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# Disulfide bonds within the α-subunit of insulin receptors in rat liver and brain membranes

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We have characterized inter- and intrasubunit disulfide bonds of insulin receptors using reductant-treated rat liver and brain membranes. In autoradiograms of $^{125}$I-insulin cross-linked to both membranes pretreated with dithiothreitol, the intensity of affinity-labeled bands of the αβ-heterodimer and α-subunit was increased. Interestingly, labeled 120 and 110 kDa bands considered to be the α-subunit in partially reduced liver and brain membranes moved to 130 and 120 kDa bands under further reduced conditions, respectively. Double electrophoresis of each partially reduced band in the presence of reductants clearly demonstrates that the α-subunit of insulin receptors contains intrasubunit disulfide bonds.

Insulin receptor; Dithiothreitol; Cross-linking; α-Subunit; Disulfide bond

## 1. INTRODUCTION

Since Massague et al. [1] proposed a model for insulin receptors consisting of two subunits, it is now believed that two α- and two β-subunits are linked by disulfide bonds to form native insulin receptors. The α-subunit ($M_r$ 125 000) is believed to represent the insulin-binding subunit on the evidence of its predominant labeling [2–4]. On the other hand, insulin-induced phosphorylation in the β-subunit ($M_r$ 95 000) has led to the speculation that this subunit may play a role in signal transduction [5–7]. Recent investigations have demonstrated more precisely the structure of insulin receptors including the amino acid sequences of the α- and β-subunits [8]. In spite of such intensive investigations, it is not yet known how linkages of α- and β-subunits by disulfide bonds affect the relationship between structure and function or the spatial orientation of both subunits. Generally speaking, disulfide bonds or sulfhydryl groups in insulin receptors have an important role in ligand binding [9,10]. The present study was therefore designed to gain better understanding of the role of disulfide linkages in the subunit structure of insulin receptors using reductant-treated rat liver and brain membranes. We describe here that there may be intrasubunit disulfide bonds within the α-subunit in addition to the known intersubunit disulfide bonds of insulin receptors.

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## 2. MATERIALS AND METHODS

### 2.1 *Materials*

The following were purchased: porcine insulin from Novo Research Institute (Copenhagen); dithiothreitol (DTT), 2-mercaptoethanol (2ME), SDS from Nakarai Chemicals (Osaka); disuccinimidyl suberate from Pierce (Rockford, IL); $^{125}$I-insulin from Dainabot (Tokyo).

### 2.2. *Preparation of liver and brain membrane particles and cross-linking of $^{125}$I-insulin to the membranes*

Liver or cerebral cortex of a decapitated male Wistar rat was finely minced and homogenized in 10 vols of 30 mM sodium phosphate buffer at pH

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7.9 using a Teflon-glass homogenizer. The 30000 × *g* pellet was then treated with various concentrations of DTT or DTT plus 2ME for 10 min at 24°C in phosphate buffer at pH 7.9 containing 10 mM $MgCl_2$ and 0.3 mM EGTA. After the membranes had been washed, cross-linking was performed by incubating these membranes in the aforementioned phosphate buffer with 0.5 nM $^{125}$I-insulin for 45 min at 20°C. Thereafter, the membranes were incubated with 0.25 mM disuccinimidyl suberate for 15 min at 4°C. Finally, the cross-linked membranes were solubilized in 62.5 mM Tris and 4.6% SDS at pH 6.8 in the presence or absence of 50 mM DTT. Electrophoresis was carried out using 5–7.5% acrylamide as described by Laemmli [11]. Double electrophoresis was performed to complete reduction of disulfide bonds in affinity-labeled membrane proteins on the first gel. Autoradiograms were obtained from the dried gels after exposure to Kodak X-Omat AR film.

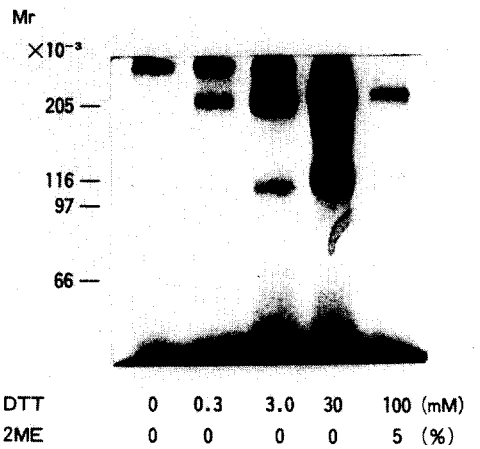


Fig.1. Effect of reductant pretreatment on labeling of insulin receptor subunits in brain membranes. Brain membranes were treated with different concentrations of reducing agents ranging from 0.3 mM DTT to 100 mM DTT plus 5% 2ME. Thereafter affinity-labled membrane protein was solubilized without further reduction and then electophoresed on a 7.5% polyacrylamide gel.

## 3. RESULTS

### 3.2 *Cross-linking of $^{125}$I-insulin to its receptors on membranes pretreated with reductants*

When brain membranes were treated with 0.3 mM DTT, the native insulin receptor complex observed in the absence of DTT was diminished concomitant with the appearance of a 210 kDa labeled species previously shown to consist of $\alpha\beta$-insulin receptor fragment and a 105 kDa labeled band considered to be free $\alpha$-subunit (fig. 1). The intensity of these two bands was increased in the dose-dependent manner of pretreated DTT. When brain membranes were treated with 30 mM DTT, the intensity of these two bands was most enhanced. Further increases in reducing agents resulted in diminished labeling of these two major bands. It should be noted that the electrophoretic mobility of the free $\alpha$-subunit was decreased in brain membranes treated with increasing concentrations of DTT. We were able to observe the same phenomenon when liver membranes were treated with DTT (not shown). This phenomenon may indicate the existence of additional disulfide bonds within insulin receptor subunits that lead to a more compact subunit structure.

To resolve this question, liver and brain membranes treated with 30 mM DTT followed by affinity labeling were solubilized and electrophoresed under reducing conditions (fig. 2). When compared with the free $\alpha$-subunit electrophoresed under nonreducing conditions, which can be seen as a 120 kDa labeled band in liver membranes or

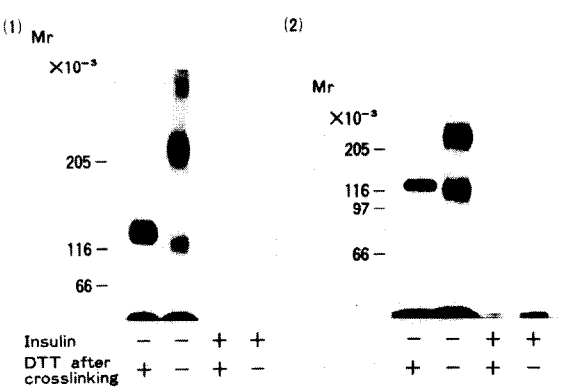


Fig.2. Effect of reductants on affinity-labeled insulin receptor in liver and brain membranes. Liver (1) and brain (2) membranes were treated with 30 mM DTT for 10 min at 24°C. Affinity-labeled membrane protein was solubilized in the absence or presence of 50 mM DTT for futher reduction and electrophoresed on 5% (1) or 7.5% (2) polyacrylamide gel.

a 110 kDa band in brain membranes, it moved to 130 kDa in the former or 120 kDa in the latter. Another major band of 220 kDa or 210 kDa disappeared under reducing conditions.

### 3.2. *Analysis of intrasubunit disulfide bonds using double electrophoresis*

In the first dimension, membranes treated with 3 mM DTT were affinity labeled and analyzed by SDS-polyacrylamide gel electrophoresis without further reduction. Then the entire lane was cut out and gel slices corresponding to the free $\alpha$-subunit were divided into two sections with the same radioactivity. Each section ground was electrophoresed on a second gel in the presence or absence of 100 mM DTT plus 5% 2ME (fig. 3). Under these conditions, the 120 kDa band yielded a 130 kDa band in liver membranes, and the 110 kDa band yielded a 120 kDa band in brain membranes, suggesting that the $\alpha$-subunit of insulin receptors on liver or brain membranes contains intrasubunit disulfide bonds. Here, the additionally reduced bands became faint in autoradiograms as noted in fig. 3, even though gel slices with the same radioactivity were applied on a second gel. We used heterogeneous iodoinsulin which was iodinated primarily on the A-chain of insulin. Since insulin consists of two disulfide-linked chains and cross-linking of $^{125}$I-insulin to its receptors occurs mainly via reaction of the B-chain amino groups [12], reductive cleavage of the A-chain from the cross-linked complex can occur. This might be the reason for the diminished radioactivity in the additionally reduced $\alpha$-subunit of insulin receptors.

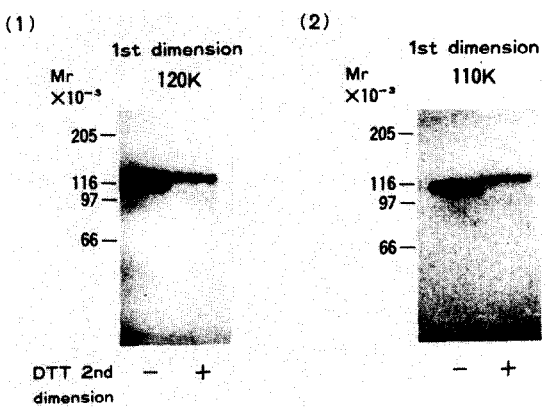


Fig.3. Comparison of partially reduced with additionally reduced insulin receptor on liver and brain membranes. $^{125}$I-insulin was cross-linked to liver (1) and brain (2) membranes pretreated with 3 mM DTT as described in section 2. Thereafter, free $\alpha$-subunit of insulin receptor resolved from the first gel was electrophoresed on a second gel in the absence or presence of 100 mM DTT plus 5% 2ME for complete reduction.

## 4. DISCUSSION

The autoradiogram of $^{125}$I-insulin cross-linked to membranes treated with increasing concentrations of DTT showed that the intensity of the $\alpha\beta$-heterodimer and free $\alpha$-subunit of insulin receptors was enhanced. In contrast, when membranes were treated with 100 mM DTT plus 5% 2ME which were the maximum amounts of reductants we used, labeling of two major bands was diminished. These findings are consistent with previous studies which have reported that DTT treatment of intact adipocytes of liver and adipocyte plasma membranes results in increased receptor affinity, while further increase in DTT concentrations is associated with decreased insulin binding [9,10]. Of particular interest is the observation that the electrophoretic mobility of free subunit in liver and brain membranes decreased after treatment of the membranes with increasing concentrations of DTT. Two-dimensional electrophoresis clearly demonstrates that the $\alpha$-subunit of insulin receptors contains intrasubunit disulfide bonds. The $\alpha$-subunit region of which the entire amino acids sequence has been recently determined is characterized by an unusually large number of cysteine residues [8]. Therefore, it is possible to speculate that there may be intrasubunit disulfide bonds at cysteine-rich $\alpha$-subunit regions. The predominant labeling of the $\alpha$-subunit by radioactive insulin derivatives in photoaffinity labeling and affinity cross-linking experiments also suggests that insulin interacts with its receptors by binding to the $\alpha$-subunit. In this study, it should be noted that partial reduction of intrasubunit disulfide bonds within the binding domain is well correlated to the increase in labeling of the $\alpha\beta$-heterodimer or free $\alpha$-subunit of the receptors. Furthermore, a recent investigation has shown that the partially reduced $\alpha\beta$ form of purified insulin receptors exhibits much higher kinase activity than intact receptor in

the $\alpha_2\beta_2$ form [13]. Taking these observations into consideration, our data raise the speculation that intrasubunit disulfide bonds within the binding domain may regulate the receptor affinity for insulin. However, at present we do not know whether the partially reduced form of the $\alpha$-subunit can be associated with the physiological sequence of events in insulin receptors. More studies are needed to clarify such important questions.

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