The Strong Fluorescence-Quenching Effect of Ergothioneine

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Ergothioneine (Erg) which possesses an imidazole-2-thione (IT) moiety had a strong quenching effect on indole fluorescence. The Stern–Volmer quenching constants (K_{sv}), which quantified its fluorescence-quenching ability were 1508 (Erg) and 517 (IT) with respect to indole at pH 5.0. Compared with the structure of IT, the K_{sv} values of ethylenethiourea (ET), which lacks a double bond between the 4 and 5 position, imidazole (IM) which lacks a sulfur atom, and mercaptoethylamine (MEA) which exhibits no thione–thiol tautomerizm were 71, 14, and 18, respectively under the same instrumental conditions. According to these results, the fluorescence-quenching effect of Erg was about 20—100 times stronger than related compounds such as ET, IM, and MEA. This physico-chemical property may help clarify the biological role of Erg.

Key words ergothioneine; fluorescence-quenching; imidazole-2-thione

Ergothioneine (Erg), a betaine of thiolhistidine possessing a imidazole-2-thione (IT) moiety, is a unique biological compound (Fig. 1). It is synthesized by a wide variety of fungi[1] and is assimilated by higher plants[2,3] and many animals including human,[4] although the biological function of Erg has not been clarified. Hartman pointed out that one primary function of Erg, both in its native form and in organisms, is its action as an interceptor of H_2O_2, some radical species, and toxic electrophilic organic molecules.[5] Heath and Toennis have postulated that it may act as a proton donor and acceptor.[6]

We have already investigated the physico-chemical properties of Erg including the thermodynamic constants during proton dissociation and its interaction with Hg^{2+}.[7,8]

In this report, we have observed that Erg has a strong quenching effect on indole (Ind) fluorescence. Fluorescence quenching reactions have been applied to study highly ordered fluorophores in biological macromolecules such as proteins.[9]

An important class of proteins containing tryptophan (Trp) residues, which have an Ind moiety in their side-chain.

Materials and Methods

Chemicals Erg and *N*-methylimidazole-2-thione (MIT) were obtained from Sigma Chemical Company. IT was prepared by the method of Stanovik, mp 225 °C (lit. 227—228 °C),[10] while 4(5)-aminoethylimidazole-2-thione (AIT) was prepared by the method of Fraser, mp 242—243 °C (lit. 244—245 °C).[11] Other compounds with quenching ability were obtained from Nakalai Tesque Co., Ltd. Trp was recrystallized from water and Ind was sublimated. All other chemicals were of special reagent grade.

Method Quenching measurements at constant pH were made on 5—7 solutions of a given material containing increasing amounts of quenchers. These were prepared by dilution of a stock solution of the fluorescent compound, quencher, KCl and buffer. KCl was used to maintain the ionic strength. Fluorescence measurements were made with a Shimadzu Corrected Recording Spectrofluorophotometer RF-540. The fluorescence spectra and their intensities were measured by exciting at 280 nm. All experiments were carried out at room temperature (22 ± 1 °C).

Results and Discussion

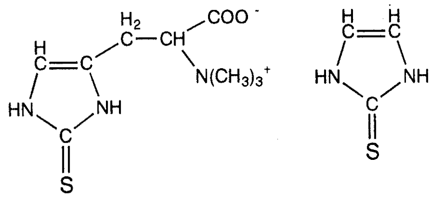
The fluorescence intensities of Ind and Trp decreased with increasing concentration of various IT derivatives and related compounds. The quenching phenomena were found to obey the Stern–Volmer quenching relationship as follows:

$$F_0/F = 1 + K_{sv}\ [Q]$$

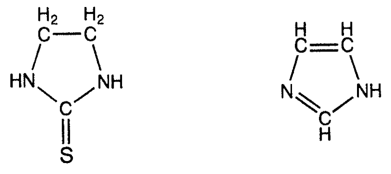
where F_0 and F are the fluorescence intensities in the absence and presence of the quencher. K_{sv} is the Stern–Volmer quenching constant.[12]

The changes in the fluorescence spectra of Ind at different concentrations of Erg are shown in Fig. 2. The relationship between F_0/F and concentration is also shown in Fig. 3 for other IT derivatives. The K_{sv} values of the IT derivatives and related compounds are listed in Table 1. Typical SH compounds such as mercaptoethanol ($HSCH_2CH_2OH$, ME) and mercaptoethylamine ($HSCH_2CH_2NH_2$, MEA) had weak fluorescence-quenching characteristics and their K_{sv} values were 5 and 20, respectively, for Trp at pH 5.0.

Histamine (HM) and imidazole (IM) which lack a sulfur atom at the 2-position of IT had a weak quenching effect at pH 5.0 (the K_{sv} values of HM and IM were 18 and 14, respectively, for Ind at pH 5.0). This phenomenon is explained by the fact that the quenching effect of the IM moiety is exhibited by formation of a charge transfer complex between protonated IM and Ind.[13] However,


Ergothioneine (Erg)

Imidazole-2-thione (IT)


Ethylenethiourea (ET)

Imidazole (IM)

Fig. 1. Structures of Erg and Related Compounds

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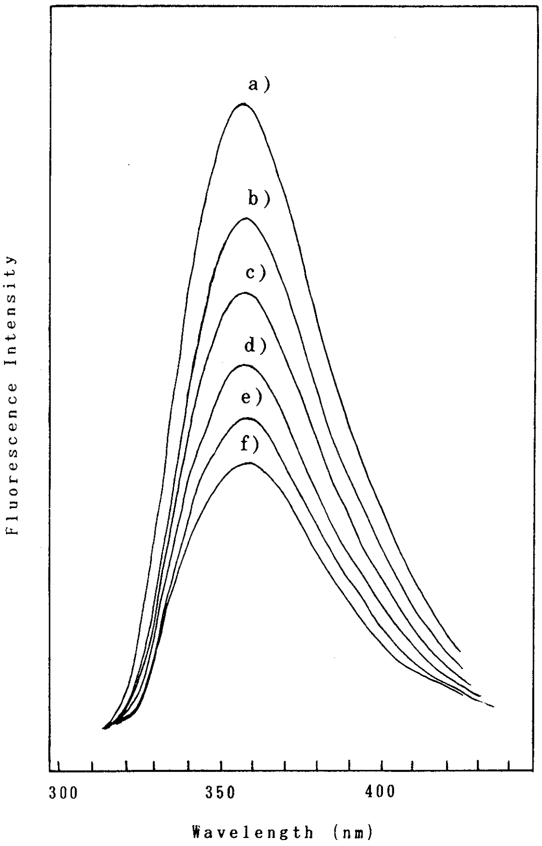

Fig. 2. The Quenching of Ind Fluorescence by Erg

The concentration of Ind was 10 μM and the pH value of the medium was 5.0. Ionic strength kept constant at 0.2 by addition of KCl. The excitation wavelength was 280 nm. The concentrations of Erg were a) 0 M, b) 200 μM, c) 400 μM, d) 600 μM, e) 800 μM, f) 1000 μM.

these quenching phenomena were not observed at pH 7.0. This suggests that the formation of a charge transfer complex was incomplete, because approximately 50% of the IM protons are dissociated at this pH (pK_a value of IM is 7.12).[14] According to these results, IM and aliphatic SH groups do not contribute to the strong fluorescence-quenching observed.

On the other hand, IT exhibited a strong quenching effect and its K_{sv} value was 457 on Trp at pH 5.0. Erg and MIT, which possess side-chains at the 4(5) position of IT, had a more potent quenching effect and their K_{sv} values were 1441 and 1319 on Trp at pH 5.0, respectively. Ethylenethiourea (ET), which lacks a double bond between the 4 and 5 positions of IT, had a moderate quenching effect (its K_{sv} value was 52 on Trp at pH 5.0).

In aqueous solution, Erg exist predominantly as the thione rather than the thiol, and the thiolate ion is present under basic conditions.[15,16] The standard redox potential of Erg at pH 7.0 is -0.06 V as opposed to -0.20 to -0.32 V for widely occurring natural thiols.[15] Because of its redox potential and propensity to tautomerize to the thione,[5] the redox reaction is suggested to be of a different type compared with natural thiols such as glutathione, and Erg shares the properties of representative thiols and thiones. This structural feature may contribute to the strong fluorescence-quenching phenomenon. As a whole, the quenching effect of Erg was 20—100 times stronger than related compounds.

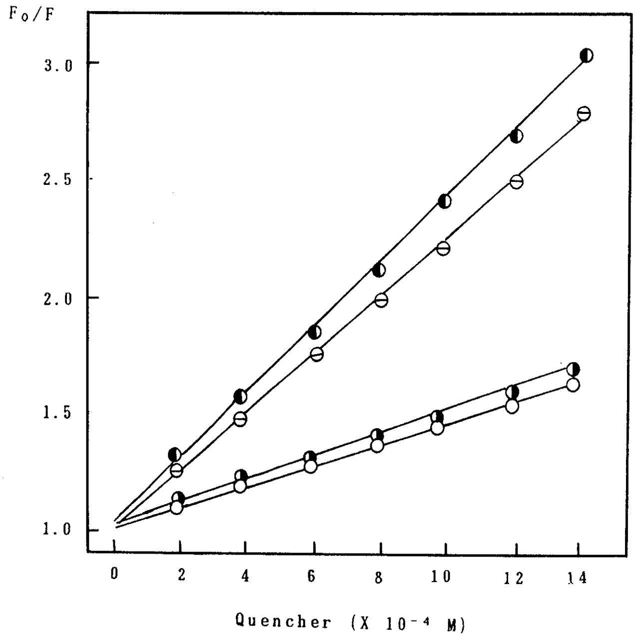

Fig. 3. Stern–Volmer Plots of the Quenching of Ind Fluorescence by Erg and Its Derivatives

The concentration of Ind was 10 μM and the pH value of the medium was 5.0. Ionic strength kept constant at 0.2 by addition of KCl. The excitation wavelength was 280 nm and the fluorescence intensities were measured at 355 nm. ◐ Erg, ⊖ AIT, ◑ IT, ○ MIT.

Table 1. Stern–Volmer Constants of Erg and Related Compounds

Compound	Ind		Try
	pH 5.0	pH 7.0	pH 5.0
Erg	1508	1399	1441 1319
AIT	1424	1119	
IT	517	451	457 442
MIT	446	357	
ET	71	50	52
HM	18(17)[a]	[b]	8
IM	14(13)[a]	[b]	6
ME			5
MEA	18		20

The concentration of Ind or Try was 10 μM. The excitation wavelength was 280 nm. The fluorescence intensities of Ind and Try were measured at 355 and 358 nm, respectively. Ionic strength kept constant at 0.2 by addition of KCl. Temperature was 25±1 °C. *a*) Ref. 19. *b*) Indicates that quenching was not observed.

Several considerations about the mechanism of fluorescence-quenching by Erg are appropriated.

1) Collisional quenching (dynamic quenching) is known to be affected by the viscosity of the medium. This phenomenon becomes weaker with increasing viscosity of the medium. The quenching using IT derivatives at various concentrations of glycerin (%), indicates an opposite effect to Ind–I$^-$[17] and Ind–ME[18] where quenching is mainly due to a collisional quenching mechanism (Fig. 4). According to these results, the collisional quenching process does not seem likely to be involved in quenching by Erg.

2) Transfer of excitation energy from the fluorophore to the IT moiety *via* a coupled electronic oscillator also does

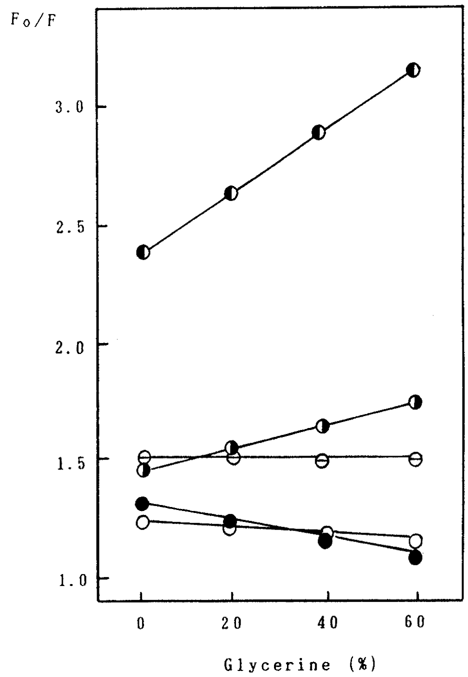

Fig. 4. Effect of Glycerin (%) on the Quenching of Ind Fluorescence by Various Compounds

The concentration of Ind was 10 μM and that of Erg or IT was 1 mM; the concentration of ET or KI was 10 mM and the concentration of MEA was 15 mM. The pH value of the medium was 5.0. The excitation wavelength was 280 nm and the fluorescence intensities were measured at 355 nm. ◐ Erg, ◑ IT, ⊖ ET, ○ MEA, ● KI.

not seen probable because of the lack of overlap of the electronic energy levels of Ind and IT groups (λ_{max} of IT is 260 nm, λ_{em} of Ind and Trp is 355 and 358 nm, respectively). The energy level of Ind or Trp emission (donor) was lower than that of IT absorption (acceptor).19)

The collisional quenching and energy transfer quenching mechanisms do not contribute to the fluorescence quenching of Ind by Erg, according to the considerations above.

Another significant mechanism associated with quenching is charge-transfer from excited Ind to quenchers. Electron acceptors such as fumarate acrylamide,20) other carbonyl compounds21) and thiourea20) have been found to be quenchers of fluorescence and quenching by these agents generally occurs primarily by interaction in the excited state. However, the quenching ability of these compounds is not so potent as that of Erg.

Following these considerations, the main mechanism of quenching by Erg remains unknown. Further investigations and more information are needed to explain this phenomena.

Erg appreciably decreases singlet oxygen generation by illuminated Rose Bengal solution, possibly by quenching the excited state of the dye molecules.22) In contrast, Erg fails to react significantly with singlet oxygen and superoxide in aqueous solutions.22) Thus, Erg might have a protective effect on the photochemical damage caused to physiologically important compounds following deactivation by photosensitizing substances.23)

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