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THE ASSOCIATION/DISSOCIATION EQUILIBRIA OF INSULIN IN THE PRESENCE OF METAL IONS:
A FLUORESCENCE ENERGY TRANSFER AND CIRCULAR DICHROISM STUDY

Abstract. The association/dissociation behaviour in the presence of co-ordinating metal ions and T-R transforming ligands is too complex for rigorous analysis. In the present study equimolar mixtures of insulin labelled with a fluorescence donor and with an acceptor group, respectively, were used to measure fluorescence resonance energy transfer as a function of concentration. These experiments were performed in the presence of 2 Zn or 2Co ions per hexamer and in the absence and presence of KSCN or 3-pentanol, respectively. The results were analysed on the basis of a monomer-dimer-hexamer model in terms of apparent equilibrium constants $K_{12}$ and $K_{26}$. The experiments were paralleled by measurements of the near-UV circular dichroism on unlabelled insulin and analysed accordingly. Both sets of experiments show that $K_{26}$ is not much more increased by Zn than by Co ions and much less than under $R_c$ conditions.

1. INTRODUCTION

The association of insulin has been the subject of several investigations (see references in Kadima et al., 1993). The techniques used so far are sedimentation equilibrium studies (Pekar and Frank, 1972; Goldman and Carpenter, 1974; Jeffrey et al., 1976; Holladay et al., 1977; Mark and Jeffrey, 1990; Brems et al., 1992), difference spectroscopy (Strazza et al., 1985), dynamic light scattering (Hvidt, 1991; Kadima et al., 1993), circular dichroism (CD) (Goldman and Carpenter, 1974; Wood et al., 1975; Pocker and Biswas, 1981; Melberg and Johnson, 1990) and fluorescence energy transfer (Hassiepen et al., 1998, 1999). Most of the investigations focussed on metal-free insulin. Metal bound insulin is of importance with respect to storage in the ß-cells as well as on the shelf and to pharmacokinetics. To the best of our knowledge neither the influence of different co-ordinating metals nor of the $T_iR_i$ and $R_s$ conformations of the insulin hexamer on its association/dissociation behaviour have been quantitatively evaluated, except in the publication of Hvidt (1991).

While the use of CD spectroscopy for the study of insulin association is well established (see above), fluorescence energy transfer was used only recently. Both techniques were applied in the present study and their respective results compared. The present approach evaluates the effect of dilution on the fluorescence of
equimolar mixtures of donor- and acceptor-labelled insulin (I_d and I_a), 2-Aminobenzoyl (Abz) and 3-nitrotyrosyl residues Tyr(NO_2), respectively, attached to the N_e-aminogroup of LysB29, served as fluorescence donor/acceptor pair. Their Förster distance (Förster, 1948) of 29 Å closely corresponds to the separation of the attachment sites in the insulin dimer and hexamer. Labelling of insulin at the ε-aminogroup of LysB29 neither affects the tertiary structure nor interferes with self-association, hence, the labelled insulins are representative of the native hormone (Hassiepen et al., 1998). Energy transfer from the donor group to the quenching acceptor group only occurs when I_d and I_a coexist as subunits in the very same oligomer. Therefore, the fluorescence intensity, normalised to the donor concentration, increases upon dilution reflecting the dissociation of the oligomers.

The same experiments were carried out using the near-UV CD spectrum to monitor the dissociation. In the case of insulin self-association, ligand induced quaternary structure formation and T→R transformation are reflected in its CD spectrum (Wood et al., 1975; Renscheidt et al., 1984; Wollmer et al., 1987). In the near ultraviolet the main band at 275 nm, the tyrosyl L_b band, increases with dimer, tetramer and hexamer formation. This increase originates in additional perturbation of the tyrosyl chromophore by chromophores in neighbouring subunits, occurring as the quaternary structure assembles (Goldman and Carpenter, 1974; Strickland and Mercola, 1976; Wollmer et al., 1977; Mercola and Wollmer, 1981) as well as in a loss of conformational mobility for chromophoric side chains buried in the interfaces between subunits. Changes around 250 nm are related to changes in the disulphide chromophores occurring upon T→R transition. There are concomitant changes also in the far ultraviolet.

To analyse the insulin association different models were used as reviewed in (Mark and Jeffrey, 1990). Basically there are two models: 1. The monomer-dimer-tetramer-hexamer model (Goldman and Carpenter, 1974; Jeffrey et al., 1976; Wollmer et al., 1980; Peck and Biswas, 1981). The association not necessarily stops at the hexamer (Brems et al., 1992). 2. The monomer-dimer-hexamer model (Pekar and Frank, 1972; Holladay et al., 1977; Hvidt, 1991). This work uses the second model. The binding metal also promotes the association of metal binding oligomers providing further binding sites for the metal. Because of these difficulties only “apparent” association constants rather than true thermodynamic parameters are obtainable. Since the results were produced for internal comparison only, this may be acceptable.

2. MATERIALS AND METHODS

2.1. Reagents and Solvents

All reagents and solvents were of analytical grade and are commercially available. Porcine Ne^{825}-2-aminobenzoyl-insulin (Ne^{825}-Abz-insulin, I_d) carrying the fluorescence donor group and porcine Ne^{825}-3-nitro-L-tyrosyl-insulin (Ne^{825}-