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QUENCHING OF INTRINSIC TRYPTOPHAN FLUORESCENCE IN MEMBRANES OF RAT PITUITARY CELLS

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Summary

The GH_4C_1 strain of hormone-producing rat pituitary cells has specific receptors for the tripeptide thyrotropin-releasing hormone (TRH). Membranes prepared from GH_4C_1 cells show intrinsic tryptophan fluorescence which was quenched by low concentrations (10—100 nM) of TRH and N^{τ}-methyl TRH but not by biologically inactive analogs of TRH. Membranes from GH_4C_1 cells were subjected to thermal denaturation. A conformational transition was noted above 40°C and an irreversible denaturation was observed at 52°C. TRH-induced quenching of intrinsic fluorescence was lost completely in membranes previously incubated for 10 min at 30°C while loss of [^{3}H]-TRH binding was only about 20% at this temperature. Collisional quenching by iodide revealed that about 38% of the tryptophanyl residues in GH_4C_1 membranes were exposed to solvent. Quenching by TRH occurred with a shift in wavelength maximum from 336 to 342 nm suggesting that few of the tryptophanyl residues quenched by the tripeptide are totally exposed. Membranes prepared from cells preincubated with 20 nM TRH for 48 h, in which TRH receptors were decreased to 30% of control values, showed no quenching of tryptophan fluorescence in response to freshly added TRH. We conclude that the TRH-receptor interaction in GH_4C_1 cells is associated with a change in membrane conformation that can be measured by differential spectrofluorometry of intrinsic tryptophan fluorescence.

Introduction

GH cells are clonal strains of rat pituitary tumor cells that synthesize and secrete prolactin and growth hormone [1] and have specific receptors for the

hypothalamic tripeptide thyrotropin-releasing hormone (TRH) [2]. In these cells, TRH stimulates the release of prolactin [3], increases the rate of prolactin synthesis [4—8], and decreases growth hormone production [4—6].

A membrane fraction prepared from GH_3 cells exhibited intrinsic tryptophan fluorescence which was quenched specifically by TRH [9]. The change in fluorescence of GH membranes induced by TRH appeared to be mediated by TRH receptors [2,6,9]. The half-maximum change in fluorescence was shown to occur at a TRH concentration of 10 nM, while half-maximum binding of TRH occurs at 11 nM TRH [2,9]. TRH analogs which did not bind to GH cells did not alter membrane fluorescence. A pituitary GH cell strain, GH_12C_1, which lacks TRH receptors, did not change membrane fluorescence on incubation with TRH. From these results, we concluded that the TRH-receptor interaction is associated with a change in overall tryptophan fluorescence in GH_3 membranes.

In this paper, we extend our previous results to a second strain of GH cells, GH_4C_1, which also possesses TRH receptors. In addition, we have measured the effects of temperature on intrinsic tryptophan fluorescence, on TRH binding, and on TRH-induced quenching of fluorescence. Finally, we have examined the collisional quenching of tryptophan fluorescence by iodide in membranes from GH_4C_1 cells.

Materials and Methods

GH cells. Three GH cell strains were used for the experiments described in this report. GH_3 and GH_4C_1 cells show the same biological responses to TRH [1—6]. GH_12C_1 cells, which do not respond biologically to TRH, do not have TRH receptors [2]. Cells were cultured using methods described previously [10]. GH_3 and GH_4C_1 cells were grown in either monolayer [10] or suspension culture [11], and GH_12C_1 cells were grown only in monolayer. Cells were harvested from monolayer by scraping with a rubber policeman or from suspension by centrifugation. Cell membrane fractions were prepared as follows. Harvested intact cell pellets ($1—10 \cdot 10^7$ cells) were washed twice with 0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.5) and then suspended in 3—5 ml of ice-cold Tris-Mg^{2+} buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.6). After 5 min at 0° C, the cells were ruptured by 25 strokes in a Dounce homogenizer (pestle A) and centrifuged for 10 min at $4000 \times g$ [2,9]. The membrane pellet was resuspended in ice-cold Tris-Mg^{2+} buffer using a vortex mixer. The concentration of the membrane suspension was determined by absorbance at 280 nm measured in a 1 cm quartz cuvette with a Cary 14 recording spectrophotometer. The suspensions were diluted to a final A_{280nm} of approx. 0.06—0.10 and kept in an ice-water bath until use.

Measurement of fluorescence. 3 ml of cell suspension was placed in each of two quartz cuvettes. A small volume of a fresh stock solution of TRH or TRH analogs was added to one cuvette and an equal volume of solvent for the peptides was added to the other cuvette. In experiments to examine quenching by salts, 2 M KI or 2 M NaCl was used instead of TRH. The solvent for TRH was Tris-Mg^{2+} buffer or distilled water, and that for salts was distilled water. The cuvettes were then incubated in an ice-water bath for the times indicated.

They were inverted occasionally during incubation and immediately before scanning.

Fluorescence was measured in a recently designed and constructed differential fluorometer [13]. The baseline value for differential fluorescence was obtained with both cuvettes containing identical membrane suspensions. This curve was subtracted from all differential fluorescence measurements in order to obtain the true difference (F_{a-b}). The emission spectrum (F_a or F_b) for each membrane suspension was read against the buffer solution. The ratio of quenching of fluorescence (δF) at maximum wavelength was calculated as follows:

$$\delta F = \frac{F_{a-b}}{F_b} \tag{1}$$

where a and b are membrane suspensions with and without quenching agents, respectively.

A fresh sample of the stock membrane suspension was used for each measurement so that an aliquot once exposed to excitation by light was discarded. Fluorescence was measured within 2—3 h after membrane preparation, and TRH concentrations and incubation time were deliberately examined in a random order. Incubation of membranes with TRH was for 20 min, because an incubation time of approx. 20 min gave the maximum δF on the fluorescence of GH_3 membranes with TRH [9]. Almost all measurements were made in duplicate or triplicate and a mean of δF and its range is given in Results.

Instrumental conditions. Instrumental conditions were as follows: excitation slit width, 3.0 mm; emission slit width, 2.7 mm; excitation wavelength, 285 nm; sensitivity, $1 \cdot 10^{-8}$ A.; full scale of Y range on recorder, 1 V/division for emission spectra and 0.5 V/division for difference spectra; scan time, 5 min/100 nm; path length of cuvette, 1 cm. Photomultiplier tube voltage was set between 680 and 780 V, since the relative fluorescence at maximum wavelength was adjusted between 80 and 100% of full scale. The temperature of cuvette chambers was 10° C, except as stated, by using a Haake thermostat bath.

Thermal experiments. A suspension of membranes was incubated in a water bath at a given temperature for the times indicated, and an aliquot of the suspension was taken for measurement of the emission spectrum at the same temperature. The temperature of the cuvettes in the fluorometer was adjusted to the same temperature as the preincubation by circulating warm water from a Haake thermostat bath through the cuvette holder. The optics and electronics of the fluorometer were cooled by a constant flow of N_2 gas.

In certain experiments, a sample of membrane suspension, which had been preincubated at a given temperature, was then cooled to 0° C before scanning or TRH binding was performed. In such experiments, fluorescence measurements were made in the order of successively increasing temperatures.

Binding of thyrotropin-releasing hormone. The binding of [^{3}H]TRH to specific receptors in membranes from GH_4C_1 cells was measured as described by Hinkle and Tashjian [2].

Results

Emission spectrum and effect of TRH on fluorescence of GH_4C_1 membranes

Fluorescence of membranes from GH_4C_1 cells at the excitation wavelength of 285 nm had a maximum at 335 nm which was contributed by tryptophan residues. Using an aqueous solution as standard, the quantum yield at 10°C was calculated by the relation:

$$Q = \frac{A}{A(\text{Trp})} \frac{A_{285\,\text{nm}}(\text{Trp})}{A_{285\,\text{nm}}} Q(\text{Trp}) \qquad (2)$$

where A was the area under the emission spectrum and $A_{285\text{nm}}$ was the absorbance at 285 nm. The value for Q(Trp) was taken as 0.20 as reported by Teale and Weber [14]. The quantum yield of GH_4C_1 membranes was thus calculated to be 0.013.

The effect of TRH on the fluorescence of GH_4C_1 membranes was determined at different TRH concentrations. Quenching was maximal at about 100 nM TRH or methyl TRH (Table I), results that were similar to those obtained with membranes from GH_3 cells [9]. Two analogs of TRH which had low affinity for the TRH receptor [6], pGlu-D-His-ProNH_2 and Pro-His-ProNH_2, produced little or no effect on membrane fluorescence (Table I). When GH_3 or GH_4C_1 cells are grown in the presence of TRH there is a decrease in the number of TRH receptors to 20—30% of control values [15]. We, therefore, examined the effect of TRH on membrane fluorescence in membranes prepared from cells grown in the presence of 20 nM TRH for 48 h in which TRH receptors were decreased to 30% of the control value. The δF when tested at TRH concentrations of 10.0, 99.9 and 990 nM was less than 0.003 in each instance. Thus, membranes prepared from cells with down-modulated receptors did not show

TABLE I

EFFECTS OF TRH AND TRH ANALOGS ON THE FLUORESCENCE OF MEMBRANES FROM GH_4C_1 CELLS

Membranes from GH_4C_1 cells were incubated for 20 min at 0°C without or with TRH or TRH analogs and fluorescence measured as described in Materials and Methods.

Peptide	No. of Expts.	Concentration of peptide (nM)	δF at 334 nm	
			Mean	Range
pGlu-His-ProNH_2 (TRH)	4	10.0	0.010	0.008—0.011
	4	99.9	0.021	0.012—0.030
	2	1010.0	0.016	0.015—0.017
pGlu-His(N^{τ}-methyl)-ProNH_2 (methyl TRH)	2	10.0	0.004	0.002—0.006
	2	101.0	0.019	0.018—0.019
	1	1000.0	0.018	—
pGlu-D-His-ProNH_2	1	10.0	0.001	—
	1	99.8	0.005	—
	1	1010.0	0.011	—
Pro-His-ProNH_2	1	10.0	0.003	—
	1	99.9	0.009	—
	1	1010.0	0.006	—

quenching of intrinsic tryptophan fluorescence in response to freshly added TRH.

Effects of temperature on the fluorescence of GH_4C_1 membranes

Suspensions of GH_4C_1 membranes were preincubated for 10 min at temperatures from 10 to 60°C and fluorescence was measured subsequently with the cuvette chambers adjusted to the preincubation temperature. As shown in Fig. 1, the relative intensity of fluorescence of GH_4C_1 membranes decreased linearly with increasing temperature, and linearity was lost at about 52°C. After incubation at 60°C for 10 min, the temperature was decreased stepwise and membrane fluorescence measured at the temperatures indicated after a

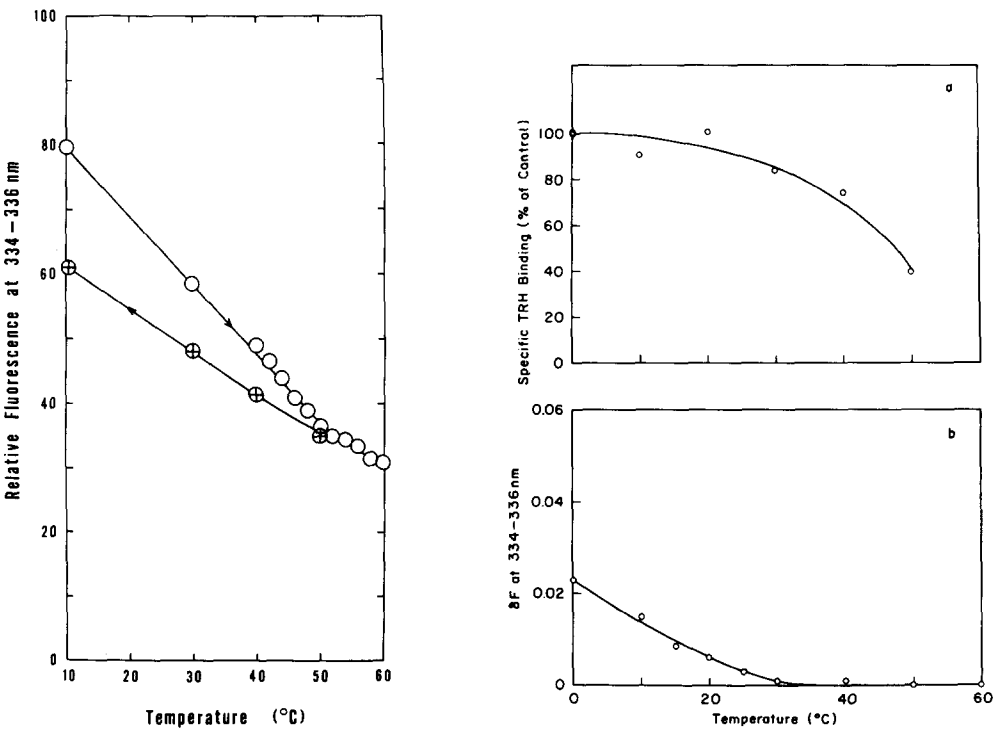

Fig. 1. Effects of temperature on the fluorescence of GH_4C_1 membranes. Suspensions of GH_4C_1 membranes were preincubated for 10 min at the temperatures indicated. Measurements of relative fluorescence were carried out in cuvettes at the same temperature as the preincubation and in the order shown by the arrows. The symbols (○ and ⊕) give the heating path and cooling paths, respectively. For samples indicated ⊕, the membranes were incubated at 60°C for 10 min, and then cooled for 5 min to 50, 40, 30 or 10°C, respectively, for scanning at that temperature.

Fig. 2. Effects of temperature on quenching of fluorescence due to TRH and on TRH binding to membranes from GH_4C_1 cells. (a) GH_4C_1 membranes were peincubated for 10 min at each of the temperatures indicated, and then specific TRH binding was measured during a 60 min incubation with [^{3}H]-TRH (25 nM) at 0°C as described in Materials and Methods. Binding obtained in membranes preincubated for 10 min at 0°C was set at 100%. Each point gives the mean of duplicate determinations which differed by less than 5%. (b) Suspensions of GH_4C_1 membranes were preincubated for 10 min at each of the temperatures indicated. The samples were cooled to 0°C and incubated for 20 min at 0°C with 100 nM TRH and measurements of fluorescence made at 13°C as described in Materials and Methods. The values shown are the means of two separate experiments in which the greatest variation from the mean value was ±0.004.

5 min equilibration. The slope of the cooling curve was the same above 52°C as that of the heating curve; however, relative fluorescence increased monotonically below 52°C and followed a different slope from the heating curve. When the suspension of GH_4C_1 membranes, which had been incubated at 60°C for 10 min, was cooled from 60 to 10°C, the intensity of emission decreased to 77% of control, and the maximum wavelength of fluorescence shifted to the red by 2–3 nm. The relative fluorescence of GH_4C_1 membranes which had been pretreated with 100 nM TRH for 20 min at 4°C had the same heating and cooling profiles as those shown in Fig. 1 (data not shown), indicating that TRH prebound to receptors did not affect the temperature-dependent alterations in intrinsic tryptophan fluorescence, and that these temperature-dependent alterations were reproducible findings. The absence of an effect of TRH could be due to dissociation of the tripeptide from its receptor at elevated temperature.

After suspensions of GH_4C_1 membranes were incubated at various temperatures for 10 min, portions of the suspensions at each temperature were cooled to 0°C, and quenching by TRH and [^{3}H]TRH binding were monitored (Fig. 2). As the temperature of the preincubation was increased, there was a progressive decrease in TRH-induced quenching (Fig. 2b). At 30°C and above, no quenching was observed. On the other hand, TRH binding was unaffected by a 10 min preincubation at temperatures up to 20°C, began to decrease at 30°C,

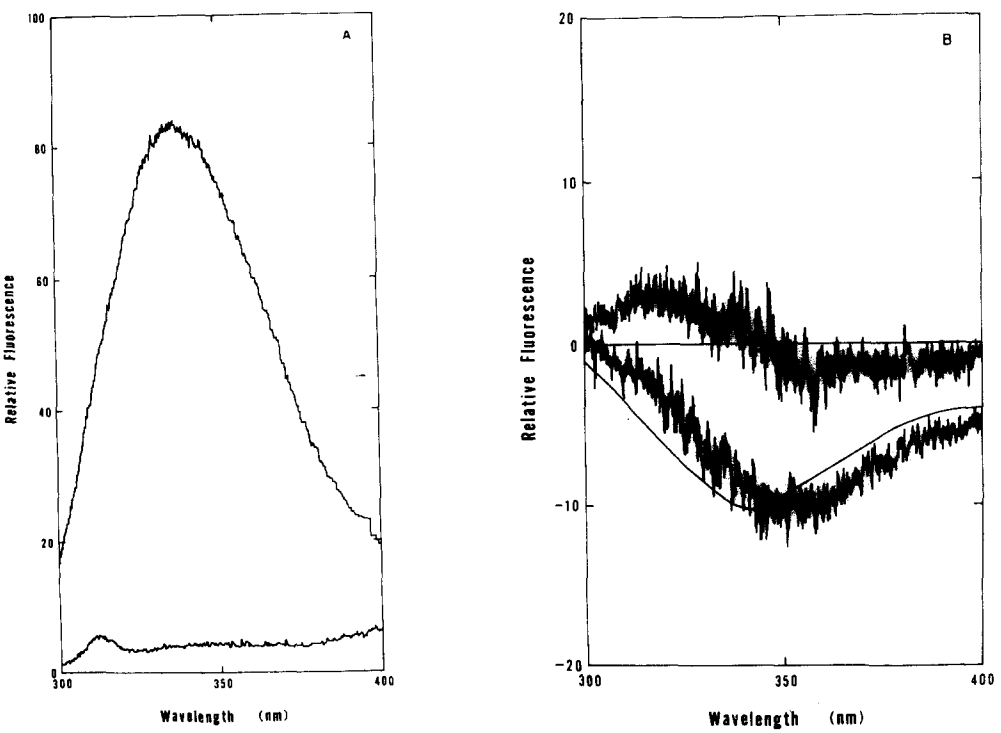

Fig. 3. (A) Instrumental plot of the emission spectrum of GH_4C_1 membranes (upper curve) and baseline (lower curve). Photomultiplier voltage was 720. The other conditions were as described in Materials and Methods. (B) Instrumental plot of the difference spectrum of GH_4C_1 membranes incubated for 5 min at 0°C with and without 0.095 M KI (lower curve) and baseline (upper curve). The lower solid line gives the true difference spectra between the instrument base line and the observed difference spectra.

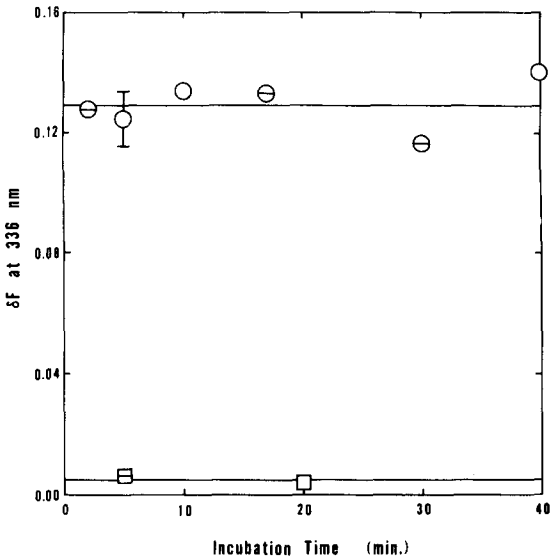

Fig. 4. Effects of duration of incubation with salts on the fluorescence of GH_4C_1 and GH_3 membranes. Suspensions of membranes were incubated at 0°C with 0.095 M KI or NaCl for the indicated times. ○ and ⊖, give the quenching by KI for GH_3 and GH_4C_1 membranes, respectively; □ and ⊟, give the results with NaCl for GH_3 and GH_4C_1 membranes, respectively. The vertical bar gives the instrumental noise.

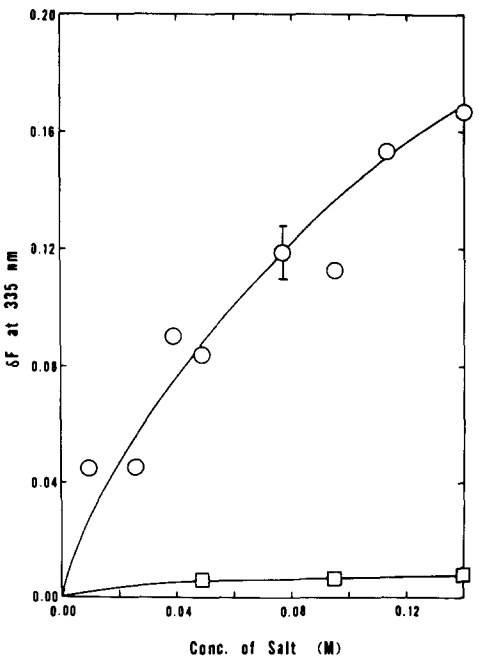

Fig. 5. Effects of salt concentration of fluorescence of GH_4C_1 membranes. GH_4C_1 membranes were incubated with KI (○———○) or NaCl (□———□) at 0°C for 5 min at the concentrations indicated. The vertical bar gives the instrumental noise.

but was still 40% of control at 50° C (Fig. 2a). Thus, quenching of fluorescence due to TRH binding was lost more rapidly as a function of temperature than was the specific binding of TRH to the membranes.

Quenching by iodide of the fluorescence of GH_3 and GH_4C_1 membranes

A suspension of GH_4C_1 membranes was incubated with 0.095 M KI at 0°C for 5 min and the relative fluorescence measured at 10°C. After incubation with KI there was quenching of fluorescence of GH_4C_1 membranes but the apparent emission wavelength maximum was not altered (Fig. 3A). However, the maximum of the difference spectrum was shifted to 342 nm from an emission maximum of GH_4C_1 membranes alone of 333 nM (Fig. 3B). Fig. 4 shows that the effect of incubation with 0.095 M I^- on the fluorescence of GH_3 and GH_4C_1 membranes was maximum by 2 min of incubation. The concentration dependence of iodide quenching is shown in Fig. 5. A concentration of 0.14 M I^- decreased relative fluorescence of GH_4C_1 membranes by 17%. The same concentrations of NaCl had little or no effect on the fluorescence of GH_3 and GH_4C_1 membranes (Figs. 4 and 5). Prebinding of TRH to its receptors caused no detecable change in subsequent iodide-induced quenching of fluorescence (data not shown).

Discussion

The wavelength of maximum fluorescence of membranes from GH cells was shifted to the blue from that of tryptophan in water at 355 nm indicating that some of the tryptophan residues in the sample are located in hydrophobic regions of the membrane [16]. Quantum yields of fluorescence were smaller than those of free tryptophan or tryptophan in simple proteins [17], suggesting that these residues in GH membranes were quenched by surrounding hydrated peptide carbonyl groups, disulfide groups or basic amino acid residues [18].

When TRH bound to membranes prepared from GH_3 or GH_4C_1 cells, there was quenching of tryptophan fluorescence. This quenching of fluorescence was dependent on the duration of incubation with TRH [9] and by the concentration of TRH. TRH analogs, which have little or no biological activity or affinity for the TRH receptor, had little or no effect on tryptophan fluorescence. GH_12C_1 cells, which lack receptors for TRH, were not quenched by TRH. Thus, the interaction of TRH with specific receptors for this tripeptide on GH_3 or GH_4C_1 cells is accompanied by the quenching of tryptophan fluorescence. Membranes prepared from GH_3 and GH_4C_1 cells do not show significant fluorometric differences, and the findings in both cell types confirm each other.

The intensity of tryptophan fluorescence in water decreases monotonically as the temperature is increased, although the shape of emission spectra is unaffected and the thermal change is reversible [19]. However, the response of tryptophan residues in proteins is more complex due to conformational changes in the protein induced by elevating the temperature. It has been reported that tryptophan fluorescence of proteins departs from the initial monotonic decrease at whatever temperature induces conformational change [19—22]. Weinryb and Steiner [22] showed that inactivated papain cooled from 80 to 20°C resulted in the recovery of about 80% (at pH 5.2) or 60%

(at pH 7.6) of the initial fluorescence. We found that at 52°C and above the decrease in fluorescence due to increasing temperature began to deviate from a straight line and that, on cooling, the return of fluorescence was not parallel to the heating curve (Fig. 1). Such findings probably indicate an irreversible denaturation of certain membrane proteins above 52°C. 77% of initial fluorescence was recovered. The data in Fig. 2 indicate that TRH-induced quenching of membrane fluorescence was lost more readily than was the decrease in TRH binding as the temperature to which the membranes were exposed was increased. This finding is consistent with our previous suggestion [9] that the quenching of fluorescence due to TRH probably reflects an overall change in membrane conformation rather than quenching of tryptophan residues in the TRH binding site per se. The greater sensitivity of fluorescence quenching by TRH than its binding to specific receptors also illustrates the utility of measurements of differential membrane fluorescence which serve to amplify the signal that results from binding of the tripeptide.

Weinryb and Steiner [22] described the temperature dependence of the quantum yield of an aromatic amino acid by

$$\ln\left(\frac{1}{Q}-1\right) = -\frac{E}{RT} + \ln\frac{f}{K_f} \tag{3}$$

assuming that K_0/K_f is small as compared with $1/Q - 1$ and that only one temperature-dependent deactivation process is operating. K_f is the (temperature-independent) rate constant for emission fluorescence, K_0 is the rate constant for the temperature-independent deactivation process, f and E are the frequency factor and activation energy, respectively, for the temperature-dependent nonradiative mechanism. In the case of GH_4C_1 membranes, $1/Q - 1$ was large in relation to K_0/K_f, which is known to be of the order of magnitude of unity [22], so that Eqn. 3 may be applied to our data.

The thermal effect on fluorescence of GH_4C_1 membranes, which was shown in Fig. 1, was replotted on the basis of Eqn. 3, and the result is shown in Fig. 6. Quantum yield for GH_4C_1 membranes at 10°C was taken as 0.013, and those at various temperatures were calculated by the relation of $Q = cF$, where c is a constant. The temperature effect can be described by three linear portions at 10—40°C, 40—52°C and 52—60°C, during the heating process, which can be ascribed to the native conformational transition and denatured conformations, respectively. The cooling and heating paths were similar between 52 and 60°C. Activation energies for native and denatured GH_4C_1 membranes were 2.9 and 2.6 kcal/mol, respectively. These values were lower than 8.1 kcal/mol found for free tryptophan [19] and similar to 2.5—3.3 kcal/mol for inactive papain [22]. Activation energy for the conformational transition region was 6.0 kcal/mol.

It is recognized that the GH_4C_1 membrane system is more complex than a free amino acid or single protein in solution and that changes in fluorescence could result from alterations in specific protein conformational changes or a change in protein lipid interactions in the membrane. Nevertheless, we believe that, as distinct transitions were noted, implying a cooperative phenomena in the membrane, such an analysis is justified.

Iodide is a selective quencher of the fluorescence of tryptophanyl side chains exposed to solvent [22—26]. The quenching by iodide of the fluorescence of

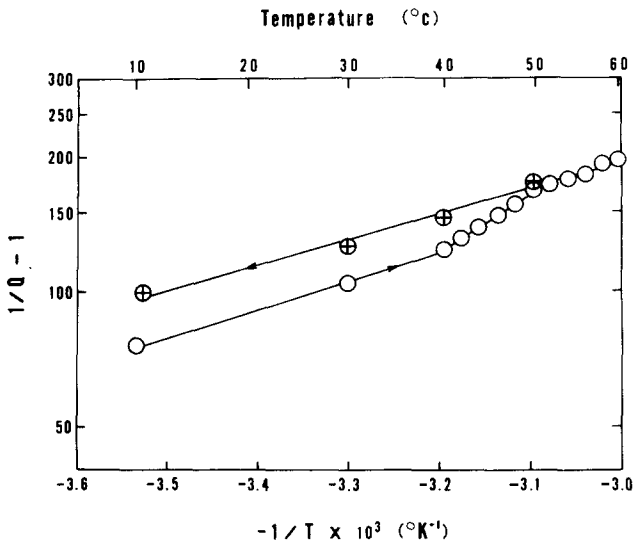

Fig. 6. Dependence of fluorescence quantum yield upon temperature. Logarithmic plot of $(1/Q - 1)$ vs. $-1/T$ for GH_4C_1 membranes. The data are taken from the experiment shown in Fig. 1.

GH_3 or GH_4C_1 membranes was rapid. Emission was quenched increasingly as the concentration of iodide was raised. Collisional quenching by iodide appears to act by a different mechanism from a quenching by TRH-receptor interaction.

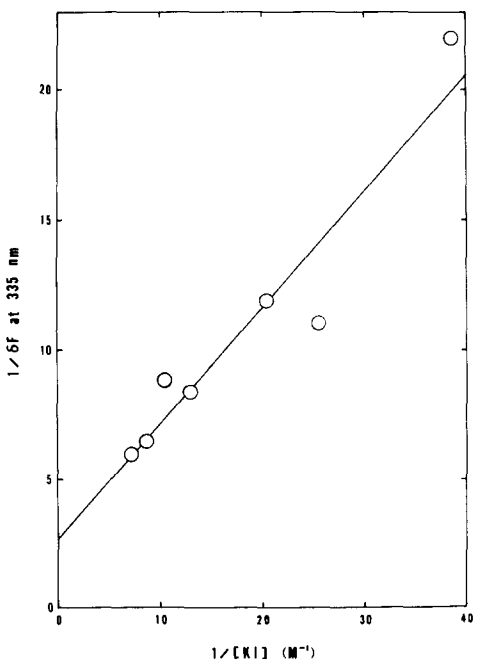

Fig. 7. Modified Stern-Volmer plot of the quenching of GH_4C_1 membrane fluorescence by iodide. The data are taken from the experiment shown in Fig. 5.

Lehrer [25] derived an equation to evaluate the number of tryptophanyl residues exposed to solvent. If f_a represents the fraction of exposed tryptophanyl side chains in protein, δF is related to f_a as follows:

$$\frac{1}{\delta F} = \frac{1}{K_Q f_a [KI]} + \frac{1}{f_a} \quad (4)$$

where K_Q is the quenching constant. This equation (Eqn. 4) may be applied to the quenching of the GH membrane system by iodide, if we define f_a as the fraction of tryptophanyl residues in membranes exposed to solvent. By plotting the data in Fig. 5 by this modified Stern-Volmer method (Fig. 7), values for $f_a = 0.38$ and $K_Q = 6.0\ M^{-1}$ were obtained, which were similar to those for native lysozyme [25]. Thus, about 38% of tryptophanyl residues in GH membranes were accessible for quenching by iodide, that is, were exposed to solvent, and this fraction was not altered by the binding of TRH to its specific membrane receptors.

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