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Fluoresceinthiocarbamyl-insulin: A potential analytical tool for the assay of disulfide bond reduction

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Abstract

We describe the synthesis of fluorescent derivatives of bovine pancreas insulin and its use as substrates of disulfide bond reduction in a spectrofluorometric assay. Amino groups of insulin were chemically modified with fluorescein isothiocyanate and proteins bearing one, two and three fluorescent groups were purified by ion-exchange chromatography. Upon incubation with dithiothreitol, di- and tri-fluoresceinthiocarbamyl-insulin evinced the highest and the lowest enhancement of fluorescence emission, whereas the mono-substituted protein had intermediate enhancement. Using di-fluoresceinthiocarbamyl-insulin, the reliability of this novel feature for the estimation of disulfide bond cleavage was assessed by (i) the separation of two fluorescent bands using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, (ii) the linear response of the fluorescence signal within a range from 0.04 to 1 μM , and (iii) the correlation of the rate of fluorescence enhancement with concentrations of dithiothreitol ranging from 0.1 to 5 mM. Moreover, di-fluoresceinthiocarbamyl-insulin was a sensitive oxidant when the catalytic capacity of thioredoxin and protein disulfide isomerase was analyzed in the presence of dithiothreitol or glutathione, as reductants. On this basis, di-fluoresceinthiocarbamyl-insulin constitutes an analytical tool to test the capacity of biochemical preparations in the reduction of disulfide bonds. © 1997 Elsevier Science Ireland Ltd.

Keywords: Disulfide bonds; Fluorescein; Insulin; Fluorometric assay; Thioredoxin; Protein disulfide isomerase

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1. Introduction

Protein disulfide oxido-reductases modulate the function of target proteins by catalyzing the change of sulfur atoms between the reduced (sulfhydryl groups) and the oxidized (disulfide bond) state [1,2]. The widely used assay for these enzymes relies on the precipitation of the B chain of insulin that takes place after the reduction of intercatenary disulfide bonds [3]. Given that this procedure requires high concentrations of insulin and signals become detectable after a prolonged lag phase, the estimation of radioactively labeled polypeptides of insulin by high-performance liquid chromatography (HPLC) [4] as well as radioimmunological and enzymatic methods improved the determination of the protein disulfide oxidoreductases [5]. However, radiometric assays are difficult to apply to a screening program because they require the hazardous separation of reaction products from unreacted substrates. Therefore, we searched for alternative substrates that overcome this disadvantage in the assay of protein disulfide oxidoreductase.

In previous studies, the fluoresceinthiocarbamyl (FTC) group was bound to amino groups of insulin for analyzing the effect on biological and immunological activities [6,7]. We reasoned that these derivatives might be used for the quantification of the reductive process if the cleavage of intercatenary cystines modifies the intensity of the fluorescence emission. Results reported herein describe the preparation of FTC-insulin and novel fluorescent features elicited by the reduction of disulfide bonds.

2. Materials and methods

2.1. Materials

Bovine pancreas insulin (henceforth insulin), fluorescein isothiocyanate isomer I (FITC), [-Cys(SO₃H)-]-A and -B chains of insulin and other biochemicals were purchased from Sigma (St. Louis, MO, USA). The concentrations of dithiothreitol (DTT) solutions were determined with the Ellman's reagent using a molar absorptivity of 14,150 mM⁻¹ cm⁻¹ for 2-nitro-5-thiobenzoate [8]. Procedures for obtaining homogeneous preparations of *Escherichia coli* thioredoxin and rapeseed protein disulfide isomerase were reported previously [9] and will be published elsewhere (A.P.H. in preparation), respectively. Glass distilled water was used throughout.

2.2. Preparation of derivatized insulins with FITC

To remove Zn²⁺, the insulin solution (10 mg/ml) was dialyzed exhaustively at 4°C against 50 mM NaHCO₃ (pH 9.2) and 1 mM ethylene diaminetetraacetic acid (EDTA). Solid FITC (2.8 mg) was dissolved in 1 ml of the dialyzate (insulin–FITC molar ratio, 1:4) and the solution was incubated, with stirring, at 28°C. As described in Section 3.1, the reaction time was adjusted to obtain a given proportion of derivatized insulins. The reaction was stopped by separating proteins from unreacted FITC on a Sephadex G-25 column (48 × 1 cm) equilibrated beforehand with 30 mM NaHCO₃ buffer (pH 9.2).

After pooling the orange-colored fractions that eluted in the void volume, proteins were (i) precipitated by addition of acetic acid (final pH of 4.0), (ii) washed with distilled water and, (iii) dissolved in 1 ml of 50 mM NaHCO₃ buffer (pH 9.2).

Solid urea was added to the sample (final concentration, 6 M) and the solution was loaded on a DEAE–cellulose column (15 × 0.7 cm). Elution was performed with 20 ml of 10 mM Tris–HCl buffer (pH 7.6), 6 M urea and 0.1 M NaCl, followed by 90 ml of a linear gradient of NaCl (0.1 to 0.8 M) in the same buffer. The presence of derivatized insulins in the effluent was monitored by diluting 0.01 ml of each fraction in 3.0 ml of 50 mM NaHCO₃ buffer (pH 9.2) and measuring the emission fluorescence at 519 nm (excitation wavelength, 495 nm).

2.3. Synthesis of di-FTC-insulin

A 3-mg amount of FITC was dissolved in 3 ml of NaHCO₃ buffer (pH 9.0) containing 21 mg of insulin (insulin–FITC molar ratio, 1:2) and the mixture was incubated for 10 h at 28°C. After loading the solution on a Sephadex G-25 column (48 × 1 cm), colored fractions that eluted in the void volume with 20 mM NaHCO₃ buffer (pH 9.0) were pooled and dialyzed for 16 h against 30 mM Tris–HCl buffer (pH 7.6) and 0.1 M NaCl. Solid urea was added to the dialyzate (final concentration, 6 M) and the solution (28 ml) was loaded on a QAE-Sephadex column (10 × 1 cm) that was washed successively with 40 ml of 0.1 M NaCl, 80 ml of a linear gradient of NaCl (0.1 to 0.8 M), and 15 ml of 1 M NaCl, all in 10 mM Tris–HCl buffer (pH 7.6) containing 6 M urea. The largest colored fraction that eluted with 0.55 M NaCl was pooled and dialyzed for 16 h against 10 mM Tris–HCl buffer (pH 7.6). Solid urea was dissolved in the dialyzate (final concentration, 6 M) and the solution was rechromatographed on a similar QAE-Sephadex column. Fractions containing di-FTC-insulin were dialyzed for 16 h against distilled water, concentrated by lyophilization to ca. 25 ml and buffered with 0.2 ml of 1 M Tris–HCl buffer (pH 7.6). The solution of di-FTC-insulin (41 μM) was stored at –15°C in aliquots of 1 ml.

2.4. HPLC analysis of di-FTC-insulin

Di-FTC-insulin (71 μg in 0.1 ml) was filtered through a 0.2-μm filter (Millipore, Bedford, MA, USA), diluted with an equal volume of 0.1% (v/v) trifluoroacetic acid in acetonitrile and injected into a 5-μm pore size column (250 × 4 mm) (Superpaq Spherisorb ODS2, Pharmacia, Uppsala) equilibrated beforehand with 0.1% (v/v) of trifluoroacetic acid in water–acetonitrile (50:50, v/v). Using an LKB apparatus with a gradient programmer, the elution of di-FTC-insulin was performed by washing for 3 min with the initial solvent and raising the concentration of acetonitrile from 50% (v/v) to 100% (v/v) in 60 min (flow rate, 1 ml min⁻¹). Separations were monitored on-column at 280 nm.

For the characterization of products originated from the reduction of di-FTC-insulin, oxidized (13.5 μg in 0.2 ml) and DTT-reduced (36 μg in 0.2 ml) forms were loaded on a 3-μm pore size column (250 × 4.6 mm) (Selectosil C4 300 Å, Phenomenex, Torrance, CA, USA), equilibrated beforehand with 20% (v/v) acetonitrile in 0.1% (v/v) trifluoro-

acetic acid. The elution was performed by increasing the concentration of acetonitrile from 20 to 70% (v/v) over 20 min (flow rate, 1 ml min⁻¹), and the absorbance of the effluent was continuously monitored at 220 nm.

2.5. Reduction of FTC-insulin

The assay was performed at 25°C in polyacrylate spectrofluorometric cuvettes (1 × 1 cm) containing 50 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.07 μM FTC-insulin and 3 mM DTT, in a final volume of 3 ml. Fluorescence emission was periodically recorded using a Jasco FP770 spectrofluorometer with monochromators set at 495 nm (excitation) and 519 nm (emission); the slit on both monochromators was 5 nm.

2.6. Absorption spectra

Absorption spectra were measured with quartz cuvettes (light path, 1 cm) containing FTC-insulin dissolved in a solution of 1 M NaOH. Wavelengths were scanned from 600 to 240 nm using a Gilford Response II spectrophotometer (slit, 0.5 nm).

2.7. SDS-PAGE

SDS-PAGE was performed at room temperature with the modular system Mini-Protean II (Bio-Rad Laboratories, Richmond, CA, USA) according to Schagger and von Jagow [10]. Proteins (5 to 40 μg) were (i) dissolved in 15 μl of 50 mM Tris-HCl buffer (pH 8), 6 M urea, 1% (w/v) SDS, 0.025% (w/v) bromophenol blue, (ii) heated at 100°C for 5 min and (iii) loaded on the polyacrylamide gel (stacking: 4% T, 2.6% C; spacer: 12.8% T, 2.6% C; resolving: 16.5% T, 2.6% C, 10% glycerol). After electrophoresis at 10 watts, the gel was stained and destained in methanol-acetic acid-H₂O (4:1:6, v/v/v) containing and lacking, respectively, 0.125% (w/v) Coomassie Brilliant Blue R-25.

2.8. Protein determination

The protein concentration of derivatized insulins was routinely determined using the method of Lowry et al. [11], calibrated with insulin.

2.9. Determination of total amino acid composition and amino-terminal sequence of di-FTC-insulin

After acid hydrolysis and derivatization with phenylisothiocyanate, the total amino acid composition of di-FTC-insulin was determined on an Applied Biosystems amino acid analyzer (model 420). The analysis of di-FTC-insulin by the method of Edman was performed on an Applied Biosystems protein sequencer (model 477A) with an on-line analyzer for liberated phenylthiohydantoin derivatives. Both instruments were operated according to manufacturer's recommendations at LANAIS-PRO, University of Buenos Aires.

2.10. Mass spectrometry

The sample of di-FTC-insulin, dissolved in methanol, was applied with the matrix (*m*-nitrobenzyl alcohol) on the fast atom bombardment tip and, subsequently, fast atom bombardment ionization in combination with mass spectrometry was performed on a ZAB-SEQ mass spectrometer at LANAIS-EMAR, University of Buenos Aires.

3. Results

3.1. Purification and characterization of FTC-insulin

In the insulin molecule, Phe-1 and Lys-29 of the B chain and Gly-1 of the A chain are potential targets for derivatization with reagents specific for amino groups. This reaction with FITC introduces a negatively charged fluoresceinthiocarbamyl group in place of a positively charged amino group, whereby the number of substituents in the insulin molecule conditions the elution profile of these derivatives in ion-exchange chromatography. On this basis, 1.7 mM insulin was incubated at 28°C in the presence of 7 mM FITC and aliquots of the reaction mixture, taken at 1, 10 and 24 h, were loaded on a DEAE-cellulose column. As shown in Fig. 1, 0.1, 0.2 and 0.35 M NaCl desorbed three fluorescent species whose $\epsilon_{495\text{ nm}}$ were 60.5, 133.6 and 195.4 $\text{mM}^{-1}\text{ cm}^{-1}$, respectively. The reasonable proximity of the ratio of molar absorptivities (0.91:2.0:2.93) to theoretical values confirmed that mono-, di- and tri-FTC-insulin eluted orderly from the anion-exchange column [7]. Although the particular experiment depicted in Fig. 1 shows that the longer the reaction time the higher the yield of tri-FTC-insulin, the proportion of a particular derivatized insulin can be varied by adjusting the pH and the insulin-FITC ratio in the reaction medium.

3.2. Reduction of FTC-insulin with DTT

At this stage, information was available on the structure, the stability and the biological activity of FTC-insulin but was lacking on the fluorescence properties of products originated from the reduction of disulfide bonds [6,7]. Notably, the emission intensity of all derivatized insulins progressively increased upon incubation with 3 mM DTT (Fig. 2); nonetheless, di- and tri-FTC-insulin evinced the highest (70%) and the lowest (10%) stimulation, respectively, whereas mono-FTC-insulin had a value that was in between these values (40%). In these experiments, the rate with which fluorescence reached the final intensity increased 8-fold for mono-FTC-insulin and 17-fold for di-FTC-insulin when the concentration of DTT was raised from 0.1 to 10 mM. Moreover, we found that excitation and emission spectra of products generated by the action of DTT were similar in maxima, but not in intensity, to those of derivatized insulins. In this context, it remains uncertain whether encounters between pendant groups on the polypeptide chain or collisional quenching account for the low fluorescence enhancement of tri-FTC-insulin relative to mono- and di-FTC-insulin.

To implement the fluorescence enhancement as an assay for measuring the reduction

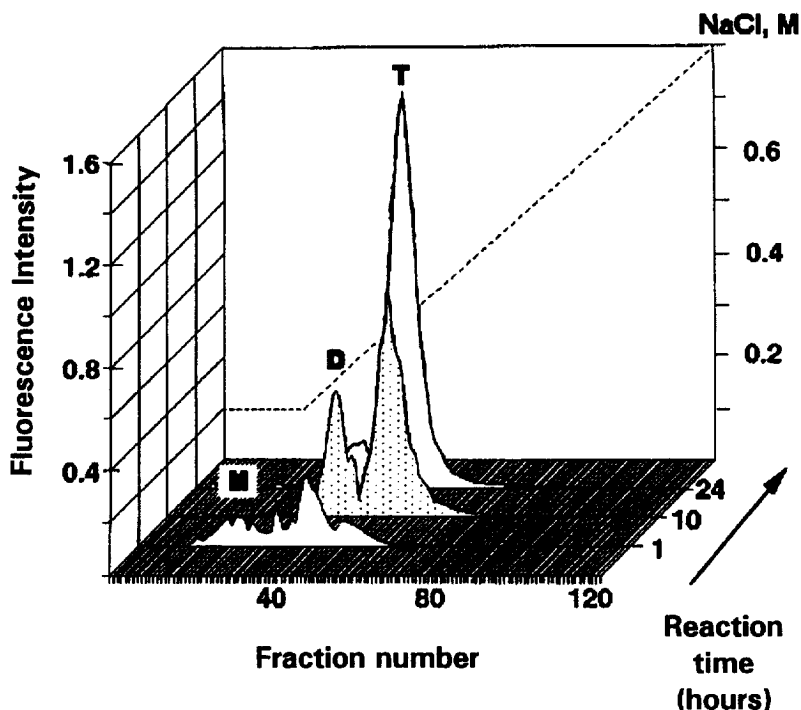


Fig. 1. Overlaid chromatograms of FTC-insulin. As described in Section 2, aliquots, withdrawn at indicated times from the solution containing insulin ($1.7 \mu\text{M}$) and FITC ($7 \mu\text{M}$), were chromatographed on columns of DEAE-cellulose. M, D, and T represent mono-, di- and tri-FTC-insulin, respectively.

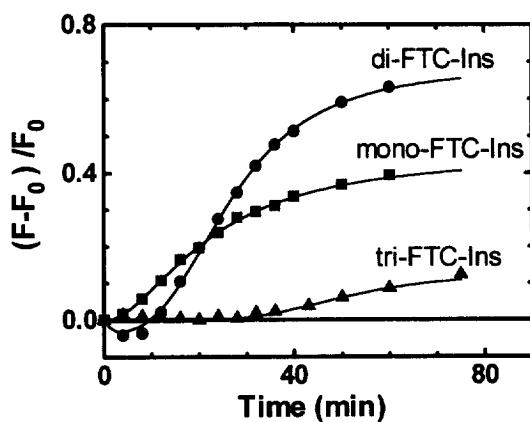


Fig. 2. Fluorescence enhancement of FTC-insulin elicited by DTT. The emission intensity at 519 nm (excitation wavelength, 495 nm) was determined in the presence of 3 mM DTT for $0.07 \mu\text{M}$ of each derivative.

of disulfide bonds requires that DTT cleaves FTC-insulin into the constituent A and B polypeptides. Congruent with this expectation, both the fluorescence emission and the elution volume of reaction products in columns of Sephadex G-25 were higher than those of non-reduced derivatives.

3.3. Purification and characterization of di-FTC-insulin

Di-FTC-insulin appeared to be suitable for routine estimations of disulfide bond cleavage because the incubation with DTT elicited the highest fluorescence enhancement. We then optimized the proportion of di-FTC-insulin in the derivatization reaction by adjusting (i) the pH to 9.0, (ii) the molar ratio of insulin-FITC to 1:2 and (iii) the reaction time to 10 h. Preparative chromatography on columns of QAE-Sephadex provided a major fraction that was judged to be 90% pure by analytical chromatography on a reversed-phase C₁₈ column (Fig. 3a). Further purification using an analogous HPLC column yielded a unique component (Fig. 3b) whose molecular mass measured by fast atom bombardment mass spectrometry was identical to the theoretical value of di-FTC-insulin (6512.3 Da).

Two complementary studies located unequivocally fluorescent labels at both N-termini of di-FTC-insulin. First, the total amino acid composition revealed one residue of glycine and phenylalanine per mole less than found in the insulin molecule, i.e. 2.7 vs. 4.1, and 1.8 vs. 3.3, respectively, whereas lysine residues remained unchanged. Second, isoleucine and valine were the first N-terminal amino acid residues of the A and B chains, respectively, in the Edman degradation. These results are congruent with a

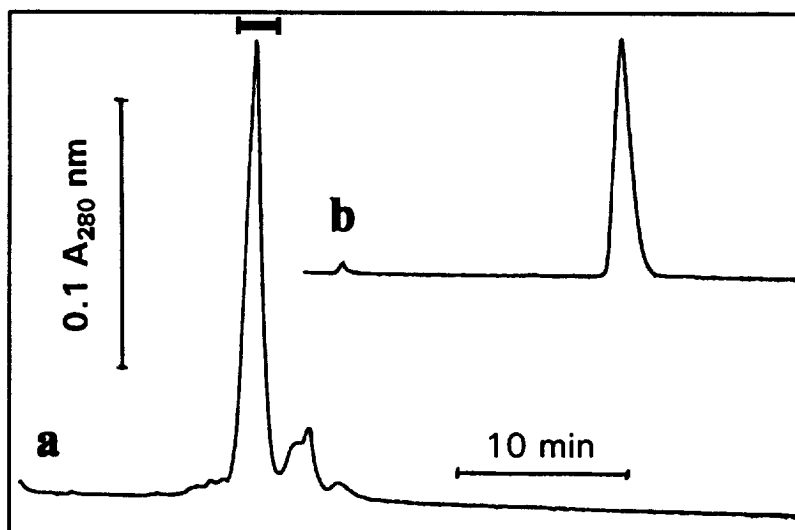


Fig. 3. Reversed-phase HPLC of di-FTC-insulin. After chromatography on columns of QAE-Sephadex, di-FTC-insulin was loaded on a Superpaq Spherisorb ODS-2 column (250 × 4 mm) and (a) eluted as described in Section 2. (b) The prominent fraction was rechromatographed on the same column.

di-FTC-insulin molecule containing fluorescein groups bound to the N-terminal glycine and phenylalanine of the A and B chains, respectively, because FTC-amino acids differ from phenylthiocarbamylated ones in the former analysis and are lost in the acid wash of the latter.

3.4. Analysis of di-FTC-insulin reduction by SDS-PAGE

The cleavage of intercatenary cystines of di-FTC-insulin (6512 Da) yields the constituent FTC-A (2729 Da) and FTC-B (3789 Da) polypeptides. To further assess the validity of di-FTC-insulin as a substrate for the reductive process, we incubated di-FTC-insulin in the presence of 10 mM DTT, stopped the reaction using 40 mM iodoacetamide, and subjected the solution to SDS-PAGE prior to (Fig. 4a) or after (Fig. 4b–c) the separation of reaction products by HPLC chromatography on a reversed-phase C_4 column. The appearance of two fluorescent species after incubation with DTT confirmed the reduction of intercatenary cystines but, surprisingly, the electrophoretic behaviour of the slow-moving band was not congruent with the expected molecular mass. The FTC-B chain, as well as the [-Cys(SO₃H)]-B polypeptide (not shown), migrated faster than di-FTC-insulin, whereas the respective A polypeptides not only moved slower than either di-FTC-insulin or insulin but also were not stained by Coomassie Brilliant Blue. Although we consistently observed the anomalous behaviour of the complex between SDS and the insulin A chain, underlying mechanisms for both lower electrophoretic mobility and resistance to staining remain unknown.

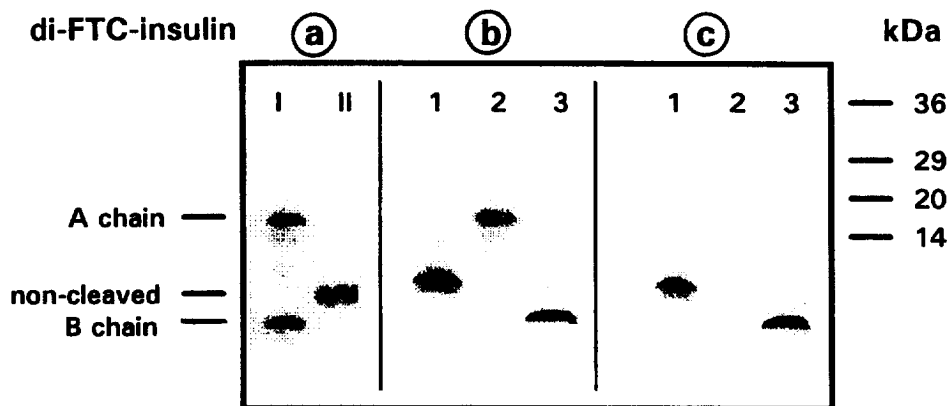


Fig. 4. SDS-PAGE of di-FTC-insulin and the products of its reduction. (a) Lane I, Di-FTC-insulin (11 μ g) was incubated with 10 mM DTT for 60 min and the reduction was stopped with 40 mM iodoacetamide. Lane II: di-FTC-insulin (8 μ g). (b) Di-FTC-insulin (13.5 μ g) and the products of its reduction (36 μ g) were purified by reversed-phase C_4 HPLC. Lane 1, di-FTC-insulin; lane 2, FTC-A chain; lane 3, FTC-B chain. Upon electrophoresis as described in Section 2, the gel was photographed under UV illumination using Polaroid film and the picture was digitized with a HP scanner. (c) After the picture (b) was taken, the gel was stained with Coomassie Brilliant Blue. The molecular masses of the calibrating proteins are indicated (kDa).

3.5. Reduction of di-FTC-insulin: Variation of fluorescence emission

At concentrations lower than 2 μM , the initial fluorescence emission (F_0) was linearly proportional to di-FTC-insulin; i.e. $F_0 = q_0 [\text{di-FTC-ins}]_0$, where q_0 is the proportionality constant for the oxidized substrate. Given that the typical standard curve for DTT-mediated enhancement of fluorescence emission was also linear with the concentration of the oxidized substrate (Fig. 5a), then $(F_f - F_0) = (q_f - q_0) [\text{di-FTC-ins}]_0$; where q_f and F_f are the proportionality constant and the final fluorescence emission, respectively, for reduced products. On this basis, it should be expected that the fluorescence enhancement relative to the basal emission is constant; i.e. $(F_f - F_0)/F_0 = (q_f - q_0)/q_0$. Data from experiments carried out at pH 7.5 with different preparations of di-FTC-insulin confirmed that $(F_f - F_0)/F_0$ was 0.85 ± 0.05 over a wide range of di-FTC-insulin concentrations (Fig. 5b). Moreover, the maximal value of the relative fluorescence enhancement varied from 1.05 at pH 6.2 to 0.85 at pH 8, even though such a change in the pH value increased the basal emission of di-FTC-insulin (F_0) 2.3-fold.

Most significantly, this feature opened a new alternative for measuring the rate of the reductive process. If 0 and 0.85 are the values of $(F - F_0)/F_0$ for the oxidized substrate and totally reduced products, respectively, then $(1/0.85) \cdot (F - F_0)/F_0 \cdot [\text{di-FTC-ins}]_0$ expresses the μmoles of di-FTC-insulin transformed. On this basis, an apparent rate of reduction could be determined from the positive slope of fluorescence enhancement vs. time because $(1/0.85) \cdot (F_2 - F_1)/F_0 \cdot [\text{di-FTC-ins}]_0$ represents the amount of di-FTC-insulin transformed in the period $t_2 - t_1$. Moreover, the advantage of data analysis with normalized values is the minimization of experimental errors when handling a large number of samples.

To analyze the suitability of di-FTC-insulin for the estimation of the rate of disulfide bond reduction, we first measured the temporal variation of the fluorescence enhancement after 0.7 μM di-FTC-insulin was treated with concentrations of DTT ranging from 0.1 to 5 mM (Fig. 6a) and, subsequently, estimated $(F_2 - F_1)/F_0$ from the positive slope

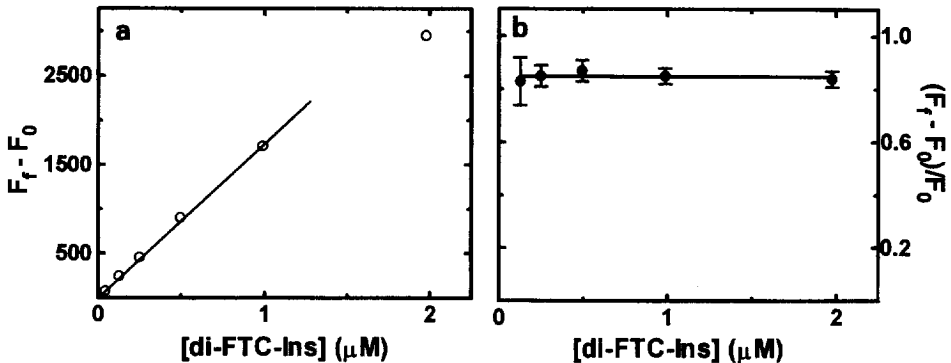


Fig. 5. Dependence of DTT-mediated fluorescence enhancement on the concentration of di-FTC-insulin. (a) After incubation for 20 min with 10 mM DTT, the emission intensity was recorded as described in Section 2. (b) Normalized data from several experiments.

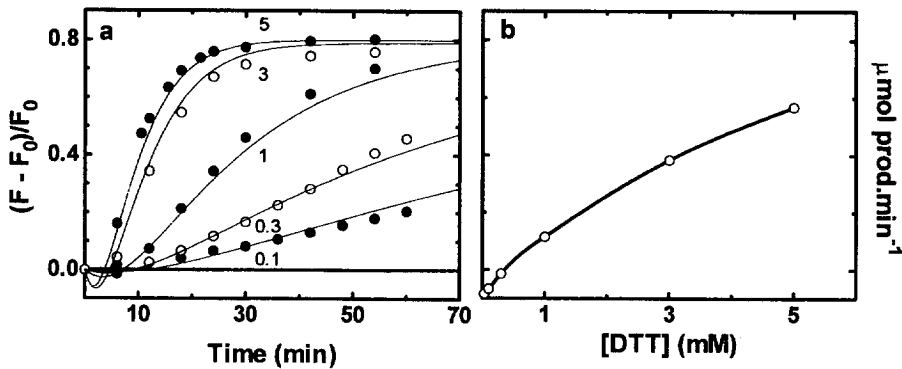


Fig. 6. Effect of DTT on fluorescence enhancement. (a) At the concentrations indicated (mM), DTT was added to 0.7 μM di-FTC-insulin and fluorescence emission was measured as described in Section 2. (b) Maximal slopes were determined for the curves shown in (a).

of the time course. As shown in Fig. 6b, the rate of di-FTC-insulin transformation measured by the novel assay was clearly proportional to the concentration of DTT. Although numerous attempts did not abolish the initial period, we found that increasing the concentration of the reductant or the pH of the reaction medium shortened the lag phase that preceded the increase in fluorescence enhancement. The mathematical description of the non-linear kinetics, indicative of intermediates that are less fluorescent than both the oxidized substrate and the reduced products, will be given elsewhere (A.P.H. submitted for publication).

3.6. Biochemical reduction of di-FTC-insulin

It is well established that the activity of protein disulfide oxidoreductases varies with the reductant. DTT reduces the unique disulfide bond of thioredoxin, whereas either DTT or reduced glutathione are effective in performing similar reactions in the active site of protein disulfide isomerase. Subsequently, these sulfhydryl groups participate in thiol/disulfide exchange with cystines of target proteins. Table 1 shows that changes in fluorescence intensity that are brought about by *Escherichia coli* thioredoxin and

Table 1
Protein disulfide oxidoreductase activity

Protein disulfide oxidoreductase	Reductant	
	DTT	Reduced glutathione
Thioredoxin	0.0196	0
Protein disulfide isomerase	0.0071	0.0109

In a 0.2-ml quartz microcuvette, *Escherichia coli* thioredoxin (0.1 μM) and rapeseed protein disulfide isomerase (1.9 μM) were incubated in 25 mM Tris-HCl buffer (pH 7.9) containing 0.5 mM EDTA, 0.35 μM di-FTC-insulin and, as indicated, 0.1 mM DTT or 5 mM reduced glutathione (final volume, 0.16 ml). The reaction was started by the addition of the reductant. Activities are expressed as μmol of product formed min^{-1} .

rapeseed protein disulfide isomerase in the presence of DTT and reduced glutathione. As expected, thioredoxin was highly efficient with the former reductant but ineffective with the latter, whereas rapeseed protein disulfide isomerase was functional with both substrates. In this context, it is important to recognize that fluorescence emissions of the oxidized substrate and reduced products were equally affected by the presence of biological macromolecules and changes in the ionic strength and pH. Nevertheless, handling data as $(F_f - F_o)/F_o$ circumvented this drawback and made the present assay effective for measuring the activity during the purification of protein disulfide oxidoreductase originated from *Escherichia coli* cells and germinated rapeseed seeds (not shown).

4. Discussion

Derivatization of primary amines with the highly fluorescent reagent FITC produces proteins with absorption and emission in the visible region, high quantum yields and resistance to photobleaching [12]. Initially, the probe was bound to insulin for analyzing the effects of such substitutions on biological and immunological activities [6,7]. Nonetheless, these derivatives appeared adequate for the estimation of disulfide bond reduction when Saito et al. [13] found that the incubation of mono-FITC-insulin with the couple glutathione/glutathione–insulin transhydrogenase decreases the proportion of the substrate that elutes from a HPLC column. The fluorescence enhancement elicited by DTT constitutes a one-stage continuous assay that avoids cumbersome separation techniques. However, before using this novel estimation of disulfide bond reduction, the following questions had to be answered:

1. Do reductants specifically cleave the derivative in the constituent polypeptides?
2. Does the spectrofluorometric assay respond to different amounts of FITC-insulin?
3. Is the derivative sensitive to the concentration of the reductant?

Affirmative answers provided by results reported herein clearly indicated that FITC-insulin is appropriate for a precise and sensitive assay of the reduction of disulfide bonds. At this stage, confronted with three potential substrates, we took into account the stability and the highest fluorescence enhancement for adopting di-FITC-insulin as the substrate in subsequent experiments.

Most often, the extreme sensitivity circumscribes fluorescence measurements to precise analytical studies because many errors intersperse in the assay during general laboratory practice. To circumvent this drawback, we chose the relative fluorescence enhancement, $(F - F_o)/F_o$, instead of the absolute value, $(F - F_o)$, because the invariability of the former over a wide range of concentrations minimizes experimental errors when handling a large number of conditions. Furthermore, this approach was extremely useful for experiments in which the basal fluorescence emission of di-FITC-insulin varies greatly in response to the composition of the reaction medium (e.g. different pH values).

We found that the temporal variation of $(F - F_o)/F_o$ can be easily adopted for

measuring the rate of the reductive process up to a 2- μ M concentration of di-FTC-insulin and a 5-mM concentration of DTT. But more important, di-FTC-insulin is an extremely sensitive oxidant for the measurement of reactions not only catalyzed by different protein disulfide oxidoreductases but also involving alternate reductants. Two features make the fluorometric estimation of protein disulfide oxidoreductase activity highly attractive; the sensitivity and the time of assay are higher and shorter, respectively, than with turbidimetry. The only disadvantage of the present system is the non-linear kinetics, which require that the estimation of velocity should be made after overcoming a lag period. Certainly, the complete reduction of three disulfide bonds in di-FTC-insulin can be strictly achieved by six different pathways. Whether the reduction catalyzed by protein disulfide oxidoreductases proceeds through a preferential pathway is not known at present. To circumvent this complexity, we have recently analyzed kinetic data according to a two-step reaction pathway in which quantum yields of putative intermediates are lower than those of both the initial oxidized substrate and the final reduced products (A.P.H. submitted for publication).

5. Simplified description of the method and its (future) applications

This report describes a simple and rapid method for obtaining homogeneous preparations of FTC-insulin. While, on the one hand, present results uncover the enhancement of fluorescence emission associated with the reduction of disulfide bonds, the analytical tool that combines the sensitivity of fluorescence spectroscopy with the extremely low concentration of di-FTC-insulin appears to be generally useful for studying the reductive process catalyzed by protein disulfide oxidoreductases.

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