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Mutations of Arg^{198} in sarcoplasmic reticulum Ca^{2+}-ATPase cause inhibition of hydrolysis of the phosphoenzyme intermediate formed from inorganic phosphate

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Abstract **Arg^{198} of sarcoplasmic reticulum Ca^{2+}-ATPase was substituted with lysine, glutamine, glutamic acid, alanine, and isoleucine by site-directed mutagenesis. Kinetic analysis was performed with microsomal membranes isolated from COS-1 cells which were transfected with the mutated cDNAs. The rate of dephosphorylation of the ADP-insensitive phosphoenzyme was determined by first phosphorylating the Ca^{2+}-ATPase with $^{32}P_i$ and then diluting the sample with non-radioactive P_i. This rate was reduced substantially in the mutant R198Q, more strongly in the mutants R198A and R198I, and most strongly in the mutant R198E, but to a much lesser extent in R198K. The reduction in the rate of dephosphorylation was consistent with the observed decrease in the turnover rate of the Ca^{2+}-ATPase accompanied by the steady-state accumulation of the ADP-insensitive phosphoenzyme formed from ATP. These results indicate that the positive charge and high hydrophilicity of Arg^{198} are critical for rapid hydrolysis of the ADP-insensitive phosphoenzyme.**

Key words: Ca^{2+}-ATPase; Site-directed mutagenesis; Arginine residue; Phosphoenzyme hydrolysis; Sarcoplasmic reticulum

1. Introduction

The Ca^{2+}-ATPase of fast twitch skeletal muscle sarcoplasmic reticulum (SERCA1a) is a 110-kDa membrane-bound protein, of which the primary structure has been determined [1]. This enzyme catalyzes ATP hydrolysis coupled to Ca^{2+} transport [2,3]. During the catalytic cycle, binding of Ca^{2+} to high affinity Ca^{2+} binding sites on the cytoplasmic side activates the transfer of the γ-phosphoryl group of ATP [4,5] to Asp^{351} [1,6–8] to form ADP-sensitive phosphoenzyme (EP), which can react with added ADP to form ATP [9]. A subsequent conformational transition of ADP-sensitive EP to ADP-insensitive EP, which cannot react with added ADP, results in Ca^{2+} release to the lumen. Finally, the latter EP is hydrolyzed to form P_i and the dephosphoenzyme. This EP can also be formed from P_i in the presence of Mg^{2+} and absence of Ca^{2+} by reversal of hydrolysis of ADP-insensitive EP [10,11]. This EP formation occurs through a magnesium-enzyme-phosphate complex that is formed by binding of Mg^{2+} and P_i to the enzyme [12,13].

It was previously shown [14–16] that F^- and Mg^{2+} bind tightly to the catalytic site of this enzyme to form a transition state analog for formation of ADP-insensitive EP from the magnesium-enzyme-phosphate complex. We utilized the protection of the catalytic site by this analog against arginine-specific chemical modification with 1,2-cyclohexanedione, and identified Arg^{198} that is involved in the modification-induced inhibition of formation of ADP-insensitive EP from P_i [17]. The results suggested that Arg^{198} is located in or close to the catalytic site in the transition state for formation of ADP-insensitive EP from the magnesium-enzyme-phosphate complex. However, it remains obscure whether Arg^{198} plays a catalytic role in the hydrolysis of ADP-insensitive EP or the 1,2-cyclohexanedione-induced inhibition is caused solely by steric hindrance.

The secondary structural model indicates that the Ca^{2+}-ATPase is composed of 10 transmembrane α-helices (M_1–M_{10}) and two main cytoplasmic domains, a small cytoplasmic loop (Ala^{132}–Asp^{237} between M_2 and M_3) and a large cytoplasmic loop (Asn^{330}–Phe^{740} between M_4 and M_5) [1]. The large cytoplasmic loop contains the phosphorylation site and the ATP binding site. The functional role of the small cytoplasmic loop is less clear, but previous studies suggested that Thr^{181} [18], Gly^{182} [18], Glu^{183} [18], Gln^{202} [19], Asp^{203} [19], Thr^{230} [19], Gly^{233} [19], Lys^{234} [20], and Arg^{236} [20] in this loop play essential roles in the conformational transition from ADP-sensitive EP to ADP-insensitive EP. The role of Arg^{198} in this loop has not yet been investigated.

In this study, we have used site-directed mutagenesis to examine the functional role of Arg^{198}. This arginine was substituted with lysine, glutamine, glutamic acid, alanine, and isoleucine. The mutated cDNAs were transfected into COS-1 cells, and hydrolysis of ADP-insensitive EP formed from P_i was analyzed with the microsomal membranes isolated from the transfected cells. The rate of this hydrolysis was reduced substantially by the glutamine, glutamic acid, alanine, and isoleucine substitutions, but to a much lesser extent by the lysine substitution. The results indicate that the positive charge and high hydrophilicity of Arg^{198} are critical for rapid hydrolysis of ADP-insensitive EP.

2. Materials and methods

2.1. Oligonucleotide-directed mutagenesis

The full-length rabbit SERCA1a cDNA, which was inserted in the modified Bluescript vector [21], was used for mutagenesis. Overlap extension PCR [22] was utilized to introduce mutations at the position of Arg^{198} in this cDNA. PCR products containing the desired mutations were obtained using SERCA1a cDNA as template. The PCR products were subcloned into the pT7Blue vector (Novagen, Madison, WI), and then the restriction *Apa*I (position 64 in the cDNA)-*Kpn*I

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Abbreviations: SERCA1a, adult fast twitch skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase; EP, phosphoenzyme; PCR, polymerase chain reaction; MOPS, 3-(*N*-morpholino)propanesulfonic acid

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(position 716 in the cDNA) fragments were excised from the vector. The restriction fragments were religated back into their original position in the full-length SERCA1a cDNA that was previously ligated into the *Eco*RI site of the pMT2 expression vector [23]. The pMT2 DNA was used for transfection of COS-1 cells [24]. Dideoxy sequencing [25] was carried out to ensure fidelity of the PCR amplification step and to confirm the presence of the correct mutations.

2.2. Cell culture, DNA transfection, and preparation of microsomal membranes

COS-1 cells were maintained in Dulbecco's modified Eagle's medium fortified with 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum, under 5% CO_2 at 37°C. Liposome-mediated DNA transfection of COS-1 cells was performed with pMT2 DNA and LipofectAMINE Plus Reagent (Life Technologies, Rockville, MD) according to protocols suggested by the manufacturer in Dulbecco's modified Eagle's medium. The cells were then cultured for 42 h at 37°C in the Dulbecco's modified Eagle's medium fortified as described above. Microsomal membranes were prepared from the cells as described by Maruyama and MacLennan [21].

2.3. Immunoblotting

Microsomal proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis according to Laemmli [26], blotted onto polyvinylidene fluoride membrane, and then incubated with VE121G9 monoclonal antibody to the rabbit SERCA1a (Affinity Bioreagents, Golden, CO). After incubation with secondary antibody (goat anti-mouse IgG-horseradish peroxidase-conjugated), the bound proteins were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala). Immunoreactivity was quantitated by densitometry. In this assay, the standard enzyme used was the deoxycholate-purified rabbit SERCA1a [27].

2.4. Ca^{2+}-ATPase activity

The rate of ATP hydrolysis catalyzed by the microsomal membranes was determined at 37°C in a mixture containing 20 μg/ml microsomal protein, 1 μM A23187, 5 mM [γ-^{32}P]ATP, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 0.1 M KCl, and 50 mM MOPS-Tris (pH 7.0). The Ca^{2+}-ATPase activity was obtained by subtracting the rate of ATP hydrolysis determined in the presence of 5 mM EGTA without added $CaCl_2$ otherwise as above. The rate of ATP hydrolysis in the absence of Ca^{2+} with wild type and mutant preparations amounted to 15–25% of the rate of ATP hydrolysis in the presence of Ca^{2+} with wild type preparations. The turnover rate was calculated as the ratio between the Ca^{2+}-ATPase activity and the content of the phosphorylation site measured as described below.

2.5. Content of phosphorylation site

Microsomal membranes were phosphorylated with $^{32}P_i$ at 25°C for 10 min in a mixture containing 25 μg/ml microsomal protein, 0.1 mM $^{32}P_i$, 10 mM $MgCl_2$, 2 mM EGTA, 40% (v/v) Me_2SO, and 50 mM MOPS-Tris (pH 7.0). It has been shown [28,29] that almost all the phosphorylation sites of the Ca^{2+}-ATPase in the fragmented sarcoplasmic reticulum are phosphorylated under these conditions. The reaction was quenched with ice-cold trichloroacetic acid containing phosphoric acid. The acid-precipitated proteins were separated at pH 6.0 by 5% SDS-polyacrylamide gel electrophoresis according to Weber and Osborn [30]. The radioactivity associated with the separated Ca^{2+}-ATPase was quantitated by digital autoradiography of the dried gels using Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo). The amount of EP formed with the expressed Ca^{2+}-ATPase (i.e. the content of the phosphorylation site) was obtained by subtracting the background digitized radioactivity with control microsomal membranes, which were prepared from COS-1 cells transfected with the pMT2 vector containing no SERCA1a cDNA. This background was less than 5% of the digitized radioactivity of EP formed with the expressed wild type Ca^{2+}-ATPase.

2.6. Phosphorylation of Ca^{2+}-ATPase from [γ-^{32}P]ATP

Microsomal membranes were phosphorylated with [γ-^{32}P]ATP at 0°C for 15 s in a mixture containing 25 μg/ml microsomal protein, 1 μM A23187, 10 μM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 0.1 M KCl, and 50 mM MOPS-Tris (pH 7.0). The reaction was quenched with ice-cold trichloroacetic acid containing phosphoric acid. The amount of EP formed with the expressed Ca^{2+}-ATPase was obtained by subtracting the background as described above. This background was less than 5% of the digitized radioactivity of EP formed with the expressed wild type Ca^{2+}-ATPase. For the determination of ADP-insensitive EP, 100 μl of the mixture containing 10 mM ADP, 6 mM EGTA, 0.1 M KCl, and 50 mM MOPS-Tris (pH 7.0) was added to 100 μl of the above phosphorylation mixture 15 s after the start of the phosphorylation. At 5 s after this addition, the reaction was quenched with the acid. The amount of EP formed with the expressed Ca^{2+}-ATPase was then determined as above. ADP-sensitive EP disappeared entirely within 5 s after the addition of ADP, whereas ADP-insensitive EP was not significantly decomposed before the acid quenching.

2.7. Dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$

Dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$ was analyzed essentially according to Andersen [31] and Chen et al. [32]. Microsomal membranes were phosphorylated with $^{32}P_i$ at 25°C for 10 min in 50 μl of the mixture containing 5 μg of microsomal protein, 1 μM A23187, 0.1 mM $^{32}P_i$, 10 mM $MgCl_2$, 2 mM EGTA, 30% (v/v) Me_2SO, and 50 mM MOPS-Tris (pH 7.0). The mixture was then cooled and diluted at 3°C by addition of 950 μl of the mixture containing 2 mM non-radioactive P_i, 9.5 mM $MgCl_2$, 0.5 mM EGTA, 0.1 M KCl (or 0.1 M LiCl in place of KCl), and 50 mM MOPS-Tris (pH 7.0). At different times after the dilution, the dephosphorylation was quenched with ice-cold trichloroacetic acid containing phosphoric acid. ^{32}P-Labeled EP remaining was then determined as described above. Kinetic measurements of the dephosphorylation were performed with a handmade rapid mixing apparatus [33] or with manual pipetting.

2.8. Miscellaneous methods

Protein concentrations were determined by the method of Lowry et al. [34] with bovine serum albumin as a standard. Data were analyzed by the non-linear least squares method as described previously [15].

Table 1
Characteristics of Ca^{2+}-ATPases mutated at Arg^{198}

Mutant	Ca^{2+}-ATPase activity (μmol/min/mg of microsomal protein)	Phosphorylation site (pmol/mg of microsomal protein)	Turnover rate (s^{-1})	EP formed from ATP	
				Total (pmol/mg of microsomal protein)	ADP-insensitive (%)
Wild type	0.425 ± 0.017	131 ± 5	53.9 ± 2.8	87.3 ± 12.7	1.6 ± 0.5
R198K	0.361 ± 0.027	115 ± 11	52.4 ± 5.7	91.6 ± 11.3	2.1 ± 1.5
R198Q	0.353 ± 0.015	141 ± 5	41.7 ± 2.0	108.0 ± 21.5	10.2 ± 1.5
R198E	0.267 ± 0.024	139 ± 3	31.9 ± 2.7	105.3 ± 10.8	21.9 ± 1.3
R198A	0.243 ± 0.014	106 ± 10	38.3 ± 3.5	79.6 ± 10.8	14.0 ± 0.7
R198I	0.288 ± 0.032	124 ± 2	38.6 ± 3.9	100.8 ± 20.2	14.9 ± 4.0

The Ca^{2+}-ATPase activity, the content of the phosphorylation site, the turnover rate, and the total amount of EP and amount of ADP-insensitive EP formed from [γ-^{32}P]ATP at steady state were determined with the microsomal membranes of COS-1 cells expressing the wild type Ca^{2+}-ATPase and mutants R198K, R198Q, R198E, R198A, and R198I, as described in Section 2. The amounts of ADP-insensitive EP are represented as percentage of the total amounts of EP. The values are the means ± S.D. for four independent experiments.

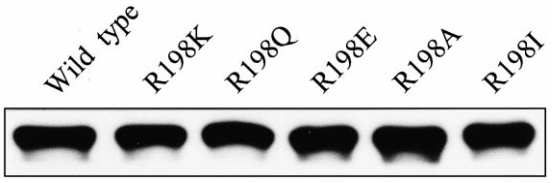

Fig. 1. Immunoblotting of microsomal membranes from COS-1 cells expressing Ca^{2+}-ATPases mutated at Arg^{198}. Expression levels of Ca^{2+}-ATPases mutated at Arg^{198} in the microsomal membranes of COS-1 cells were determined by immunoblotting as described in Section 2. Each lane contains 1 μg of microsomal protein. The relative expression levels of mutants R198K, R198Q, R198E, R198A, and R198I normalized to the wild type Ca^{2+}-ATPase (100%) are 83, 87, 93, 109, and 93%, respectively.

3. Results

3.1. Expression of Ca^{2+}-ATPases mutated at Arg^{198}

Fig. 1 shows immunoblots of the wild type Ca^{2+}-ATPase and five substitution mutants R198K, R198Q, R198E, R198A, and R198I which were expressed in the microsomal membranes of COS-1 cells. As shown, all the mutants were expressed to approximately the same level as that of the wild type. The expression levels of the mutants were estimated to be between 83 and 109% of the wild type level, indicating that expression was not impaired by the substitutions.

3.2. Ca^{2+}-ATPase activity, content of phosphorylation site, and turnover rate

The Ca^{2+}-ATPase activities of the microsomal membranes from COS-1 cells expressing the wild type Ca^{2+}-ATPase and the mutants were determined at 37°C in the presence of K^+ (Table 1). The contents of the phosphorylation site of the expressed Ca^{2+}-ATPase in the microsomal membranes ranged from 106 to 141 pmol/mg microsomal protein. This indicates that the wild type Ca^{2+}-ATPase and the mutants were expressed at similar levels, in agreement with the above conclusion obtained by immunoblotting.

The turnover rate of the mutant R198K was almost the same as that of the wild type, while the turnover rates of the mutants R198Q, R198A, and R198I were substantially lower than that of the wild type. The turnover rate was further reduced in the mutant R198E.

Table 2
Rate constants for dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$

Mutant	Rate constants (s^{-1})	
	$+K^+$	$-K^+$
Wild type	0.68 ± 0.01	0.085 ± 0.006
R198K	0.57 ± 0.05	0.070 ± 0.002
R198Q	0.35 ± 0.03	0.038 ± 0.006
R198E	0.26 ± 0.03	0.019 ± 0.002
R198A	0.30 ± 0.01	0.025 ± 0.003
R198I	0.26 ± 0.01	0.026 ± 0.004

The first-order rate constants for dephosphorylation of ADP-insensitive EP were determined with the wild type Ca^{2+}-ATPase and mutants R198K, R198Q, R198E, R198A, and R198I in the presence of KCl ($+K^+$) or in the presence of LiCl without added KCl ($-K^+$), as described in Fig. 3. The values are the means ± S.D. for four independent experiments.

3.3. ADP-insensitive EP formed from ATP

The total amount of EP and the amount of ADP-insensitive EP at steady state were determined at 0°C under conditions otherwise similar to those used for determination of the Ca^{2+}-ATPase activity (Table 1). All the mutants were phosphorylated with ATP at levels similar to that of the wild type. EP formed from ATP was almost completely ADP-sensitive in the mutant R198K as well as in the wild type. In contrast, ADP-insensitive EP accumulated to a considerable extent in the mutant R198Q, to a larger extent in the mutants R198A and R198I, and to the largest extent in the mutant R198E.

3.4. Dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$

Dephosphorylation of ADP-insensitive EP was examined by first phosphorylating the Ca^{2+}-ATPase with $^{32}P_i$ in the absence of K^+ and then diluting the sample with a large volume of a solution containing non-radioactive P_i and KCl (or LiCl in place of KCl). This dilution terminated phosphorylation of the Ca^{2+}-ATPase with $^{32}P_i$ and allowed dephosphorylation of ^{32}P-labeled EP to proceed. Typical examples of the digitized autoradiographs monitoring dephosphorylation of ^{32}P-labeled EP in the absence of K^+ are shown in Fig. 2. It is evident from visual inspection that decays of ADP-insensitive EP in the mutants R198E and R198I were considerably slower than that observed with the wild type. Inspection further reveals that the rate of decay of ADP-insensitive EP in the mutant R198K was not very different from that in the wild type.

The time courses of dephosphorylation of ADP-insensitive

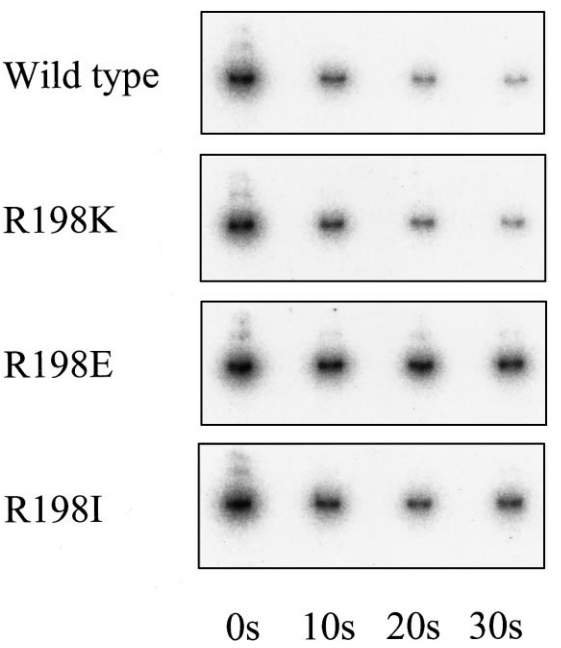

Fig. 2. Autoradiographs showing dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$. The wild type Ca^{2+}-ATPase and mutants R198K, R198E, and R198I were phosphorylated with $^{32}P_i$ as described in Section 2. The phosphorylation mixture was then diluted with non-radioactive P_i in the presence of LiCl without added KCl. Acid quenching was performed immediately before the dilution (0 s) or at the times indicated after the dilution. Digitized autoradiographs of the radioactivity associated with the Ca^{2+}-ATPase band on the gel are shown.

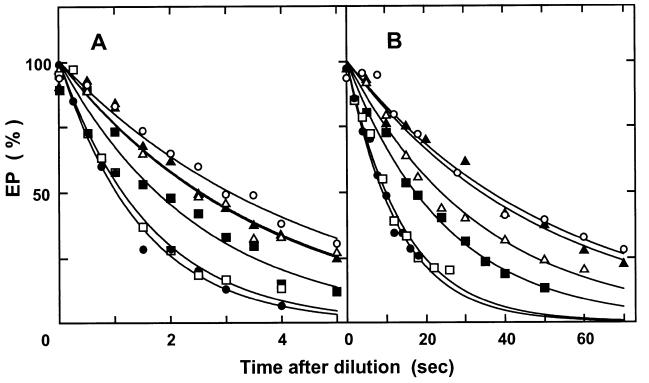

Fig. 3. Kinetics of dephosphorylation of ADP-insensitive EP formed from $^{32}P_i$. The wild type Ca^{2+}-ATPase (●) and mutants R198K (□), R198Q (■), R198E (○), R198A (△) and R198I (▲) were phosphorylated with $^{32}P_i$ as described in Section 2. The phosphorylation mixture was then diluted with non-radioactive P_i in the presence of KCl (A) or in the presence of LiCl without added KCl (B). Acid quenching was performed immediately before the dilution (0 s) or at the times indicated after the dilution, and the amount of ^{32}P-labeled EP remaining was determined. Solid lines show least squares fit to single exponentials. The first-order rate constants obtained from solid lines in A are 0.68 (wild type), 0.61 (R198K), 0.40 (R198Q), 0.23 (R198E), 0.28 (R198A), and 0.27 (R198I) s^{-1}, and those in B are 0.078 (wild type), 0.071 (R198K), 0.041 (R198Q), 0.019 (R198E), 0.030 (R198A), and 0.021 (R198I) s^{-1}. The values obtained from the zero time intercepts are normalized to 100%. These values were in close agreement with the contents of the phosphorylation site in the microsomal membranes used.

EP formed from $^{32}P_i$ in the wild type Ca^{2+}-ATPase and in the five substitution mutants were determined in the presence or absence of K^+ by quantitation of the digitized autoradiographs of ^{32}P-labeled EP remaining after dilution with non-radioactive P_i (Fig. 3). The dephosphorylation proceeded with first-order kinetics. The rate constants obtained by least squares fit to single exponentials are listed in Table 2. The rate of dephosphorylation in the presence or absence of K^+ in the mutant R198K was nearly equivalent to that in the wild type, as expected from the autoradiographs in Fig. 2. The rate of dephosphorylation was substantially reduced in the mutant R198Q, more strongly reduced in the mutants R198A and R198I, and most strongly reduced in the mutant R198E. The substitution-induced reductions in the rate of dephosphorylation in the absence of K^+ were somewhat more pronounced than those in the presence of K^+.

4. Discussion

The present results show that the rate of dephosphorylation of ADP-insensitive EP is substantially reduced by the substitution of Arg^{198} with glutamine, more strongly with alanine and isoleucine, and most strongly with glutamic acid, but to a much lesser extent with lysine (Figs. 2 and 3, Table 2). These findings led to the conclusion that the positive charge and high hydrophilicity of Arg^{198} are important for rapid hydrolysis of ADP-insensitive EP.

The substitution-induced reductions in the rate of dephosphorylation of ADP-insensitive EP (Table 2) correspond well to the substitution-induced reductions in the turnover rate and to the substitution-induced steady-state accumulations of ADP-insensitive EP in the presence of K^+ (Table 1). This is reasonable because ADP-insensitive EP should not appreciably accumulate in the presence of K^+ when hydrolysis of ADP-insensitive EP is not inhibited [35] as observed with the wild type Ca^{2+}-ATPase and the mutant R198K, and because inhibition of hydrolysis of ADP-insensitive EP should cause a reduction in the turnover rate accompanied by accumulation of ADP-insensitive EP in the presence of K^+ [35] as observed with the mutants R198Q, R198A, R198I, and R198E.

Arg^{198} is catalytically non-essential for hydrolysis of ADP-insensitive EP, because the substitution-induced inhibition of dephosphorylation of ADP-insensitive EP is incomplete in any of the five substitution mutants tested. Instead, it is very likely that the positive charge and high hydrophilicity of Arg^{198} critically contribute to a suitable conformation of the catalytic site for hydrolysis of ADP-insensitive EP.

The role of Arg^{198} for hydrolysis of ADP-insensitive EP shown in the present study is consistent with our previous findings obtained from the chemical modification of Arg^{198} [17] that this residue is located in or close to the catalytic site when the enzyme is in the transition state for formation of ADP-insensitive EP from the magnesium-enzyme-phosphate complex (reversal of hydrolysis of ADP-insensitive EP). It is also compatible with the previously reported findings that the enzyme is protected against tryptic cleavage at Arg^{198} by formation of ADP-insensitive EP from P_i [36] as well as by formation of the magnesium-enzyme-vanadate complex [37] that is a transition state analog for formation of ADP-insensitive EP from the magnesium-enzyme-phosphate complex [38,39].

The data show that all the mutants are phosphorylated at levels similar to that of the wild type at a low ATP concentration (10 μM) (Table 1). Therefore, it is unlikely that Arg^{198} is critically involved in ATP binding or in the subsequent phosphoryl transfer from ATP to the enzyme.

Arg^{198} is conserved in all sarco(endo)plasmic reticulum Ca^{2+}-ATPases [1,40–42], but not in other P-type ATPases including Na^+,K^+-ATPase [43,44], H^+,K^+-ATPase [45,46], and plasma membrane Ca^{2+}-ATPase [47]. It is likely, therefore, that the functional role of Arg^{198} shown in this study is specific to sarco(endo)plasmic reticulum Ca^{2+}-ATPases.

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