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Iodination-induced alterations in biochemical properties of human placental insulin receptor

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Insulin receptors from human placenta have been labeled by using an oxidative iodination procedure (iodogen-mediated or chloramine-T-mediated), Bolton-Hunter reagent or [^{3}H]acetic anhydride. The oxidative iodination procedure reduces the affinity for ^{131}I-insulin and the receptor protein becomes fragmented into smaller pieces with an $s_{20,w}$ value of 5–6. However, treatment with Bolton-Hunter reagent or [^{3}H]acetic anhydride does not alter the K_d of ^{131}I-insulin binding and the $s_{20,w}$ value remains unchanged with respect to the native receptor. It is proposed that for labeling multisubunit sulfhydryl-linked protein drastic oxidative iodination procedures should be avoided.

Protein iodination; Iodogen; Chloramine-T; Bolton-Hunter reagent; Protein acetylation; Insulin receptor

1. INTRODUCTION

In vitro labeling of proteins by iodination with ^{125}I is a general method for the detection and labeling of nanogram quantities of purified proteins. Iodination of proteins can be achieved by oxidative methods like iodogen- [1] or chloramine-T-induced ^{125}I incorporation into tyrosine residues [2]. However, Bolton-Hunter reagent-induced labeling is mediated by formation of an active ester derivative [3] at NH_2-terminal residues such as lysine. There are reports that oxidative labeling of proteins leads to alterations in biological properties of the protein. EGF when labeled by chloramine-T and ^{125}I undergoes structural modification and thereby forms a covalent complex with cell surface EGF receptor [4].

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Abbreviations: BSA, bovine serum albumin; PEG, polyethylene glycol 6000; EGF, epidermal growth factor

In the course of my early studies with human placental insulin receptor, it was observed that the sedimentation properties of labeled receptor on a 5–20% sucrose gradient change significantly depending on whether it is labeled by an oxidative iodination procedure or by Bolton-Hunter reagent (unpublished). Here, evidence is presented that oxidative iodination leads to fragmentation of insulin receptor and loss of insulin-binding affinity within the physiological concentration range. However, labeling of receptor by Bolton-Hunter reagent or acetylation of the receptor by [^{3}H]acetic anhydride [5] does not alter its biological and physicochemical properties.

2. MATERIALS AND METHODS

Human placental insulin receptor was purified by the method of Siegel et al. [6] using an acid-urea elution procedure. Bolton-Hunter reagent (NEN) was dried inside the vial by means of a gentle stream of dry N_2 gas and then mixed with 2 μg of purified insulin receptor in 100 mM phosphate

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buffer (pH 7.5) in final volume of 10–20 μl. This mixture was placed on ice for 2–4 h and then the reaction was stopped by the addition of 500 μl of 200 mM glycine-borate buffer (pH 8.4). Labeled protein was filtered on a Sephadex G-50 column which was pre-equilibrated using 0.2% gelatine in 100 mM phosphate buffer. Labeled insulin receptor eluted in the void volume and was collected and concentrated by dialysis vs 40% sucrose in 100 mM phosphate buffer (pH 7.5).

Iodogen-induced labeling of insulin receptor was achieved by mixing 2 μg purified receptor, 500 ng iodogen coated inside a glass tube and 1 mCi $Na^{125}I$ on ice [1,6]. Labeled protein was eluted by gel filtration on a Sephadex G-50 column pre-equilibrated with 0.1% BSA. ^{125}I-labeled receptor was concentrated by dialysis vs sucrose.

Chloramine-T-induced labeling of insulin receptor was performed by mixing 2 μg purified receptor, 1 mCi $Na^{125}I$ and 5 μl chloramine-T (1 mg/ml) in a final volume of 100 μl in 100 mM phosphate buffer (pH 7.5). After mixing briefly at room temperature 5 μl Na metabisulfite was added (1 mg/ml). The whole mixture was then filtered on a Sephadex G-50 column, and labeled protein was isolated and concentrated from the void volume after dialysis vs 40% sucrose.

Porcine insulin was labeled with ^{131}I (carrier-free, NEN) by the chloramine-T procedure as described by Siegel et al. [2] to a specific activity of 1000–1500 cpm/fmol.

[^{3}H]Acetic anhydride (NEN, 50 mCi/mmol) was dissolved in anhydrous dioxane (0.5 mCi) and mixed with 2 μg insulin receptor in 0.3 M Na phosphate buffer (pH 7.2) at room temperature for 30 min. Labeled receptor was separated from unreacted label by gel filtration on a Sephadex G-50 column preequilibrated with 0.1% BSA in 100 mM phosphate buffer. Acetylated receptor collected in the void volume was concentrated by dialysis vs 40% sucrose. This preparation contained almost 100% trichloroacetic acid-precipitable radioactivity [5]. However, no attempt was made to calculate the specific activity because of the presence of Triton X-100 micelles in the mixture.

Scatchard analysis of ^{131}I-insulin binding to labeled insulin receptor was performed as described by Siegel et al. [6]. Only the high-affinity region of the binding isotherm was utilised to calculate the K_d [6]. PEG-precipitable ^{131}I-insulin bound to ^{125}I-receptor was counted in an Intertechnique multichannel counter.

Sucrose density gradient sedimentation of labeled insulin receptor was performed by using a 5–20% linear sucrose gradient prepared in 50 mM phosphate buffer (pH 7.5) containing 0.1% BSA and 0.2% Triton X-100. Gradients were run in a Beckman SW50.1 rotor at 4°C for 16 h at 42000 rpm. Internal standards used were catalase (11.3 S), fumarase (8.9 S) and hemoglobin (4.5 S). About 1×10^6 cpm labeled receptor (either ^{14}C or ^{125}I) were loaded on each gradient and fractions were counted for radioactivity after the run. The $s_{20,w}$ values presented are apparent sedimentation coefficients and are not corrected for Triton X-100 micelles bound to each preparation [6,8].

3. RESULTS AND DISCUSSION

Fig.1 demonstrates that when insulin receptor is labeled by Bolton-Hunter reagent or acetylation, its sedimentation coefficient remains the same as that of the native insulin receptor ($s_{20,w}$ = 10.8 S). This indicates that even when bulky side chains like diiodo-*p*-hydroxyphenylpropionic acid or small groups such as acetyl groups are covalently linked with insulin receptor, its conformational perturbation is minimum and the $s_{20,w}$ value remains unchanged. However, when receptor is exposed to

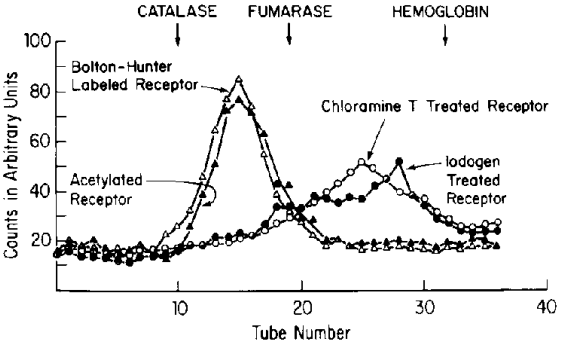

Fig.1. Sedimentation pattern of labeled insulin receptor on a 5–20% sucrose gradient. Placental insulin receptors labeled by means of various procedures were loaded on the top of a 5–20% linear sucrose gradient in a Beckman SW50.1 rotor. After 16 h of spinning at 42000 rpm, fractions were collected and counted for radioactivity. The large peak ahead of catalase has an $s_{20,w}$ value of 10.8 S where unlabeled receptor also migrates [6].

Table 1

Effects of various labeling procedures on the biochemical properties of human placental insulin receptor

Labeling procedure	% trichloroacetic acid-precipitable counts	K_d (nM)	Apparent $s_{20,w}$ (S)
Control receptor	–	3.5	10.9
Bolton-Hunter treatment	43	3.3	10.8
Iodogen treatment	51	60.0	6–5
Chloramine-T treatment	49	30.0	6–5
Acetic anhydride treatment	100	3.1	10.8

Labeled receptor was allowed to interact with ^{131}I-insulin at various concentrations and the specific amount of insulin bound was determined by PEG precipitation assay [6]. Data were analysed by Scatchard plot and high-affinity K_d values were evaluated from the plot. Apparent $s_{20,w}$ values were evaluated from fig.1. 1 μl of reaction mixture after iodination was precipitated by 10% trichloroacetic acid with BSA as carrier and the fraction of counts in the precipitate was determined. This roughly reflects the degree of ^{125}I incorporation into the protein

drastic oxidizing agents like chloramine-T or iodogen the molecule breaks down to smaller pieces and bands around $s_{20,w}$ values of 5–6 S. Since human insulin receptor is a multisubunit molecule [7] whose subunits are held together by S–S bonds, it seems logical to propose that oxidation during iodination destroys these bonds and that the protein is fragmented.

This oxidation damage to the receptor is also reflected in the loss of affinity for ^{131}I-insulin. Receptors labeled by Bolton-Hunter reagent or [^{3}H]acetic anhydride retain their K_d for insulin of around 2–3 nM while receptors labeled by iodogen and chloramine-T have much higher K_d values clearly indicating altered conformation and/or higher-order structure of the molecule.

During the course of this investigation, I have observed that a substantial amount of Triton X-100 which is tightly bound with the receptor [6] also undergoes iodination when the receptor is exposed to iodogen or chloramine-T. In fact, when Triton X-100 alone is exposed to these reagents in the presence of Na^{125}I, a substantial amount of trichloroacetic acid- and PEG-precipitable counts can be obtained (unpublished). This makes quantitation of the specific activity of protein labeling very difficult. In summary, labeling of S-S linked multisubunit proteins should be carried out using Bolton-Hunter reagent or through acetylation by acetic anhydride and oxidative procedures should be avoided.

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