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Fluorescence energy transfer between ionophore, A23187, and membrane proteins of isolated outer and cytoplasmic membranes of a Gram-negative bacterium

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When tryptophan residues of the proteins of outer and cytoplasmic membranes of a modeprately halophilic bacterium, *Pseudomonas halosaccharolytica* ATCC 29423, were excited at the wavelength 270 nm, tryptophan emission was observed at 330 nm. Adding the calcium ionophore, A23187, to both suspensions, the tryptophan emission at 330 nm decreased and the ionophore emission at 430 nm increased. Thus, when the calcium ionophore was increased in both suspensions, the ionophore emission increased with excitation of membrane tryptophan, that is, the fluorescence energy was transferred from tryptophan to the calcium ionophore. Using the Förster equation the critical distance was calculated to be 52 Å. As this distance is considerable compared with the diameter of the membrane protein molecules, the ionophore cannot be bound to the membrane proteins. It is probably located in the lipid bilayers.

Introduction

Calcium ionophore, A23187, seems to act as a carrier of calcium through the lipid membranes. Hyono et al. [1] found that A23187 transports calcium as a complex of molar ratio [A23187]/[calcium] = 2 : 1, but on lowering the ionophore concentration, the ratio becomes 1 : 2. Molecular structures of the complex in solid crystals or in solutions are known [2–5]. Complexes of this ionophore with another divalent cation, Mg^{2+}, and monovalent cations, Na^+, K^+, etc. were also reported [6,7]. Case et al. [8] found that a cationic form of A23187 carry some current but most A23187 transport Ca^{2+} by electrically neutral form. Pringle and Hidalgo [9] reported that this ionophore has no effects on either the rotational motion of Ca^{2+}-ATPase, in the membranes or on the mobility of the lipid acyl chains.

Calcium concentrations inside the cells of usual bacteria are maintained at less than 10^{-6} M by the calcium efflux through Ca^{2+}-ATPase even if the concentration of 10^{-3} M Ca^{2+} outside the cells is exceeded. On the contrary, A23187 would then act as an influx carrier of Ca^{2+} through the lipid bilayers. Verjovski-Almeida [10] obtained evidence for a fluorescence energy transfer between ATPase and another ionophore, X537A, in the sarcoplasmic reticulum. We studied the relations between membrane proteins and A23187 using separated outer and cytoplasmic membranes of a moderately halophilic Gram-negative bacterium, *Pseudomonas halosaccharolytica* ATCC 29423. We used this bacterium as we are studying the biophysical properties of its membranes [11–15]. We found no direct interaction between the Ca^{2+} efflux by the membrane proteins and Ca^{2+} influx by A23187, however, we did obtain evidence for a fluorescence

energy transfer from the tryptophan residue of membrane proteins to A23187, in both the outer and cytoplasmic membranes of this bacterium.

Materials and Methods

Preparation of outer and cytoplasmic membranes. Moderately halophilic bacterium, *Pseudomonas halosaccharolytica* ATCC 29423, was grown in HP 101 medium containing 2 M NaCl at 30°C [1], and its outer and cytoplasmic membranes (OM and CM) were prepared by the method of Hiramatsu et al. [16]. The harvested cells were suspended in 50 mM Tris-HCl buffer (pH 7.8, containing 20% sucrose and DNAase), stirred for 1 h and then centrifuged at $10000 \times g$. The supernatant and precipitate containing outer and cytoplasmic membranes, respectively, were treated with lysozyme, washed and the membranes separated by sucrose discontinuous density gradient centrifugation in 50 mM Tris-HCl buffer, in a Hitachi RPS 40T rotor at 36000 rpm. The outer and cytoplasmic membranes were recovered from the 50 and 30 per cent sucrose layers, respectively, and then dialyzed against cold 5 mM Tris-HCl buffer (pH 7.8).

Reagents. Calcium ionophore, A23187, was purchased from CalBiochem-Behring Corp. DNAase and lysozyme were products of Sigma Chemical Co. Most chemicals were special grade products from Wako Pure Chemical.

Absorption spectra. Absorption spectra of suspensions of outer membrane and cytoplasmic membrane in 50 mM Tris-HCl buffer (pH 7.8) and A23187 in ethanol/50 mM Tris-HCl buffer (pH 7.8) solution were all from a Hitachi 220A Spectrophotometer (the ratio of ethanol to Tris-HCl buffer was 9 : 1 (v/v)). Absorption spectra of both suspensions contained a large amount of scattering background.

Fluorescence analyses. Suspensions of outer membrane and cytoplasmic membrane were diluted with 50 mM Tris-HCl buffer (pH 7.8) to the appropriate concentrations of tryptophan for fluorescence analyses. The stock solution of A23187 was prepared by dissolving the ionophore in ethanol ($1 \cdot 10^{-3}$ M) and a small amount of the stock solution was added to the membrane suspension (not exceeding 1/200 of the suspension volumes). Fluorescence emission spectra were obtained using a Hitachi 650-60 Fluorescence Spectrophotometer and a 1 cm square cell (excitation wave length 270 nm, both slit widths of excitation and emission; 10 nm). When the content of A23187 in the membrane suspension was gradually increased, the tryptophan emission at 330 nm decreased, whereas the emission of A23187 at 430 nm increased. Emission and excitation spectra were automatically corrected using Rhodamine-B solution.

Results

Optical observation of outer and cytoplasmic membranes

For light scattering by the suspensions of outer and cytoplasmic membranes, absorption spectra of the suspensions increased steeply at shouter wave length and there was a clear shoulder at 270 nm. This corresponded to absorption of the membrane protein (tryptophan), as shown in Fig. 1. When membrane suspensions were excited at a 270 nm, strong fluorescence was observed at 330 nm tailing to 300 nm. Though a tyrosine fluorescence should appear to wavelength 300 nm, the main emission at 300 nm is the tryptophan tail and the tyrosine emission would be negligible. Excitation spectra (emission; 330 nm) of both membrane suspensions coincided with the tryptophan absorption spectrum.

Absorption and fluorescence of A23187

Ionophore, A23187, in ethanol/50 mM Tris-HCl buffer (pH 7.8) solution had three main ultraviolet absorption spectra at 230, 300 and 370 nm, in the presence of Ca^{2+}. The absorbance at 270 nm of A23187 (10^{-6}M in ethanol) was less than 0.02. The fluorescence of this ionophore was observed at 430 nm, and the excitation spectra coincided with its three absorption band spectra.

Fluorescence energy transfer

When both membrane suspensions in the absence of calcium ionophore A23187 were excited at 270 nm, only tryptophan fluorescence was observed, while, in the presence of A23187, tryptophan fluorescence at 330 nm decreased and the ionophore fluorescence appeared at 430 nm. With increase in the ionophore concentrations in

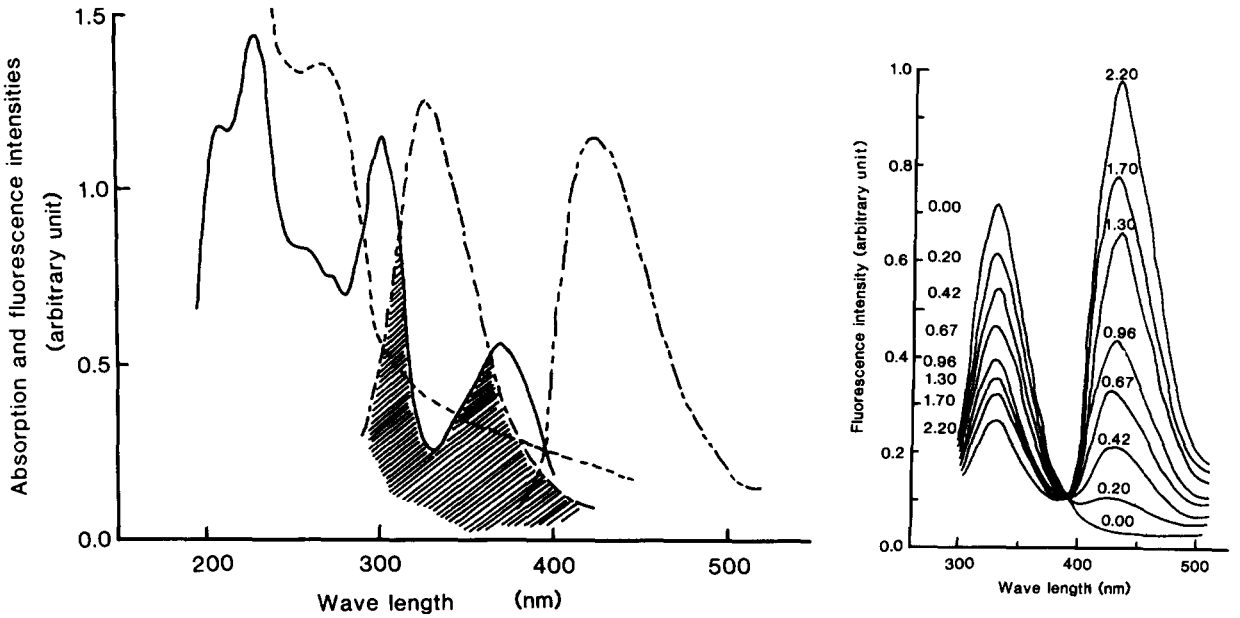
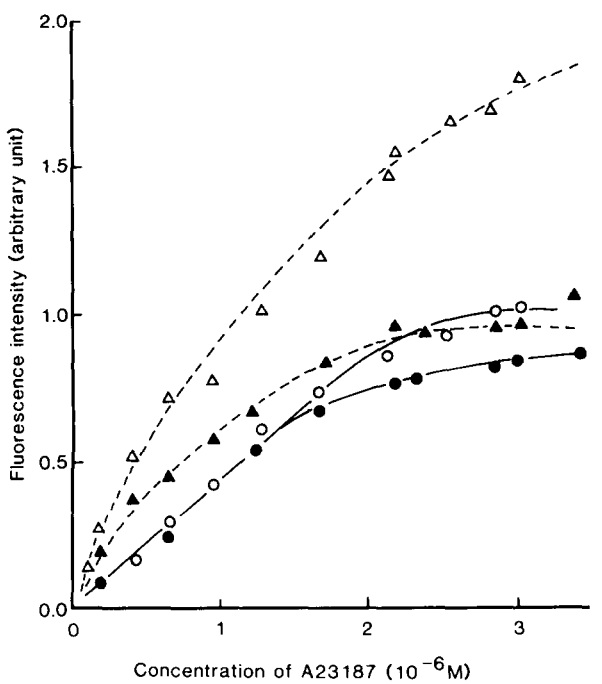


Fig. 1. Schematic representation of the relation between absorption and fluorescence spectra (in arbitrary unit). ————; absorption spectrum of A23187, 10^{-5} M in ethanol/50 mM Tris-HCl buffer (pH 7.8) solution in the presence of Ca^{2+} (the ratio of ethanol to Tris-HCl buffer was 9:1 (v/v)), - - - - - -; absorption spectrum of isolated outer membrane in 50 mM Tris-HCl buffer, — - — -; fluorescence spectrum of tryptophan of membrane proteins in the suspension of outer membrane, —'- - —; fluorescence spectrum of A23187 added to the suspension of outer membrane (excitation, 375 nm). Shadowed area is the overlap between fluorescence of tryptophan and absorption of A23187 (almost equal to the excitation spectrum of A23187).

Fig. 2. Fluorescence spectra of the suspension of cytoplasmic membranes in 50 mM Tris-HCl buffer (pH 7.8) in the absence of NaCl. The concentration of ionophore A23187 (μM) was increased step by step as shown in this figure. Excitation wavelength; 270 nm.



each membrane suspension, the intensity of tryptophan fluorescence at 330 nm decreased, while the intensity of ionophore fluorescence at 430 nm increased, as shown in Fig. 2. The relation between the decrease of tryptophan fluorescence and the increase of ionophore fluorescence is shown in Figs. 3 and 4. In the absence of Ca^{2+} in both suspensions, fluorescence intensities at 430 nm (excitation, 270 nm) were at first in proportion to the concentration of A23187 and then gradually increased with direct excitation of absorption of A23187 itself at 270 nm. Irrespective of the presence of Ca^{2+}, the increasing rate of the fluores-

Fig. 3. Relation between fluorescence intensity (excitation; 270 nm, emission; 430 nm) and concentration of A23187 in the suspensions of both isolated membranes. Buffer: 50 mM Tris-HCl (pH 7.8), closed and open symbols present values of outer and cytoplasmic membranes and triangles and circles represent values in the presence of 2 mM Ca^{2+} and in the absence of Ca^{2+}, respectively, in this and the subsequent figure.

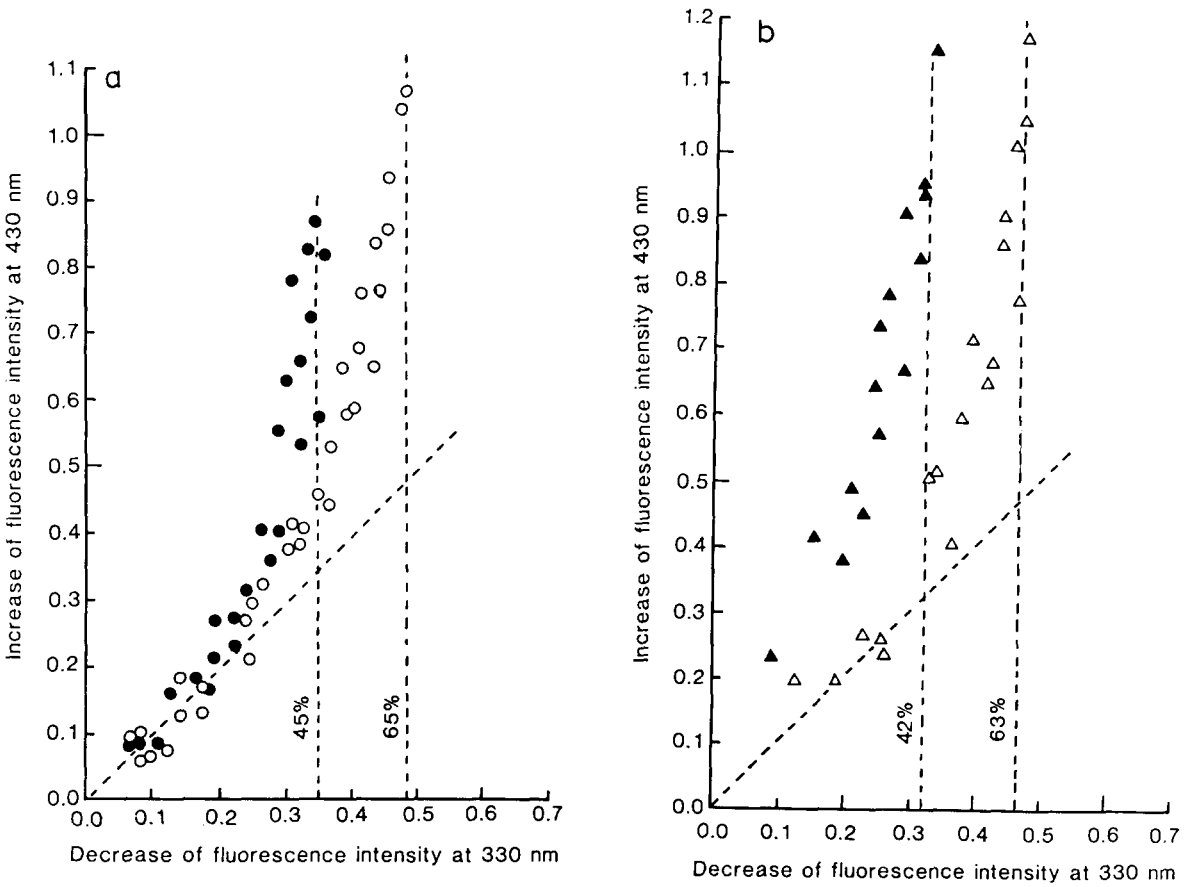
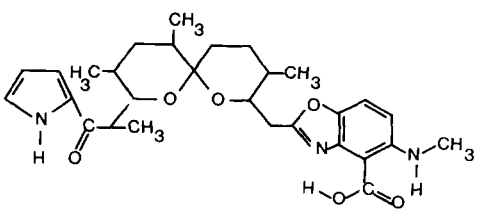


Fig. 4. Relation between fluorescence intensities of A23187 at 430 nm and of tryptophan at 330 nm. (a) Without Ca^{2+}, (b) with 2 mM Ca^{2+}. At the most, 45% and 65% tryptophan residues in outer and cytoplasmic membrane proteins can transfer their fluorescence energy to A23187, respectively. Both ordinate and abscissa units are the same as the ordinate unit in Fig. 3.

cence intensities at 430 nm with increasing concentrations of A23187 was reduced to the rate of the direct excitation of A23187 at 270 nm (Fig. 3). The relation between increase of fluorescence intensity at 430 nm and decrease of fluorescence intensity at 330 nm is shown in Fig. 4. Fluorescence (430 nm)-temperature curves obtained at the elevating temperature all but coincided with the curves obtained at the reducing temperature (data not shown). With suspensions of outer and cytoplasmic membranes, the plots of logarithm of fluorescence intensity to reciprocal of absolute temperature were all compared of two straight lines with a breakpoint (Fig. 5). The temperature at these breakpoints was much the same as the phase transition temperature and also the same as the growth temperature of this bacterium [12–15].

Discussion

Calcium ionophore A23187 contains two chromophores, ketopyrrole and 4-carboxy, 5-aminomethylbenzoxazole. The absorption band of the former is located at a wavelength of 200–250 nm, whereas the latter is found at 200–400 nm. The presence of inner filter effects can be ruled



A23187

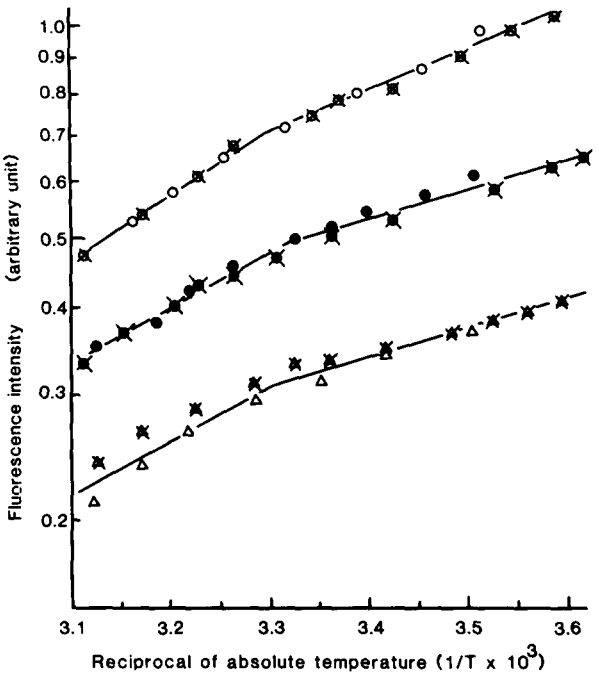


Fig. 5. Variation of fluorescence intensity at 430 nm by reducing and elevating the temperature. Ordinate: logarithm of fluorescence intensity; abscissa: reciprocal of temperature (absolute temperature). Uncrossed and crossed symbols represent the curves on reducing and elevating temperature, and open and closed circles represent the curves of outer and cytoplasmic membranes, respectively. Membrane suspensions represented by open and closed circles contained no NaCl, whereas the outer membrane suspension (open crossed and uncrossed triangles) contained 2 M NaCl.

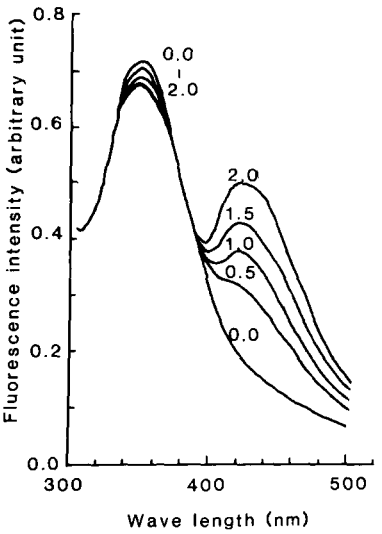


Fig. 6. Fluorescence spectra of the suspension of intact cells of this bacterium in 50 mM Tris-HCl buffer (pH 7.8), containing 2 M NaCl. A23187 was increased from 0 to 2 μM as shown in this figure. Excitation: 270 nm. See legend of Fig. 2.

out as the absorbance at 270 nm of 10^{-6} M A23187 was less than 0.02 though the slope in Fig. 3 becomes concave downward with increasing concentrations of A23187. Moreover, the fluorescence of tryptophan did not decrease below the lower limits, as shown in Fig. 4, and all the fluorescence spectra in Fig. 2 intersect at a point at 390 nm just like an isosbestic point. On carrying out the same experiment on the suspension of intact cells of this bacterium we observed only a little fluorescence decrease at 330 nm but observed rapid increase at 430 nm (Fig. 6). Thus, A23187 scarcely entered in the membranes of intact cells of this bacterium and the fluorescence energy transfer was not observed between proteins of intact cell membranes and A23187.

Assuming that the geometrical arrangement of two chromophores, tryptophan and benzoxazole, is perfectly random, the critical distance, R_0, of excitation energy transfer between them is given by the Förster equation [17]:

$$R_0^6 = \frac{3000 \ln 10}{32 \pi^6 n^4 N} \int \frac{F(\bar{\nu}) \epsilon(\bar{\nu})}{\bar{\nu}^4} d\bar{\nu} \quad (1)$$

where n, N and $\bar{\nu}$ are the refractive index of the medium, Avogadro's number and wave number, respectively, and $F(\bar{\nu})$ and $\epsilon(\bar{\nu})$ are the normalized fluorescence energy distribution and the molar extinction coefficient at wave number $\bar{\nu}$, respectively. (R_0 is the critical transfer distance for which excitation transfer and spontaneous deactivation of sensitizer are of equal probability.) The critical distance, R_0, between tryptophan and benzoxazole of A23187 calculated by this equation is 52 Å, a distance probably too far to directly bind A23187 to the membrane protein. As shown in Fig. 4, though the fluorescence intensity of A23187 at 430 nm increases upto an unlimited extent with increase in the concentration of A23187 in both membrane suspensions, tryptophan fluorescence intensity at 330 nm did not decrease below a certain proportion, that is, only 65% of tryptophan in cytoplasmic membrane and 45% of tryptophan in outer membrane can transfer the excitation

energy to A23187, regardless of whether or not the suspensions contain Ca^{2+}.

Though both quantum yields of tryptophan fluorescence and benzoxazole fluorescence are generally about 20%, the quantum yields of this excitation energy transfer would be close to 100% because the decrease of tryptophan fluorescence is almost equal to the increase of A23187 fluorescence (initial angle is almost 45° in Fig. 4), while the fluorescence increase at 430 nm contains the intrinsic fluorescence of A23187 excited directly at 270 nm of the higher excited state of A23187. If after the emission of tryptophan this fluorescence were firstly absorbed by A23187 and then A23187 emitted its fluorescence, then the quantum yield of A23187 fluorescence would not be 100% but rather 20%. Thus, Förster's energy transfer must be accomplished without emission of the tryptophan's fluorescence. The quantum yield of the emission of A23187 (430 nm) on exciting tryptophan at 270 nm would depend solely on the quenching of radiationless transition at the excited state of tryptophan. Accordingly, the step of fluorescence energy transfer is scarcely affected by temperature. With decreasing tempeorature, the fluorescence intensity at 430 nm increased gradually, as shown in Fig. 5. There is a clear breakpoint at the temperature around the growth temperature of this bacterium. Here, the membrane proteins would change from liquid to solid like the membrane lipid bilayers of this bacterium [12–15]. The slope for the liquid state in Fig. 5 is steeper than the slope for the solid state, that is, the activation energy of radiationless transition in the liquid state would be larger than that in the solid state.

The coupling energy between tryptophan and A23187 is weak and the critical distance of the energy transfer is about 52 Å. Therefore, the calcium ionophore A23187 would transport calcium ions without disturbance of membrane proteins in the form of a electrically neutral complex of two ionophores and one calcium ion through the membrane lipid bilayers.

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References

1 Hyono, A., Hendriks, T., Daemen, F.J.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta 389, 34–46
2 Chaney, M.O., Demarco, P.V., Jones, N.D. and Occolowitz, J.L. (1974) J. Am. Chem. Soc. 96, 1932–1933
3 Deber, C.M. and Pfeiffer, D.R. (1976) Biochemistry 15, 132–141
4 Smith, G.D. and Duax, W.L. (1976) J. Am. Chem. Soc. 98, 1578–1580
5 Brasseur, R., Deleers, M., Malaisse, W.J. and Ruysschaert, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2895–2897
6 Pfeiffer, D.R. and Lardy, H.A. (1976) Biochemistry 15, 935–943
7 Pfeiffer, D.R., Taylor, R.W. and Lardy, H.A. (1978) Ann. N.Y. Acad. Sci. 307, 402–423
8 Case, G.D., Vanderkool, J.M. and Scarpa, A. (1974) Arch. Biochem. Biophys. 162, 174–185
9 Pringle, M.J. and Hidalgo, C. (1982) Biophys. J. 37, 633–636
10 Verjovski-Almeida, S. (1981) J. Biol. Chem. 256, 2662–2668
11 Ohno, Y., Yano, I. and Masui, M. (1979) J. Biochem. 85, 413–421
12 Hyono, A., Kuriyama, S., Hara, H., Yano, I. and Masui, M. (1979) FEBS Lett. 103, 192–196
13 Hyono, A., Kuriyama, S., Hara, H., Yano, I. and Masui, M. (1980) J. Biochem. 88, 1267–1274
14 Hara, H., Hyono, A., Kuriyama, S., Yano, I. and Masui, M. (1980) J. Biochem. 88, 1275–1282
15 Kuriyama, S., Hara, H., Hiramatsu, T., Hyono, A., Yano, I. and Masui, M. (1982) Can. J. Biochem. 60, 830–837
16 Hiramatsu, T., Yano, I. and Masui, M. (1980) FEMS Microbiol. Lett. 7, 289–292
17 Förster, T. (1959) Discuss. Faraday Soc. 27, 7–17