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# Studies on the iodination of a *ras* protein and the detection of *ras* polymers

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## Abstract

Several methods for the iodination of recombinant v-H-*ras* protein were compared. The Iodobead method gave greatest incorporation of radioactivity with minimal modification of the *ras* protein. Upon treatment of the *ras* protein with [$^{125}$I] NaI and an Iodobead, radioactivity was initially incorporated into a 22 kDa species with a pI of 5.2, then predominantly into a 23 kDa species with a pI of 5.4. The specific activity of [$^{125}$I]*ras* was $6 \times 10^6$ cpm/pmol total *ras* protein. Iondination did not alter the biological activity of the *ras* protein as judged by its ability to bind GTPγS and induce maturation of *Xenopus laevis* oocytes. It is concluded that while iodination alters the apparent molecular weight and pI of *ras*, presumably by the oxidation of one or more classes of amino acids, this does not affect the biological function of the protein. The *ras* protein, radioactively-labelled with iodine using the Iodobead method, should be suitable for studies of protein-protein interactions involving *ras*. Treatment of iodinated *ras* with the chemical cross-linking agent disuccinimidyl suberate revealed the presence of several minor high molecular weight protein species. This result shows that, in a dilute solution of purified *ras* protein, the monomeric form is in equilibrium with small amounts of polymeric forms. (Mol Cell Biochem **137**: 75–83, 1994)

*Key words: ras*, iodination, GTPγS binding, oocyte maturation, polymeric forms

*Abbreviations:* DSS, Disuccinimidyl Suberate; GTPγS, Guanosine 5′-[γ-thio] triphosphate; ATPγS, Adenosine 5′[γ-thio] Triphosphate

## Introduction

The mammalian *ras* proteins are monomeric (low molecular weight) GTP binding proteins which are activated by the displacement of GDP and 'switched off' by the hydrolysis of this bound GTP. The *ras* proteins are components of several major signalling pathways which carry information from protein-tyrosine kinase receptors on the plasma membrane to the nucleus and other intracellular locations (reviewed in [1–3]). The protein-tyrosine kinase receptors include those for the growth factors EGF, NGF, PDGF, and the hormone insulin. The *ras* signalling pathways are present in many types of cells [1–3]. In order to achieve their cellular functions, the *ras* proteins interact with a number of other proteins in the *ras* signalling pathways. These other proteins include the *ras* guanine nucleotide exchange factor/SOS, the *raf*-1 kinase and the *ras* GTP-ase activating proteins, p120 *ras* GAP and neurofibrin [1–5]. There are probably other, so far unidentified, down-stream effector proteins in addition to *raf*-1 kinase [2]. In addition to proteins which are directly part of the *ras* signalling pathway, *ras* proteins also bind to farnesyl transferase, which plays a critical role in the post-translational modification of *ras* (reviewed in [1] and [4]).

Protein-protein and protein-peptide interactions are an essential component of the normal cellular functions of the *ras* protein and may also be necessary for some approaches

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to the pharmacological alteration of the function of the *ras* oncogene [6]. Thus further elucidation of the mechanisms of action of the *ras* protein and of the normal and pathological regulation of its activities will require studies of the molecular events which occur when *ras* interacts with other proteins.

The use of radioactively-labelled iodinated *ras* offers one way in which the interaction of *ras* with other proteins can be monitored. This approach has already proven useful [7] (Chataway, T.K. and Barritt, G.J. unpublished results). However, the iodination of any protein can potentially alter its chemical and biological functions [8, 9]. The aim of the present work was to determine the best method for the iodination of a *ras* protein, and to elucidate the effect of iodination on some properties of *ras*. Since it has been suggested that *ras* can form dimers [10] the iodinated *ras*, together with a chemical cross-linking agent, were employed to search for polymeric forms of *ras*. The results have shown that the method of choice for the iodination of *ras* is the Iodobead (Chloramine B) method [11]. While iodination was found to alter the apparent molecular weight and the pI of the *ras* protein, it did not affect biological activity assessed by the ability of *ras* to induce the maturation of oocytes. The results also provide evidence for the presence of small amounts of polymeric *ras* in dilute preparations of the pure protein.

## Materials and methods

*Materials*

*E. coli* transfected with the v-Ha-*ras* oncogene was a gift from Dr. A. Wittinghofer, Max-Planck-Institut fur medizinische Forschung, Heidelberg, West Germany, and cDNA which encodes histidine-tagged v-Ha-*ras* (six histidine residues at the amino terminus) was a gift from Drs. A.J. Polverino and M. Wigler, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York. Iodobeads and Iodogen were obtained from the Pierce Chemical Company, Illinois, USA; carrier-free [$^{125}$I] NaI (17.4 Ci/mg) and [$^{35}$S]GTPγS (1000–1500 Ci/mmol) from Du Pont, Wilmington, USA; disuccinimidyl suberate (DSS), Chloramine T, lactoperoxidase and molecular weight markers from the Sigma Chemical Company, St. Louis, Missouri, USA; enzymobeads from Bio-Rad Laboratories, Richmond, CA, USA; and Sephadex G100 and Mono Q were from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. All other chemicals and materials were of the highest grade commercially available.

*Purification of* ras

The v-H-*ras* protein was purified from *E. coli* transfected with the v-H-*ras* oncogene [12]. The purification involved chromatography on DEAE cellulose (Whatman DE52) and Sephadex G100 columns followed by FPLC on a Mono Q column. The pure *ras* protein (5.5 mg/ml) was dissolved in 64 mM Tris-HCl (pH 7.6) which contained 1 mM dithiothreitol, 1 mM $NaN_3$, 10 mM $MgCl_2$, 20 mM GDP and 25% (w/v) glycerol, and stored at –70°C. Protein concentrations were determined by the method of Lowry *et al.* [13] as modified by Peterson [14].

Purified his-*ras* was prepared under native (non-denaturing) conditions by a modification of the methods of Janknecht *et al.* [15] and Shibuya *et al.* [16] according to the manufacturer's instructions for the use of Pro-Bond resin (Invitrogen, San Diego, CA). The bacterial extract (from 4 L of culture), containing his-*ras* protein in 50 mM Tris-HCl (pH 7.0), 100 mM NaCl, 10 mM imidazole and 0.25 mM phenylmethanesulfonyl fluoride, was applied to 10 ml of Pro-Bond resin and incubated for 2 h at 4°C. Elution was performed by increasing concentrations of imidazole in 50 mM Tris HCl (pH 6.6). The purity of his-*ras*, as assessed by SDS PAGE, was greater than 95%.

*Iodination methods*

1. *Iodobeads* – Iodobeads (N-chloro-benzene sulfonamide (chloramine B) bound to a polystyrene bead) were used as described by Markwell [11]. Five microlitres of *ras* (28 μg) were diluted with 95 μl of 100 mM sodium phosphate buffer (pH 7.4) which contained 1 mCi of carrier-free [$^{125}$I] NaI. A single washed Iodobead was added, and the reaction allowed to proceed for 5 min at room temperature. The reaction mixture was removed from the Iodobead and placed in another tube which contained 2 μl of 500 mM dithiothrietol and 1 μl of 100 mM NaI. Fresh [$^{125}$I] *ras*, specific activity $6.05 \pm 0.55 \times 10^6$ cpm/pmol, was prepared each day. Free $^{125}I^-$ was not separated from the [$^{125}$I] *ras* because in preliminary experiments it was found that greater than 50% of the iodinated protein was lost during the separation procedure. In the presence of dithiothreitol, free $^{125}I^-$ did not result in the detectable labelling of other proteins (results not shown).
2. *Chloramine T* – Iodination was performed at room temperature by the method of Hunter and Greenwood [17] as described by Harlow and Lane [18]. The reaction mixture (160 μl) contained 30 μg *ras* in 250 mM sodium phosphate buffer (pH 7.4), 1 mg/ml or 0.01 mg/ml Chloramine T and 1 mCi of [$^{125}$I]NaI.
3. *Iodogen (direct exposure)* – Iodination was performed by the method of Fraker and Speck [19] as described by Harlow and Lane [18]. The reaction mixture (40 μl), containing 28 μg of *ras* and 0.6 mCi of [$^{125}$I]NaI in 500 mM sodium phosphate buffer (pH 7.4), was added to an Eppendorf tube coated with 50 ng of Iodogen. Following a 20

min incubation at room temperature, the reaction mixture was transferred to a tube containing 2 µl 500 mM dithiothreitol and 1 µl 100 mM NaI.

4. *Iodogen (indirect exposure)*—Iodination with Iodogen was performed as described above except that the oxidation of [$^{125}$I]NaI by Iodogen was initiated in the absence of *ras*. The reaction products were then added to the *ras* protein in two successive steps. The reaction mixture (40 µl, containing 0.6 mCi of [$^{125}$I]NaI in 500 mM sodium phosphate buffer (pH 7.4), was added to an Eppendorf tube coated with 50 ng of Iodogen. Following a 5 min incubation at room temperature, 20 µl of the 40 µl-reaction mixture was added to a separate tube which contained 12 µg of *ras* in 10 µl of 500 mM sodium phosphate buffer (pH 7.4). After 5 min, the remaining 20 µl of the 40 µl-reaction mixture was added to the tube which contained the *ras* protein and incubated for a further 5 min. The reaction was stopped by the addition of 2 µl of 500 mM dithiothreitol.
5. *Lactoperoxidase*—Iodination was performed by the method of Marchalonis [20] as described by Harlow and Lane [18]. The reaction mixture contained 12 µg of *ras* in 20 µl of 137 mM NaCl which contained 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.7 mM $KH_2PO_4$ (pH 7.2) (Phosphate-Buffered Saline) containing 10 units of lactoperoxidase and 0.5 mCi of [$^{125}$I]NaI. One microlitre of 0.0015% (v/v) $H_2O_2$ in Phosphate-Buffered Saline was added each minute over a four minute period. The reaction was terminated by addition of 1 µl of 500 mM dithiothreitol.
6. *Enzymobeads*—Iodination [20, 21] was performed at room temperature according to the manufacturer's (Bio-Rad) instructions. The reaction mixture consisted of 100 ng of *ras* in 100 µl of 100 mM sodium phosphate buffer (pH 7.2), which contained 0.5 mCi [$^{125}$I]NaI and 0.25% (w/v) β-D-glucose. The reaction time was 25 min and the reaction was terminated by the addition of 5 µl of 1 M dithiothreitol.
7. *Sodium hypochlorite* – Five microlitres of *ras* in 40 mM MOPS-NaOH (pH 7.5) and 100 µCi [$^{125}$I]NaI was placed at the bottom of one well in a 96 well microtitre plate (Neogh, S., personal communication). A glass coverslip was placed over the well and sealed in place with vacuum grease. The inner surface of this coverslip was coated with 1 µl of 1% (w/v) sodium hypochlorite spread evenly over the surface. After incubation at room temperature for periods of between 1 and 10 min, the reaction was terminated by the addition of 186 mM Tris-HCl (pH 6.7) which contained 30% (w/v) sucrose, 6% (w/v) sodium dodecylsulphate (SDS), 162 mM dithiothreitol and 0.003% (w/v) bromophenol blue (Sample Buffer).

*SDS-PAGE and Western blotting*

SDS-PAGE was performed by the discontinuous buffer system of Laemmli [22] using a mini gel apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The locations of radioactively-labelled proteins were detected by autoradiography. In cross-linking experiments, most of the [$^{125}$I] *ras* and free [$^{125}$I]NaI which migrated near the dye front were allowed to run off the end of the gel. Hence the location of [$^{125}$I] *ras* is not shown in the photographs. For Western blots, proteins were separated on SDS-PAGE and transferred to nitrocellulose filters [23]. The ability of species of *ras* protein to interact with an antibody against *ras* proteins was tested by the double antibody method, using a mouse monoclonal antibody against *ras* and reagents supplied by the Cetus Corporation, Emeryville, California, USA.

*Two dimensional electrophoresis*

Two dimensional electrophoresis was performed with a Pharmacia Multiphor II electrophoresis unit according to Görg *et al.* [24] and to manufacturers instructions. Immobiline Drystrips pH 4–7 (Pharmacia LKB Biotechnology) were employed in the first dimension and SDS-PAGE in the second dimension. Two microlitres of the protein sample were mixed with 25 µl of 8 M urea which contained 0.02% (w/v) 2-mercaptoethanol, 0.02% (w/v) Pharmalyte 3–10, 0.005% (w/v) Triton X-100 and 0.001% (w/v) bromophenol blue, added to an Immobiline Drystrip, and electrophoresed for 16 h. The strips were equilibrated for 10 min at room temperature in 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% (w/v) glycerol, 1% (w/v) SDS (Equilibration Buffer) which also contained 16 mM dithiothreitol. After equilibration, the strips were transferred to Equilibration Buffer which contained 250 mM iodoacetamide and 0.001% (w/v) bromophenol blue for a further 10 min at room temperature. The strips were overlaid onto a 12% plyacrylamide gel (16 cm × 18 cm), covered with low-melting point agarose (pH 6.8), and electrophoresed at 200 volts. Silver staining [25] was performed according to the manufacturer's instructions for Immobiline Drystrips.

*Treatment with the cross-linking agent disuccinimidyl suberate*

[$^{125}$I] *ras* (2.8 µg, 10 µl) was added to 40 µl of 100 mM sodium phosphate buffer (pH 7.2), which contained 0.5 mM EDTA, 0.5 mg/ml bovine serum albumin (Sigma fraction V), 10 mM dithiothreitol and 0.005% (w/v) sodium cholate. After preincubation at 30°C for 30 min, 17 µl of the solution was added to 50 µl of 20 mM MOPS-NaOH (pH 7.2) which contained 1 mM $MgCl_2$, 0.1 mM EDTA, 200 mM sucrose, 20 µg/ml antipain, 20 µg/ml leupeptin, 40 µg/ml *para*-methylsulfonyl fluoride, 0.5 mM phosphotyrosine,

0.5 mM phosphothreonine, 0.5 mM phosphoserine and 1 mM dithiothreitol, and incubated at room temperature for 30 min. The final concentrations of *ras*, EDTA and $Mg^{2+}$ were 1.8 μg/ml, 0.1 mM and 1.0 mM, respectively, and the final volume of the incubation mixture was 55 μl. Disuccinimidyl suberate (DSS) (82 mM in dimethysulphoxide) was then added to give a final concentration of 0.75 mM and the mixture incubated for a further 30 min at room temperature. The reaction was terminated by the addition of 25 μl of 186 mM Tris-HCl (pH 6.7) which contained 30% (w/v) sucrose, 6% (w/v) sodium dodecylsulphate, 162 mM dithiothreitol and 0.003% (w/v) bromophenol blue.

*Binding of [$^{35}$S]GTPγS to* ras

The binding of [$^{35}$S]GTPγS to *ras* was measured by the method of Satoh *et al.* [26]. The results are expressed as mole [$^{35}$S]GTPγS bound per mol of *ras* (22 kDa plus 23 kDa species). The sum of the amounts of 22 kDa and 23 kDa species of *ras* present in untreated and in Iodobead-treated samples were determined by separating the protein species by SDS-PAGE, staining the proteins with Coomassie Blue and quantitating the amount of protein on the gel. Quantitation was performed by laser densitometry using an Ultrascan densitometer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) at 633 nm. The sum of the areas under the absorbance peaks which corresponded to the 22 kDa species and 23 kDa species of *ras* was a linear function of the amount of protein applied to the gel up to 6 μg protein per lane.

*Bioassay of* ras

The effect of iodination on the biological activity of *ras* was assessed by testing the ability of iodinated *ras* proteins to induce the maturation of *Xenopus laevis* oocytes. For these experiments, histidine-tagged v-Ha-*ras* (6 histidine residues at the amino terminus) was used. The histidine tag enabled rapid purification of milligram quantities of *ras* while maintaining its biological activity. Purified his-*ras* was dialysed with 50 mM Tris-HCl (pH 7.4) which contained 100 mM NaCl and 5 mM $MgCl_2$. Iodination of his-*ras* and bovine serum albumin (dissolved in the Tris-NaCl-$MgCl_2$ buffer) were performed as described above, except that the reaction mixture contained 200 μg of protein, and NaI was substituted for [$^{125}$I]NaI. Analaysis of the iodinated *ras* by SDS-PAGE revealed complete conversion of the 22 kDa species to the 23 kDa species. Iodinated his-*ras* or untreated his-*ras* was diluted with 50 mM β-glycerophosphate pH 7.0 to the required concentration for injection. Stage VI oocytes were obtained from *Xenopus laevis* as described by Wu *et al.* [27] with the exception that oocytes were treated with 2 mg/ml collegenase for 60 min at 17°C to aid dissection. His-*ras* or bovine serum albumin (50 nl) were injected to oocytes as described by Wu *et al.* [27]. Oocytes were inspected for signs of germinal vesicle breakdown at 42 and 64 h. Oocytes which exhibited a white spot at the animal pole were scored as positive for germinal vesicle breakdown [27].

*Presentation of results*

Except where indicated otherwise, the results shown represent those obtained for one of at least three experiments which gave similar results.

# Results

*Comparison of different methods of iodination*

Analysis of purified v-H-*ras* by SDS-PAGE revealed a major band of protein with an apparent molecular weight of about 22 kDa and a barely-visible minor band with a higher molecular weight (about 23 kDa) (Fig. 1A, lane 1). Preliminary experiments showed that the iodination of *ras* using [$^{125}$I] NaI led to the incorporation of radioactivity into the 23 kDa band as well as the 22 kDa band (results not shown). As described below, it is considered likely that the 23 kDa band represents a chemically-modified species of the *ras* protein. The percentage of 23 kDa species formed was used to monitor the degree of modification of *ras* induced by

*Table 1.* Comparison of different methods for the iodination of *ras*. The iodination of *ras*, SDS-PAGE of iodinated *ras*, measurement of the amount of radioactivity incorporated into the 22 and 23 kDa species (determined by laser densitometry), and measurement of the amounts of protein (laser densitometry) in these two species were conducted as described in Materials and methods. The proportion of 23 kDa species formed was estimated from the amounts of radioactivity and protein found in the 22 and 23 kDa species. The amount of radioactivity incorporated after Iodobead treatment was $6.05 \pm 0.55 \times 10^6$ cpm/pmol

| Method | Incorporation of radioactivity (relative to that for Iodobeads) | Proportion of 23 kDa species formed |
|---|---|---|
| Iodobead | Large | Small |
| Iodogen (direct exposure) | Moderate | Moderate |
| Iodogen (indirect exposure) | Small | Small |
| Chloramine T | Poor | None |
| Lactoperoxidase | Poor | None |
| Enzymo beads | Very poor | None |
| Milton's reagent | Large | High |

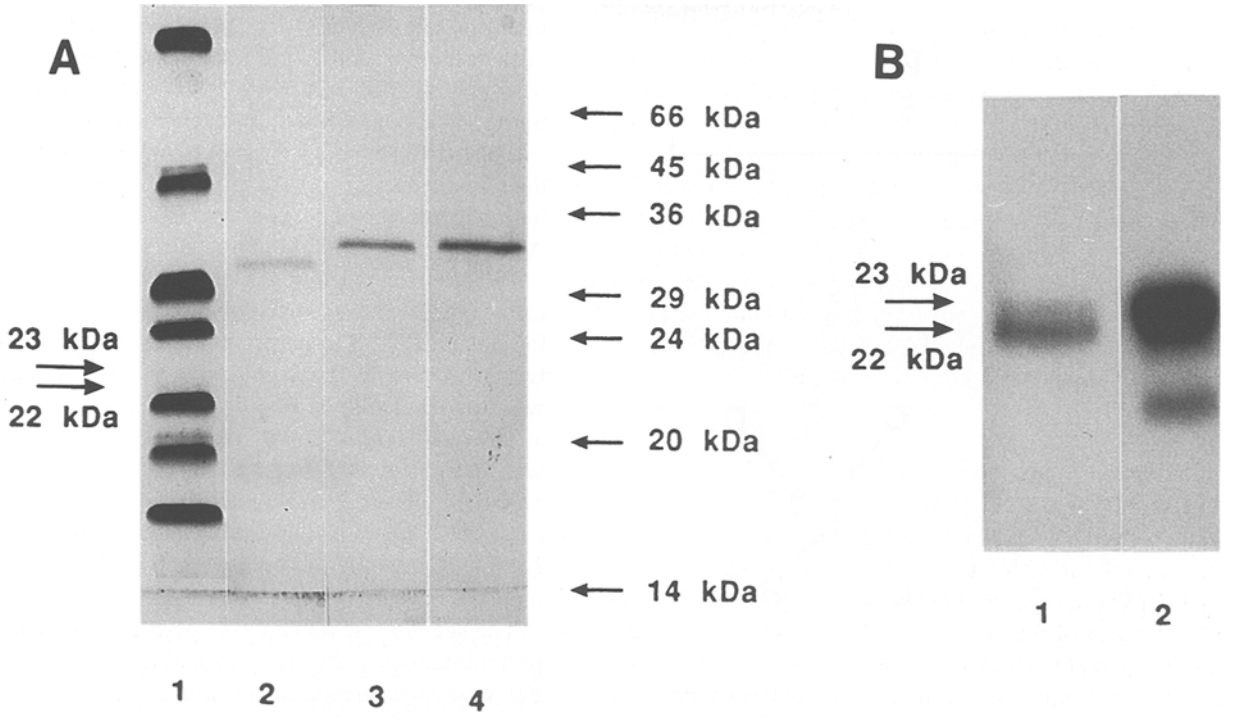


*Fig. 1.* Effect of iodination of the *ras* protein on the protein species detected by Coomassie Blue (A) and autoradiography (B) after electrophoresis on SDS-PAGE. (A) A Coomassie Blue stain of the protein species present in purified *ras* before exposure to an Iodobead (in the absence of NaI) (lane 1) and 30 sec (lane 2) or 5 min (lane 3) after exposure to the Iodobead. Molecular weight markers are shown in lane 4. SDS-PAGE was performed using a 12% gel. (B) An autoradiograph of [$^{125}$I] *ras* after iodination (Iodobead and $^{125}$I ) for 30 sec (lane 1) and 5 min (lane 2). SDS-PAGE was performed using a 12% gel.

iodination when several methods of iodination were compared. Chloramine T, lactoperoxidase and Enzymo beads induced little conversion of the 22 to 23 kDa species but gave a poor incorporation of radioactivity (Table 1). Iodogen and Milton's reagent gave moderate or large incorporation of radioactivity but also substantial conversion of the 22 to the 23 kDa species of *ras*. In the case of Iodogen, generation of the 23 kDa species was not diminished when *ras* was allowed to react with oxidised iodine in the absence of Iodogen (i.e. after removal of the oxidising agent) (Table 1, Iodogen, indirect exposure). It was concluded that the Iodobead method is the best one available for the iodination of *ras*. Subsequent studies were conducted using this method.

### *Characterisation of* ras *iodinated using Iodobeads*

When the *ras* protein was iodinated for 30 sec using the Iodobead method, most of the incorporated radioactivity was found in the 22 kDa species with a small amount in the 23 kDa species (Fig. 1B, lane 1). After iodination for 5 min the total amount of radioactivity incorporated was substantially increased. The majority of the radioactivity was found in the 23 kDa species with a small amount in the 22 kDa species and in another minor band at 20 kDa (Fig. 1B, lane 2). Autoradiography for a shorter period of time (data not shown) confirmed that the major band of radioactivity had a molecular weight of 23 kDa while only small amounts of radioactivity were present in the bands with molecular weights of 22 and 20 kDa.

The increase in molecular weight observed following the treatment of *ras* with an Iodobead and [$^{125}$I]NaI could have been due to an effect of the Iodobead (e.g. the oxidation of *ras* by chloramine B) or to the covalent attachment of molecules of iodine to the *ras* protein. To distinguish between these possibilities, purified *ras* protein was exposed to an Iodobead in the absence of NaI, subjected to analysis by SDS-PAGE, and the protein species detected using Coomassie Blue. A time-dependent increase in the amount of protein in the 23 kDa molecular weight band was observed (Fig. 1A, lanes 2 and 3, cf. lane 1). There was also some loss of protein, which varied between 10 and 30% of total protein. After transfer of the proteins from the gel to a nitrocellulose membrane, both the 23 and 22 kDa bands were found to react with an antibody directed against *ras* (results not shown). Bands with intensities similar to those shown in Fig. 1A (lanes 1–3) were obtained when the experiment was repeated with the inclusion of NaI (unlabelled) in the incubation medium

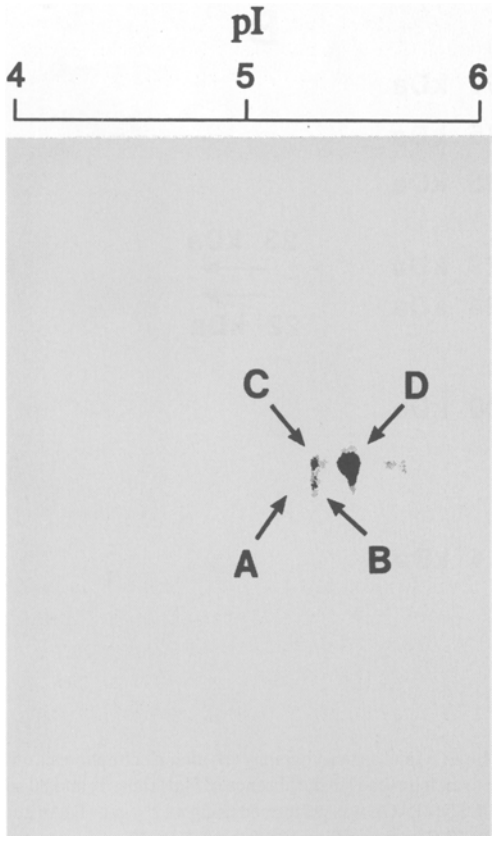


*Fig. 2.* A two dimensional electrophoretogram of the protein species present after the treatment of *ras* with an Iodobead (in the absence of NaI). The *ras* protein was treated with an Iodobead, subjected to two-dimensionsal electrophoresis, and proteins detected using a silver stain, as described in Materials and methods. Arrows B, C and D refer to the protein species detected in iodinated *ras*. Arrow A refers to the position of a protein species detected in untreated *ras* but not observed in iodinated *ras*. (The electrophoretogram for untreated *ras* is not shown.)

(results not shown).

The effect of Iodobead treatment on the *ras* protein was further evaluated using two dimensional electrophoresis. Figure 2 shows the pattern of protein species observed after iodination of *ras* with an Iodobead (in the absence of NaI) and separation of the proteins by two dimensional electrophoresis. A major spot was observed at position D and minor spots at positions B and C. When untreated *ras* was subjected to two dimensional electrophoresis on a separate gel, two protein species were detected (results not shown). These were a major protein species which migrated at a pI and molecular weight equivalent to that indicated by arrow B in Fig. 2, and a minor species which migrated to the position indicated by arrow A in Fig. 2. Thus iodination caused the appearance of a new major protein spot (D) and a new minor protein spot (C) (Fig. 2), a decrease in the amount of protein the position B and a complete removal of protein from position A. Each of the new spots induced by iodination (spots C and D) had an apparent molecular weight which was about 1 kDa larger than spots A and B, respectively, in the untreated *ras* from which they were derived, and exhibited an increase in pI of about 0.15 when compared with spots A and B, respectively, in untreated *ras*.

To determine whether iodination of *ras* affects its biological activity, two tests were conducted. In the first, the binding of [$^{35}$S] GTPγS to the protein species present after 5 min treatment of *ras* with iodobeads (in the absence of [$^{125}$I]NaI) was compared with its binding to untreated *ras*. When tested at 100 nM, 500 nM, 1 μM, 10 μM or 50 μM-GTPγS, there was no significant difference in the amounts of [$^{35}$S]GTPγS bound by untreated and treated *ras* proteins. The amounts of [$^{35}$S]GTPγS bound to untreated *ras* were 0.05 ± 0.02, 0.25 ± 0.03, 0.29 ± 0.02, 0.41 ± 0.05 and 0.42 ± 0.09 moles per mole of *ras*, respectively.

The second test was an assessment of the ability of *ras* proteins to induce the maturation of *Xenopus laevis* oocytes. For these experiments recombinant histidine-tagged *ras* (his-*ras*) was employed since this can be prepared and purified much more efficiently than the native *ras* protein. Both untreated and iodinated *his-ras* induced germinal vesicle breakdown when compared with the control protein, iodinated-bovine serum albumin (Table 2). When tested at three different concentrations, and at 42 and 72 h after the microinjection of *ras* protein, the proportion of injected oocytes exhibiting germinal vesicle breakdown in the presence of iodinated his-*ras* (iodinated) was similar to that for oocytes treated with his-*ras* (non-iodinated) (Table 2).

### *Detection and stabilisation of polymeric forms of* ras

To determine whether purified *ras* can form polymers, [$^{125}$I] *ras* was treated with the chemical cross-linking agent disuccinimidyl suberate (DSS) in order to stabilise any high molecular weight forms which might be present. Figure 3A (lane 2 cf. lane 1) shows an autoradiograph of [$^{125}$I] *ras* which has been treated with DSS and subjected to SDS-PAGE on a 12% gel. In addition to the major band of radioactivity at 22 kDa, a number of other radioactive species were observed. These were a minor band at 40 kDa, a band at about 100 kDa, and material with very high molecular weights which did not enter the gel. Further analysis of cross-linked [$^{125}$I] *ras* by SDS-PAGE on a 7% acrylamide gel revealed that the 100 and 40 kDa bands are composed of multiple radioactively-labelled species (Fig. 3B). These results suggest that the *ras* protein aggregates to form dimers, tetramers, and higher polymeric species which are stabilised by the cross linking agent. Comparison of the amount of radioactivity in all the

*Table 2.* The maturation of *Xenopus laevis* oocytes induced by untreated his-*ras* and by iodinated his-*ras*. The purification of his-*ras*, the iodination of his-*ras* and bovine serum albumin using an Iodobead and unlabelled NaI, the microinjection of protein to oocytes, and the measurement of germinal vesicle breakdown were conducted as described in Materials and methods. In this experiment, the iodination of *ras* resulted in complete conversion of the *ras* protein to the 23 kDa species and the loss of 10% of the total protein. The results, which were obtained with a single preparation of iodinated his-*ras* protein, are expressed as the number of oocytes which exhibited germinal vesicle breakdown compared with the total number of oocytes tested

| Protein injected | Amount injected (ng) | Number of mature oocytes exhibiting germinal vesicle breakdown | |
|---|---|---|---|
| | | 42 h after injection | 64 h after injection |
| his-*ras* (untreated) | 5 | 0/7 | 0/7 |
| | 50 | 2/6 | 4/6 |
| | 100 | 6/6 | 6/6 |
| Iodinated his-*ras* | 5 | 0/7 | 0/7 |
| | 50 | 1/6 | 4/6 |
| | 100 | 6/6 | 6/6 |
| Iodinated bovine | 100 | 0/7 | 0/7 |

high molecular weight forms of [$^{125}$I] *ras* with the amount of radioactivity in the 22 kDa form indicates that less than 0.03% of the total amount of radioactivity is present in higher molecular forms.

## Discussion

In this study, established methods for the iodination of proteins were compared for their ability to iodinate the *ras* protein. None gave sufficient incorporation of [$^{125}$I] (i.e. a sufficiently high specific activity) without altering the apparent molecular weight of the *ras* protein. It was concluded that the Iodobead (Chloramine B) method is the best since this procedure gave minimal alteration of the physical properties of the *ras* protein with maximal incorporation of radioactivity. While the low incorporation of radioactivity observed with Chloramine T was somewhat unexpected in view of the avid ability of this reagent to catalyse the incorporation of iodine into proteins [28], results similar to those re-

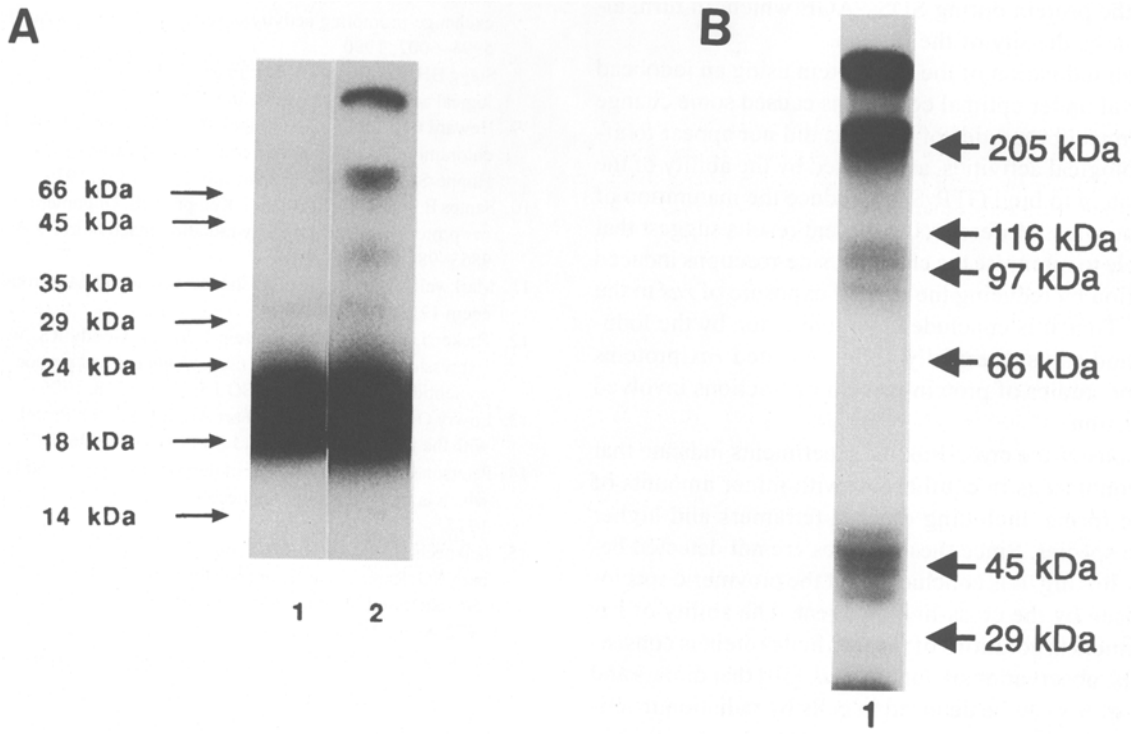


*Fig. 3.* Stabilisation of minor polymeric forms of the *ras* protein by treatment of [$^{125}$I]*ras* with a cross linking agent. (A) An autoradiograph of [$^{125}$I] *ras* incubated with 0.75 mM DSS (lane 2) or vehicle (lane 1), as described in Materials and methods, then subjected to SDS-PAGE using a 12% gel. (B) An autoradiograph of [$^{125}$I] *ras* incubated with 0.75 mM DSS, as described in Materials and methods, then subjected to SDS-PAGE using a 7% gel.

ported for Chloramine T in Table 1 were obtained in three separate experiments. These were conducted with the same batch of Chloramine T. Thus the possibility that greater incorporation of radioactivity would be observed with another batch of Chloramine T cannot be excluded.

The observation that the treatment of *ras* with an iodobead in the absence of NaI alters the apparent molecular weight of the protein indicates that the observed change in apparent molecular weight is due to the action of N-chloro-benzene sulphonamide (Chloramine B) bound to the Iodobead rather than to the covalent attachment of molecules of iodine to the *ras* protein. The loss of protein observed during treatment with an Iodobead may have been due to the absorption of *ras* protein to the walls of the reaction tube and/or to absorption to the Iodobead itself. Another possibility is the oxidation of tryptophan residues in the *ras* protein which may lead to an increase in the susceptibility to hydrolysis of the *ras* polypeptide chain [29].

The change in the apparent molecular weight of *ras* from 22 to 23 kDa is most likely due to the oxidation of methionine and/or other residues, such as cysteine, leading to an alteration in the electrophoretic properties of the protein. Thus in some proteins iodination can lead to the oxidation of susceptible residues such as methionine [8, 9]. While the mechanism for the change in electrophoretic properties is not known, it could involve a change in the amount of SDS bound to the protein during SDS-PAGE which, in turn, alters the charge density of the protein.

Although iodination of the *ras* protein using an iodobead and [$^{125}$I]NaI under optimal conditions caused some change in the chemical properties of *ras*, this did not appear to affect its biological activities, as assessed by the ability of the treated protein to bind GTPγS and induce the maturation of *Xenopus* oocytes. Moreover, the present results suggest that it is possible to minimise the chemical side-reactions induced by iodination by reducing the time of exposure of *ras* to the Iodobead. Thus it is concluded that iodination by the Iodobead method can successfully yield iodinated *ras* proteins suitable for studies of protein-protein interactions involved in *ras* function.

The results of the cross-linking experiments indicate that the *ras* monomer is in equilibrium with minor amounts of polymeric forms, including dimers, tetramers and higher polymeric species. Since these species are not detected before cross-linking, it is concluded that the ploymeric species are stabilised by the cross-linking agent. The ability of *ras* to form dimers in solutions of the purified protein is consistent with the observation of Santos *et al.* [10] that dimers and tetramers of *ras* can be detected in cells by radiation inactivation analysis.

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