FLUORESCENCE QUENCHING OF Ca^{2+}-ATPase IN BILAYER VESICLES BY A SPIN-LABELED PHOSPHOLIPID

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1. Introduction

Fluorescence quenching in natural and model membranes has recently been shown to be useful for examining membrane properties [1–5]. Molecules containing nitroxide groups [6] quench the fluorescence of a wide variety of fluorophors. The quenching occurs when the nitroxide is close (< 4–6 Å) to the fluorophor. Perylene fluorescence in bilayer vesicles and tryptophan fluorescence in red blood cell ghosts [2,3] can be quenched by spin-labeled fatty acids. The availability of spin-labeled phospholipids makes possible the detection of differences in fluorescence quenching among phospholipid species. Two different approaches to this binding problem are to measure either the relative fluorescence quenching of different spin-labeled phospholipids which are analogs of the phospholipid species of interest, or else to use one spin-labeled phospholipid and to measure its displacement by non-spin-labeled phospholipids. The latter approach, which we describe here, avoids the question of whether the spin-labeled phospholipid has the same protein-binding properties as does the analogous non-labeled phospholipid. In this way measurements can be made to evaluate the binding strengths of different phospholipids to an intrinsic membrane protein. We report here experiments which utilize a spin-labeled phosphatidylcholine to detect binding differences between DMPC and egg PC to the Ca^{2+}-ATPase of sarcoplasmic reticulum.

To measure the quenching of an isolated intrinsic membrane protein by spin-labeled phospholipid, it is necessary to alter the native phospholipid composition of the purified protein by the incorporation of the exogenous phospholipid into the membrane. The Ca^{2+}-ATPase of sarcoplasmic reticulum is an intrinsic membrane protein which can be reconstituted with a new phospholipid population by several methods [4,7–11]. Incubation of Ca^{2+}-ATPase in the presence of a sufficient amount of the detergent potassium cholate equilibrates the endogenous phospholipid with a large excess pool of exogenous phospholipid [7–10]. Upon removal of cholate from solubilized phospholipid small unilamellar bilayer vesicles form [12].

2. Experimental

Ca^{2+}-ATPase was purified by the method in [13]. The 1-oxyl-2,2-dimethyloxazolidine derivative of 8-keto-palmitic acid was synthesized as in [14]. Spin-labeled phosphatidylcholine ((7,6) PC) was synthesized by condensation of the spin-labeled palmitic acid with lyso-phosphatidylcholine derived from egg yolk as in [15]. Ca^{2+}-ATPase was incorporated into phospholipid vesicles by a modification of the method in [10]. Small unilamellar phospholipid vesicles were made by sonication in a bath sonicator. Samples of sonicated phospholipid vesicles were solubilized in 1% (w/v) potassium cholate, pH 7.8. After a 30 min incubation purified Ca^{2+}-ATPase was added to each

Abbreviations: DMPC, dimyristoyl phosphatidylcholine; egg PC, egg phosphatidylcholine; (7,6)PC, 1-acyl-2-[5-(6′-carboxyhexyl)-5-octyl-1-oxyl-2,2-dimethyloxazolidine]-phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine

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sample. After another 30 min incubation an aliquot of each sample was diluted 100-fold in 20 mM Tris–Cl (pH 7.5). Fluorescence of the samples was measured using a Perkin-Elmer MPF-3 spectrofluorimeter operating in ratio mode. Samples were exposed to excitation at 290 nm and emission was measured at 340 nm, with excitation and emission slits set at a nominal bandwidth of 10 nm. Vesicle size was measured by gel permeation chromatography on a 30 × 1.5 cm Sepharose 4B column. ^{1}H NMR studies were performed using a Varian CFT-20 NMR Spectrometer operating at 79.54 MHz, equipped for variable temperature operation.

3. Results and discussion

Since the nitroxide moiety might perturb the phospholipid bilayer structure, especially in the high concentrations used for fluorescence quenching experiments, preliminary studies were conducted to determine some physical properties of aqueous dispersions of (7,6) PC. Sepharose 4B chromatography of sonicated dispersions of (7,6) PC indicated that the phospholipid is in the form of small unilamellar vesicles, of approximately the same size as egg PC vesicles. When Ca^{2+}-ATPase was reconstituted with (7,6) PC by cholate dilution, the protein-containing phospholipid vesicles chromatographed on Sepharose 4B as a dispersion of homogeneous size with a profile similar to that in [12] for small unilamellar vesicles prepared from egg PC by removal of cholate with Sephadex G-50. ^{1}H NMR resonance studies of linewidth and signal intensity (E. L. and G. W. F., unpublished observations) indicated that in unsonicated dispersions at 23°C the (7,6) PC molecules have significant mobility relative to DPPC dispersions (which are in the gel state at 23°C). These studies also showed that there is no sharp transition between 23°C and 88°C for the (7,6) PC. Finally, the ATPase activity of the enzyme could be detected in (7,6) PC: in 49 mol% (7,6) PC–49 mol% egg PC, ATPase activity is 9 μmol/min/mg and in 98 mol% (7,6) PC, ATPase activity is 4 μmol/min/mg. (The other 2 mol% of phospholipid is endogenous sarcoplasmic reticulum phospholipid associated with the purified Ca^{2+}-ATPase.) These values compare favorably with those in [8] for the activity of Ca^{2+}-ATPase in saturated phospholipids.

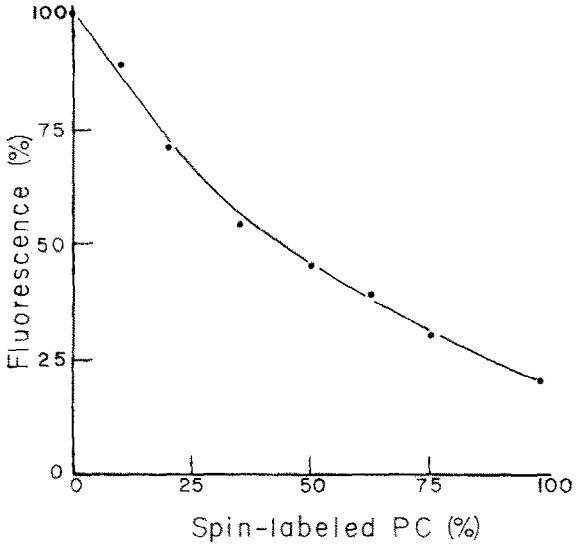

Fig.1. Quenching of Ca^{2+}-ATPase fluorescence by incorporation of (7,6) PC. Samples consisting of 12 μg Ca^{2+}-ATPase were reconstituted with 0.2 μmol total of egg PC and (7,6) PC in 2 ml 20 mM Tris–Cl (pH 7.5). Fluorescence was measured at 23°C.

The effect on protein tryptophan fluorescence of the incorporation of increasing mol% of (7,6) PC in egg PC is illustrated in fig.1. Significant quenching is observed with as little as 10 mol% (7,6) PC. The quenching is not linear with mol% spin label. There is more quenching per spin-labeled PC at low mol% of spin label. About 80% of the fluorescence was quenched with 98 mol% (7,6) PC. At 98 mol% (7,6) PC there is little or no shift of the fluorescence excitation or emission spectra of the enzyme. A simple explanation for the high degree of quenching is that most tryptophans are in close proximity to the phospholipids surrounding the protein. It is also possible that fluorophors far from the phospholipid transfer their excitation energy to tryptophans near the phospholipids. In fig.1 the ratio of total phospholipid to protein was ~15 : 1 (w/w). In experiments where Ca^{2+}-ATPase was incorporated into vesicles containing only spin-labeled phospholipid, about 75% quenching of protein fluorescence was obtained at a 5 : 1 ratio of phospholipid to protein. At higher ratios of phospholipid to protein little additional quenching was obtained.

Since the phospholipid immediately surrounding

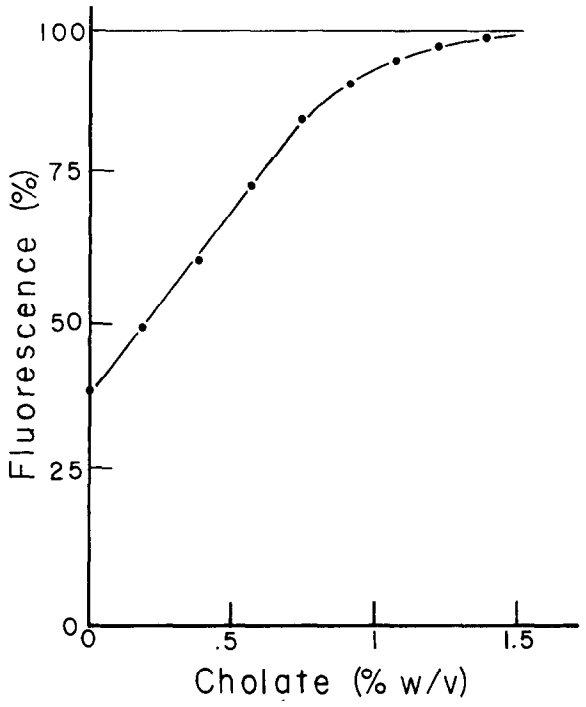

Fig.2. Effect of cholate addition on quenching of Ca^{2+}-ATPase fluorescence by (7,6) PC. Potassium cholate (pH 7.8) was titrated into a sample consisting of 12 μg Ca^{2+}-ATPase reconstituted as in fig.1 with 0.1 μmol each of DMPC and (7,6) PC. Fluorescence was measured after each addition of cholate.

the intrinsic membrane protein is believed to be relatively immobile [16,17] and since the effective viscosity of a phospholipid bilayer is high relative to that of H_2O [18], the quenching probably represents a static process over the tryptophanyl fluorescence lifetime of 2–5 ns. In addition, quenching arises only from bound phospholipids surrounding the protein. The shape of the quenching versus mol% (7,6) PC curve depends on several factors including the distribution of tryptophan residues and the binding isotherm for each phospholipid.

The effect of the addition of cholate on the fluorescence of Ca^{2+}-ATPase reconstituted with (7,6) PC is shown in fig.2. The fluorescence quenching by (7,6) PC can be abolished by the addition of cholate. This effect, caused by the delipidation of the enzyme by the detergent [19], shows that the binding of the (7,6) PC to the Ca^{2+}-ATPase is reversed upon addition of cholate.

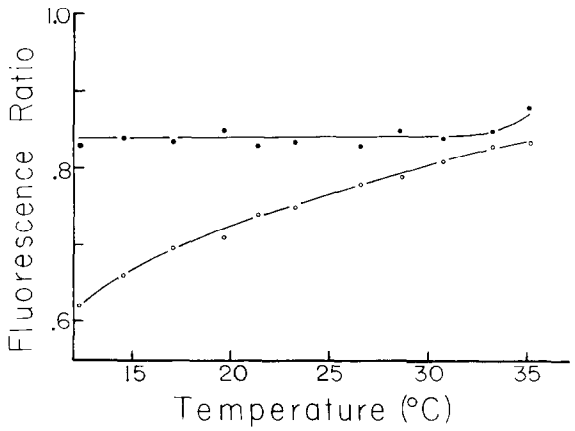

Fig.3. Temperature dependence of quenching of Ca^{2+}-ATPase fluorescence in reconstituted samples containing 10 mol% (7,6) PC. Samples were prepared as in fig.1. The fluorescence ratio was calculated from the ratio of fluorescence in samples with 10 mol% (7,6) PC to fluorescence in samples with no (7,6) PC. (•) Fluorescence ratio with 10 mol% (7,6) PC in egg PC. (○) Fluorescence ratio with 10 mol% (7,6) PC in DMPC.

A comparison of quenching in mixtures of (7,6) PC with either egg PC or DMPC is shown in fig.3. Between 12°C and 35°C, the temperature range examined, the (7,6) PC quenches fluorescence more strongly in vesicles containing DMPC than in vesicles containing egg PC. The quenching in the sample containing egg PC is not temperature dependent. The quenching by (7,6) PC in the sample containing DMPC is significantly enhanced at lower temperatures. As DMPC-containing vesicles are cooled below their transition temperature the local phospholipid environment of the Ca^{2+}-ATPase, i.e., the layer of bound phospholipid, is enriched in (7,6) PC. This is consistent with the observation [20] that the Ca^{2+}-ATPase is found in an environment of phospholipids in the liquid crystal state rather than in phospholipids in the gel state in systems where both phases coexist. There seems to be no sharp change in quenching at the transition temperature of DMPC (24°C). This may be due to the broadened phase transition of small unilamellar vesicles relative to the transition in large multilamellar vesicles [21], an effect of the (7,6) PC on the transition temperature of the vesicles or an effect of residual cholate in the vesicles. There could also be a perturbation of the local phospholipid environment by the Ca^{2+}-ATPase which results in a very broad phase transition, or no

phase transition at all, in the vicinity of the enzyme [16]. The difference in the level of quenching by (7,6) PC with egg PC as compared to (7,6) PC with DMPC indicates that egg PC excludes more (7,6) PC from the environment of the Ca^{2+}-ATPase than does DMPC, i.e., egg PC 'binds' more tightly to the enzyme than DMPC. Some of this difference in 'binding' is a consequence of the phase transition of DMPC vesicles. However, the difference in quenching persists at temperatures above the phase transition of DMPC, and so may represent a difference in the intrinsic affinity of the Ca^{2+}-ATPase for egg PC and for DMPC.

In summary, these quenching measurements are sensitive to the phospholipid composition of the immediate environment around the Ca^{2+}-ATPase, when the protein is incorporated into a bilayer vesicle. These experiments provide a method for determining differences in relative binding strengths of different phospholipid to an intrinsic membrane protein. Other factors, such as the phase transition behavior of a phospholipid, can also affect which phospholipids are bound to the protein. Finally it should be noted that although the differences in the phospholipids used here were in the fatty acyl chains, the binding of phospholipids with different headgroups could also be measured using this technique.

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References

[1] Koblin, D. D., Kaufmann, S. A. and Wang, H. H. (1973) Biochem. Biophys. Res. Commun. 53, 1077–1083.
[2] Bieri, V. G. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta 389, 413–427.
[3] Bieri, V. G. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta 406, 415–423.
[4] Hardwicke, P. M. D. (1976) Eur. J. Biochem. 62, 431–438.
[5] Shinitzky, M. and Rivnay, B. (1977) Biochemistry 16, 982–986.
[6] Green, A. J., Singer, L. A. and Parks, J. H. (1973) J. Chem. Phys. 58, 2690–2695.
[7] Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974) Proc. Natl. Acad. Sci. USA 71, 622–626.
[8] Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974) Biochemistry 13, 5501–5507.
[9] Racker, E. (1972) J. Biol. Chem. 247, 8198–8200.
[10] Racker, E., Chien, T.-F. and Kandrach, A. (1975) FEBS Lett. 57, 14–18.
[11] Racker, E. and Eytan, L. (1973) Biochem. Biophys. Res. Commun. 55, 174–178.
[12] Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322–331.
[13] MacLennan, D. H. (1970) J. Biol. Chem. 245, 4508–4518.
[14] Hubbell, W. L. and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326.
[15] Boss, W. F., Kelley, C. U. and Landsberger, F. R. (1975) Anal. Biochem. 64, 289–292.
[16] Hesketh, F. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C. and Warren, G. B. (1976) Biochemistry 15, 4145–4151.
[17] Longmuir, K. J., Capaldi, R. A. and Dahlquist, F. W. (1977) Biochemistry 16, 5746–5755.
[18] Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochemistry 12, 521–528.
[19] Knowles, A. F., Eytan, E. and Racker, E. (1976) J. Biol. Chem. 251, 5161–5165.
[20] Kleeman, W. and McConnell, H. M. (1976) Biochim. Biophys. Acta 419, 206–222.
[21] Kantor, H. L., Mabrey, S., Prestegard, J. H. and Sturtevant, J. M. (1977) Biochim. Biophys. Acta 466, 402–410.