et al. (1987) used this approach to prepare \([^{3}H]_{2}^{1}\text{Phe}^{1}\) proinsulin. Internally labelled GH has been produced previously by incubating pituitary glands with radioactive amino acids (e.g. Wallis et al. 1978), but the specific radioactivity of material produced in this way was very low. An alternative approach involves the use of a bacterial strain expressing GH, developed using recombinant DNA techniques. Here we present a study in which methods were developed to allow incorporation of \(^{35}\text{S}\), supplied as inorganic sulphate, into an ovine GH analogue (oGH1) expressed in Escherichia coli (Wallis & Wallis, 1990). Renaturation and purification of the oGH1 so-produced was achieved by adapting our previously developed procedure to handle very small quantities (<50 \(\mu\)g) of highly radioactive material (6–27 \(\mu\)Ci/\(\mu\)g). The ability of the labelled hormone to interact specifically with receptors and antibodies was investigated, as was its stability.

MATERIALS AND METHODS

Materials

Carrier-free \(\text{Na}_{2}^{35}\text{SO}_{4}\) was obtained from ICN Flow (High Wycombe, Bucks, U.K.). Lysozyme (chicken egg white), DNase I, Sepacryl S-100 HR, bovine serum albumin (BSA; Cohn Fraction V), cyanogen bromide-activated Sepharose 4B, isopropyl \(\beta\)-D-thiogalactopyranoside (IPTG) and phenyl-methylsulphonylfluoride (PMSF) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). A monoclonal antibody against bovine GH (GH1/D4) was purified by ammonium sulphate precipitation from mouse ascites fluid. The hybridoma producing this antibody was developed in our laboratory by Dr K. P. Ray (Thorpe et al. 1990). Monoclonal antibodies OA11, OA12 and OA16, raised against ovine GH (Aston et al. 1987), were a gift from Dr R. Aston. Human GH was a gift from Wellcome Laboratories, Beckenham, Kent, U.K.

**Growth of bacteria**

Plasmid pOGHe101 includes the coding sequence for an ovine GH variant, oGH1, which has an eight amino acid extension on the N terminus of ovine GH and shows full growth-promoting activity (Wallis & Wallis, 1990). The oGH1 coding sequence is inserted under the control of the lac promoter. *E. coli* strain JM109, harbouring plasmid pOGHe101, was grown in a minimal medium (M9 salts plus 2·5 \(\mu\)g thiamine/ml and 20 \(\mu\)g thymine/ml; Miller, 1972), containing 0.01% ampicillin and 0·2% galactose as carbon source. The sulphate concentration (as \(\text{MgSO}_{4}\)) was varied from 0 to 2 \(\text{mM}\), with \(\text{MgCl}_{2}\) being added to maintain a total \(\text{Mg}^{2+}\) concentration of 2 \(\text{mM}\). For most of the work described here, growth medium contained 100 \(\mu\)M \(\text{MgSO}_{4}\) and medium used for labelling contained no added unlabelled sulphate. Bacteria were grown in a shaking water bath at 37 °C. oGH1 expression was induced by adding IPTG to a final concentration of 0·67 \(\text{mM}\), 2–20 h before harvesting.

**Labelling of bacteria**

For labelling at high specific radioactivity, bacteria were grown in minimal medium containing 100 \(\mu\)M \(\text{MgSO}_{4}\) and no labelled sulphate. IPTG was added 2 h before harvesting; induction before adding the label was intended to maximize the incorporation of the label into GH, though this may have been at the cost of somewhat decreased specific radioactivity. After harvesting and washing with medium containing no added sulphate, bacteria (equivalent to 1 ml of a culture with an optical density at 600 nm of 0·4 units) were incubated in 0·2 ml minimal medium (no unlabelled sulphate) containing 0·67 mM IPTG and 3–30 mCi Na\(_{2}^{35}\text{SO}_{4}\)/ml for 15 h at 37 °C.

To follow the progress of the labelling and purification procedures, samples were taken for scintillation counting or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at various stages. These samples amounted to no more than 5% of the starting material in total, and no correction has been made for these losses in

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calculating yields and recoveries (Table 1). For all centrifugation steps during the purification, 3 min at low speed (6500 r.p.m., ~2000 g) on an MSE Micro Centaur bench centrifuge was used. For scintillation counting a Wallac 1409 counter was used, and samples were mixed with 0.5-1 ml scintillation fluid (Optiphase Safe; Wallac, Milton Keynes, Bucks, U.K.), after solubilization with 0.1 ml Soluene (Canberra Packard, Pangbourne, Berks, U.K.) for 60 min at 60°C in the case of bacteria or inclusion bodies.

Lysis of bacteria and preparation of $^{35}$SJoGH1-containing inclusion bodies

After labelling, bacteria were recovered by centrifugation, washed with 100 mM NaCl in Tris–HCl buffer (10 mM Tris adjusted to pH 8.0 with HCl) and lysed using lysozyme (Marston, 1987). The washed pellet was suspended in 1 ml lysis buffer cooled to 0°C (50 mM Tris, 1 mM EDTA, 100 mM NaCl, adjusted to pH 8.0 with HCl), and PMSF (30 μl; 50 mM in methanol) and lysozyme (27 μl; 10 mg/ml in lysis buffer) were added. After 30 min at 0°C, 1:3 mg deoxycholic acid were added and the mixture was incubated for 75 min at 37°C with shaking. DNase (7 μg; 1 mg/ml in lysis buffer) was added and after 1 h at 20°C the mixture was frozen/thawed three times and centrifuged. The pellet (primarily inclusion bodies) was washed twice with 10 mM Tris–HCl, pH 8.0, containing 100 mM NaCl.

Solubilization of inclusion bodies and renaturation of $^{35}$SJoGH1

The inclusion body pellet (in a 1.5 ml microcentrifuge tube) was dissolved in 0.2 ml 6 M guanidinium chloride (GuCl) in ethanolamine–HCl buffer (50 mM ethanolamine adjusted to pH 9.3 with HCl) containing 5 mM dithiothreitol, and left for 75 min at 20°C under N2. Reduced glutathione (GSH; 5 μl, 410 mM) and oxidized glutathione (GSSG; 5 μl, 205 mM) (both in the same GuCl–ethanolamine–HCl buffer) were added and the tube was flushed with N2, capped and left for 45 min at 20°C. Ethanolamine–HCl (420 μl, 50 mM, pH 9.3) containing 63 μg sucrose/420 μl was added slowly, with mixing (giving 10% (w/v) final concentration of sucrose). Again the tube was flushed with N2, capped and stood for 3 h at 20°C. A pinhole was made in the cap and the tube was left at 4°C for 2-4 h. The cap was then removed and air-oxidation was completed by standing at 4°C for 11-16 h. Ethanolamine–HCl buffer (0.34 ml, 50 mM, pH 9.3) was added, with mixing; the mixture was centrifuged, and the supernatant was applied to a Sephacryl S-100 column (see next section).

Chromatography on Sephacryl S-100 HR

Renatured labelled oGH1, still in 0.5-1.0 m GuCl, was fractionated by gel filtration on a column of Sephacryl S-100 HR (47 cm × 0.78 cm2), equilibrated and eluted with Tris–HCl buffer (25 mM Tris adjusted to pH 7.6 with HCl) containing 0.1% BSA, 0.02% NaN3 and 0.05% Tween 20 (20°C; flow rate about 7.5 ml/h). Fractions (approximately 0.5 ml) were collected, and 2 μl samples of these were subjected to scintillation counting. Fractions were stored at 4°C and 1.5 ml 50 mM PMSF in methanol was added to those fractions that were to be purified further.

Immuoaffinity purification

A monoclonal antibody against bovine GH (GH1/D4) was coupled to cyanogen bromide-activated Sepharose 4B using a method based on that of Szewczuk & Prusak (1985). Cyanogen bromide-activated Sepharose 4B (0.3 g) was swelled, washed and suspended in coupling buffer (0.25 m NaHCO3 adjusted to pH 9.5 with NaOH). Monoclonal antibody GH1/D4 (20.7 mg), dissolved in coupling buffer, was added to the cyanogen bromide-activated Sepharose 4B (final volume about 6 ml), followed by mixing for 22 h at 4°C. The Sepharose 4B (to which about 7.7 mg of antibody had bound) was recovered by centrifugation, washed with coupling buffer containing 0.5 m NaCl, and then suspended in 0.5 m ethanolamine–HCl buffer, pH 9.5, to destroy any remaining active groups (2 h at 20°C). After further washing, the matrix was suspended in 4 ml storage buffer (0.8% NaCl in sodium phosphate buffer, pH 7.6, 50 mM with respect to phosphate, containing 0.02% NaN3 and 0.5 mM PMSF) and stored at 4°C. The material prepared in this way could be reused at least three times. After GH was stripped from the antibody with 3.6 m NaSCN (see below), the antibody– Sepharose 4B affinity matrix was washed with storage buffer, and again stored at 4°C.

Anti-bovine GH–Sepharose 4B (1 ml; approximately 1.9 mg antibody bound to 75 mg (dry weight) Sepharose 4B) was mixed with 0.5-2.0 ml (16 × 106-100 × 106 d.p.m.) of the $^{35}$SJoGH1 produced following chromatography on Sephacryl S-100 (see above). PMSF was added to give a concentration of 0.5 mM, and the mixture was stirred for 1-2 h at 20°C, by which time maximal binding of labelled protein had been achieved. The affinity matrix was then transferred to a small column and washed with about 6 ml storage buffer. The column was eluted with NaSCN, in storage buffer, usually 1.5 m (3 ml), 2.0 m (3 ml) and 3.6 m (2 ml). The bulk of the label was eluted in
TABLE 1. The progress of purification of [\(^{35}\)S]oGH1 through one experiment, showing the recovery of [\(^{35}\)S] and the yield and purity of [\(^{35}\)S]oGH1

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Total (^{35})S (mCi)</th>
<th>Recovery of (^{35})S (%)</th>
<th>(^{35})S in oGH1* (mCi)</th>
<th>Recovery of [(^{35})S]oGH1* (%)</th>
<th>Purity of [(^{35})S]oGH1* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original incubation medium</td>
<td>3-72</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Labelled bacteria</td>
<td>1-46</td>
<td>39</td>
<td>0-23</td>
<td>100</td>
<td>16</td>
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<tr>
<td>Inclusion bodies</td>
<td>0-62</td>
<td>17</td>
<td>0-175</td>
<td>74</td>
<td>28</td>
</tr>
<tr>
<td>Main peak after first gel filtration step</td>
<td>0-146</td>
<td>3-9</td>
<td>0-115</td>
<td>49</td>
<td>79</td>
</tr>
<tr>
<td>Main peak after immunoaffinity purification and second gel</td>
<td>0-074</td>
<td>2-2</td>
<td>0-072</td>
<td>33</td>
<td>97</td>
</tr>
</tbody>
</table>

*Based on results of sodium dodecyl sulphate-polyacrylamide gel electrophoresis/autoradiography.

about 1 ml 1·5 mM NaSCN, which was transferred rapidly (<45 min) to a column of Sephacryl S-100 (55 cm x 0·78 cm\(^2\)), equilibrated and eluted with the column buffer described previously. Fractions (about 0·6 ml) were collected, and samples (2 µl) were removed for scintillation counting. The main peak was pooled and stored at 4°C after adding PMSF to give a final concentration of 0·5 mM.

SDS-PAGE and autoradiography

[\(^{35}\)S]oGH1, labelled E. coli, and samples taken during the course of the purification were subjected to SDS-PAGE using the method of Laemmli (1970) (reducing conditions; 12·5% acrylamide). Gels were stained with Kenacid Blue, destained, dried and subjected to autoradiography for 1–14 days (using Fuji X-ray film, RX). Quantification of autoradiograms was carried out using a laser densitometer (Ultrascans XL; Pharmacia/LKB, Milton Keynes, Bucks, U.K.).

Antibody-binding studies

The ability of [\(^{35}\)S]oGH1 to bind to polyclonal and monoclonal antibodies was studied, using a rabbit anti-bovine GH polyclonal antibody and three anti-ovine GH monoclonal antibodies (OA11, OA12, OA16; Aston et al. 1987) prepared from mouse ascites fluid. [\(^{35}\)S]oGH1 (4–10 nCi/tube) and increasing concentrations of antibodies were incubated in radioimmunoassay buffer (50 mM NaH\(_2\)PO\(_4\) adjusted to pH 7·6 with NaOH, containing 0·6 mM merthiolate, 0·05% BSA and 0·05% Tween 20), final volume 0·3 ml, in polypropylene LP3 tubes (Luckhams, Burgess Hill, Sussex, U.K.) for 18 h. In the case of the study with monoclonal antibodies, the buffer also contained 0·15 mM PMSF. Antibody-bound oGH1 was separated from unbound hormone by precipitation with polyethylene glycol 6000 (Desbuquois & Aurbach, 1971), followed by centrifugation (1000 g, 4°C, 20 min). Precipitates were solubilized by adding 0·1 ml Soluene and standing for 5 min; 1 ml scintillation fluid was then added, followed by scintillation counting.

Receptor-binding studies

Binding of [\(^{35}\)S]oGH1 to GH receptors was carried out using a plasma membrane preparation prepared from the liver of a pregnant rabbit as described by Webb et al. (1986). [\(^{35}\)S]oGH1 (approximately 10 nCi/tube) was incubated with plasma membranes (0–300 µg protein/tube) in Tris-HCl buffer (25 mM Tris, 0·6 mM merthiolate, 0·1% BSA, 10 mM CaCl\(_2\), 0·1 mM PMSF, adjusted to pH 7·6 with HCl). For each receptor concentration, non-specific binding was determined in the presence of 1 µg human GH. The final volume was 0·5 ml/tube, and incubation was carried out for 18 h at 20°C in polypropylene LP3 tubes. Samples were then centrifuged (1000 g, 20°C, 20 min), supernatants were removed and precipitates were dissolved in 0·1 ml Soluene (30 min, 20°C) and subjected to scintillation counting.

Radioimmunoassay; determination of specific radioactivity

To determine the specific radioactivity of [\(^{35}\)S]oGH1, the hormone content of each preparation was determined by radioimmunoassay for
ovine GH1, using the method of Ray & Wallis (1982) and oGH1 as standard, and compared directly with the corresponding radioactivity determined by scintillation counting.

RESULTS AND DISCUSSION

Labelling of oGH1

In order to maximize incorporation of $^{35}$SO$_4^{2-}$ into bacterial proteins (including oGH1), bacteria (E. coli JM109/pOGHe101) were grown on minimal medium containing a lowered sulphate concentration. The maximum yield of bacteria obtained was approximately halved when minimal medium containing 2 mM MgSO$_4$ was used instead of the enriched growth medium used previously (Wallis & Wallis, 1990), but after induction with IPTG the proportion of bacterial protein that was oGH1 (20–30% as assessed by SDS-PAGE) was unchanged. When the sulphate concentration was lowered, bacterial growth was unaltered at sulphate concentrations above about 100 $\mu$M (data not shown) and oGH1 continued to make up 20–30% of bacterial proteins. Minimal medium containing this sulphate concentration was therefore used subsequently for growth of bacteria.

When trace amounts of Na$_2^{35}$SO$_4$ (20 $\mu$Ci/ml) were included in the growth medium $^{[35S]}$oGH1 was synthesized as expected, and labelled oGH1 represented 20–30% of the labelled bacterial protein (assessed by SDS-PAGE and autoradiography) (Gwilliam et al. 1991). However, when larger amounts of Na$_2^{35}$SO$_4$ were used (1-10 mCi/ml) bacterial growth was stopped completely, presumably due to radiation damage to the repair-deficient bacterial strain. In order to produce $^{[35S]}$oGH1, bacteria were therefore grown in minimal medium (100 $\mu$M sulphate) containing no label, and then incubated in minimal medium containing no unlabelled sulphate and 3–30 mCi Na$_2^{35}$SO$_4$/ml. Under these conditions $^{35}$S was incorporated into oGH1 although bacterial growth stopped; presumably the processes involved in the biosynthesis of amino acids and proteins are less sensitive to radiation damage than is replication. Label incorporated into oGH1 using this procedure represented 15–20% of the label present in all bacterial proteins. Incorporation was normally carried out for about 15 h at 37 °C in a shaking water bath, using bacteria that had been induced with IPTG for 2 h prior to the addition of label. However, variation of these times did not cause large differences in the amount of label incorporated or the proportion in oGH1. Under these conditions, label incorporated into bacteria represented 20–50% of that in the incubation medium.

Isolation, solubilization and renaturation of $^{[35S]}$oGH1 in inclusion bodies

When oGH1 is synthesized in E. coli it is largely deposited in insoluble inclusion bodies (Schoner et al. 1985; Wallis & Wallis, 1990). The procedure used to obtain correctly folded and purified oGH1 from such inclusion bodies was based on that described previously for larger-scale preparation (Wallis & Wallis, 1990), but with modifications to allow handling of small amounts of highly radioactive material. Release of inclusion bodies from bacteria was achieved by lysozyme treatment. This is not thought to be more efficient than sonication, as used previously, but involves fewer problems of radioactive contamination of the equipment used and its surroundings. After recovery of inclusion bodies by centrifugation, they were solubilized using 6 M GuCl containing 5 mM dithiothreitol, as usual, but a 50:50 mixture of reduced and oxidized glutathione was then added (based on Wingfield et al. 1987). This provides a more controlled environment for air oxidation of disulphide bridges during renaturation in a very small volume. Alteration of the ratio of oxidized to reduced glutathione over a moderately wide range did not affect the yield of renatured protein substantially. Stepwise dilution of the GuCl solution of oGH1 was similar to that used previously. Salts and reducing agents were subsequently removed by gel filtration (the first Sephacryl S-100 column, see below) rather than dialysis. This was more convenient for the small highly radioactive sample at this stage. Table 1 shows the recovery of $^{35}$S and the yield and purity of oGH1 achieved at various stages of the isolation procedure for one preparation, and Fig. 1 shows SDS-PAGE/autoradiography of various fractions.

Further purification of $^{[35S]}$oGH1

After renaturation, the $^{[35S]}$oGH1 (still dissolved in 0.5–1.0 M GuCl) was centrifuged (removing 2–10% of the labelled material) and the supernatant was fractionated on a Sephacryl S-100 gel filtration column. The elution profile for such a column is shown in Fig. 2a. The 'monomeric' labelled oGH1 peak (fractions 37–41 in Fig. 2a, as shown by SDS-PAGE/autoradiography) accounted for 30–45% of the radioactivity recovered from the column. It was important to process $^{[35S]}$oGH1 in bacteria to the chromatographic stage as rapidly as possible; delays of a few days (e.g. inclusion bodies stored

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were electrophoresis (SDS-PAGE)/autoradiography of $^{35}$S-oGH1 at various stages of purification. Samples of fractions at various stages of the purification scheme were subjected to SDS-PAGE followed by autoradiography. Lane 1, labelled bacteria; lane 2, supernatant fraction following inclusion body preparation; lane 3, inclusion body fraction; lane 4, $^{35}$S-oGH1 peak from the first S-100 column (see Fig. 2a); lane 5, $^{35}$S-oGH1 peak from the second S-100 column (see Fig. 2b).

Frozen) led to a marked decrease in the size of the oGH1 peak (and an increase in the amount of material of higher $M_r$), probably due to radiation damage caused by storage of highly labelled protein in the very concentrated form of inclusion bodies.

The monomeric $^{35}$S-oGH1 peak from Sephacryl S-100 was treated with protease inhibitor (PMSF) and then subjected to immunoaffinity chromatography. This step replaced the ion-exchange chromatography previously used for the isolation of oGH1 (Wallis & Wallis, 1990), which proved difficult to scale down to deal with microgram quantities of labelled material. Seventy to eighty per cent of the $^{35}$S-labelled material from the peak from gel filtration bound to the immunoaffinity matrix, which was in good accord with the purity of the oGH1 at this stage, as judged by SDS-PAGE/autoradiography. Eighty to ninety per cent of the bound label was eluted from the column with 1·5 M NaSCN, and the remainder was eluted by higher concentrations of NaSCN. The material eluted with 1·5 M NaSCN was subjected to a second gel filtration step on Sephacryl S-100 (Fig. 2b), which

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**FIGURE 1.** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)/autoradiography of $^{35}$S-oGH1 at various stages of purification. Samples of fractions at various stages of the purification scheme were subjected to SDS-PAGE followed by autoradiography. Lane 1, labelled bacteria; lane 2, supernatant fraction following inclusion body preparation; lane 3, inclusion body fraction; lane 4, $^{35}$S-oGH1 peak from the first S-100 column (see Fig. 2a); lane 5, $^{35}$S-oGH1 peak from the second S-100 column (see Fig. 2b).

**FIGURE 2.** Chromatographic purification of $^{35}$S-oGH1. (a) Gel filtration of renatured $^{35}$S-oGH1 on Sephacryl S-100. The inclusion body fraction was dissolved in 6 M guanidinium chloride, renatured as described in the text, and applied to a column of Sephacryl S-100 (47 cm x 0·78 cm$^2$), equilibrated and eluted with 25 mM Tris–HCl, pH 7·6, containing 0·1% bovine serum albumin, 0·02% NaN$_3$ and 0·05% Tween 20. Fractions (0·62 ml) were collected at a flow rate of 7·5 ml/h, and a 2 µl sample from each was subjected to scintillation counting. (b) Gel filtration of $^{35}$S-oGH1 following immunoaffinity purification. The main peak from immunoaffinity purification was applied to a column of Sephacryl S-100 (55 cm x 0·78 cm$^2$) using conditions as for (a). A 2 µl sample from each fraction was subjected to scintillation counting.
served to remove NaSCN and to give some further purification (possibly mainly separating monomeric oGH1 from polymerized material formed in the previous step). It was important to minimize the time that the labelled oGH1 was exposed to 1·5 M NaSCN, transferring material eluted from the affinity column to the gel filtration column within about 45 min. Delays at this stage, or use of higher concentrations of NaSCN to remove oGH1 from the affinity column or of a somewhat higher pH for the elution buffer, led to substantial aggregation (especially dimerization) of the protein.

It should be noted that the two gel filtration steps were carried out in buffer containing 0·10% BSA and 0·05% Tween 20. This minimizes loss of labelled protein due to non-specific adsorption, but does, of course, mix the purified [$^{35}$S]oGH1 with unlabelled protein. Similar additions are normally made when handling $^{125}$I-labelled proteins. Yields of material achieved in the chromatographic steps are shown in Table 1, and Fig. 1 shows the behaviour of material at various stages on SDS-PAGE/autoradiography.

**Characterization of [$^{35}$S]oGH1**

Figure 1 (lane 5) shows that labelled oGH1, following the second gel filtration step, ran as a major component on SDS-PAGE (corresponding to 97% of the radioactivity detected by autoradiography), with a minor component running slightly behind it.

Labelled oGH1 bound to a polyclonal antibody to bovine GH with a maximum bindability of 93% (Fig. 3a). This is similar to values obtained in our laboratory with $^{125}$I-labelled bovine GH. [$^{35}$S]oGH1 bound to monoclonal antibodies raised against ovine GH with bindabilities of 62–80% (Fig. 3b). Binding of $^{125}$I-labelled bovine GH to these antibodies was 54–89% under comparable conditions (data not shown). With both types of labelled GH the lowest bindability was seen using antibody OA16; the explanation for this is not yet clear.

The [$^{35}$S]oGH1 also bound to a microsomal membrane preparation from the liver of a pregnant rabbit (Fig. 4). Binding achieved with 300 µg receptor protein/tube was 69% (57% specific binding; Fig. 4a), and this appeared to be below the maximum (plateau) value (i.e. bindability), which was estimated to be about 84% by using a double reciprocal plot. With $^{125}$I-labelled bovine GH, maximum binding to rabbit liver receptors in our laboratory is normally in the range 40–55% (specific binding 30–45%) (Cadman & Wallis, 1981; Webb et al. 1986). The bindability of the [$^{35}$S]oGH1 shown in Fig. 4a is thus substantially greater than that normally achieved with $^{125}$I-labelled bovine GH. This was confirmed by experiments in which the binding of [$^{35}$S]oGH1 and $^{125}$I-labelled bovine GH to receptors was compared directly, as illustrated in Fig. 4b.
Figure 4. Binding of labelled GH to rabbit liver receptors. (a) $^{35}$S-oGH1 (13 μCi/μg; 10.6 nCi) was incubated with various amounts of a preparation of receptors prepared from the liver of a pregnant rabbit, in a total volume of 0.5 ml for 18 h at 20 °C. Bound and free $^{35}$S-oGH1 were separated by centrifugation. Total binding (●) and non-specific binding (■) are shown. (b) $^{125}$I-labeled bovine GH (●) and $^{125}$I-labelled bovine GH (▲) were incubated with receptors, as for (a). Specific binding is shown.

The specific radioactivity of $^{35}$S-oGH1 prepared as described here was determined by measuring GH concentration in a radioimmunoassay, and matching this with the radioactivity measured by scintillation counting. It varied from 6 to 27 μCi/μg. As expected, the value obtained for specific radioactivity was correlated with the amount of radioactivity added to the original 0.2 ml incubation medium, the highest specific radioactivity (27 μCi/μg) being achieved with 3.7 mCi/0.2 ml. The highest specific radioactivity obtained is close to the range that we normally obtain by labelling with $^{125}$I (35-80 μCi/μg). The specific radioactivity that can be achieved is limited by the need to grow bacteria in unlabelled medium prior to incubation in label. Addition of a higher concentration of Na$_2$SO$_4$ to the incubation medium or alteration of labelling conditions might give an increase in the specific radioactivity achieved, but the material as produced should be satisfactory for most of the purposes for which it is required.

The stability of $^{35}$S-oGH1 was studied by rechromatography of stored material on Sephacryl S-100 (conditions as for Fig. 2b). Material stored at $-30$ °C was slightly more stable than that stored at $4$ °C; after 3 months at $-70$ °C, 72% of the label ran as monomer, compared with 67% after storage at 4 °C. The stability of $^{35}$S-oGH1 was much greater than that of $^{125}$I-labelled bovine GH, which showed only 39% running as monomer after 3 months of storage at 4 °C. Previous studies (Wallis et al. 1980; Cadman, 1981) have shown that for $^{125}$I-labelled GH and related hormones freezing and thawing leads to aggregation, and storage at 4 °C is preferable to storage frozen.

Conclusions
The objective of this work was to produce an internally labelled GH derivative that would be equivalent or superior to $^{125}$I-labelled material in terms of ligand binding, but would potentially avoid the disadvantages associated with external labelling (see the Introduction). The results shown here indicate that this has been achieved. $^{35}$S-oGH1 has been purified and shown to bind to antibodies to GH in a manner similar to that achieved with $^{125}$I-labelled GH. Binding to rabbit liver receptors was substantially greater than that achieved with $^{125}$I-labelled material. The specific radioactivity of the $^{35}$S-oGH1 is somewhat lower than that achieved by labelling with $^{125}$I, but it is likely that it could be increased if necessary. Further work is needed on the ligand-binding properties of $^{35}$S-oGH1, but the studies carried out so far suggest that it should have significant advantages compared with $^{125}$I-labelled material.

The procedure for production of $^{35}$S-oGH1 is more complex than that used for the preparation and purification of $^{125}$I-labelled GH, but it can readily be completed within 3 days, with production of up to 150 μCi $^{35}$S-oGH1. The substantially greater stability of $^{35}$S-oGH1 compared with $^{125}$I-labelled GH should help offset any inconvenience involved in its preparation.
The oGH1 labelled in this study differs from pituitary-derived GH by having an N-terminal extension of eight amino acids derived from β-galactosidase and linker regions. It is fully active in biological and ligand-binding assays (Wallis & Wallis, 1990) and therefore suitable for biological studies. It is thought likely that the method described here can be readily extended to the production of other labelled GH analogues, and to 'authentic' GH, though it should be noted that pituitary GH itself is a heterogeneous material (e.g. Lewis, 1992) and the concept of a single 'normal' GH molecule is misleading.

The internally labelled oGH1 prepared in this study is thus apparently suitable for the detailed study of GH-ligand interactions and also of the in-vivo and in-vitro metabolism and processing of this GH analogue. Such studies are now underway in our laboratory. The basic approach used here would seem to be potentially applicable to the labelling of other eukaryotic proteins that can be produced in E. coli, and to production of proteins internally labelled with ¹⁴C or ³H.

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REFERENCES


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