# TRANSFER OF RADIOIODINE FROM IODINATED ONCORNAVIRUS PROTEINS TO UNLABELED PROTEIN CARRIER DURING ROUTINE PROCEDURES FOR PEPTIDE MAPPING

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SUMMARY. Denaturation of radioiodinated oncornavirus proteins by performic acid oxidation, a routine procedure in peptide mapping studies, results in the unexpected release of 60-80% of the iodine label. In addition, if unlabeled carrier protein is present during oxidation, the iodine label is efficiently transferred to the carrier protein. Radioiodine release and transfer was not detected when proteins were denatured by reduction and carboxymethylation. These results indicate that reduction and carboxymethylation are the preferred procedures for denaturing proteins prior to chemical or enzymatic cleavage and peptide analysis.

In recent years, classical techniques of protein chemistry have been applied to the study of micro- and nanogram quantities of proteins. These studies depend greatly on the use of radioisotopes or fluorescent labels (1). One radiolabeling method which is becoming increasingly popular is the chemical (2) or enzymatic (3) attachment of radioiodine to protein tyrosine. This procedure results in high specific activity radiolabeled protein without the expense and complications of producing internally labeled proteins from radioactive amino acids.

In the course of doing routine tryptic peptide fingerprinting of $^{125}$I-labeled oncornavirus proteins, we observed that there were more labeled peptides than could be accounted for by the known tyrosine content of these proteins. This observation led us to investigate the possibility that the iodine label was being transferred from the iodinated oncornavirus protein to the BSA[1] carrier which had been added during the performic acid oxidation procedure prior to digestion by trypsin. The unexpected finding that the radioiodine

[1]Abbreviations: BSA - bovine serum albumin; gp - glycoprotein; 2-ME - 2-mercaptoethanol; Pr-RSV-C - Prague strain of Rous sarcoma virus, subgroup C; SDS - sodium dodecyl sulfate; SDS-PAGE - polyacrylamide gel electrophoresis on SDS-containing gels; TCA - trichloroacetic acid





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was indeed transferring to BSA under these oxidation conditions prompted us to examine other routine laboratory procedures in which carrier proteins are usually employed. This report describes some specific conditions under which iodine transfer can occur.

MATERIALS AND METHODS. Chloramine-T-mediated iodination of gp35. The minor glycoprotein (gp35) of Pr-RSV-C (a gift from R. Green of this laboratory) was iodinated by dissolving 80 µg of the protein in 0.5 ml of 0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS and 1 mCi Na$^{125}$I. The iodination was carried out by adding three 20 µl samples of freshly prepared cholaramine-T (1 mg/ml) at ten min intervals. After a total reaction period of 30 min, 5 µl of 10% 2-ME was added to stop the reaction. The iodinated protein was then separated from unreacted $^{125}$I by desalting on a column (0.9x15 cm) of Sephadex G-25 eluted with 0.01 M sodium phosphate, pH 7.2, containing 0.1% SDS.

Performic acid oxidation. Protein was oxidized with performic acid as described by Hirs (4). Briefly, protein was suspended in 10 µl of formic acid and 2 µl of anhydrous methanol in a 5 ml Reactivial (Pierce, Rockford, Ill.) and cooled at -5° for 30 min. Twenty µl of performic acid was added and the reaction mixture was kept at -5° for 150 min. At the end of this time the reaction mixture was diluted with 800 µl of cold deionized water and lyophilized twice.

Reduction and carboxymethylation of protein disulfide bonds. To reduce disulfide bonds, 100 µl of $^{125}$I-gp35 and 10 µl of carrier BSA (1 mg/ml) were dissolved in 1.0 ml of 0.5 M Tris-HCl, pH 8.6, containing 0.4% 2-ME and incubated at 45° for 1 hr. Carboxymethylation was then carried out by adding iodoacetamide to a final concentration of 0.15 M and incubating in the dark at 45° for 10 min, at which time 30 µl of 2-ME was added to terminate the alkylation. In order to obtain the carboxymethylated proteins in a buffer compatible with our SDS-PAGE system, the above reaction mixture was desalted through a 10 ml column of Sephadex G-25. After elution with 1 mM sodium phosphate, pH 7.2, containing 0.1% SDS, the protein peak fractions then were pooled, lyophilized to dryness, and the residue dissolved in one-tenth the original volume of distilled water.

TCA precipitation of proteins. Twenty µl of $^{125}$I-gp35 and 100 µl of carrier BSA (1 mg/ml) were mixed with 0.9 ml 12% TCA and incubated at 4°C for 10 min. The precipitate was then pelleted by centrifuging at 8000 x g for 20 min. To remove residual TCA prior to solubilization of the pellet for SDS-PAGE, the protein pellet was washed once each with 0.5 ml cold ethanol; diethylether mixture (1:1) and then with 0.5 ml diethylether. The dry pellet then was dissolved for SDS-PAGE as described below.

Solubilization of proteins for SDS-PAGE. The procedure for SDS-PAGE sample preparation has been described previously (5,6). Briefly, protein samples were prepared for SDS-PAGE by dissolving in 100 µl of 0.01 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 1% 2-ME and heating in a boiling water bath for 3-5 min. After cooling, sucrose crystals were added to a final concentration of about 10% to facilitate sample layering, and 1 µl of 0.5% bromphenol blue (in 0.02 N NaOH) was added as a visual tracking dye. To test specifically for transfer of radioiodine label under these solubilization conditions, 10 µl of $^{125}$I-gp35 and 10 µl carrier BSA (1 mg/ml) were mixed in the above buffer, heated and analyzed by SDS-PAGE.

SDS-polyacrylamide gel electrophoresis and analysis of gels. The procedure for SDS-PAGE, gel fractionation and analysis of radioactivity have been described previously (5,6).

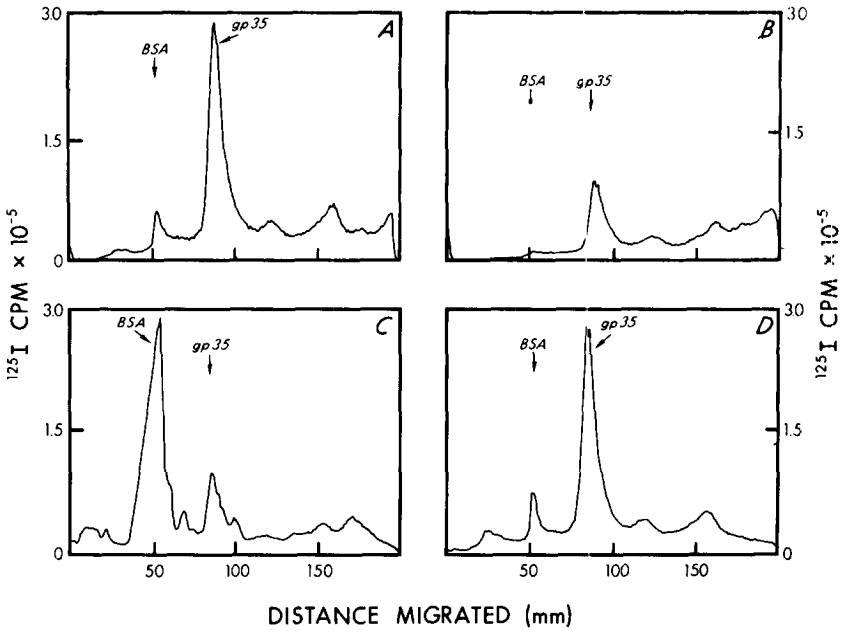


Fig.1. SDS-PAGE of $^{125}$I-gp35 from Pr-RSV-C after (A) being mixed with carrier BSA and solubilized for electrophoresis, (B) carrier-free performic acid oxidation, (C) performic acid oxidation in the presence of BSA carrier and (D) reduction and carboxymethylation in the presence of carrier BSA. All procedures are described in Materials and Methods. The radioactivity scale is adjusted to represent an equal quantity of $^{125}$I-gp35 used in each procedure. The positions of iodinated BSA and gp35 from parallel gels are indicated.

RESULTS. Initially, $^{125}$I-gp35 from Pr-RSV-C was solubilized and co-electrophoresed with unlabeled BSA as described in Materials and Methods. Under these conditions (Fig. 1A), the iodine label migrates predominantly in the position of gp35 (fraction 90), with minor peaks (less than 10% of the total recovered label) appearing at the position expected for BSA (fraction 50) and at positions characteristic of smaller molecular weight proteins. Since these minor peaks also are present on gels of $^{125}$I-gp35 which contain no BSA carrier (data not shown), they probably represent minor contaminants and breakdown products in this particular gp35 stock solution. Taken together, these observations argue against any significant transfer of iodine label during solubilization or electrophoresis. Quantitation of the radioactivity

recovered on the gel further revealed that 86% of the applied label was recovered; the 14% not recovered presumably represents iodide which was noncovently bound to protein or released from unstable sulfenyl iodide bonds (8). Free iodide migrates at a rate of 5.5 cm/hr under the conditions used here and is thus rapidly eluted from the end of the gel.

A standard procedure for denaturing proteins prior to chemical or enzymatic digestion in peptide mapping studies is to dissociate and oxidize disulfide bonds by treatment with performic acid (4). When $^{125}$I-gp35 was oxidized with performic acid and then subjected to analysis by SDS-PAGE (Fig. 1B), only 26% of the applied radioactivity was recovered relative to the control gel in Fig. 1A. More interesting, however, was the finding that when the performic acid oxidation was carried out in the presence of BSA carrier, 85% of the $^{125}$I label was recovered on the gel, but the majority (54%) electrophoresed in the position of BSA (Fig. 1C). These data suggest that under conditions of performic acid oxidation, $^{125}$I label is released from gp35 and becomes bound to both BSA and gp35. Since the BSA carrier is present in much greater amounts than gp35, the re-iodination favors attachment to BSA.

An alternative procedure for dissociating the blocking disulfide bonds in proteins prior to digestion with trypsin is reduction with 2-ME and carboxymethylation with iodoacetamide (8). As shown in Fig. 1D, reduction and carboxymethylation of $^{125}$I-gp35 in the presence of carrier BSA results in minimal (<1-2%), if any, transfer of iodine label; only about 10% of the radioactivity was recovered in the position of BSA. These data indicate that reduction and carboxymethylation are the preferred procedures for disrupting disulfide bonds when using iodine radiolabeled proteins.

The release of iodine label under acidic conditions prompted us to test for a similar release and transfer during TCA precipitation, which is used routinely for assaying proteins for covalently bound $^{125}$I and for purifying iodinated proteins from SDS solutions. A sample of $^{125}$I-gp35 was mixed with BSA carrier and precipitated with TCA as described in Materials and Methods.

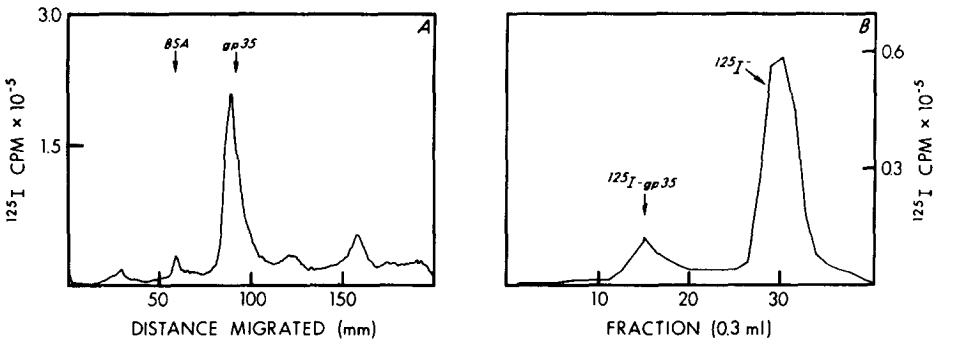


Fig.2. TCA precipitation of $^{125}$I-gp35 mixed with carrier BSA. (A) SDS-PAGE of TCA precipitated protein. (B) Gel filtration of TCA supernatant on a column of Sephadex G25. All procedures are described in Materials and Methods.

The resultant protein precipitate and TCA supernatant were then analyzed by SDS-PAGE (Fig. 2A) and by gel filtration (Fig. 2B), respectively. The data indicate that about 85% of the input protein was precipitated with less than a 10% release of iodine label, and no detectable transfer of label to the carrier BSA. Davison (1) has also reported a lack of iodine label transfer when precipitating protein with acidified methanol, but did not assay for iodine release. It would seem advisable to check for iodine release and transfer, particularly when more rigorous precipitation conditions are employed.

DISCUSSION. Peptide mapping of radioiodinated proteins has become increasingly popular during the last several years, particularly with viral proteins and glycoproteins (1,9-11). Prior to chemical or enzymatic cleavage of the radioiodinated protein, the standard procedure has been to dissociate protein disulfide linkages and block the free sulfhydryl groups by performic acid oxidation. However, the results described here indicate that performic acid oxidation is not a viable procedure for radioiodinated proteins. If the labeled protein is used carrier free, a substantial amount (about 60-80%) of the label is released from the protein resulting in very poor yields. Furthermore, if carrier protein is added, the label is transferred to the protein carrier thereby rendering the material useless for analysis. Thus it appears that

reduction and carboxymethylation are the preferred procedures for denaturing iodinated proteins prior to chemical or enzymatic digestion. These results also suggest that previous peptide mapping studies which employed performic acid oxidation of iodinated proteins in the presence of carrier should be carefully evaluated.

The unexpected finding of radioiodine release and transfer during performic acid oxidation emphasizes the necessity of checking for similar occurences in other routine laboratory procedures. Bogdanove and Stash (12) have reported that iodinated proteins apparently release label as iodide and volatile iodine during storage, however, the mechanism of this release is uncertain. It is known that the amino acid residues primarily iodinated are tyrosine, histidine and cysteine (8). Sulfenyl iodides are usually unstable and decompose rapidly in aqueous solutions to release iodide (8). However, it appears unlikely that the predominant source of iodide release during performic acid oxidation (75% of total) could be from sulfenyl iodides. In fact, only 14% of the label was not covalently bound to protein when the gp35 was solubilized with 2-ME and SDS and subjected to electrophoresis, conditions which would completely dissociate sulfenyl iodide bonds. Hence it appears more likely that the label is being released from tyrosine and histidine under acidic conditions. Since transfer to BSA carrier can occur, the iodine label evidently must establish an equilibrium between free iodide and covalent attachment to protein. When only trace quantities of protein are present, the label is released as free iodide; when carrier protein is present in a large excess, the equilibrium then favors attachment to protein.

Perhaps it should also be emphasized that the release of radioactive iodine from iodinated proteins during routine laboratory procedures also poses a potential health hazard, and that all necessary precautions should be taken to minimize the risk of exposure to laboratory personnel.

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