Biochemical and Biophysical Research Communications **261,** 41–45 (1999)
Article ID bbrc.1999.0908, available online at http://www.idealibrary.com on IDEAL®

Retinol, a Probe of Conformational Changes in Protein Disulfide Isomerase

Jorge E. Churchich
Department of ABCT, Hong Kong Polytechnic University, Hong Kong

Received May 24, 1999

Nanosecond and steady fluorescence techniques have been employed to study the interaction of retinol with protein disulfide isomerase (PDI). Retinol binds tightly to PDI; and the rotational correlation time (θ = 36 ns) corresponds to a monomeric subunit of 55 kDa. The enzyme does not undergo aggregation in the presence of low molecular weight peptides. Under denaturing conditions; presence of 0.75 M Gnd HCl, the fluorescence yield of bound retinol is enhanced, suggesting stronger interactions of exposed hydrophobic groups of the protein with retinol. Based on far UV CD and fluorescence measurements of the protein in the presence of Gnd HCl, it is proposed the existence of molten globule intermediates during the unfolding of PDI.

Protein disulfide isomerase (PDI) catalyses disulfide bond formation and isomerization within the endoplasmic reticulum (1, 2, 3). It acts also as a subunit of prolyl-4-hydroxylase (4) and triacylglycerol transfer protein (5) in mammalian cells. The multifunctional protein of 55 kDa displays chaperone activity *in vitro* and *in vivo* (6). It has been reported that the interaction between PDI and insulin is influenced by certain oestrogens (7); hence the existence of an oestrogen-receptor like domain in the isomerase has been proposed (8). More recently it has been shown that 17-β-oestradiol specifically inhibits the binding of some peptides to PDI by interfering with its peptide binding site (9). However other hydrophobic molecules recognize PDI, but they do not elicit any inhibition of either glutathione-dependent reduction of insulin or APTase activity. *trans*-Retinol belongs to the class of hydrophobic molecules which bind tightly to PDI without interfering with the catalytic properties. It is the main purpose of this work to report the interaction of retinol with PDI and to demonstrate that the bound ligand can be used as a probe of conformational changes of the enzyme. Nanosecond fluorescence spectroscopy is used to assess the state of aggregation of PDI in the absence and presence of low molecular weight peptides, whereas steady fluorescence spectroscopy is applied to the detection of native-like intermediates of the enzyme (molten globule) during the denaturation induced by guanidine chloride (Gnd HCl).

Abbreviations used: protein disulfide isomerase, PDI; guanidinium chloride, Gnd HCl.

EXPERIMENTAL PROCEDURES

Protein disulfide isomerase (PDI) was purified by the method described in reference (10) with small modifications. Fresh porcine livers (600 g) were homogenized in 0.1 M sodium phosphate (pH 7.5) containing 1% Triton X-100 and 5 mM EDTA. After centrifugation, the supernatant was treated with ammonium sulphate and the fractions obtained between 55–85% saturation were suspended in 25 mM citrate buffer (pH 5.3), dialysed against the same buffer (buffer A), applied to CM-Sephadex C-50 column and eluted with the same buffer. Fractions displaying PDI activity were pooled, dialysed against 20 mM sodium phosphate (pH 6.3) (buffer B) and applied to a DEAE-Sepharose fast flow column which was eluted using a linear gradient of 0-0.7 M NaCl in buffer B.

The concentration of PDI was determined using absorbance at 280 nm = 1 for 1 mg protein/ml. The activity of PDI was determined using the insulin reduction assay as described in reference (2). The purified enzyme was kept at 4°C.

Denaturation of PDI. For denaturation experiments, each sample was prepared by diluting 0.2 ml of protein stock solution to 2 ml with Gnd HCl containing buffer. The buffer was 10 mM Tris/HCl (pH 7.6). Fluorescence and circular dichroism (CD) measurments were carried out at 25°C after 1 h incubation in the presence of Gnd HCl. This time of incubation was sufficient to achieve equilibrium.

Spectroscopy. CD spectra were recorded on a Jasco (J-40) spectropolarimeter using a 0.2 cm cell. Protein concentration ranged from 0.035 to 0.1 mg/ml, and spectral data were acquired over the range 250-195 nm.

Emission spectra were recorded in a Perkin-Elmer LS-50B spectrofluorimeter. The excitation and emission slits were set at 2.5 nm. Emission anisotropy measurements were performed in a modified SLM polarization apparatus. Polarized fluorescence was measured with a precision of $\pm$0.005.

Fluorescence decay measurements were made using the monophotonic technique on an Ortec Model 3200 nanosecond spectrometer. The excitation band was set at 340 nm, and the emission was filtered through a glass filter (Corning C-S-3-70). Fluorescence decay measurements were corrected for the finite duration of the exciting light pulse using the method of Demas and Adamson (11). Nanosecond emission anisotropy was determined from the fluorescence decay

0006-291X/99 $30.00

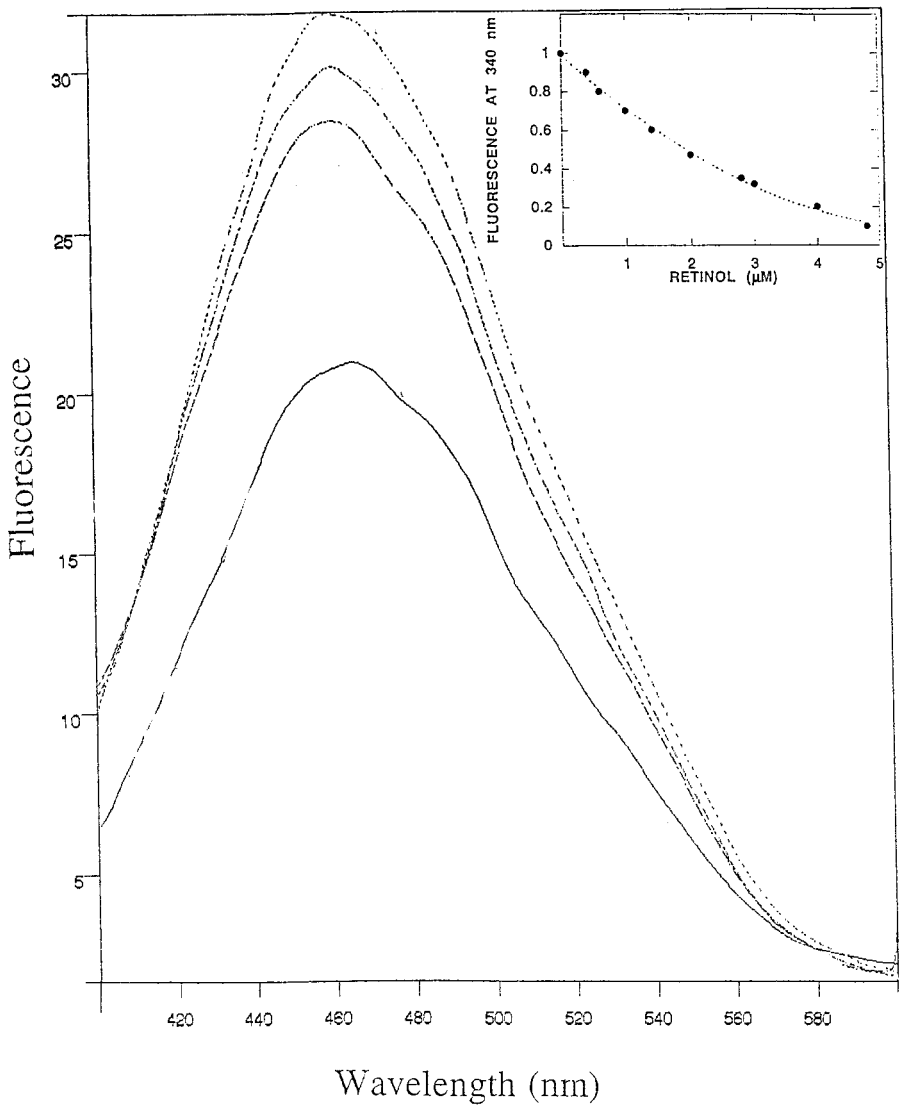

FIG. 1. Fluorescence characteristics of *trans*-retinol in the absence and presence of PDI at pH 7.6 in Tris/HCl buffer. Excitation wavelength, 340 nm; excitation and emission slits, 2.5 nm. From bottom to top; emission spectra of free retinol (10 μM); emission spectra of PDI (4 μM) + retinol (10 μM) in the absence and presence of 1 and 0.75 M Gnd HCl. Inset: titration of PDI (2 μM) with retinol. Quenching of protein fluorescence was monitored at 340 nm upon addition of increasing concentrations of retinol. Excitation 295 nm.

curves of the polarized components Fv(t) and Fh(t), parallel and perpendicular, respectively, to the plane of the incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission. The exciting light was set at 340 nm and the emission was passed through a combination of a glass filter and an interference cut-off filter. Transmission was observed at wavelengths longer than 460 nm. The emission anisotropy is an explicit function of time (12) given by the following relationship: A(t) = D(t)/S(t) (Equation [1]). For a rigid spherical macromolecule, the emission anisotropy is described by a single exponential function.

Materials. Porcine livers were obtained from a local slaughter house. DEAE-Sephacel, DEAE-Sepharose, CM-cellulose and Sephadex-G-25 were obtained from Pharmacia Biotechnology. Insulin, GSH, NADPH, *trans*-retinol and the proteins bovine serum albumin and β-lactoglobulin were purchased from Sigma. The polypeptide mastoparan (ILE-ASN-LEU-LYS-ALA-LEU-ALA-ALA-LEU-ALA-LYS-LYS-ILE-LEU) was obtained from Sigma.

RESULTS AND DISCUSSION

Nanosecond Spectroscopy

The binding of all trans retinol to PDI was assessed by fluorescence spectroscopy by measuring either the emission of bound retinol excited at 340 nm or the quenching of protein fluorescence excited at 295 nm. A typical emission spectra of retinol in the absence and presence of PDI is shown in Fig. 1. The fluorescence enhancement detected over the spectral region 400-500 nm is comparable to that observed when retinol is allowed to interact with either bovine serum albumin or β-lactoglobulin at a mixing molar ratio of 1 mol of ligand per mol of protein (13). Binding of retinol to PDI

TABLE 1

Spectroscopic Properties of Bound Retinol

Sample	Solvent	Decay time (ns)	Anisotropy	Rot. correlation time (ns)
Retinol	Glycerol	2.3	0.33	
Retinol + PDI	Tris/HCl (pH 7.6)	6.20	0.29	36
Retinol + PDI	Tris/HCl (pH 7.6)	6.10	0.30	40

also elicits quenching of protein fluorescence as indicated by the titration results included in Fig. 1, inset. Under this set of experimental conditions, the stoichiometry of binding approaches a value of 1 mol of retionol/per mol of PDI (55 kDa). Since retinol is immobilized by PDI, as indicated by the emission anisotropy value of 0.29 (Table 1), it is possible to use the hydrophobic molecule in time-dependent emission anisotropy studies designed to investigate the rotational dynamics of PDI.

The rotational correlation time of (θ) of a macromolecule is proportional to the volume of the rotating unit (V) at fixed viscosity (η) and the temperature of the solution according to Equation [2].

$$\theta = \frac{\eta V}{kT}. \qquad [2]$$

If PDI undergoes a process of reversible aggregation in solution induced by changes in protein concentration, then the rotational correlation time would increase as the volume of the rotating unit is increased. For time dependent emission anisotropy measurements retinol bound to PDI was repetitively excited by nanosecond light pulses; and the components of the fluorescence intensities Fv(t) and Fh(t) were recorded and analyzed as indicated in "Experimental Procedures." The function S(t) is characterized by the fluorescence lifetime τ_S whereas the function D(t) is characterized by the lifetime τ_D if both lifetimes decay in a monoexponential manner, then the rotational correlation time is given by Equation [3].

$$\theta = \frac{\tau_S \tau_D}{\tau_S - \tau_D}. \qquad [3]$$

The results of a typical time-dependent emission anistropy experiment experiment conducted with retinol-PDI is given in Fig. 2, where it can be seen that S(t) and D(t) decay in a monoexponential manner with fluorescence lifetimes τ_S = 6.20 ns and τ_D = 5.30 ns, respectively. Substituting these values in equation 3, one obtains a rotational correlation time of 36 ns for PDI. The measured rotational correlation time is comparable with the value expected for an hydrated particle of 55 kDa which deviates from spherical shape. Under identical experimental conditions, a rotational correlation time of 40 ns was obtained for bovine serum albumin (Table 1). The effect of the polypeptide mastoparan of the state of aggregation of PDI was also investigated by time-dependent emission anisotropy. When the concentration of the polypeptide (0.1 mM) was above the concentration required to saturate the protein (9), the rotational correlation remained practically constant, suggesting that the polypeptide does

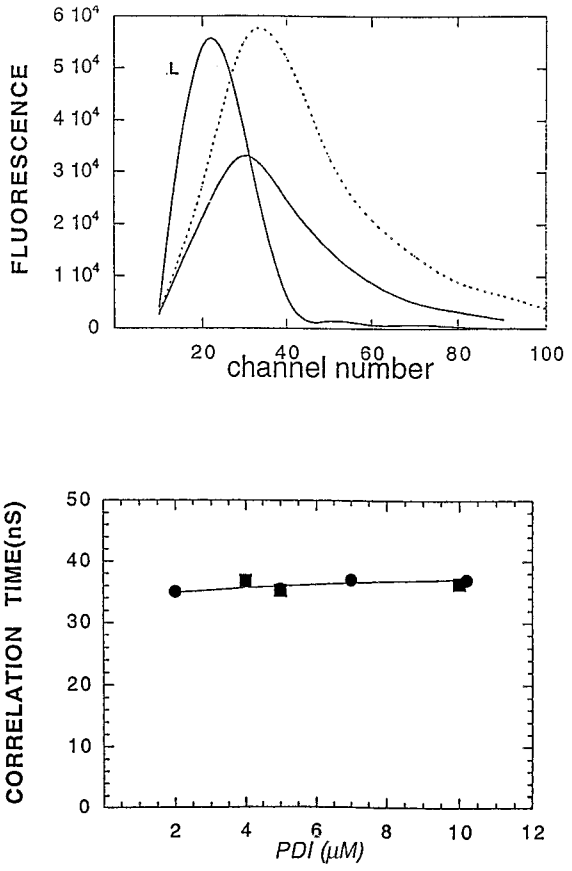

FIG. 2. Time dependent emission anisotropy measurements. Decays of the functions S(t) (---) and D(t) (—) of retinol (5 μM) bound to PDI (5 μM) excited at 340 nm. The curve for the excitation flash is included in the figure (L). Time scale is 0.578 ns/channel. Inset changes in rotational correlation time (θ) in the absence (●) and presence (■) of the polypeptide mastoparan. The buffer is 0.1 M Tris/HCl (pH 7.6).

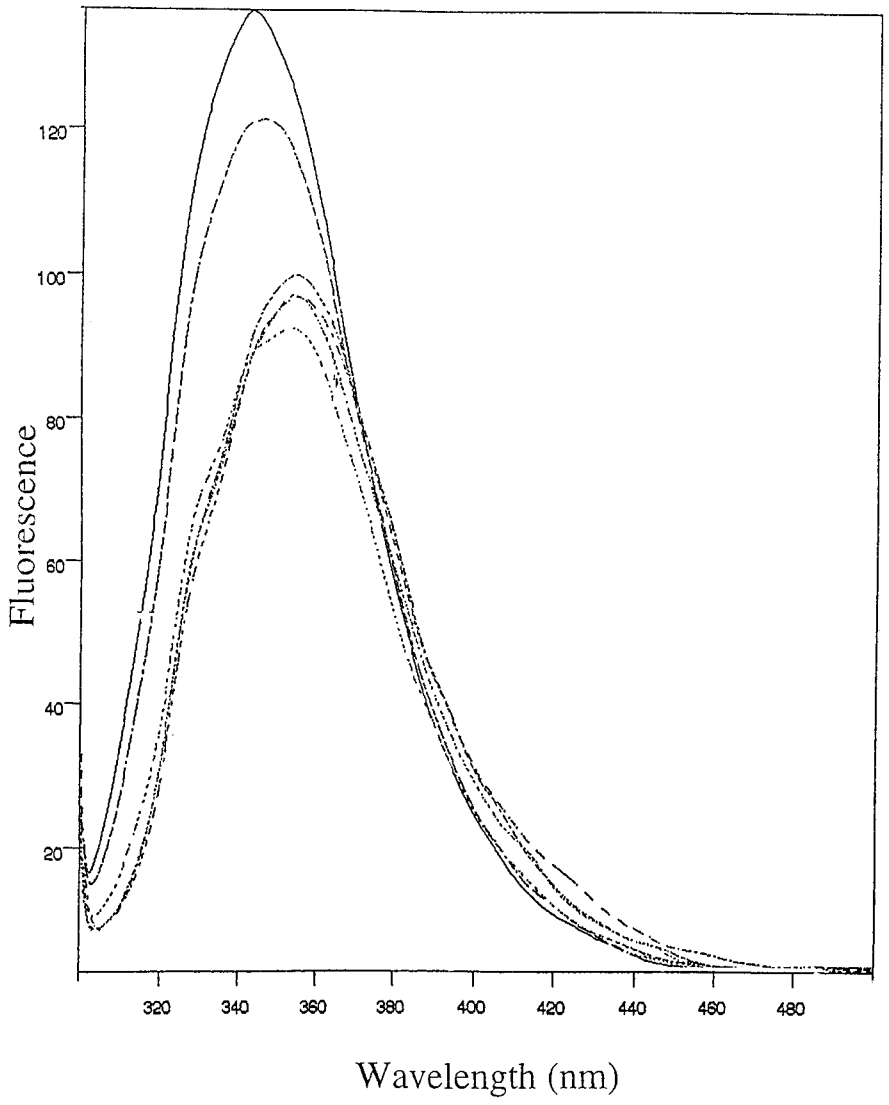

FIG. 3. Emission spectra of PDI in the presence of increasing concentrations of Gnd HCl in 10 mM Tris/HCl (pH 7.6). From top to bottom; emission spectra of PDI (2 μM) in the presence of 0, 0.8, 1, 6, 3, 4, and 5 M Gnd HCl. Excitation wavelength, 295 nm; slits for excitation and emission 2, 5 nm.

not stimulate dimerization or the formation of high molecular weight aggregates.

Native-like Intermediates during the Folding of PDI

The effect of guanidinium chloride (Gnd HCl) on the conformation of PDI was investigated by steady-state fluorescence. PDI contains several tryptophanyl groups distributed among distinct domains; and upon excitation at 295 nm shows a structureless emission band centered at 340 nm. Addition of increasing concentrations of Gnd HCl brings about a decrease in the fluorescence intensity together with a progressive red shift in the band position of the emission spectra (Fig. 3). Over the Gnd HCl concentration range of 0.2-1 M, the maximum of emission is shifted from 340 to 346 nm, whereas above 1.2 M, the tryptophanyl residues displayed an emission maximum at 350 nm, indicating that most of the tryptophanyl groups have been exposed to the solvent. Further increase in Gnd HCl concentration to the 5 M level resulted in small decrease in the fluorescence yield. When the unfolding of the protein was studied by CD spectroscopy, it was found that the ellipticity at 222 nm, -10200 $dgcm^2dmol^{-1}$, remained practically invariant upon changing the concentration of denaturant from 0.2 to 1.2 M, in agreement with results published in the literature (14).

A dramatic decrease in ellipticity takes place at concentrations of Gnd HCl above 1.2 M (19), and the ellipticity reaches a value of -1000 $degcm^2dmol^{-1}$ in

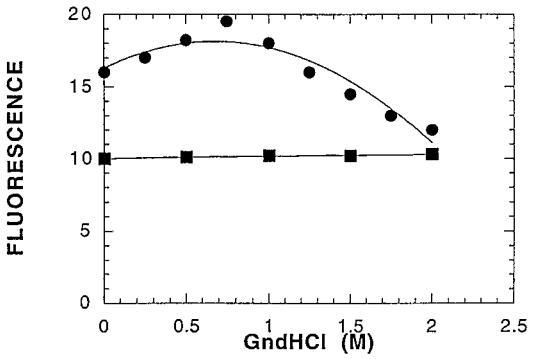

FIG. 4. Fluorescence intensity of retinol bound to PDI as a function of Gnd HCl concentration (●). The fluorescence intensities were recorded at 470 nm upon excitation at 340 nm. Retinol concentration (5 μM), PDI 5 μM. The buffer is 0.1 M Tris/HCl (pH 7.6). Included is the effect of Gnd HCl on the fluorescence emitted by free retinol (■).

the presence of 5 M Gnd HCl. The results of the fluorescence measurements, taken together with the CD results, indicate that tryptophanyl groups become exposed to the relaxing solvent at low concentrations of Gnd HCl when the secondary structure of the protein remains practically invariant.

When the unfolding of PDI in the presence of equimolar amounts of retinol was investigated by measuring the emission of the hydrophobic chromophore excited at 340 nm, it was found that increasing concentrations of Gnd HCl from 0.2 to 0.75 M caused the fluorescence emitted by retinol to increase in the manner represented in Fig. 4. Above 0.75 M, the fluorescence undergoes a gradual decrease until it reaches the level of free retinol in 2 M Gnd HCl. These results are interpreted to mean that native-like conformations of PDI (molten globule) exist during the earlier stages of the unfolding process. These results are different from those reported by Morjana *et al.* (14), who have postulated the existence of partially folded conformations of PDI at guanidinium chloride concentrations ranging from 3 to 4 M at pH 7.5.

In conclusion, retinol (all-*trans*) can be used as a probe of conformational changes of PDI in solution. The state of aggregation, i.e., the dynamic interactions of monomeric subunits at neutral pH, is easily monitored by rotational correlation time measurements. The same experimental approach could be used to study the association of PDI with denatured protein substrates.

Under denaturation conditions leading to exposure of hydrophobic amino acids of the proteis, the fluorescence emitted by bound retinol allows the identification of molten globule intermediates. It remains to be investigated whether retinol has any effect on the chaperone function of PDI under *in vitro* and *in vivo* conditions.

ACKNOWLEDGMENTS

I thank Miss Belinda Cheung for her help in the purification of PDI. The support given by Professor Albert S. Chan is also appreciated.

REFERENCES

1. Goldberg, R. F., Epstein, C. J., and Anfinsen, C. B. (1963) *J. Biol. Chem.* **238,** 628–635
2. Gilbert, H. F. (1998) *Methods Enzymol.* **290,** 26–50.
3. Ferrari, D. M., and Soling, H. D. (1999) *Biochem. J.* **339,** 1–10
4. Pihiajaniemi, T., Hellakoski, T., Tasenen, K., Myllyla, R., Huhtala, M. L., Koivu, J., and Kivirikko, K. I. (1987).
5. Wetterau, J. R., Combs, K. A., Spinner, S. N., and Joiner, B. J. (1980) *J. Biol. Chem.* **265,** 9800–9807.
6. Song, J. L., and Wang, C. C. (1995) *Eur. J. Biochem.* **231,** 312–316.
7. Tsibris, J. C., Hunt, L. T., Ballego, G., Barker, W. C., Toney, L. J., and Spellacy, W. N. (1989) *J. Biol. Chem.* **264,** 13967–13970.
8. Yamauchi, K., Yamamoto, T., Hayashi, H., Koya, S., Takikawa, H., Toyoshima, K., and Horiuchi, R. (1987) *Biochem. Biophys. Res. Commun.* **146,** 1485–1492.
9. Klappa, P., Stomer, T., Zimmerman, R., Ruddock, L. W., and Freedman, R. B. (1998) *Eur. J. Biochem.* **254,** 63–69.
10. Hillson, D. A., Lambert, N., and Freedman, R. B. (1994) *Methods Enzymol.* **107,** 281–294.
11. Demas, J. N., and Adamson, A. W. (1971) *J. Phys. Chem.* **75,** 2463–2467.
12. Jablonski, A. (1960) *Bull. Acad. Polon. Sci.* **8,** 259–264.
13. Georghiou, S., and Churchich, J. E. (1975) *J. Biol. Chem.* **250,** 1149–1151.
14. Morjana, N. A., McKeone, B. J., and Gilbert, H. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2107–2111.