THE INTERACTION OF B29-FLUORESCEINTHIOCARBAMYL-INSULIN WITH ADIPOCYTE MEMBRANES

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1. Introduction

A growing body of evidence implicates a hydrophobic region on the surface of the insulin molecule as the site which binds to the insulin receptor [1–3]. Direct evidence for this is difficult to obtain partly because of the chemical problems associated with making insulin derivatives in which this surface is specifically modified. However the B29 lysine residue lies close to the putative binding site and has been modified to produce $N^{\epsilon B29}$-fluoresceinthiocarbamyl-insulin which has a biological activity of ~30% [4]. It occurred to us that interpretable changes in fluorescence might result when this insulin derivative interacted with its receptor. We report here that the fluorescence polarisation is decreased either by the presence of rat adipocyte membrane fragments or by the presence of urea. We interpret this to mean that in the native state the fluorescein moiety interacts with the receptor binding surface of insulin so that its rotational mobility is restricted but that the fluorescein moiety has to swing away from the insulin surface before interaction with the insulin-receptor can occur.

2. Experimental

The synthesis of $N^{\epsilon B29}$-fluoresceinthiocarbamyl-insulin (FTC-insulin) involved the protection of α amino groups with citraconic anhydride, reaction of the purified product with fluorescein isothiocyanate and hydrolysis of the citraconyl residues. Analysis of the product showed that it ran as a single band on cellulose acetate electrophoresis at pH 8.6 and on paper in 33% acetic acid. Sulphitolysis yielded unmodified A-chain tetra-*S*-sulphonate and mono-FTC B-chain di-*S*-sulphonate. The absence of fluorescein attached to the α amino group of the B1 residue was confirmed by the stability of the B-chain to treatment with trifluoroacetic acid. Its molar decadic absorbtivity at 497 nm was 5.81 $m^2 . mol^{-1}$. The FTC-insulin was bioassayed as in [5]. It produced the same maximum stimulation of glucose metabolism as native insulin, and had ~30% of its activity.

For the preparation of membrane fragments, adipocytes were prepared as in [6] from the epididymal fat pads of 8 fed Wistar rats weighing 100–200 g. The cells were washed 3 times with and finally suspended in Krebs bicarbonate buffer containing 50% $CaCl_2$ specified [7]. The suspension was subjected to sonication for 3 periods of 15 s using an MSE probe-type sonicator set for maximum resonance through the suspension. The sonicated cells were centrifuged for 15 min at 56 000 × *g* with a Beckman L2-65B centrifuge. The pellet of membranes was washed twice with Tris–HCl buffer (pH 7.4) and resuspended in 2 ml of the same buffer by homogenising with a Teflon homogeniser rotating at 1750 rev./min. Samples of the suspension (0.5 ml) were dispensed into small tubes and sometimes stored frozen at –24°C for a maximum of 5 days. Frozen membranes were always used within 3 h of thawing. After staining with 1,8-anilinonaphthalene sulphonate the membranes appeared in a fluorescence microscope as large open sacks ~33–25% the size of whole

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adipocytes. Their protein:cholesterol:phospholipid ratio was 2.7:0.8:1. The method in [8] was used to show that the membranes bound ^{125}I-labelled insulin specifically and that their insulin binding capacity was negligibly affected by freezing for ≤5 days.

Fluorescence polarisation was measured with a Hitachi MPF-2A spectrofluorimeter using excitation and emission wavelengths of 460 and 520 nm. The polarisation, corrected for instrumental polarisation, was calculated as in [9]. No measurements were recorded until a steady signal was observed (~5 min after relevant additions).

3. Results

The fluorescence polarisation of 6 nM fluorescein in Tris–HCl buffer (pH 7.4) at 25°C was found to be 0.010 and to be unchanged by the presence of membranes or of 3.6 M urea. This shows that the fluorophore does not itself react with the membranes and that its fluorescence polarisation is negligibly affected by any change in the viscosity of the solution caused by this concentration of urea.

The fluorescence polarisation of 12 nM FTC-insulin at 25°C was found to have a mean value of 0.185 in 3 expt and 0.148 at 37°C (table 1). We have shown that FTC-insulin at 100-times this concentration elutes from a column of Sephadex G100 with M_r 6000 so that the hormone is monomeric under the conditions of these experiments. The addition of 30 μl membrane suspension to 2 ml FTC-insulin solution reduced the polarisation to a mean value of 0.146 at 25°C and to 0.095 at 37°C (table 1) Student's t-test shows that these differences are significant at the 95% confidence level. In the presence of 3.6 M urea the polarisation at 25°C was reduced to 0.077.

Table 1
Change in fluorescence polarisation of FTC-insulin caused by membranes

Membrane prep.	Temp. (°C)	Polarisation	
		In buffer alone	With membranes
A	25	0.184 ± 0.032 (6)	0.122 ± 0.037 (5)
B	25	0.179 ± 0.014 (5)	0.160 ± 0.013 (3)
C	25	0.191 (2)	0.155 (2)
C	37	0.148 ± 0.027 (6)	0.095 ± 0.035 (6)

4. Discussion

In the absence of changes in fluorescence lifetime, a reduction in fluorescence polarisation is indicative of an increase in the vibrational and rotational mobility of the fluorophore. The difference between the polarisation of free fluorescein and of FTC-insulin in the presence of urea reflects the restrictions to fluorophore mobility caused by its covalent attachment to a macromolecule. Because the polarisation of native FTC-insulin is higher than of FTC-insulin in urea solution we suggest that the B29 lysine residue carrying the fluorescein moiety is positioned in such a way as to bring the fluorescein into contact with the surface of the insulin, and that the interaction energy between the fluorophore and the insulin surface is reduced in the presence of urea.

Examination of the crystal structure of insulin [1] shows that the positions of the C-terminal residues of the B-chain are not well defined and that more than one structure is possible. It can be inferred from this that this region is very flexible when insulin is in aqueous solution, especially when it is monomeric. It follows that the fluoresceine moiety attached to the B29 lysine residue would be able to adopt a number of positions some of which might involve interaction with the surface of the protein.

Inspection of a model of the insulin molecule suggests two possible ways in which the fluorescein moiety could be brought into favourable contact with the protein surface. One way would be to bring it into a hydrophobic pocket between the B26 tyrosine and A3 valine residues; the other would involve rotating the B29 lysine residue in the opposite direction so that the fluorescein moiety interacts with the faces of the aromatic residues B25 phenyl alanine and A19 tyrosine. Both surfaces are thought to be part of the binding area by which insulin interacts with its receptor [1]. If this is so and if the fluorescein moiety interacts with the protein in either of the ways suggested then the FTC-insulin cannot bind to its receptor unless the B29 lysine residue rotates to remove the contact between the fluorophore and the protein. The requirement that such a rotation occurs before the FTC-insulin can bind is consistent with the observed reduction in biological activity since it would decrease the affinity of FTC-insulin for its receptor. Furthermore the consequences of rotation, which would necessarily leave the fluorophore exposed to solvent, are consistent with the

observation that the fluorescence polarisation of FTC-insulin is decreased when binding to the receptor occurs.

Our results therefore add to the evidence that the insulin monomer interacts with its receptor in the plasma membrane thought a binding site on the insulin surface which includes the aromatic residues B25 phenyl alanine, A19 tyrosine, B24 phenyl alanine and B16 tyrosine.

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