Low Temperature Molecular Adaptation of the Skeletal Muscle Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase 1 (SERCA1) in the Wood Frog (Rana sylvatica)*

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We have compared the primary sequence and enzymatic properties of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPases from a cold-tolerant frog Rana sylvatica with those of a closely related cold-intolerant frog, Rana clamitans. Sarcoplasmic reticulum isolated from leg muscles of both species contains a major protein (~100 kDa) that reacts with a monoclonal antibody against sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase type 1 (SERCA1). The apparent molecular mass of R. sylvatica SERCA1 is 115 kDa, whereas that of R. clamitans is 105 kDa. However, the deduced amino acid sequences obtained from cDNAs do not indicate a difference in molecular weight, thus suggesting post-translational protein modification of R. sylvatica SERCA1. Comparison of the temperature dependence of both ATP hydrolysis and Ca\(^{2+}\) transport indicates that R. sylvatica SERCA1 exhibits significantly lower activation energy below 20 °C and an ~2-fold greater Ca\(^{2+}\)-ATPase activity near 0 °C. Furthermore, R. sylvatica SERCA1 exhibits simple Michaelis-Menten kinetics with ATP and Ca\(^{2+}\) as opposed to the two-site ATP kinetics and positive cooperativity with Ca\(^{2+}\) observed for R. clamitans and mammalian SERCA1s. Cooperativity has been linked to protein-protein interaction in SERCA1, and this property may be altered in R. sylvatica SERCA1. Primary sequence comparison shows that R. sylvatica SERCA1 exhibits seven unique amino acid substitutions, three of which are in the ATP binding domain. We also report for the first time the presence of alternative splicing in the frog, resulting in isoforms SERCA1a and SERCA1b. Thus, it appears that the low temperature muscle contractility of R. sylvatica can be explained partially by significant functional and structural differences in SERCA1.

There has been considerable interest in the mechanism by which the wood frog, Rana sylvatica, survives freezing. This frog is one of the few vertebrate species that can be frozen solid and revived upon thawing (1). R. sylvatica survives freezing by producing very high cryoprotectant concentrations of glucose in vital organs (2, 3). Furthermore, R. sylvatica mates and lays eggs in water temperatures approaching 0 °C. Because R. sylvatica muscle functions efficiently near 0 °C, the Ca\(^{2+}\) pump must also be functional at low temperatures. However, although the effects of freezing/thawing on several enzymes and physiological processes have been studied (4–6), no studies on the wood frog skeletal muscle Ca\(^{2+}\)-ATPase (SERCA1) have been reported. The kinetic properties and amino acid sequence of SERCA1 from Rana esculenta (European green frog) have been reported (7, 8) and are quite similar to those of the rabbit. However, R. sylvatica muscle could not function at near freezing temperatures if its Ca\(^{2+}\)-ATPase had the same properties as the rabbit because rabbit SERCA1 is almost totally inactive at 0 °C (9). Kossler and Kuchler (10) concluded that the main cause of severe inhibition of muscle contraction in the rat at 6 °C is the temperature-dependent inhibition of Ca\(^{2+}\) uptake and release. A similar conclusion was reached for another frog Rana temporaria at 10 °C (11). Thus, SERCA1 is likely to have altered properties in the skeletal muscle of the cold-tolerant frog R. sylvatica.

There is a vast amount of knowledge concerning structure/function relationships for SERCA1 because it is one of the most highly studied membrane-associated proteins. Structure/function relationships have recently been reviewed by MacLennan et al. (12). The protein was first purified in 1970 by MacLennan (13), and the sequence was deduced from the cDNA by MacLennan et al. (14). The complete three-dimensional structure has recently been obtained at 2.6 Å resolution by x-ray crystallographic analysis of protein crystals (15). This recent advance has confirmed the three-dimensional folding of the domains that had previously been proposed based on protein chemistry and analysis of two-dimensional arrays in membranes. An astonishing proportion of the amino acid residues predicted to be involved in SERCA1 function has been modified by site-directed mutagenesis and the effects on the function of the expressed protein were determined (12). However, there is no direct structural information regarding temperature dependence of the pump.

In the present study, we have compared structural and func-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ298901 for R. sylvatica and AJ298902 for R. clamitans SERCA1s.

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1 The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SR, sarcoplasmic reticulum; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; ds, double-stranded; FSBA, 5’-p-fluorosulfonfonylbenezoyladenosine.
tional properties of SERCA1s from R. sylvatica with those of Rana clamitans, a closely related frog living in the same environment that is cold-sensitive and cannot function at 0 °C. Our results indicate that there are significant differences in the kinetic properties of SERCA1 between these two species, including temperature dependence of ATP hydrolysis and Ca2⁺ transport, kinetics with ATP, and cooperativity of Ca2⁺/kinetins. We also report the complete amino acid sequences of these two SERCA1s.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**R. sylvatica and R. clamitans were collected from local ponds in accordance with the guidelines provided by the Institutional Animal Care and Use Committee. The chemicals used for SDS-polyacrylamide gel electrophoresis and immunoblotting were obtained from Bio-Rad. Chemicals, reagents were purchased from PerkinElmer Life Sciences. Monoclonal anti-SERCA1, clone VE121G9 was from Affinity BioReagents, Inc. (Golden, CO) as were the monoclonal anti-SERCA2 (MA3-910) clone ID8 and polyclonal anti-antibodies to anti-SERCA3 (PAI-910) antisera. All three antibodies exhibit cross-reactivity with several mammalian species. Restriction endonucleases were supplied by New England Biolabs, Inc. (Beverly, MA). The Expand® Long Distance PCR kit, which uses a mixture of Taq and Pwo polymerases, was from Roche Molecular Biochemicals. AFLP® Express Sequencing kit and cyanin dATP-Labeling Mix as well as cyanin-labeled sequencing primers were purchased from Amersham Pharmacia Biotech. Plasmid preparations were carried out using Qiagen columns (Qiagen, Hilden, Germany). Restriction fragments used for subcloning procedures were gel-purified with the QIAEX II Gel Extraction kit. pGEM®-T Easy vector (Promega, Madison, WI) was chosen for direct subcloning of all 5’ and 3’ RACE-PCR products. Gene-specific oligonucleotides were from Life Technologies, Inc. Other molecular biology grade chemicals were purchased from Sigma.

**Preparation and Analysis of Sarco/plasmatic Reticulum (SR)—**Leg muscles were removed from euthanized R. sylvatica captured in late February. Leg muscles were dissected, separated into SR fractions, homogenized using a hand held homogenizer, and centrifuged for 10 min at 100,000 g at 4 °C. Supernatants were used for determination of Ca2⁺ transport. SR membranes were purified from the homogenized muscle by sucrose density centrifugation using the procedure described by Bull et al. (16). SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (17), and immunoblotting was done as described earlier (18). Ca2⁺-ATPase activity was assayed using a system with ATP hydrolysis coupled to NADH oxidation by pyruvate kinase and lactate dehydrogenase. The components were the same as the coupling components, the assay contained 10 mM Tes buffer at pH 7.4, 0.1M KCl, 0.1M MgCl₂, EGTA, Mg²⁺, ATP, temperature and pH using the Winnmax program available on the world wide web (Chris Patton, Stanford University). Ca2⁺-transport was measured using the Ca2⁺-sensing dye, Rhod2, as described previously (20). The buffer components were the same as the coupling components listed above for the Ca2⁺-ATPase assay except that MgCl₂ was eliminated, and 2.5 μM Rhod2 was added. ATP (5 mM) was added as the MgATP complex. The assay was carried out at various temperatures in a PerkinElmer LS50B Luminescence Spectrometer with excitation at 553 nm and emission at 576 nm. Before the addition of membranes (a final concentration of 0.1 mg/ml), 0.5 mM monolatios of CaCl₂ were added to calibrate the assay and allow for the determination of Ca2⁺ transport rates. The addition of BrA235C at the end of each assay released all sequestered Ca2⁺ (20). Kinetic data were analyzed using Enzfitter© software (version 1.05), whereas curve fitting was accomplished using the Slide Write Plus© software (version 5.0). Protein concentration was estimated using biocinchonic acid (BCA Protein Assay kit, Pierce) with bovine serum albumin as a standard.

**Construction of Wood and Green Frog cDNA Libraries—**Uncloned adaptor-ligated double-stranded (ds) cDNA libraries were constructed using the Marathon™ cDNA amplification kit from CLONTECH (Palo Alto, CA) according to manufacturer's instructions. Total RNA was isolated from either an entire wood frog (weighing 1 g) at an early age or a green frog skeletal muscle (weighing 1 g) using the guanidinium thiocyanate/CsCl purification method (21). 10 μg of total RNA was used for each first-strand synthesis. The Marathon™ cDNA synthesis primer (52-mer) was extended with Moloney murine leukemia virus reverse transcriptase for 1 h at 42 °C, after which second-strand synthesis was carried out by adding an enzyme mixture containing RNase H, Escherichia coli DNA polymerase I, and E. coli ribonuclease H to 18 °C. A blunt-end of the ds-cDNA was performed with T4 DNA polymerase and CaCl₂ at 16 °C for 45 min. ds-cDNAs were purified by phenol/chloroform extraction precipitated by 95% ethanol in the presence of 4 M ammonium acetate. The pellet was washed with 80% ethanol and dissolved in sterile water. The Marathon™ cDNA adaptor was ligated to ds-cDNAs overnight at 16 °C using T4 DNA ligase.

**Rapid Amplification of 3’ and 5’ cDNA Ends by PCR (5’ and 3’ RACE-PCR)—**3’ and 5’ RACE-PCR amplifications were performed using the adaptor-ligated ds-cDNAs as template DNAs, a mixture of Taq and Pwo (with a proofreading activity) polymerases, a common Marathon adaptor-primer 1, and an internal gene-specific primer. For the 3’ RACE-PCR, a gene-specific primer PH+ (5’-CTCTGATAGACTGG-TACTCTGACCAC-3’) was derived from the nucleotide sequence coding for the phosphorylation site (5’-S2KGTGTT/PTT-3’) of the R. esculenta SERCA1a (7). The PH+ primer corresponds to nucleotides 1275–1301 in the R. esculenta cDNA sequence (numbered according to the sequence deposited under GenBank™ accession number X63099) and to nucleotides 1251–1277 and 1254–1280, respectively, in R. sylvatica and R. clamitans nucleotide sequences described in this study. However, the 3’ RACE-PCR of the R. esculentaSERCA1a cDNA in cold °C, in both R. sylvatica and R. clamitans cDNA sequences. The 3’ RACE-PCR amplifications with the PH+/adapter primer 1 primer set were carried out for 30 cycles, each cycle consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, and 4 min of extension at 68 °C. PCR products were size-fractionated by 1% agarose gel electrophoresis, visualized by ethidium bromide staining, gel-purified, and subcloned into the pGEM®-T Easy vector. For each frog, several clones were partially sequenced. To perform the 5’ RACE-PCR reactions, an antisense primer (5’R-) common for both frog cDNAs, was derived from the partially sequenced 5’-GTCCGACAGTTGGTGGCATCAG-3’. This primer corresponds to the inverse complement of nucleotides 1431–1458 and 1434–1461, respectively, in R. sylvatica and R. clamitans. Cloned fragments were sequenced using the adaptor primer 15’/R- primer set were performed using the above mentioned cycle conditions. The 5’ RACE-PCR products were also subcloned in pGEM®-T Easy vector. The composite wood and green frog cDNA nucleotide sequences in both strands were determined completely using several gene-specific primers and the A.L.F.® Express DNA Sequencing Kit (Amersham Pharmacia Biotech). The sequencing strategy is shown in Fig. 1. For further applications, cDNA clones containing the entire coding regions were obtained by PCR amplifications using the following pair of primers: cdNA+ (5’-GGCTGAGAGGGAGAGGGAGGAGGAAACGAC-3’) corresponding to nucleotides 189–216 and 191–219, respectively, in wood and green frog cdNAs and cdNA− (5’- TTATCCTCTAACGGTAGTTACGGCGAACA-3’) corresponding to the inverse complement of nucleotides 3181–3186 and 3186–3191, respectively, in R. esculenta and R. clamitans sequences. The cycle conditions were the same as above except for a longer extension time (5 min) per cycle) for the inverse complement of nucleotides. The amplified cDNA products (3.2 kilobase pairs) were subcloned in either pCR®3.1 (for green frog cdNA) or NOl cut and dephosphorylated pCDNA3 (for R. sylvatica cDNA) expression vectors (Invitrogen, San Diego, CA). The accuracy of the nucleotide sequence was verified by complete sequencing of several clones for each frog SERCA cDNA.

**Reverse Transcriptase-PCR Analysis of R. sylvatica SERCA1 Splice Variants—**The primers used to analyze the possibility of an alternative splicing event of the SERCA1 primary transcript are as follows: a 5’ primer, SPLICE+ (5’-GTAGAACGTITGGTGGTGTACTG-3’) corresponding to nucleotides 3093–3116 and a 3’ primer, SPLICE− (5’-GCTGAGCTTCTTCTTGTTGTTTAT-3’) corresponding to the inverse complement of nucleotides 3252–3250 in wood frog SERCA cDNA. The adapter-ligated ds-cDNA served as template DNA. PCR amplification was carried out for 35 cycles, each cycle consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The PCR products were subcloned in the pGEM®-T Easy vector, and several positive clones were sequenced.

**RESULTS**

**Analysis of R. sylvatica and R. clamitans SR—**SDS-polyacrylamide gel analysis of a Coomassie Blue-stained gel is shown in Fig. 1. The major protein in the SR of both R. sylvatica and R. clamitans is ~100 kDa. Both preparations contain lesser amounts of several other proteins. Immunoblot

9312 R. sylvatica SERCA1 Functions at Low Temperature
analysis with monoclonal anti-SERCA1 reveals that the major 100-kDa proteins are indeed SERCA1 (Fig. 1B). Antibodies against SERCA2 and SERCA3 (see “Experimental Procedures” for details) did not recognize any frog proteins. SERCA1 in R. sylvatica exhibits a molecular mass of 115 kDa, whereas that of R. clamitans is ~105 kDa. Thus, the SERCA1s differ in apparent mass between the two frog species. This is not a general property because the other SR proteins evident in Fig. 1A exhibit the same molecular weights. This difference in apparent mass could indicate the presence of post-translational modifications that affect the enzymatic properties of the proteins.

Kinetic analyses were carried out to determine whether there are functional differences between the two SERCAs. The specific activities at 37 °C of two preparations of R. sylvatica sarcoplasmic reticulum were 0.8 μmol (used in Figs. 1 and 2A) and 3.0 μmol of ATP hydrolyzed/min/mg (used in Figs. 2B, 3 and 4), whereas the specific activity of a single R. clamitans preparation was 2.7. These values are similar to those observed for rabbits (13).

Because we hypothesize that R. sylvatica SERCA1 is more active at 0 °C than that of R. clamitans, we analyzed the temperature dependence of SERCA1 Ca$^{2+}$-ATPase activity. ATP hydrolysis activity was determined at saturating ATP (10 mM) and Ca$^{2+}$ (100 μM), and the results were analyzed using the Arrhenius plot after calculating the relative activities normalized to the maximum rate at 37 °C. As shown in Fig. 2A, R. sylvatica SERCA1 has a higher relative activity at all temperatures tested, and activities for the two frogs at the two lowest temperatures (6 and 2 °C corresponding to 3.584 and 3.636 × 10$^{-2}$ 1/K) are significantly different at the p < 0.005 level t test. Calculation of the activation energies by linear fitting below 20 °C yields a significantly higher value for the green frog (28 ± 1.8 kcal/mol (mean ± S.E.) than for the wood frog (23.7 ± 0.6). Extrapolation to 0 °C indicates that the wood frog will exhibit a 2.1-fold higher activity than the green frog. Thus, although the activity is low, the wood frog exhibits significantly higher activity.

Fig. 2B shows a similar analysis for Ca$^{2+}$ transport data. Between 13 and 30 °C, the activation energy for Ca$^{2+}$ transport is again higher for the green frog, 21.7 ± 0.6 versus 19.0 ± 0.1 kcal/mol (mean ± S.E.), yielding an extrapolated value for Ca$^{2+}$ transport in the wood frog at 0 °C that is 1.8-fold greater than in the green frog. This result suggests a similar coupling of ATP hydrolysis to Ca$^{2+}$ transport over a wide range of temperatures.

Next, the dependence of ATP hydrolytic rate on ATP concentration was determined. Fitted plots of wood and green frog kinetics are shown in Fig. 3. The R. clamitans SERCA1 data fit a two-site model with $K_m$ of 0.005 and 0.34 m. The correlation coefficient ($r^2$) for the two-site model is 0.999, whereas attempting to fit the data to Michaelis-Menten kinetics yields a relatively poor correlation coefficient of 0.77. Thus, the green frog exhibits ATP kinetics quite similar to that described for rabbit SERCA1 (19). This behavior has been ascribed to half of the site reactivity in a SERCA1 dimer or additional ATP binding sites on a single SERCA1 monomer (22). Interestingly, the R. sylvatica SERCA1 data can be fitted with a simple single-site Michaelis-Menten model yielding a relatively poor correlation coefficient of 0.77. Thus, the green frog SERCA1 has a higher relative activity at all temperatures tested, and activities for the two frogs at the two lowest temperatures (6 and 2 °C corresponding to 3.584 and 3.636 × 10$^{-2}$ 1/K) are significantly different at the p < 0.005 level t test. Calculation of the activation energies by linear fitting below 20 °C yields a significantly higher value for the green frog (28 ± 1.8 kcal/mol (mean ± S.E.) than for the wood frog (23.7 ± 0.6). Extrapolation to 0 °C indicates that the wood frog will exhibit a 2.1-fold higher activity than the green frog. Thus, although the activity is low, the wood frog exhibits significantly higher activity.

The kinetics of stimulation of ATP hydrolysis by Ca$^{2+}$ was also analyzed, as shown in Fig. 4. As indicated by the error bars in the plot, there is a significant difference in the Ca$^{2+}$ dependence of Ca$^{2+}$-ATPase activity for the two frogs at both low and high Ca$^{2+}$ concentrations. The similarity in activities near the center of the plot indicate similar apparent affinities for Ca$^{2+}$ (0.5 μM). Fitting the plots to a model of positive cooperativity yields a Hill coefficient of 1.5 ± 0.2 (mean ± S.D.) for the green frog indicating a positive cooperativity, a model that has been well established for rabbit SERCA1 (23). In contrast, the wood frog data yield a Hill coefficient of 0.9 ± 0.2 (mean ± S.D.) indicating a lack of cooperativity. This result suggests a loss of protein-protein interactions in R. sylvatica SERCA1 because the positive cooperativity of Ca$^{2+}$ binding to rabbit SERCA1 has been shown to be lost when the protein is dissociated to monomers in C$_{12}$E$_8$ (23).

Characterization of cDNA Clones Coding for Wood and Green Frog SERCAs—To obtain the nucleotide sequence of the two
frog SERCA cDNAs, we have employed the RACE-PCR strategy (24), which allows the amplification of both 3' and 5' cDNA ends. To conduct the 3' RACE reactions, a gene-specific oligonucleotide (PH+) was designed from the phosphorylation site of the known sequence of the previously cloned cDNA of the European frog (*R. esculenta*). For both frog SERCA cDNAs, 3' RACE-PCR products (2.3 kilobase pairs) were obtained and sequenced. From the obtained sequence, a new primer (5'R-) was generated to amplify the 5' cDNA ends. The resulting PCR products (1.5 kilobase pairs) were subcloned and sequenced. Using several other sequencing primers, the 3' and 5' overlapping PCR products were completely sequenced in both strands. The composite nucleotide sequences of *R. sylvatica* and *R. clamitans* SERCA cDNAs were determined (data not shown) (GenBank™ accession numbers AJ298901 and AJ298902). The sequencing strategy is shown in Fig. 5. Wood and green
frog SERCA cDNAs are, respectively, 3790 and 3793 nucleotides in length. The nucleotide difference is due to the presence of three more nucleotides in the 5'-untranslated region of \( R. \) clamitans cDNA (206 versus 203 nucleotides in the wood frog sequence). The nucleotide sequence (AGGATGG) surrounding the translation initiation codon (ATG) is found in an acceptable context characteristic of initiation of eukaryotic translation (25). Two adjacent consensus polyadenylation signals (ATTA/AA/26) in the 605-nucleotide 3'-untranslated region of both frog SERCA cDNAs are located, respectively, 18 and 12 nucleotides upstream of the beginning of the poly(A) tract.

Amino Acid Sequence Analysis and Comparison with Other SERCA Pumps—The primary translation products deduced from the two composite nucleotide sequences are 994 amino acids in length. The predicted molecular masses of \( R. \) sylvatica and \( R. \) clamitans SERCA proteins are, respectively, 109.7 and 109.3 kDa. In Fig. 6, the deduced amino acid sequence of \( R. \) sylvatica SERCA is compared with the sequences of other SERCA pumps expressed in skeletal muscle from \( R. \) clamitans, \( R. \) esculenta, and rabbit. In pairwise comparisons, \( R. \) sylvatica SERCA exhibits 98% (982 of 994 amino acids) overall identity with green frog SERCA and 95% (948 of 994 amino acids) and 88% (879 of 994 amino acids) identity with \( R. \) esculenta and rabbit SERCA1a, respectively. The deduced \( R. \) sylvatica SERCA sequence clearly matches that of rabbit fast-twitch skeletal muscle isoform (SERCA1a) at two characteristic points of deviation between rabbit SERCA1a and SERCA2a (expressed in cardiac and slow-twitch muscles) isoforms. One of them is represented by the COOH terminus (ARNYLEG994), whereas the other consists of a 5-amino acid stretch (AAVGN941) located COOH-terminal to Arg905. Therefore, based on these considerations and the overall sequence identity, we suggest that the nomenclature used for \( R. \) sylvatica and \( R. \) clamitans \( \text{Ca}^{2+} \)-ATPases that are encoded by the cloned cDNAs be SERCA1a as it was proposed earlier for \( R. \) esculenta (7). The hydrophobic putative transmembrane domains (M1-M10), the phosphorylation site (residues 349-355), as well as sites known to bind fluorescein isothiocyanate or the ATP analogues FSBA and CIRATP in SERCA1 (27-29) are conserved in \( R. \) sylvatica SERCA1a. There are five nonconservative amino acid substitutions (Asn, Gln, Ala, Ser, and Leu) characteristic for \( R. \) sylvatica (Fig. 6 and Table I), respectively, at positions 26 (NH2 terminus region), 546, 547, 643 (nucleotide binding domain), and 838 (transmembrane domain M7). In the other SERCA pumps, these positions are occupied by highly conserved residues: Pro56 or Leu56, Leu546, Ser547, Gly548, and Met838.

Alternative Processing of the Wood Frog SERCA1 Primary Transcript—So far, no information suggesting the alternative splicing of SERCA1 primary transcript in frog species has been reported. We are now providing the first evidence for the expression of two SERCA1 splice variants in a frog species, \( R. \) sylvatica, by means of the PCR strategy using SPLICE+ and SPLICE− as primers and the adaptor-ligated ds-cDNA as template. After amplification, two products of 159 and 117 base pairs were detected by 1.8% agarose gel electrophoresis (data not shown). Subcloning and subsequent sequencing confirmed that the 159-base pair PCR fragment corresponded to SERCA1a, whereas the second smaller fragment represented a novel splice variant, SERCA1b. The retention or exclusion of an optional exon (Fig. 7) gives rise, respectively, to SERCA1a and SERCA1b splice variants. The insertion of the optional exon in wood frog SERCA1a occurs immediately after
nucleotide 2980 (relative to the ATG codon), which, interestingly, represents the point of divergence between the different splice variants in the related SERCA1, SERCA2, and SERCA3 genes. In turn, the nucleotide sequence divergence in SERCA1a splice variants is reflected at the protein level by a generation of SERCA1 isoforms with different COOH termini. Both isoforms present identical amino acid sequences up to amino acid 993 (Glu), after which the last residue (Gly994) in SERCA1a is replaced in SERCA1b by a tetrapeptide stretch (DKPQ997). The significance of the different COOH termini has still to be investigated.

**DISCUSSION**

The results presented herein may provide at least a partial explanation for the unusual low temperature muscle contractility of the wood frog *R. sylvatica*. *R. sylvatica* SERCA1 at saturating levels of ATP and Ca\(^{2+}\) exhibits a 2-fold higher Ca\(^{2+}\)-ATPase activity and an estimated 1.5-fold higher Ca\(^{2+}\) transport rate at 0 °C than *R. clamitans* (Fig. 2, A and B). Furthermore, *R. sylvatica* SERCA1 at 0.5 mM ATP has an activity 1.6-fold higher than that of *R. clamitans* (Fig. 3). Together, these differences in enzymatic activity may enable *R.
sylvatica SR to maintain muscle contractility near 0 °C in contrast to R. clamitans. The fact that excitation/contraction coupling is maintained in the wood frog at 0 °C signifies that very low Ca$^{2+}$-ATPase activity at this temperature (1.5% of the activity at 37 °C) is sufficient for SR function. The published physiological experiments indicate that in both rat and frog, loss of muscle function at low temperature results from the diminution of Ca$^{2+}$ release and re-uptake (10, 11). However, it is possible that the lower activity of green frog SERCA1 at 0 °C (0.7% of the activity at 37 °C) is not the rate-limiting step that prevents muscle function at this temperature in this frog.

Measurements of temperature dependence of rabbit SERCA1 activity in native SR membranes indicate an apparent discontinuity at −20 °C (32, 33), and the data shown in Fig. 2A can also be fit with two linear segments and a break at 20 °C. It was originally thought that this discontinuity in temperature dependence of ATP hydrolysis was a direct indicator of an abrupt change in the fluidity of the SR membrane (33). Subsequent work indicated that a lipid phase transition does not occur at 20 °C, and it was proposed that the discontinuity was a result of changes in lipid cluster formation (34) or in a tightly associated lipid annulus (35). In contrast, Dean and Tanford (19) showed that the discontinuity at 20 °C was maintained in the absence of phospholipid, which argues against any specific effect of lipid on SERCA1 at 20 °C. Nevertheless, it is clear that the activation energy for Ca$^{2+}$-stimulated ATP hydrolysis is higher for R. clamitans SERCA1 than for R. sylvatica below 20 °C (28 versus 23.7 kcal/mol). These values are in the published range for rabbit, lobster, scallop, and winter flounder (32, 36–38). The Arrhenius plots of ATPase activity versus temperature for the cold-tolerant scallop, lobster, flounder, and trout are similar to that of the rabbit, but they show modest shifts to lower activation energies below 20 °C as is reported here for R. sylvatica. Interestingly, trout SERCA1 showed a nearly constant activation energy from 4 to 37 °C for phosphoenzyme formation from phosphate resulting in much higher levels of this activity at 4 °C than that observed for rabbit. The temperature dependence of phosphoenzyme formation in lobster, scallop, winter flounder, or frog has not been reported. Similarly, the cooperativity of kinetics with Ca$^{2+}$ and ATP observed to be different in R. sylvatica compared with R. clamitans or rabbit has not been described for lobster, scallop, or winter flounder. Kalabokis and Hardwicke (36) concluded that the lipid environment contributed to the differences in properties observed between rabbit and scallop SERCA1 because scallop SERCA1 solubilized in C12E8 exhibited an Arrhenius plot similar to that of solubilized rabbit SERCA1. However, in the trout it was implied that differences in the temperature dependence of phosphoenzyme formation reside in the protein itself and not in the lipid environment due to the inability of C12E8 to alter the temperature dependence of phosphoenzyme formation (37). Thus, the role of the phospholipid environment in altering temperature dependence of SERCA1 in the SR of cold-tolerant species is not established. This is an area for future work on R. sylvatica SR.

Several lines of evidence indicate that SERCA1 is aggregated in its native SR and that this interaction has a functional significance. Intrinsic fluorescence, fluorescence energy transfer, chemical cross-linking, and analytical ultracentrifugation all indicate a propensity for aggregation of SERCA1 (30). The positive cooperativity of Ca$^{2+}$ binding has been proposed to be a result of protein-protein interactions (23, 30). Similarly, Dean et al. (19, 31) have shown that solubilization of SERCA1 with the nonionic detergent C12E8 results in the formation of an
active monomer but that multiple site ATP kinetics is lost in the monomer. Because cooperativity of Ca$^{2+}$ activation and multiple site ATP kinetics are lost in R. sylvatica, it is possible that there is a change in protein-protein interactions in comparison with R. clamitans that results from amino acid differences between the two frogs.

As a first step in reaching a molecular understanding of the enzymatic differences between R. sylvatica and R. clamitans SERCA1s, we report the complete amino acid sequences for the two proteins. Comparisons between R. sylvatica, R. clamitans, and R. esculenta indicate that there are seven amino acids in SERCA1 that are unique to R. sylvatica and, therefore, may be related to the functional differences noted above (Table I). Three of the seven amino acid substitutions (Asn$^{546}$, Ala$^{547}$ and Ser$^{643}$) that are unique to R. sylvatica when compared with the two other frogs are located in the ATP binding domain. Because the ATP kinetics are different in R. sylvatica in that Michaelis-Menten kinetics are exhibited, it appears likely that these mutations contribute to different enzymatic behavior exhibited by R. sylvatica SERCA1. None of these three amino acids has been examined by site-directed mutagenesis (12). It is possible that site-directed mutagenesis of single amino acid residues will result in changes in ATPase kinetics or that more than one concomitant change is required to achieve this effect.

Three of the remaining four amino acid changes in R. sylvatica are not in regions of the SERCA1 sequence with predicted functional roles. Glu$^{383}$ is located at the beginning of an NH$_2$-terminal α-helix that has no known functional role. Leu$^{838}$ is located in the M7 transmembrane segment that is thought to be located on the outer surface of the proposed Ca$^{2+}$ channel and to have no direct role in Ca$^{2+}$ transport (15). Ser$^{877}$ is located in the extracellular loop between M7 and M8. Mutagenesis of this residue has no effect on activity (12). Ser$^{650}$ (Thr$^{650}$ in R. sylvatica) was originally proposed to be in the nucleotide binding domain (14), but recent determination of the structure of SERCA1 at 2.6-Å resolution indicates that this residue is in the phosphorylation domain (15). This position has not been examined by site-directed mutagenesis.

Three distinct genes code for SERCA pumps in higher vertebrates: SERCA1 (ATP2A1), SERCA2 (ATP2A2), and SERCA3 (ATP2A3) (12). More isoform diversity is brought about by alternative processing of the SERCA primary transcript as follows: adult SERCA1a and neonatal SERCA1b (both expressed in fast-twitch skeletal muscle) (39); SERCA2a (cardiac/slow-twitch skeletal muscle) and SERCA2b (ubiquitously expressed) (12); and SERCA3a, SERCA3b, and SERCA3c (broad cellular expression) (40). The alternative splicing mechanism governing the processing of rabbit SERCA1 pre-mRNA appears to be regulated developmentally. Both isoforms present identical amino acid sequences up to amino acid 993, after which the last amino acid (Gly$^{994}$) in SERCA1a is replaced by a charged tetrapeptide, DKPQ, in SERCA1b isoform. Remarkably, this finding demonstrates that the alternative splicing of the SERCA1 gene is conserved among amphibians and mammals. The physiological significance of the two splice variants is not clear yet. Moreover, COS-1 cell expression studies revealed no functional differences between rabbit SERCA1a and SERCA1b isoforms (41). Furthermore, it is surprising that there are no reports in the literature indicating the expression of a SERCA2 gene in frogs, because the expression of a unique SERCA gene resembling the SERCA2 gene was also documented in invertebrates such as the crustacean Artemia franciscana (42) and the insect Drosophila melanogaster (43). Despite our efforts (Western blot analyses using anti-SERCA2 antibodies and reverse transcriptase-PCR studies using primers flanking nonconserved nucleotide sequences among invertebrate and higher vertebrate SERCAs), we were not able to demonstrate the existence of a SERCA2-like gene in R. sylvatica. However, we cannot rule out the possibility that the epitope for the anti-SERCA2 antibody is modified in the frog protein or that the SERCA2 gene may still be expressed but at much lower mRNA levels than SERCA1.

In conclusion, the molecular adaptation of SERCA1 in the skeletal muscle of R. sylvatica allows this frog to exploit a unique habitat for amphibians in freshwater temporary ponds in late winter. The unusual kinetic properties of R. sylvatica SERCA1 almost certainly result from the seven amino acid residues that are unique to this species. Future studies will shed light on the role of these amino acids in temperature dependence, kinetics with ATP, Ca$^{2+}$ cooperativity, and protein-protein interactions.

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