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Binding of Fluorescein Isothiocyanate to Insulin: A Fluorimetric Labeling Study

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The determination of the fluorophore to the protein molar ratio has been studied using fluorescence spectroscopy. The tyrosine fluorescence is measured from insulin (Ins) solutions at wavelengths $\lambda_{ex}/\lambda_{em} = 276/300$ nm and from fluorescein isothiocyanate (FITC) solutions at $\lambda_{ex}/\lambda_{em} = 494/518$ nm. Series of solutions prepared from insulin and FITC are tested for conjugation, recording their fluorimetric intensities. Fluorimetric titrations with different formal concentrations are followed either by intrinsic and extrinsic emission intensities at $\lambda_{ex}/\lambda_{em} = 276$ or 494/518 nm and by their typical emission spectra at pH 9.0. All results denoted a binding ratio of 3 moles of FITC/mole of Ins.

KEY WORDS: Fluorescent probe; labeling; conjugation; insulin.

INTRODUCTION

Most of biomolecules have low intrinsic emission in the visible region, a reason why typical fluorophores have been used to probe the biomolecules extrinsic emission. These fluorophores, such as dansyl chloride, fluoresceins and rhodamines, form covalent conjugate ion to biomolecules, including proteins, nucleic acids, drug analogs, hormones and growth factors [1–3]. The molar ratio of fluorophore to protein, f/p, is a quantitative mean that shows the extend of binding, thus the labeling of the protein, used in fluorimetric and immunofluorimetric determinations [4].

Fluorescein isothiocyanate (FITC) is known as the amine reactive fluorescent probe. Isothiocyanate group covalently binds with primary and secondary amine groups of biomolecules. In this study, insulin (Ins) was chosen as a model protein for fluorimetric labeling study because of its pharmacological importance, relatively small size, several reactive groups and characterized structure and function [5–7]. Insulin is a polypeptide hormone that is produced by the β cells of the pancreatic islets. Insulin-fluorescein is commercially available and used in a number of applications. Insulin has been previously labeled with FTIC by use of HPLC and electrospray mass spectrometry [8], capillary electrophoresis with laser induced fluorescence [9]. This study is based on fluorimetric titration with different concentrations of insulin and FITC followed directly either by intrinsic and extrinsic emission intensities at $\lambda_{ex}/\lambda_{em} = 276$ or 494/518 nm.

EXPERIMENTAL

Reagents

Insulin (Bovine pancreas, 27.3 USP units/mg, contained 0.5% Zn, Sigma) was used without further purification to prepare 0.4 mg/mL (6.45×10^{-5} M) stock solution of the hormone. FITC stock solution was prepared from fluorescein 5-isothiocyanate (Isomer I, Sigma) as 0.32 mg/mL (8.22×10^{-4} M). Both stock solutions were prepared in 0.06 M sodium phosphate buffer solution (PBS) at pH 7.4. 0.15 M NaCl (Sigma) and 0.5 M Na_2HPO_4 (Sigma) solutions were prepared to add all conjugation solutions. Deionized water was used for the

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preparation of solutions. Working solutions were prepared daily from stock solutions by appropriate dilution.

Apparatus

The fluorescence measurements were made on a *Jasco Model FP-550* spectrofluorometer interfaced with an IBM computer. The instrument has a xenon lamp, a dual monochromator. Monochromatic readings were taken from the digital display with 0.25 s time constant and with 3 nm bandwidth on excitation side, 5 nm on the emission side. All studies were made using a quartz cell or flow cell with pathlength of 10 mm. IR spectra were recorded by Mattson 1000 FTIR Spectrometer. The electropherograms were obtained using Beckman Capillary Electrophoresis (P/ACE System 5000) equipped with 488 nm line of an Ar^+ ion laser and fluorescence detection (CE-LIF) system. With this system separations took place in a 35 cm long fused silica capillary for 15 min. Fluorescein disodium was used as internal standard. The capillary was rinsed for 4 min with the 20 mM borate buffer and 20% acetonitrile (ACN) at pH 8.5.

Procedure

The fluorimetric titration method was used for conjugation of FITC and insulin [10]. Two sets of experiments were performed in each case: one without Ins (containing buffer instead of Ins), which serves as control; and one containing Ins. A fixed concentration of Ins solution was titrated with an increment of FITC solution each time at pH 9.0. FITC solutions were added to mixture of insulin solution, 0.5 M Na_2HPO_4 and 0.15 M NaCl. The *intrinsic* fluorescence intensities were measured from Ins at wavelengths $\lambda_{ex}/\lambda_{em} = 276/300$ nm and from FITC solutions at $\lambda_{ex}/\lambda_{em} = 494/518$ nm. The *extrinsic* fluorescence intensities were measured from FITC-Ins conjugation solutions at wavelengths $\lambda_{ex}/\lambda_{em} = 276/518$ nm. All studies were performed at room temperature with continous shaking of the reaction mixture. The experiments were repeated and found to be reproduciple within experimental errors.

RESULTS AND DISCUSSION

FITC is used extensively to prepare of biomolecule conjugate for use in immunofluorescence assays. The synthesis of FITC and similar derived reagents in the bottom ring gives two isomers (the 5 and 6 isomers). The 5 isomer or isomer I of FITC is commonly used in applications, probably because it is the easier of two isomers to isolate in

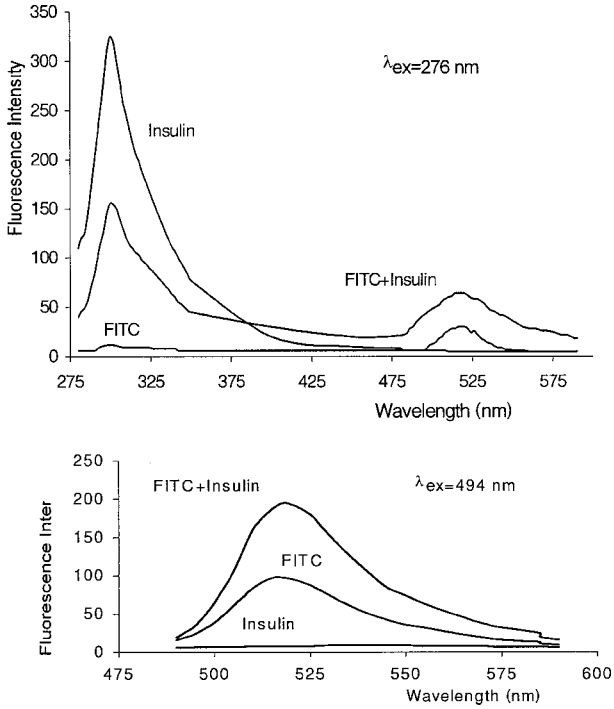

Fig. 1. Emission spectra of insulin in the presence and absence of FITC. *Top*: 276 nm excitation; *bottom*: 494 nm excitation.

pure form. Fluorescence intensity of FITC increases with increasing pH. Neutral molecule and partially monoanion are present at pH 7.0. Fluorescence of FITC above pH 7.0 strongly depends on dianion form [11]. Insulin molecule has two chains, 21 amino acid residues in the chain A and 30 residues in chain B. Insulin fluorescence completely depends on the four tyrosine residues (A14, A19, B16, B26) because it lacks tryptophan [12].

Fig. 1 (top) shows that in conjugation solution, both insulin and FITC were excited at 276 nm. Addition of FITC to the Ins results in a 50% decrease of protein fluorescence and about twofold enhancement of the FITC

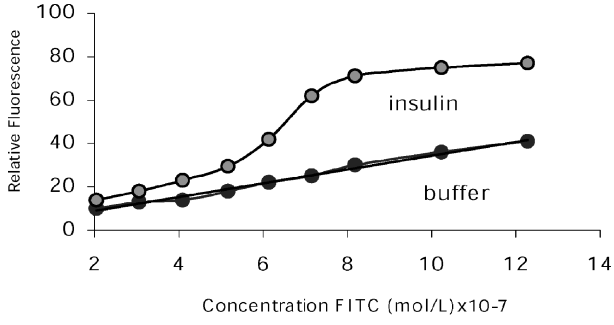

Fig. 2. Fluorescence intensity of FITC titrated into buffer and a solution of insulin.

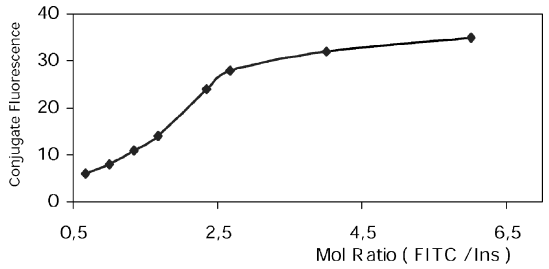

Fig. 3. Dependence of fluorescence intensity of FITC-Ins conjugation on FITC/Ins molar ratios. Insulin concentration was 3.1×10^{-7} M.

fluorescence. The emission spectra of FITC shows that its emission is more intense in the presence of Ins. This can be seen from the increased intensity at about 520 nm with 276 nm excitation (Fig. 1, top). It is not clear from these data whether the quantum yield of FITC has increased on binding or energy transfer caused the increased FITC emission. It is possible to excite FITC alone, and not the Ins, using excitation at 494 nm (Fig. 1, bottom). These spectra show that the emission of FITC is about twofold higher in the presence of protein. This can also be understood by the examination of the titration of Ins with FITC. Fig. 2 shows that titration curves plotted as the relative fluorescence intensity against concentration of FITC in buffer and insulin solution at $\lambda_{ex}/\lambda_{em} = 494/518$ nm. In the absence of protein, the emission increases linearly with FITC concentration. In the presence of Ins, the intensity slowly increases at the beginning of titration then it begins to increase more rapidly upon the addition of 6.0×10^{-7} M FITC. The increase in the intensity of FITC is thus due to binding of FITC to Ins which results an increase in the quantum yield of FITC.

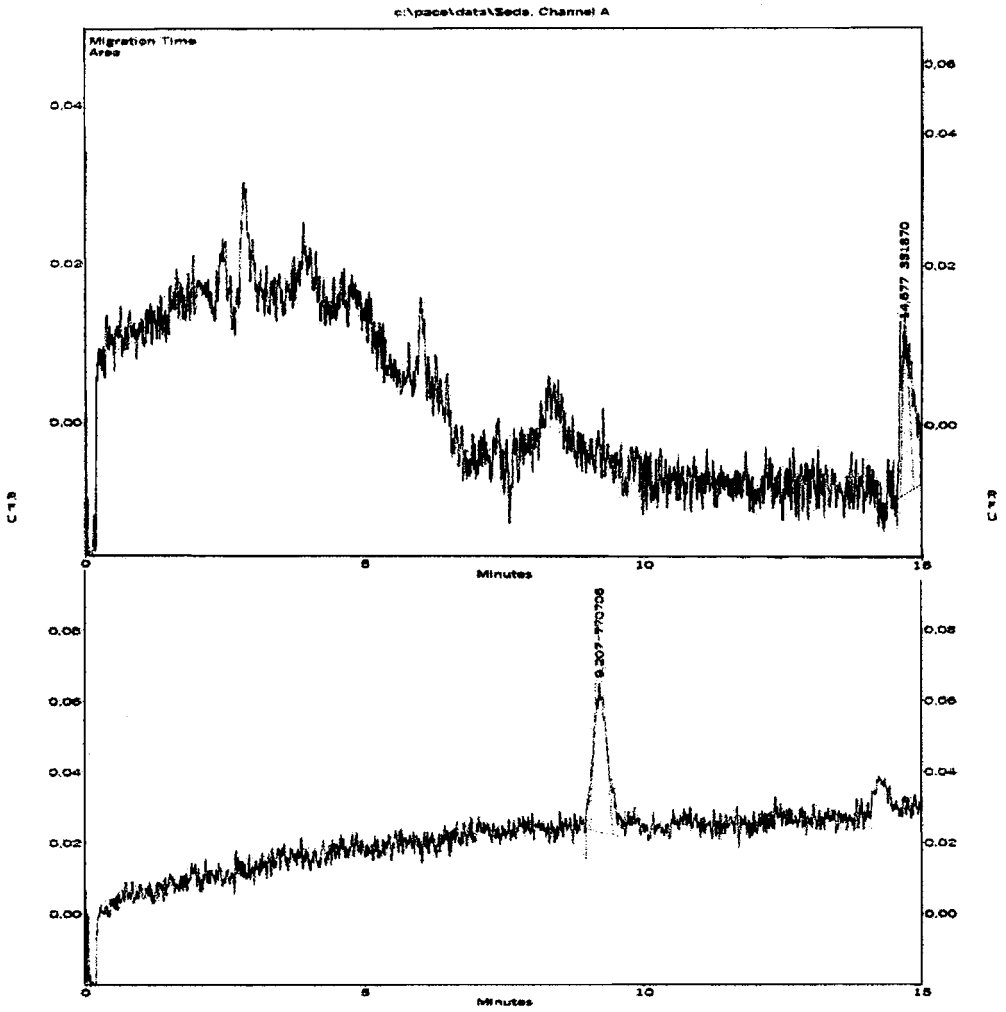

Fig. 4. Elecropherograms of 0.32 mg/mL FITC solution (bottom) and FITC-Ins conjugation at molar ratio of 3:1 (top) in run buffer, 20 mM borate, pH 8.5, with 20% ACN.

To obtain binding stoichiometry of FITC with Ins we studied the dependence of FITC-Ins conjugation fluorescence intensity, at $\lambda_{ex}/\lambda_{em} = 276/518$ nm, from the FITC/Ins ratio at the constant insulin concentration (3.1×10^{-7} M) (Fig. 3). We obtained one break in the titration curve at the ratio of 3 moles FITC to 1 mole Ins. Further addition of Ins solution does not lead to the essential enhancement of fluorescence intensity because of the formation of conjugation.

The formation of binding between FITC and insulin was also evidenced independently using infrared (IR) and capillary electrophoresis (CE) techniques. In CE experiments, FITC and FITC-Ins conjugation electropherograms were compared under the same experimental conditions. Fig. 4 contains electropherograms of 0.32 mg/mL FITC solution (bottom) and FITC-Ins conjugation at molar ratio of 3:1 (top). In examining the electropherograms on time coordinate, increasing number of peaks around 7.0 min were observed while the FITC peak at 9.2 min vanished. These peaks around 7.0 min are due to an increase in the number of derivatization of conjugated protein. A positive charge associated with the protein is replaced by a negative charge at the pH 8.5 of the conjugation/run buffer. Since amine group of insulin is positively charged, whereas fluorescein is negatively charged. FITC is incorporated into insulin through amine residues. Vanishing CE peak of FITC and appearance of new peaks around 7.0 min clearly indicate the binding of FITC to insulin.

IR spectra of insulin, FITC and FITC-Ins conjugation form were also recorded by FTIR with KBr. FITC spectrum has adsorption bands at 3069 cm^{-1} (O—H streching), 2038 cm^{-1} (N═C═S isothiocyanate, streching), 1600 cm^{-1} (C═O streching), 1469 cm^{-1} (C═C streching), 1275 cm^{-1} (C—H bending and C—N streching). Insulin spectrum has adsorption bands at 3400–3100 cm^{-1} (characteristic N—H streching), 1712 cm^{-1} (C═O streching), 1250–1010 cm^{-1} (C—N streching). FITC-Ins conjugation form has different and characteristic adsorption band at 1357 cm^{-1} (N—CS—N streching). It shows clearly that isothiocyanate group (—N═C═S) of fluorescein forms thiourea (N—CS—N) on reaction with amine residues of insulin.

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