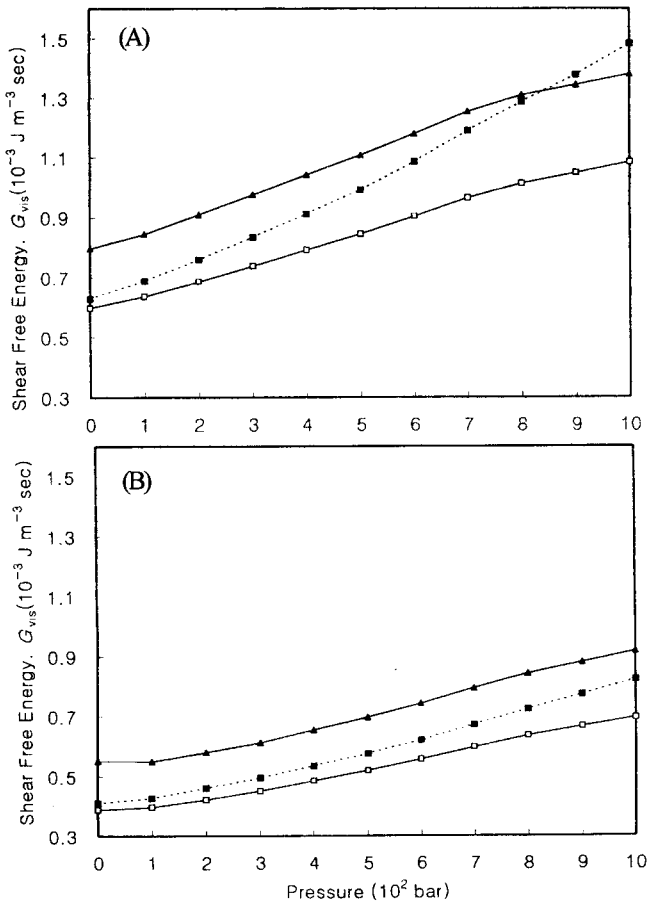

Figure 1. The Shear Free Energies of Fluids as a Function of Pressure at 20 °C (A) and 60 °C (B): – □ – Toluene, ···■··· Benezene, –▲– Chlorobenzene.

As the pressure is raised, the density of a fluid is increased and the intermolecular distance is decreased. Therefore, the free volume of the fluid is diminished and molecules get more tightly packed at high pressure.[6] This molecular event manifests as the increment in the shear free energy. The free volume reduction rate of benzene is greater than that of substituted benzenes as the pressure is raised. The slopes of the curves in Figure 1 indicate the trend. At low temperature, the free volume reduction, *i.e.*, molecular packing against the viscous flow, is more effectively enhanced for benzene as compared to substituted benzenes. At high temperature, however, increased thermal motion acts against molecular packing. Therefore, favorable packing of benzene does not result in excessive shear free energy over that of chlorobenzene upto 1,000 bar at 60 °C.

We can quantitatively understand the molecular interaction potential and molecular kinetic energies by examining the viscosity, a simple bulk property, at the molecular level. A detailed evaluation of molecular interactions can be performed through molecular simulations. A set of molecular dynamics simulations and complementary experiments with a diversity of substituted benzenes under extended pressure and temperature conditions are in progress.

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Resonance Energy Transfer in the Equilibrated Unfolding States of Human Tumor Necrosis Factor-α

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The protein folding study has attracted wide attentions in recent years as one of the most puzzling problems in the respect of the intermediates involved in the kinetic and equilibrium folding pathways.[1] The human Tumor Necrosis Factor-α (hTNF-α), a cytokine which has potent roles in a wide range of cell regulatory, immune and inflammatory functions,[2] is ideal for folding studies because of its interesting trimeric native structure and β-sheet property.[3] For these reasons, hTNF-α has been extensively investigated with regard to its structure, thermodynamics and kinetics of folding and unfolding.[4]

Resonance energy transfer is widely used in studies of

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biomolecular structures and dynamics.[5,6] It provides information about the interatomic distances on the order of 10-100 Å, and thus it is suitable for the investigation of spatial relationship in proteins.[5,6] For this purpose, we have investigated the equilibrium unfolding of wild type hTNF-α during denaturation at different guanidine hydrochloride (GdnHCl) concentrations (0-4.2 M) with Förster resonance energy transfer by the steady-state fluorescence spectroscopy.

Energy transfer rate, k_T, by dipolar interaction is described by the Förster rate equation as follows,[5,6]

$$k_T = 1/\tau_d\,(R_0/r)^6 \qquad (1)$$

and the critical transfer distance R_0 can be expressed by the following equation.[5,6]

$$R_0 = 9.79 \times 10^3 (\chi^2 n^{-4} J\ \Phi_d)^{1/6} \quad \text{(in Å)} \qquad (2)$$

where τ_d is the donor lifetime in the absence of acceptor, r is the donor-acceptor distance, and R_0 is the Förster critical distance at which the energy transfer rate is equal to the decay rate. The Förster distance is related to the orientation factor, χ^2, between donor and acceptor, and the spectroscopic properties of the donor and the acceptor. Φ_d is the quantum yield of the donor in the absence of the acceptor. The index of refraction (n) is generally taken to be 1.4 in aqueous solution.[5,6] J is the overlap integral between the donor and acceptor,[5,6]

$$J = \int \varepsilon_a(\lambda)\, F_d(\lambda)\, \lambda^4\, d\lambda \qquad (3)$$

where $F_d(\lambda)$ is the peak-normalized fluorescence spectrum of the donor. $\varepsilon_a(\lambda)$ is the molar absorption coefficient of the acceptor.

The efficiency of energy transfer between donor-acceptor pair is another important physical quantity which is defined as e=1-(I_{da}/I_d)=1-(τ_{da}/τ_d), where I_{da} and I_d are donor's fluorescence intensities in the presence and the absence of the acceptor, respectively. e becomes one half when r is R_0 and r may be obtained from R_0 and e using the following expression which is derived by substituting Eq. (1) into above equation of the transfer efficiency.[5,6]

$$r = R_0\,(e^{-1}-1)^{1/6} \quad \text{(in Å)} \qquad (4)$$

The hTNF-α contains the intrinsic fluorescence probe of two tryptophan residues and seven tyrosine residues per monomer, therefore tryptophans were used as acceptor and tyrosines as donor in this measurement. That is, we could use the phenomena that the absorption spectra of the tryptophan residues extensively overlap with the fluorescence emission of the tyrosine residues resulting in possible resonance energy transfer between excited tyrosines and tryptophans in this protein. In applying Förster energy transfer theory to this protein, however, there is a problem that we cannot spectroscopically separate the donor-acceptor pair. In other words, since tryptophan absorbs light at any wavelength where tyrosine absorbs, tyrosine as a donor cannot be excited selectively. To circumvent these difficulties, the method suggested by Josef Eisinger was used in order to evaluate the parameters of Förster resonance energy transfer.[7] The core of this method lies in the procedure of extracting the fluorescence spectrum of the only Tyr residues by subtracting the factored fluorescence spectrum of the Trp residues excited at 295 nm from the fluorescence spectrum of the Tyr and the Trp residues simultaneously excited at 280 nm. In this procedure, the former excited at 295 nm was factored to be equal to the red edge of the latter excited at 280 nm. This procedure is reasonable because the fluorescence spectrum of the tyrosine residues contributes little to the red edge of the fluorescence spectrum excited at 280 nm. This corrected spectrum was used to obtain the fluorescence quantum yield of the Tyr residues in the absence of the Trp residues indirectly from Eq. (5).

$$\frac{A_{tyr}}{A_{trp}} = \frac{f_{tyr}(280\text{ nm})(1-e_{tyr}\,\Phi_{tyr})}{[f_{trp}(280\text{ nm})+e_{tyr}\,f_{tyr}(280\text{ nm})]\,\Phi_{trp}} \qquad (5)$$

A_{Tyr} and A_{Trp} are the areas of the deconvoluted fluorescence spectra of the Tyr and the Trp residues excited at 280 nm, respectively. In Eq. (5), energy transfer efficiency(e_{Tyr}) was obtained from Eq. (6).

$$\Phi_{TNF-\alpha} = \Phi_{Trp}\,[f_{trp}(\lambda)+e_{tyr}\,f_{trp}(\lambda)] \qquad (6)$$

$\Phi_{TNF-\alpha}$ and Φ_{Trp} which are fluorescence quantum yields of TNF-α and the tryptophan residues in hTNF-α, respectively, are wavelength-independent as obtained experimentally. In addition, to determine the fluorescence quantum yield of each residue in this protein, we have utilized the tryptophan as a reference compound of known quantum yield (Φ=0.14 at 25 °C) by the optically dilute method.[8]

In order to ensure the equilibrium had been reached, protein solutions were incubated for 24 hours at ambient temperature in 50 mM sodium phosphate buffer (pH 7.6) containing the desired concentration (0-4.2 M) of GdnHCl. The protein concentrations were about 0.125 mg/mL (2.40 μM as trimer) for the steady-state fluorescence spectroscopy. The wild type hTNF-α was supplied by Hanhyo Institute of Technology and all the chemicals used in the experiments were of analytical grade.

The steady-state fluorescence spectra excited at 280 nm and at 295 nm were obtained using a fluorescence spectrophotometer (Hitachi F-4500). (data not shown) Some of the light was absorbed by the tyrosine excited at 280 nm, whereas, at 295 nm, tryptophan is the only absorbing chromophore. The excitation and emission bandwidths were both 10 nm. Spectra were collected at ambient temperature.

Analysis of the fluorescence spectra showed that, during 0 M-0.9 M [GdnHCl], there were only changes in fluorescence intensities without spectral shift at 328 nm and, above 1.2 M [GdnHCl], the spectra were gradually red-shifted to the spectral maximum of 350 nm at 4.2 M [GdnHCl] while the intensities decreased to 40% of that for the native state.[10] Since the spectroscopic properties of fluorophores such as spectral shape or maximum peak may change in different environments,[5] these results raised the possibility of the environmental change near the aromatic residues above 1.2 M GdnHCl concentration solution.

Table 1 presents the result of calculations for Förster energy transfer parameters between the tyrosine residues and the tryptophan residues in the hTNF-α at the different GdnHCl concentrations including the native state. The determined average distances between two kinds of residues were approximately 8-10 Å in various GdnHCl concentration solutions. These distance values of the narrow

Table 1. Parameters for the Förster resonance energy transfer in hTNF-α at the different GdnHCl concentrations

[GdnHCl]	Φ_{trp}[a] (280 nm)	Φ_{trp}[a] (295 nm)	Φ_{tyr}[a] (280 nm)	e[b]	J[c] $\times 10^{-16}$ (cm^3 M^{-1})	R_0[d] (Å)	r[e] (Å)
0 M	0.031	0.038	0.022	0.533	3.724	9.9	9.7
0.6 M	0.050	0.062	0.053	0.496	2.308	10.6	10.6
0.9 M	0.031	0.040	0.035	0.463	1.924	10.3	10.5
1.2 M	0.031	0.044	0.020	0.228	1.447	8.3	10.2
1.5 M	0.031	0.042	0.028	0.341	2.471	9.6	10.8
1.8 M	0.046	0.061	0.038	0.375	1.343	9.2	10.0
2.1 M	0.057	0.063	0.063	0.633	1.706	10.4	9.5
4.2 M	0.039	0.046	0.038	0.598	1.559	9.4	8.8

[a] $\Phi_x(y)$ is the fluorescence quantum yield of x residue excited at y wavelength. [b] e is the efficiency of energy transfer from the tyrosine residues to the tryptophan residues. [c] J is the spectral overlap integral between the tyrosine fluorescence and the tryptophan absorption. [d] R_0 is the Förster distance; χ^2 was taken to be two-thirds and n=1.4. [e] r is the determined average distance between the tyrosine residues and the tryptophan residues.

range which are insensitive to GdnHCl concentrations can result from the intensity-averaging nature of steady-state fluorescence technique which originates from the distribution of the various donor-acceptor pairs along or between the polypeptide chains.

In the native state, however, this average distance between the donor-acceptor pair was significantly smaller than that obtained from the X-ray crystallographic data by about 10 Å.[3] Therefore, it is plausible to say that the resonance energy transfer between two kinds of fluorophores which are distributed in this solvated protein is not the only effective pathway in the fluorescence quenching processes. According to the previous reports including model studies, tyrosine fluorescence can be absent or minimal in proteins for a variety of reasons such as energy transfer, quenching by nearby groups and besides, peptide quenching has a major influence on the fluorescence lifetime in proteins.[5,9] Thus, it is reasonable to suggest that the quenching of the tyrosine fluorescence in hTNF-α was due to not only energy transfer to the tryptophan residues but also quenching by nearby groups on the peptide chain. In addition, we may consider the determined average distances about 8-10 Å as the effective distances at which energy transfer can practically occur.

Figure 1 shows the curves of the calculated energy transfer efficiencies (e) and Förster critical distances (R_0) at the various GdnHCl concentration solutions. Although the variance of the determined average distances (r) were not articulate, however, on the basis of our results, we could deduce the structural variation near the donor-acceptor pairs using these two parameters (e, R_0). From overall respects, both parameters showed the abrupt decrease at about 1.2 M GdnHCl concentration and increased again up to 2.1 M GdnHCl. As mentioned before, since these two parameters are related to the energy transfer efficiency, we could say that the dramatic effect of disturbing the energy transfer between the pairs was found at about 1.2 M GdnHCl concentration solution. Therefore it is plausible to consider that this phenomenon is reflecting the phase transition of internal structure in hTNF-α affected by the different solvent

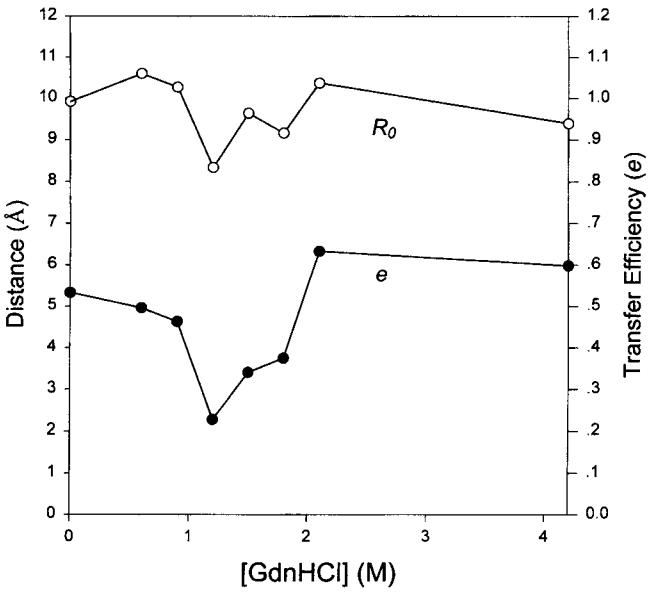

Figure 1. The variations of both energy transfer efficiency (e) and Förster critical distance (R_0) depending on the GdnHCl concentration.

environment. In addition, these observations are also consistent with the previous results for the existence of the partially folded structure showing the internal mobility from 1.2 M GdnHCl concentration which was investigated by the iodide quenching experiments and the time-resolved fluorescence anisotropy study.[10] Furthermore we can consider that the variable values of the orientation factor (χ^2) affected by the relative motion of the donor-acceptor pair may change transfer efficiency.

As a rule the three dimensional structure of a protein is fully determined by its amino acid sequence and the solvent environments containing both local next-neighbor (short-range) interactions between the groups closed in the primary structure and nonlocal through-space (long-range) interactions determining the tertiary structure.[1] Although our data do not provide all the detailed equilibrium unfolding behaviour in hTNF-α, we can suggest that the abrupt decrease of the energy transfer efficiency at about 1.2 M GdnHCl presents the possibility of the beginning region of internal structure change in the spatial relationship to the secondary and the tertiary structure induced by the variance of both the short-range and the long-range interaction. Particularly, it is worthwhile to note that such Förster resonance energy transfer study of protein dynamics can show the possibility of understanding about the internal structure changes as well as the usual distance information between the donor-acceptor pairs.

Futhermore, the mutated hTNF-α which includes the phenylalanine in the position of the tryptophan (W114) is being investigated for the unfolding and refolding dynamics with the fluorescence kinetic study.

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Insoluble Polymer Catalysts for Photooxidation of Amine

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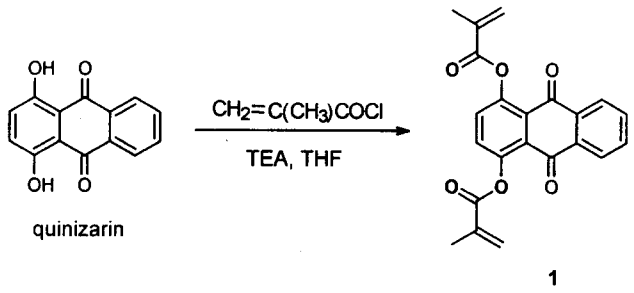
Recently, organic reactions on polymer supports have gained great attention as tools for generating diverse compounds.[1-5] A key strength of the solid phase organic synthesis is the relatively easy work-up and purification steps: simple filtration allows separation of reagents, starting materials, and solvents from the desired products. We have been interested in designing efficient and inexpensive polymer catalysts which participate in photoinduced electron transfer process. We have, especially, focused on developing cross-linked polymer systems which are insoluble in common organic solvents so that we can easily remove the polymer catalysts by filtering. As preliminary results, we now report the preparation of cross-linked polymers having anthraquinone group and their application to photoinduced oxidation of benzylamine.

The anthraquinone-containing dimethacrylated quinizarin monomer QDMA **1** used in this study was prepared in a high yield from commercially available quinizarin by treatment with methacryloyl chloride (Scheme 1). The presence of methacryloyl moieties in the monomer **1** was evidenced by the appearance of ^{1}H NMR resonances for the vinyl protons at 5.89 and 6.42 ppm. The pale-yellow solid, QDMA **1** (70%, mp 110-112 °C) was stable over months at room temperature: IR (KBr) ν(cm^{-1}) 2978 (aliphatic C-H), 1745, 1676 (C=O), 1322, 1132 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.18 (s, 6H, $2CH_3$), 5.89 (s, 1H, C=CH_2), 6.42 (s, 1H, C=CH_2), 7.48 (s, 2H, Ar-H), 7.25 (q, 2H, Ar-H), 8.16 (q, 2H, Ar-H).

Polymerization of the monomer **1** in the presence of *N,N'*-azobisisobutyronitrile (AIBN) in 1,4-dioxane for 12h at 60 °C provided the cross-linked polymer $\mathbf{P_1}$ in 75% yield. The monomer **1** was also copolymerized with an equivalent molar ratio of methyl methacrylate (MMA) to give polymer $\mathbf{P_2}$ in 91% yield (Scheme 2). The cross-linked polymer $\mathbf{P_1}$ and $\mathbf{P_2}$ were insoluble in organic solvents. IR (KBr) ν (cm^{-1}): polymer $\mathbf{P_1}$, 2929 (aliphatic C-H), 1743, 1677 (C=O), 1321, 1116 (C-O); polymer P_2, 2953 (aliphatic C-H), 1738, 1679 (C=O), 1321, 1113 (C-O).



Scheme 1

The polymers were ground in a crucible and sieved through 150-μm sieve. The powders which passed the sieve were collected and further ground using a ball-mill machine. The resulting solid polymers were subjected to extensive extraction with hot MeCN in a soxhlet apparatus to remove unreacted monomers, oligomers and soluble impurities. The average size of the particle obtained was *ca.* 10 μm.

In order to investigate photochemical reactions of the po-