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Pyruvate kinase from the thermophilic eubacterium *Bacillus acidocaldarius* as probe to monitor the sodium concentrations in the blood

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Abstract

We describe the isolation and characterization of a pyruvate kinase from the thermophilic eubacterium *Bacillus acidocaldarius*. This protein appears to be a tetramer composed of four 55-kDa subunits. The intrinsic tryptophan fluorescence of this protein is quenched by approximately 20% upon binding sodium, which occurs with a dissociation constant near 15 mM. Importantly, the intrinsic fluorescence of this pyruvate kinase does not appear to be affected by potassium, magnesium, and calcium at the concentrations found in whole blood. It appears that this pyruvate kinase can provide the basis for a selective protein sensor for sodium with minimal interference from other cations.

Keywords: Pyruvate kinase; *Bacillus acidocaldarius*; Fluorescence; Protein sensors; Sensing; Sodium; Potassium; Lifetimes; Time-resolved fluorescence

1. Introduction

The isolation and characterization of microorganisms living well above 100°C have posed intriguing questions about the biological means and mechanisms of survival in environmental conditions where other life-forms are rapidly killed. These microorganisms are also important as sources of thermostable enzymes for use in biotechnology because of their high resistance to heat, organic solvents and detergents. In fact, one main reason for great interest in thermo-resistant enzymes is their use in bioprocessing, such as the acceleration of reaction rates catalyzed by en-

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zymes, the preservation from microbial contaminations, and the sterilization of the end products in the course of the reaction [1–3]. Characterization of thermostable enzymes is also of interest in studies of the molecular basis of protein stability, which is a central problem in protein biochemistry.

Enzymes from thermophiles have an optimal activity temperature around the optimal growth temperature of the organism from which they are derived. However, we have recently demonstrated with β-glycosidase isolated from *Sulfolobus solfataricus,* with an optimal activity over 95°C, that the enzyme K_m does not vary from 10 to 90°C [4]. This result suggests that other thermophilic enzymes will display binding of substrates and cofactors at ambient temperature, suggesting their use as protein biosensors.

In our laboratories we have developed methodology for the preparation of adequate quantities of proteins from extremophiles microorganisms by isolating and cloning their genes following by expressing them in *E. coli* and/or yeast [5–7]. Because of their thermostability, the expressed proteins can be conveniently purified by using thermal denaturation steps that can easily be scaled-up in an industrial process [8].

Practical biosensors require that the proteins be stable under a wide range of environmental conditions, as their replacement accounts for most of the operating costs. Chemical modification of enzymes to make them more stable and specific is likely to become very important in the next generation of biosensors. The use of proteins or enzymes from extreme thermophilic organisms is a possible alternative [8].

Pyruvate kinase (PK) (ATP-pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) catalyzes the essentially irreversible transphosphorylation from phosphoenolpyruvate (PEP) to ADP, a reaction that requires magnesium and potassium ions [9–11]. However, although the vast majority of pyruvate kinases studied to date require activation by monovalent cations, some microbial pyruvate kinases lack this property [12–16]. In particular, Laughlin et al. [17] recently reported that the pyruvate kinase from *Corynebacterium glutanicum* and *Escherichia coli*, which do not require K^+ for activation, possess the replacement of Glu 117–Lys 117 in the cation-binding site. The proximity of Glu 117 to the potassium-binding site in the rabbit pyruvate kinase, and the conservation of the binding site in the two bacterial enzymes which lack a dependence on monovalent cations, suggested that a protonated ε-amino group of Lys 117 in these bacterial enzymes may provide an 'internal monovalent cation' [17]. This suggested to us that pyruvate kinases from other thermophilic organisms may show specific binding of sodium or potassium.

Measurements of sodium and potassium in blood are a routine part of clinical blood analysis, and it would be valuable to have simple optical methods for rapid point-of-care testing. A variety of fluorescent probes have been developed which respond to Na^+ and/or K^+ [18–26]. Most of these responses are based on partially selective binding of these cations to crown ethers [27,28], and most display association constants suitable for intracellular measurements when the Na^+ and K^+ concentrations are near 6 and 120 mM, respectively (Table 1). In blood the extracellular concentrations of Na^+ and K^+ are near 140 and 4 mM, respectively. Measurement of Na^+ and K^+ in blood is particularly difficult given the 25-fold excess of chemically similar potassium ions. Because of this difficulty, we are only aware of one report describing the use of fluorescent probes to measure potassium at the concentration present in blood [29]. Absolute specificity for either cation seems unlikely, so that additional methods to determine Na^+ or K^+ in blood are useful for correction for the non-specific responses of the Na^+ or K^+ sensors.

Table 1
Apparent cation dissociation constants and Hill coefficients for pyruvate kinase[a] from *Bacillus acidocaldarius*

Cation	K_D (mM)	n
Na^+	27	1.08
K^+	–	–
Ca^+	2.1	1.44
Mg^+	2.1	1.97

[a]Determined from the steady state intensities at 350 nm, 295 nm excitation.

In the present report we describe the purification and characterization of a pyruvate kinase from *Bacillus acidocaldarius* [30].

The enzyme molecular mass, determined by gel filtration, was 240 kDa. Moreover a band corresponding to a molecular mass of approximately 55 kDa was obtained by SDS-PAGE, indicating that this pyruvate kinase is a tetramer, composed of four similar subunits, each of which possesses a molecular weight of approximately 55 kDa. The enzyme was very stable respect to the temperature and exhibited the interesting ability to bind sodium, magnesium and calcium ions, but the binding of Mg^{2+} and Ca^{2+} appears to occur at cation concentrations higher than present in whole blood. Additionally, titration by potassium ions did not result in any variation of the fluorescence features of the enzyme. This suggested using the fluorescent properties of *Bacillus acidocaldarius* pyruvate kinase (PK) to monitor sodium in blood under physiological conditions.

2. Materials and methods

2.1. Organism and growth

Bacillus acidocaldarius, a thermoacidophilic eubacterium with optimal growth temperature at 60°C at pH 4.0, was originally isolated from hot springs at Agnano, near Naples (Italy). The microorganism was grown at 60°C with an aeration flux of 200 ml min^{-1} (broth l)$^{-1}$ in a 150-l stainless steel prototype fermentor supplied by Bioindustrie Mantovane (Italy). The fermentor was inoculated to 1:20 v/v with a 16-h broth culture. The complex culture medium contained (g l^{-1}): KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 1.3; $MgSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot H_2O$, 0.15; yeast extract, 1.0; Casaminoacids, 1.0; Sucrose, 2.0. The pH was adjusted to 4.0 with 0.1 M H_2SO_4. The biomass obtained after a 19-h growth was 1.5 g l^{-1} wet wt. Cells were harvested by centrifugation at 20 000 rev. min^{-1} (14°C) in the logarithmic phase of growth, using a Padberg model Z41 continuous-flow centrifuge. Cell past was stored at −80°C until use.

2.2. Assay method

The pyruvate kinase activity was monitored by using the method described by Bucher et al. [31] following the changes in the absorbance at 340 nm due to the NADH reduction. The enzymatic assays were carried out at 65°C in sealed quartz cuvettes.

2.3. Purification of pyruvate kinase

All procedures were performed at room temperature, except for the preparation of cell extract which was run at 4°C.

2.4. Preparation of cell extract

Frozen cells (50 g) were broken with 60-g glass beads Type I (Sigma) plus 100 ml of 20 mM Tris–HCl, 10 mM KCl, 1.0 M NaCl (pH 7.5) in a refrigerated Sorvall Omni-Mixer for three cycles 5.0 min at high speed with 10-min intervals. The glass beads were removed by centrifugation at 3000 rev. min^{-1} for 20 min in a Beckman rotor. The supernatant was centrifuged at 20 000 rev. min^{-1} for 90 min at 4°C. The supernatant represented the crude extract.

2.5. Precipitation by ammonium sulfate

Solid ammonium sulfate (516 g l^{-1}) was added to the previous supernatant to obtain 75% saturation; the pH was maintained at 7.5 by the use of 100 mM Tris–HCl (pH 7.5). The precipitate was redissolved in 10 ml of 10 mM Tris–HCl (pH 7.5) and dialyzed twice against 1000 ml of 10 mM Tris–HCl (pH 7.5) for 24 h at 4°C.

2.6. Affinity chromatography

The enzyme solution was loaded at a flow rate of 30 ml h^{-1} on a Blue-Sepharose CL6B column (Pharmacia), previously equilibrated with 10 mM Tris–HCl (pH 7.5). The column was washed with 100 ml of buffer Tris–HCl (pH 7.5) and a step of 2 mM NADH in the same buffer was applied to the column.

2.7. Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the pyruvate kinase purity as well as information on the subunit molecular weight. A 10% (w/v) separation slab gel was used with Laemmli's continuous buffer system [32]. Phosphorylase *b* (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa), were used as standards. Proteins were stained with Coomassie Brilliant Blue R-250.

2.8. Molecular mass determination

In order to estimate the molecular mass of the pyruvate kinase, gel filtration was carried out by the procedure described by Andrews [33]. An aliquot of 0.2 ml of the enzyme solution (2.0 mg ml^{-1}) was loaded on a Superdex G-200 column by a FPLC system, at a flow rate of 0.6 ml min^{-1}. The column was previously equilibrated with 50 mM sodium phosphate, 0.15 mM NaCl (pH 6.8) and calibrated with the following standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

2.9. Spectroscopy measurements

Steady-state emission spectra were measured on a SLM AB2 spectrofluorometer, 295 nm excitation. For intensity measurements during titrations with cations the intensities were measured at 350 nm.

Frequency-domain (FD) intensity and anisotropy decays were measured with instrumentation described previously [34,35]. Excitation was obtained with a cavity dumped rhodaminer 6G dye laser which was frequency doubled to 297 nm. The emission was measured with magic angle polarizer conditions using a 345-nm cut-off filter and a Corning 7–60 color glass filter.

3. Theory and data analysis

3.1. Time-resolved measurements

The frequency-domain intensity decays were analyzed in terms of a multi-exponential decay

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i are the amplitudes; τ_i the decay times are $\sum \alpha_i = 1.0$ [35]. The frequency domain anisotropy decay data were analyzed in terms of the multi-correlation time model

$$r(t) = r_0 \sum_i g_j \exp(-t/\theta_j) \quad (2)$$

where θ_j are the correlation times which display an amplitude r_{0j}. The sum of the r_{0j} values is the observed time-zero anisotropy

$$r(0) = r_0 \sum_j g_j \quad (3)$$

where g_j is the amplitude associated with the rotational correlation times.

Least-squares analysis was performed according to Lakowicz and co-workers [36,37]. For fitting and calculating of the goodness-of-fit χ^2_R the errors in phase ($\delta\phi$) and modulation (δm) were taken as $\delta\phi = 0.3°$ and $\delta m = 0.008$.

3.2. Sodium binding constants

The frequency-domain data for different Na^+ concentrations, with and without a presence of K^+, were globally fitted to the triple-exponential fluorescence decay model. For these fits the lifetimes were taken as global parameters and the amplitudes varied at each cation concentration.

The apparent sodium dissociation constants K_D were obtained from the fractional amplitudes (α_i), by assuming a two-state system. In the absence of sodium the fluorescence decay of the protein is characterized by a set of global lifetimes τ_i and amplitudes α_i^0. In the presence of a saturating concentration of Na^+ the fluorescence is de-

scribed by the same set of lifetimes with amplitudes α_i^{sat}. At intermediate sodium concentrations, we observe a linear combination of these states, i.e. the amplitudes α_i follow the formula,

$$\alpha_i = f^0\alpha_i^0 + (1-f^0)\alpha_i^{sat} \quad (4)$$

The fraction f^0 of the free protein is described by

$$f^0 = \frac{1}{1+[Na^+]/k_D} \quad (5)$$

By combining Eqs. (4) and (5) we obtained the fractional amplitudes α_i as a function of the sodium concentration

$$\alpha_i = \frac{\alpha_i^0 + \alpha_i^{sat}[Na^+]/K_D}{1+[Na^+]/K_D} \qquad i = 1,2 \quad (6)$$

$$\alpha_3 = 1 - \alpha_1 - \alpha_2 \quad (7)$$

Fitting of the recovered α_i values from the global fitting of the experimental data by Eq. (6) yields a global value for K_D. This secondary fitting procedure was repeated for data sets corresponding to a different fixed K^+ concentrations giving dependence of sodium K_D on the K^+ concentration.

The steady-state fluorescence titration curves were also fit using an equation similar to Eq. (6),

$$\frac{I}{I_0} = \frac{1+(I^{sat}/I^0)([Na^+]/K_{d,app})^N}{1+([Na^+]/K_{d,app})^n} \quad (8)$$

where I^0 and I^{sat} is a fluorescence intensity of PK in the absence and presence of a saturating concentration of the particular ion, respectively, and n is a Hill coefficient to account for an apparent cooperativity of the ion binding.

4. Results

Emission spectra of pyruvate kinase are shown in Fig. 1. The emission maximum is characteristic of partially shielded tryptophan residues. The

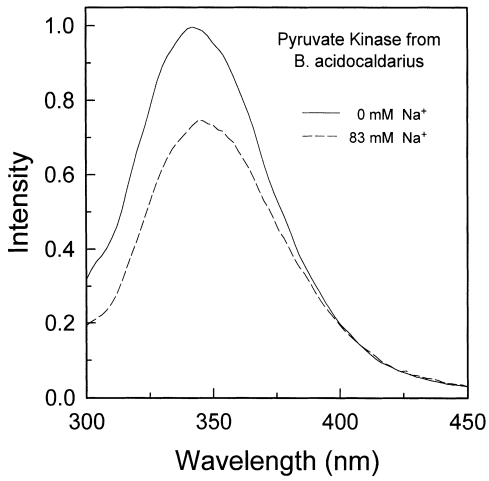

Fig. 1. Steady-state fluorescence spectra of pyruvate kinase from *Bacillus acidocaldarius* in absence (solid line) and presence (dashed line) of 83 mM NaCl.

shoulder near 305 nm may reflect some tyrosine emission, but such emission would be unusual for excitation at 295 nm. The steady state intensity of pyrvuate kinase decreases by approximately 25% upon saturation with sodium.

The cation-dependent intensities were used to determine the apparent cation binding constants of pyruvate kinase (Fig. 2 and Table 1). These data (Fig. 2) were fit to Eq. (7). One notices that K^+ binding is very weak. This result is in agreement with the probable substitution of a lysine at position 117 which serves as an internal cation [17]. Mg^{2+} and Ca^{2+} also caused a decrease in the emission intensity of pyruvate kinase. Comparison of typical cation concentrations in blood (see Table 2), reveals that only sodium is present in concentrations adequate to alter the emission intensity of pyruvate kinase. Hence, this pyruvate

Table 2
Typical concentrations of cations inside cells and in whole blood

Cation	Intracellular (mM)	Whole blood, extracellular (mM)
Na^+	4–10	135–148
K^+	100–140	3.5–4.5
Ca^+	50–200 nM	4.5–5.5 nM
Mg^+	0.5–2	–

kinase, or this pyruvate kinase labeled with an extrinsic fluorophore, may provide a protein biosensor specific for sodium.

We questioned whether the intrinsic tryptophan decay of pyruvate kinase changed upon binding of cations. Fig. 3 shows the frequency domain intensity decays for PK with increasing concentrations of Na^+. The frequency responses with Na^+ shift to higher frequencies which reflect a decrease in the mean lifetime on sodium binding (Table 3). The data for all the sodium concentrations were fit globally assuming the decay times were independent of Na^+ binding, but the amplitudes were variable. The data were well matched under this assumption (Table 3). However, we note that we may obtained a similar fit using the α_i values and global sodium-dependent lifetime.

We also examined the frequency response of PK in the presence of potassium (Fig. 4). In this case the intensity decay was essentially unchanged at 200 mM K^+, in agreement with the absence of an intensity change shown in Fig. 2.

The effects of sodium binding on the intensity decay of PK are best seen by examining the Na^+-dependent amplitudes. These amplitudes are shown in Figs. 5–7 for three concentrations of K^+. In all cases we observed a Na^+-dependent decrease in the amplitudes of the two longer

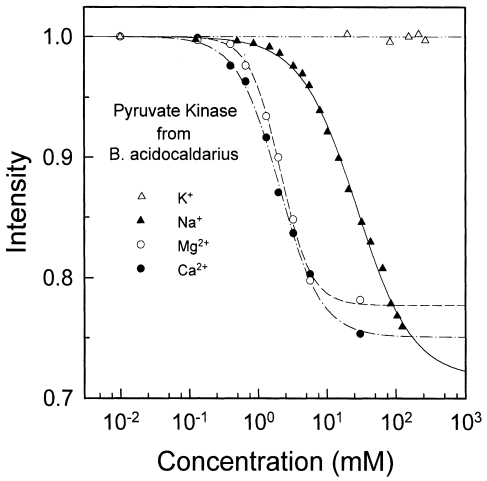

Fig. 2. Steady-state fluorescence titration curves of pyruvate kinase from *Bacillus acidocaldarius*. The lines represent the best fit of the data to Eq. (7).

decay times of 2.6 and 6.7 ns, and an increased amplitude of the short component with a 0.47 ns decay time. In the presence of Na^+ one or more of the tryptophan residues appear to move closer to some quenching moiety in the protein.

We used the amplitudes in Figs. 5–7, and Eq. (6), to determine the apparent Na^+ dissociation constants for PK. The dissociation constant is near 15 mM, and decreases on the increasing the

Table 3
Fluorescence anisotropy decay of pyruvate kinase from *Bacillus acidocaldarius* in the absence and presence of Na^+ ions

$[Na^+]$	0 mM		80 mM
τ_1 (ns)[a]		0.47 (0.02)[d]	
τ_2 (ns)[a]		2.6 (0.1)[d]	
τ_3 (ns)[a]		6.7 (0.2)[d]	
α_1	0.314 (0.009)[d]		0.567 (−0.008, +0.007)[d]
α_2	0.47 (0.01)[d]		0.347 (0.007)[d]
α_3	0.21 (0.02)[d]		0.086 (−0.008, +0.009)[d]
ϕ_1 (ns)	0.038 (0.003)[e]		0.056 (0.003)[e]
ϕ_2 (ns)	26.5 (1.1)[e]		26.2 (1.5)[e]
r_0[b]	0.28		0.28
g_2	0.428 (0.002)[e]		0.399 (0.003)[e]
χ^{2c}_R	1.05		1.57

[a] Global parameters from fitting of 15 frequency-domain decay curves.
[b] A fixed parameters for $\lambda_{exc} = 297$ nm.
[c] For $\delta\theta = 0.2°$ and $\delta m = 0.005$.
[d] Standard deviations were estimated by a Monte-Carlo method for $\delta\phi = 0.3°$ and $\delta m = 0.008$.
[e] Standard deviations were estimated by a Monte-Carlo method for $\delta\phi = 0.2°$ and $\delta m = 0.005$.

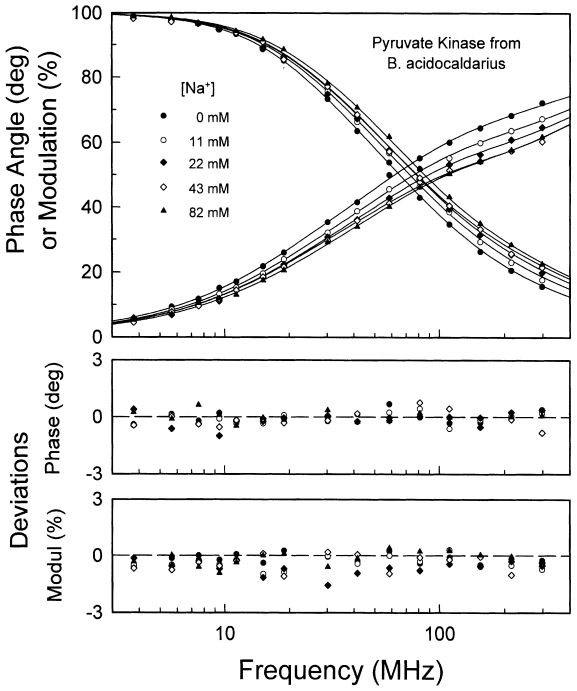

Fig. 3. Global frequency-domain fluorescence decay curves of pyruvate kinase from *Bacillus acidocaldarius* as a function of sodium concentration. Solid lines represent the best global fit of the data.

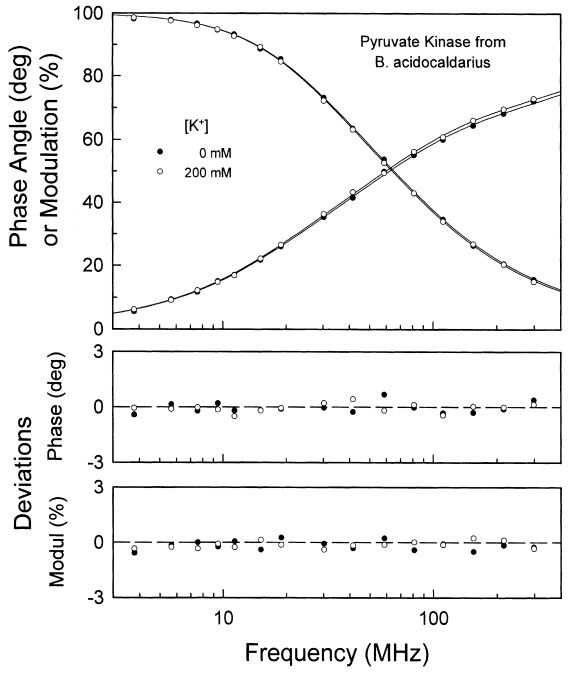

Fig. 4. Frequency-domain fluorescence decay curves of pyruvate kinase from *Bacillus acidocaldarius* in the presence (○) and absence (●) of 200 mM K^+. Solid lines represent the best global fit of the data. The χ^2_R value was 2.4 for a global fit of 15 data sets, five sets each for potassium concentrations of 0, 20 and 200 mM. In each set the sodium concentrations were 0, 11, 22, 43 and 82 mM. Only the extreme curves for 0 mM Na^+ are shown.

potassium concentration. In particular, the data shown in Figs. 5–7 point out that PK has an apparent Na^+ dissociation constant of 8 mM and 4 mM in the presence of 20 and 200 mM potassium, respectively. The absence of a strong effect of potassium over a wide range of concentrations indicates that PK can be used as a sodium sensor without significant interference from potassium. Additional studies are needed to determine whether other species in blood affect the binding of sodium to PK.

We also examined the FD anisotropy decay of PK with 0 and 80 mM Na^+ (Fig. 8). The tryptophan anisotropy decays were similar, suggesting little changes in protein dynamic upon Na^+ binding. However, the intrinsic tryptophan fluorescence is only sensitive to dynamic processes occurring on a timescale comparable to its decay time. For a molecule the size of PK, 240 kDa, the overall rotational correlation time is expected to

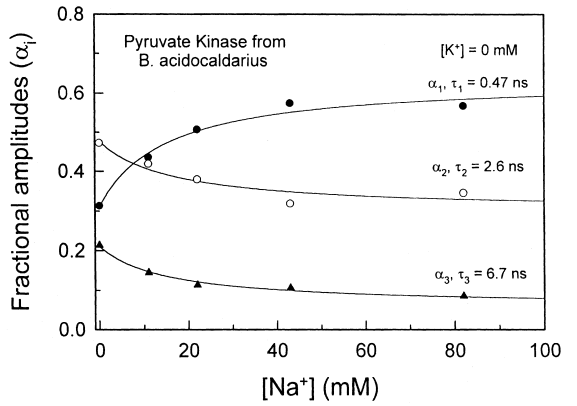

Fig. 5. Global fractional amplitudes of pyruvate kinase from *Bacillus acidocaldarius* as a function of sodium concentration. Solid lines represent a best global fit of the amplitudes to Eq. (6).

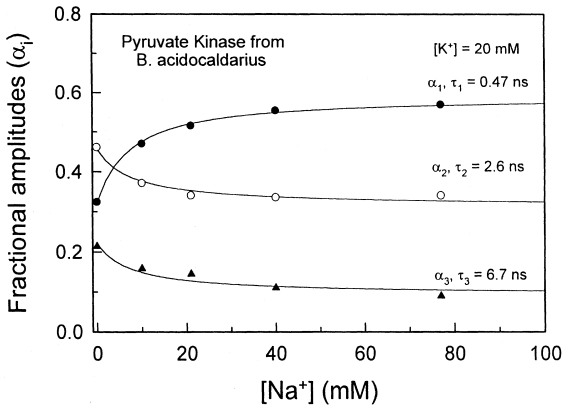

Fig. 6. Global fractional amplitudes of pyruvate kinase from *Bacillus acidocaldarius* as a function of sodium concentration in a presence of 20 mM K^+. Solid lines represent a best global fit of the amplitudes to Eq. (6).

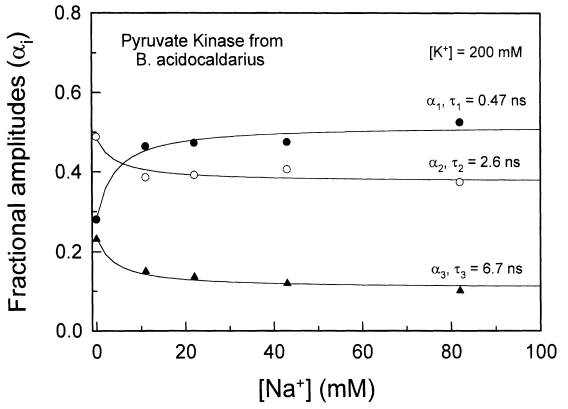

Fig. 7. Global fractional amplitudes of pyruvate kinase from *Bacillus acidocaldarius* as a function of sodium concentration in a presence of 200 mM K^+. Solid lines represent a best global fit of the amplitudes to Eq. (6).

be near 120 ns. The FD anisotropy data revealed a dominant correlation time near 26 ns (Table 3), which is too small for overall rotational motion, but comparable to that expected for motions of the 55-kDa monomers. If changes in structure or dynamics of regions of the protein occur upon Na^+ binding, such changes may be observed using longer decay time probes.

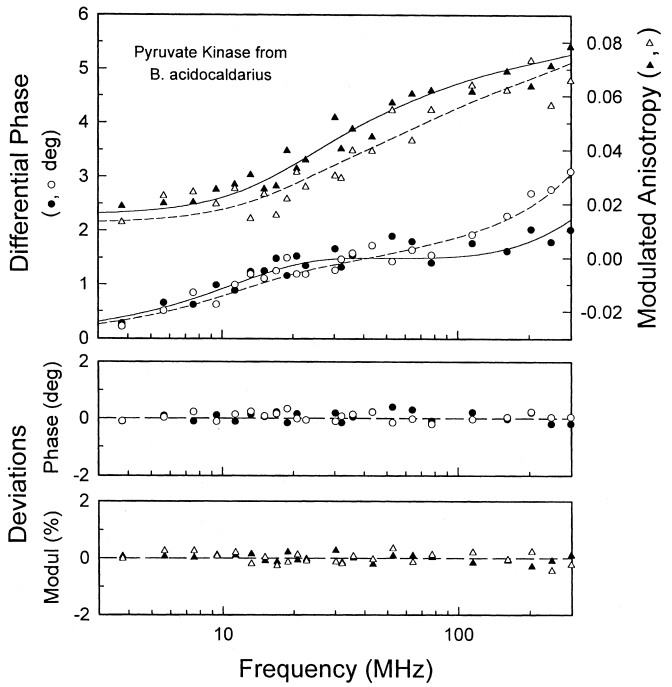

Fig. 8. Frequency-domain fluorescence anisotropy decay of pyruvate kinase from *Bacillus acidocaldarius* in the presence (opened symbols) and absence (closed symbols) of 80 mM Na^+. Solid lines represent the best fit of the data. $\delta\phi = 0.20$, $\delta m = 0.005$ and $\chi^2_R = 1.05$ and 1.57 at 0 and 80 mM.

5. Discussion

In the preceding section, we showed that the pyruvate kinase from *Bacillus acidocaldarius* was specific for sodium at the concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} present in whole blood. It seems probable that with cloning and insertion of cysteine residues for labeling, one could develop a protein sensor for blood sodium. However, the more important clinical need is for a potassium sensor. Blood potassium is routinely measured for hypertensive screening. Present technology does not provide rapid point-of-care measurements of K^+, and the measurements are more often performed in a central clinical laboratory.

The present report represents just the first step in developing a protein sensor for cations in blood. A number of pyruvate kinases are known, and most exhibit a requirement for monovalent cations [38–40]. It is also known that substitution of various charged amino acids can change the requirement for monovalent cations [40]. In summary, the high stability of thermophilic proteins is a valuable characteristic of proteins used as sensors [41]. The stability of the thermophilic pyruvate kinase, and their cation specificity, make these proteins ideal candidates for a new class of ion sensors.

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