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Time-resolved fluorescence of erythrocyte plasma membrane Ca^{2+}-ATPase in different functional states

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Abstract

The fluorescence decay of the plasma membrane calmodulin-activated Ca^{2+}-ATPase from the erythrocyte was measured for the first time. The availability of a novel procedure for on-line blank subtraction in frequency-domain lifetime data acquisition (G.G. Reinhart, B. Feddersen, D. Jameson and E. Gratton, Biophys. J. 57 (1990) 189a) permitted the elimination of background interference from detergent-solubilized purified plasma membrane ATPase samples. The fluorescence decay of the erythrocyte Ca^{2+}-ATPase was measured in the absence of Ca^{2+}, or in the presence of Ca^{2+} or Ca^{2+} plus calmodulin. In the three different experimental conditions the fluorescence decay was very heterogeneous and could be best described by Lorentzian distributions of lifetime values. In the absence of Ca^{2+} the decay was described by a broad lifetime distribution centered at 4.4 ns with a width of 3.2 ns, indicating heterogeneity of tryptophan microenvironments in the ATPase. Calcium ion binding promoted an 11% increase in the center and a 27% decrease in the width of the distribution. By contrast, addition of calmodulin in the presence of Ca^{2+} caused a 15% decrease in the center of the distribution, revealing structural difference between calmodulin-activated and Ca^{2+}-activated states of the ATPase. These results indicate the usefulness of on-line blank subtraction in frequency-domain lifetime measurements to investigate conformational changes in detergent-solubilized membrane protein samples.

Keywords: Erythrocyte plasma membrane; Ca^{2+}-ATPase; Conformational states; Calmodulin; Fluorescence lifetimes

1. Introduction

The kinetics of the erythrocyte plasma membrane Ca^{2+}-pumping ATPase have been well characterized both in the native membrane and in affinity-purified detergent-solubilized preparations [1,2]. However, detailed structural data on the conformational changes undergone by the ATPase during catalysis and on the correlation between conformational changes and function are still lacking.

Similarly to other transport ATPases, erythrocyte Ca^{2+}-ATPase undergoes isomerization between two major conformational states E_1 and E_2 [1,2], depending on Ca^{2+} binding to high-affinity sites on the enzyme. Fluorescence intensity changes have been used to follow Ca^{2+} binding

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to the ATPase [3]. The activity of the erythrocyte ATPase is further regulated by calmodulin (CAM) [1–4], and steady-state fluorescence measurements have been used to measure binding of CaM to the ATPase [5,6].

The purpose of this work was to measure the fluorescence decay of the erythrocyte Ca^{2+}-ATPase in different functional states, in order to assess the effects of activation by Ca^{2+} or CaM on the conformation of the ATPase. Such measurements had not been possible up to this date due to interference from background fluorescence in detergent-solubilized purified ATPase samples. A recent method for on-line blank subtraction in frequency-domain data acquisition [7] was used to permit accurate fluorescence lifetime measurements. The ATPase fluorescence decay was adequately described with Lorentzian lifetime distributions. Additions of Ca^{2+} or Ca^{2+} plus CaM to the ATPase produced significant changes in the fluorescence decay, revealing structural differences between these activated states.

2. Materials and methods

2.1 Materials

Ca^{2+}-ATPase was purified from erythrocyte ghosts [8] using $C_{12}E_8$ instead of Triton X-100. Bovine brain CaM was from Sigma. $C_{12}E_8$ was from Nikkol (Japan). All reagents were of the highest analytical grade available. Free Ca^{2+} concentrations were calculated as in [9].

2.2 Sample preparation

Ca^{2+}-ATPase was diluted to 40 μg/ml in 130 m*M* KCl, 4 m*M* EDTA, pH 7.4 and 0.05 mg/ml final $C_{12}E_8$, immediately before use. Where indicated, 50 μM free Ca^{2+} or 50 μM Ca^{2+} plus 0.57 μM CaM (sufficient to promote full activation of the ATPase, ref. [6]) were added. Samples were purged with argon and the cuvette was sealed to prevent photooxidation. Fluorescence emission spectra were recorded on an ISS (Champaign, IL) GREG-PC spectrofluorometer. No spectral changes were observed after lifetime measurements. Ca^{2+}-ATPase activity measurements [8] revealed that activity was fully preserved after lifetime measurements in the presence of EDTA, whereas in the presence of Ca^{2+} activity was significantly inhibited. Therefore, individual fresh samples were used for each experiment.

2.3 Lifetime measurements

Phase-modulation data were acquired at 22°C as described elsewhere [10] at 12 frequencies from 6 to 220 MHz. The accuracy in lifetime determinations, based on instrument response with standard deviations (σ) of $\pm 0.3°$ and ± 0.004 for measurements of phase angles and modulation ratios, respectively, was ± 0.1 ns (see also refs. [11] and [12]). Measurements were repeated at least three times with different ATPase preparations, yielding reproducible results. On-line blank subtraction was based on the determination of the background waveform and subtraction from the sample waveform [7]. Analysis of the ability of the background subtraction procedure to recover correct lifetimes indicated that this procedure is effective with samples containing background fluorescence ranging from 5 to 90% of the total signal intensity (G.D. Reinhart, P. Marzola, D.M. Jameson and E. Gratton, personal communication). In our case, background fluorescence was approximately 25–30% of the total intensity.

2.4 Data analysis

The Global Analysis software used was kindly provided by Drs. J. Beechem and E. Gratton (Laboratory for Fluorescence Dynamics, University of Illinois). Data were fitted with sums of exponential decays or continuous lifetime distributions [13]. Goodness of fit was estimated by calculation of reduced χ^2_R values, as:

$$\chi^2_R = \frac{1}{(2n - f - 1)} \times \sum \left[\frac{(\phi_m - \phi_c)^2}{\sigma^2_\phi} + \frac{(M_m - M_c)^2}{\sigma^2_M} \right] \quad (1)$$

where ϕ_c and M_c are the calculated values and ϕ_m and M_m are the measured values of phase and modulation at a given frequency; n is the number of frequencies; f is the number of fitting parameters. The phase method selects for the shorter-lived lifetimes, and the modulation method selects for the longer-lived ones. Thus, in heterogeneous systems the apparent lifetimes τ_p and τ_m obtained from phase and modulation measurements are smaller and larger than the average lifetime, respectively [14]. This and other systematic errors intrinsic to the phase-modulation technique contribute to the increase in minimized χ^2_R values [11,15]. Thus, statistical significances of different fits were not assessed by the absolute χ^2_R values. Rather, significances in improvements in fits when passing from a simpler to a more complex decay model were judged by calculating the F-statistics [16], as:

$$F = \frac{\left[\left(\chi^2_{R1} \cdot df_1\right) - \left(\chi^2_{R2} \cdot df_2\right)\right] / \left(df_1 - df_2\right)}{\chi^2_{R2}} \quad (2)$$

where χ^2_{R1} and df_1 are the χ^2_R and the degrees of freedom of the fit for the simpler model (i.e., the one with fewer fitting parameters). Confidence intervals for choosing a given model were obtained from F-statistics tables.

3. Results and discussion

Figure 1(A) shows experimental data obtained in multifrequency phase-modulation measurements with blank-subtraction for erythrocyte Ca^{2+}-ATPase in the unligated state, in the presence of Ca^{2+} or in the presence of Ca^{2+} plus CaM. Solid lines passing through data points were calculated with Lorentzian distribution or triple-exponential fits (see below). For either distribution or triple-exponential fits the residuals in phase angles and modulation ratios were randomly scattered about zero throughout the frequency range used (Fig. 1, panels B and C). Therefore, the choice of which model gave a better description of the fluorescence decay was based on the reasons presented below.

Table 1 summarizes the analysis of phase-modulation data for Ca^{2+}-ATPase with different decay models. Single-exponential decay analysis was inadequate for fitting the data, as evidenced by the very high χ^2_R values obtained (Table 1). Anal-

Table 1

Lifetime data analysis [a]

Additive	Single exponential	Double exponential		Triple exponential		Unimodal Lorentzian distribution
EDTA	$\tau = 4.3$ $\chi^2_R = 260.3$	$\tau_1 = 6.1$ $\tau_2 = 1.4$ $\chi^2_R = 18.64$	$f_1 = 0.80$	$\tau_1 = 9.6$ $\tau_2 = 3.6$ $\tau_3 = 0.5$ $\chi^2_R = 6.73$	$f_1 = 0.35$ $f_2 = 0.60$	$c = 4.4$ $w = 3.2$ $\chi^2_R = 7.09$
50 μM Ca^{2+}	$\tau = 4.7$ $\chi^2_R = 139.0$	$\tau_1 = 7.8$ $\tau_2 = 2.7$ $\chi^2_R = 16.57$	$f_1 = 0.59$	$\tau_1 = 11.8$ $\tau_2 = 3.8$ $\tau_2 < 0.1$ $\chi^2_R = 4.55$	$f_1 = 0.28$ $f_2 = 0.71$	$c = 4.9$ $w = 2.4$ $\chi^2_R = 6.22$
50 μM Ca^{2+} with 0.57 μM CaM	$\tau = 3.9$ $\chi^2_R = 271.0$	$\tau_1 = 5.6$ $\tau_2 = 1.2$ $\chi^2_R = 17.73$	$f_1 = 0.78$	$\tau_1 = 12.2$ $\tau_2 = 3.8$ $\tau_3 = 0.6$ $\chi^2_R = 1.54$	$f_1 = 0.20$ $f_2 = 0.72$	$c = 4.2$ $w = 2.6$ $\chi^2_R = 1.06$

[a] Data were acquired in the indicated experimental conditions as described in Section 2.2, and were analysed with different fluorescence decay models. τ_i: ith fluorescence lifetime component (in nanoseconds) recovered in multiexponential analysis; f_i: fractional intensity associated with the i^{th} lifetime component; c and w are the center and width values (in nanoseconds) of the Lorentzian lifetime distribution, respectively.

ysis with two or three exponential decays led to marked decreases in χ^2_R (Table 1), representing in either case a significant improvement (at a confidence level above 0.99) over single-exponential analysis. Triple-exponential analysis fit the data well, but a very complex pattern of changes was observed in lifetimes and fractional intensities when comparing the results obtained in different experimental conditions (Table 1). The existence of seven tryptophan residues in the ATPase [17] possibly explains the complexity in the fluorescence decay, and suggests that continuous distributions of lifetimes [18] could be useful to describe the fluorescence decay. Analysis with a Lorentzian distribution of lifetimes yielded similar χ^2_R values to those obtained with triple-exponential analysis (Table 1). Attempts to fit the fluorescence decay with bimodal Lorentzian distributions resulted in modest decreases in χ^2_R (not shown) as compared to χ^2_R values obtained in the unimodal Lorentzian analysis. *F*-statistics analysis indicated confidence levels of only 70–80% of significance in improvements in fit when passing from unimodal to bimodal Lorentzian distributions. In addition, attempts to use other

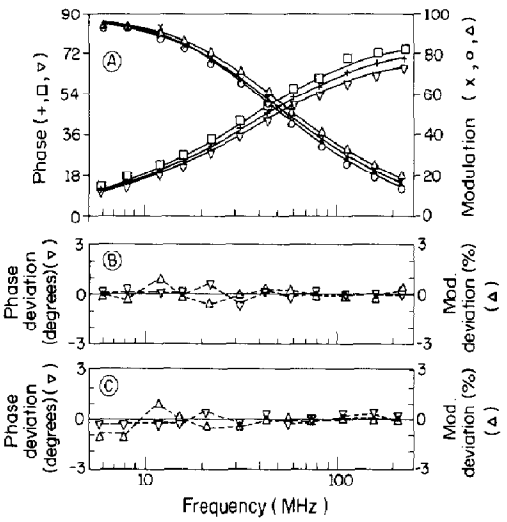

Fig. 1. (A) Multifrequency phase-modulation plot for erythrocyte ATPase. Data were acquired under the following experimental conditions: (+, ×) EDTA; (□, ○) 50 μM free Ca^{2+}; (▽, △) 50 μM free Ca^{2+} plus 0.57 μM CaM. Panels (B) and (C) show residual plots for Lorentzian distribution and triple-exponential fits, respectively, for (Ca^{2+} + CaM)-activated ATPase.

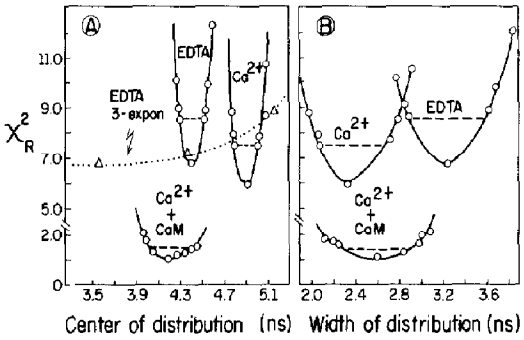

Fig. 2. χ^2_R surface analysis for parameters recovered in Lorentzian distribution fits. Dashed lines indicate the range of values encompassed by one standard deviation about the minima. Panel (A): centers of distributions; for comparison, triangles represent the surface obtained for the major (3.6 ns) component in the triple-exponential analysis of data for Ca^{2+}-free ATPase. Panel (B): widths of distributions.

distribution functions (e.g., Gaussian or gamma) yielded worse fits than those obtained with Lorentzian distributions.

It should be noted that despite the similarity in χ^2_R values obtained for unimodal Lorentzian and triple-exponential analysis, the distribution analysis uses only two fitting parameters (center and width of distribution), while triple-exponential analysis uses five fitting parameters. In order to choose between triple-exponential and distributed analysis we evaluated the accuracy and resolvability of the lifetimes obtained in the different experimental conditions tested. χ^2_R surfaces [15] associated with each fitting parameter recovered in the Lorentzian distribution fits were examined (Fig. 2). The sharp χ^2_R surfaces obtained indicate that recovered parameters were well-defined, and small deviations about the minima led to statistically significant increases in χ^2_R. Uncertainties in parameter determinations (dashed lines in Fig. 2, corresponding to the range of values encompassed by a 67% confidence interval that the recovered parameter lies within that range) were less than 4% for distribution centers (panel A) and less than 12% for widths (panel B). For comparison, Figure 2(A) also shows χ^2_R surface (triangles) obtained for the major lifetime component in the triple-exponential analysis of Ca^{2+}-free ATPase; it can be seen that this surface is strikingly flatter than the cor-

responding surface for the distribution analysis, indicating much larger uncertainties in parameter determination.

When lifetime measurements were performed without using the blank-subtraction procedure, recovered lifetimes were poorly resolved as indicated by χ^2_R surface analysis and were dominated by significant and very heterogenous contributions from background fluorescence (not shown). Thus, χ^2_R surface analysis indicated that the blank-subtraction procedure was necessary to eliminate background interference and to permit accurate measurements of the ATPase fluorescence decay (Fig. 2). Blank subtraction is a routine procedure in time-domain fluorometry. By contrast, despite the increasing application of frequency-domain fluorometry in the investigation of complex biological systems, the on-line procedure for fluorescence background elimination that we used here was only developed very recently [7]. As shown here, this procedure should be useful for the study of membrane proteins in detergent solution by frequency-domain fluorometry, which is generally hampered by the presence of background fluorescence contributions originating from impurities or decomposition of detergents or phospholipids.

Figure 3 shows the fluorescence lifetime distributions recovered from the data of Fig. 1. The solid line is the curve obtained in the absence of Ca^{2+}; the fluorescence decay was very heterogeneous, and was best described with a Lorentzian distribution centered at 4.4 ns with a width of 3.2 ns. It is interesting to note that the lifetime

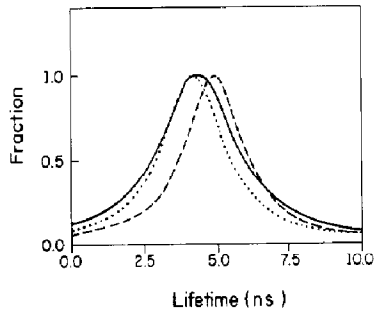

Fig. 3. Fluorescence lifetime distributions for erythrocyte ATPase. Experimental conditions: EDTA (solid line), 50 μM free Ca^{2+} (dashed line), 50 μM free Ca^{2+} plus 0.57 μM CaM (dotted line).

distribution width of 3.2 ns found for the erythrocyte ATPase is the same as width reported for sarcoplasmic reticulum ATPase, which contains 13 tryptophan residues [11]. The broad lifetime distribution observed in the erythrocyte ATPase likely indicates heterogeneity of microenvironments detected by the seven tryptophan residues. Addition of Ca^{2+} (dashed line) promoted an 11% increase in the average lifetime (center of distribution) and a 27% decrease in the width of the distribution (Fig. 3, Table 1). These results are in excellent agreement with those reported for sarcoplasmic reticulum Ca^{2+}-ATPase [11], and suggest that the conformational changes induced by Ca^{2+} in these two ATPases may be similar. The Ca^{2+}-induced increase in lifetime is in qualitative agreement with Ca^{2+}-induced increases in erythrocyte ATPase fluorescence intensity [3]. It has recently been shown [5] that Ca^{2+} enhances the quenching of fluorescence of erythrocyte Ca^{2+}-ATPase by hydrophillic quenchers, possibly reflecting an increase in exposure of tryptophan residues to the solvent. Thus, the increase in fluorescence lifetime promoted by Ca^{2+} binding (this report) may be due to removal of tryptophan residues from the vicinity of amino-acid sidechains that quenched the fluorescence within the apoprotein. It remains to be seen whether the decrease in heterogeneity of the decay (width of the distribution) promoted by Ca^{2+} is related to the internal dynamics of the protein [19] or to decreased heterogeneity of microenvironments detected by tryptophan residues in the ATPase.

Addition of CaM to the ATPase in the presence of Ca^{2+} (Fig. 3, dotted line) produced a 15% decrease in the average lifetime, without affecting the width of the distribution compared to that observed in the presence of Ca^{2+} alone. The decrease in lifetime may be due to interaction of CaM with a tryptophan residue which is known to be located within the CaM-binding domain of the ATPase [20]. Interaction of CaM with this tryptophan residue has been suggested by fluorescence quenching measurements [5].

In conclusion, this work shows that it is possible to make accurate measurements of the fluorescence decay of detergent-solubilized ATPase by the use of a blank-subtraction procedure for

frequency-domain lifetime measurements. The ATPase fluorescence decay was very heterogeneous and was described with Lorentzian lifetime distributions. Our results show for the first time that the fluorescence decay of a plasma membrane CaM-regulated ATPase is affected differently by the addition of Ca^{2+} alone or Ca^{2+} plus CaM to the ATPase. This finding is in line with recent circular dichroism results [5], which showed that binding of Ca^{2+} or Ca^{2+} plus CaM promoted different changes in secondary structure of the ATPase. These data lead us to conclude that interaction with CaM produces significant changes in the tertiary structure of the ATPase molecule which may be related to a distinct activation state of this enzyme.

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