Biochimica et Biophysica Acta, 1027 (1990) 264–267
Elsevier

BBAMEM 74998

Oxidation of sulfhydryl groups and inhibition of the (Ca^{2+} + Mg^{2+})-ATPase by arsenazo III

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(Received 29 December 1989)
(Revised manuscript received 23 April 1990)

Key words: Calcium ion indicator; Sulfhydryl group oxidation; Arsenazo III; ATPase, (Ca^{2+} + Mg^{2+})-

In the presence of divalent cations, the metallochromic Ca^{2+} indicator arsenazo III is reduced by sulfhydryl groups to form an azo anion radical. Reduced arsenazo III is reoxidized back to its original state by oxygen. The formation of the arsenazo III azo anion radical in the presence of sarcoplasmic reticulum vesicles leads to the rapid inhibition of the (Ca^{2+} + Mg^{2+})-ATPase. These data indicate that several factors should be considered when arsenazo III is used as a Ca^{2+} indicator; (1) Functionally important sulfhydryl groups may be oxidized by arsenazo III; (2) the generation of free radicals by arsenazo III reduction may be toxic to the system being studied; (3) the absorbance spectrum of arsenazo III is altered when reduced by sulfhydryl groups.

Introduction

Arsenazo III is a metallochromic indicator commonly used to monitor changes in the Ca^{2+} concentration [1,2]. It is commonly believed that arsenazo III is relatively nontoxic and nonreactive in biological systems [1,2]. However, when using arsenazo III to study Ca^{2+} transport by sarcoplasmic reticulum vesicles in the presence of sulfhydryl compounds such as dithiothreitol or 2-mercaptoethanol, we observed a change in the arsenazo III absorbance spectrum that was not related to changes in the Ca^{2+} concentration but was due to arsenazo III reduction. In addition, in the presence of sulfhydryl compounds, arsenazo III inhibited Ca^{2+} transport by sarcoplasmic reticulum. Because arsenazo III is an important Ca^{2+} indicator used to monitor Ca^{2+} uptake and release by sarcoplasmic reticulum both in situ and in vitro, we have investigated the reaction between arsenazo III and sulfhydryl groups.

Docampo et al. [3] have reported that arsenazo III undergoes a one electron reduction in the presence of rat liver microsomes and NAD(P)H to produce an azo anion radical. The azo anion radical can react with oxygen to regenerate arsenazo III and a superoxide radical. We report here that sulfhydryl compounds also reduce arsenazo III to form free radicals which alters the absorbance spectrum of arsenazo III and inactivates the (Ca^{2+} + Mg^{2+})-ATPase.

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Materials and Methods

Materials

All sulfhydryl compounds were obtained from Aldrich (Milwaukee, WI). All other reagents were purchased from Sigma (St. Louis, MO).

Preparation of sarcoplasmic reticulum vesicles

Low-density sarcoplasmic reticulum vesicles were isolated from the back and hind leg skeletal muscles of Sprague-Dawley rats (200–300 g) as previously described [4].

Assays

The Ca^{2+}-dependent ATPase activity of the sarcoplasmic reticulum was monitored using a coupled enzyme assay as described by Warren et al. [5]. The concentration of sulfhydryl groups was assayed spectrophotometrically using the reagent 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman [6].

Results

Change in the arsenazo III spectrum caused by sulfhydryl compounds

Ca^{2+} binding to arsenazo III causes an increase in the arsenazo III absorbance at 600 nm and 660 nm. The addition of sulfhydryl compounds (dithiothreitol, 2-

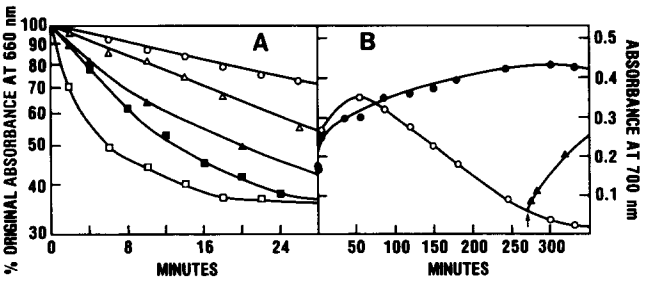

Fig. 1. Rate of change of the arsenazo III absorbance at 660 and 700 nm following the addition of sulfhydryl compounds. (A) The change in the absorbance of 20 μM arsenazo III at 660 nm was measured in a solution containing 1.0 mM $CaCl_2$ and 10 mM Hepes (pH 7.0) at 37 °C following the addition of 20 mM dithiothreitol (■), 20 mM 2-mercatoethanol (○), 20 mM cysteine (▲), 20 mM 3-mercaptopropionate (△), or 20 mM 2-aminoethanethiol (□). (B) The absorbance change of 50 μM arsenazo III at 700 nm was measured in a solution containing 1.0 mM $CaCl_2$ and 10 mM Hepes (pH 7.0) at 37 °C following the addition of 40 mM 2-aminoethanethiol. One sample was continuously exposed to air by stirring (●) while the other sample was kept anaerobic under nitrogen (○). Exposure of the anaerobic sample to air after 270 min (▲) is also shown.

aminoethanethiol, 2-mercaptoethanol, cysteine and 2-mercaptopropionate) to the arsenazo III-Ca complex causes a decrease in the absorbance at both 600 and 660 nm and an increase at 700 nm (Figs. 1 and 2). The rate of absorbance decrease at 660 nm of 20 μM arsenazo III (in the presence of 1 mM Ca^{2+}) is directly proportional to the concentration of 2-mercaptoethanol between 5 and 100 mM. Between 10 and 50 °C, the rate of absorbance change at 660 nm following the addition of 20 mM 2-mercaptoethanol or 2-aminoethanethiol increased 2.2 fold for every 10 degree increase in temperature. In the absence of Ca^{2+} or other divalent cations, sulfhydryl compounds have no effect on the arsenazo III spectrum.

The change in absorbance spectra of the arsenazo III-Mg complex (data not shown) and the arsenazo

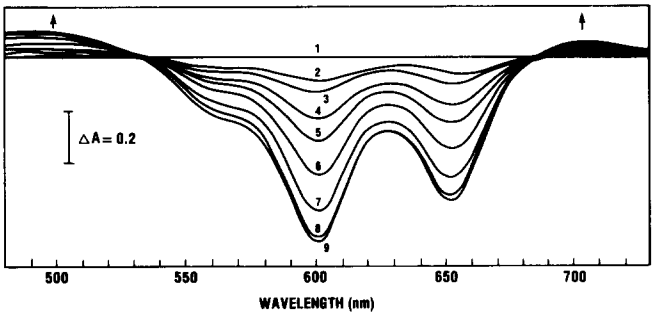

Fig. 2. Change in the arsenazo III spectrum upon the addition of aminoethanethiol. The sample and reference cuvette contained 20 μM arsenazo III, 1.0 mM $CaCl_2$ and 10 mM Hepes (pH 7.0). The difference absorbance spectrum was recorded before (trace 1) and at various times following the addition of 3 mM 2-aminoethanethiol to the sample cuvette. (trace 2, 0.5 min; 3 trace, 1.5 min; 4 trace, 6.0 min; 5 trace, 10.0 min; 6 trace, 19.0 min; 7 trace, 40 min; 8 trace, 120 min).

III-Ca complex (Fig. 2) following the addition of sulfhydryl compounds are similar to the change observed by Docampo et al. [3] when arsenazo III is reduced by NAD(P)H in the presence of rat liver microsomes to form an azo anion radical. They reported a decrease in the absorbance of the arsenazo III-Mg complex in the 540 to 680 nm region upon reduction of arsenazo III while an increase in the absorbance at 700 nm was correlated to the formation of an azo anion radical [3]. The reduction of the arsenazo III-Mg complex by the sulfhydryl groups gave the same spectral change as that reported by Docampo et al. [3] indicating the formation of an azo anion radical. With arsenazo III-Ca, a similar increase in the absorbance at 700 nm (Fig. 1B and 2) upon the reduction of arsenazo III with sulfhydryl compounds was observed indicating the formation of a similar azo anion radical. The change in the arsenazo III-Ca spectrum between 480 to 680 nm was similar to that observed when the Ca^{2+} concentration of the solution is lowered.

The spectral changes caused by sulfhydryl compounds are reversible. In the presence of O_2, the original arsenazo III absorbance spectrum returns following the complete oxidation of the sulfhydryl compound.

Under anaerobic conditions, the increase in the absorbance of 50 μM arsenazo III at 700 nm following the addition of 40 mM 2-aminoethanethiol reaches a maximum after about 50 min and then slowly decreases until the absorbance in the entire visible range disappears (Fig. 1B). This secondary absorbance change is also reversed by exposure to oxygen. Under aerobic conditions, the decrease in the absorbance at 700 nm after 50 min was not observed. The disappearance of the visible spectrum under anaerobic conditions is probably due to either a second slower reduction of the azo anion radical or reactions between azo anion radicals to form derivatives such as the hydrazine [8].

As stated above, in the absence of Ca^{2+} or other divalent cations, none of the sulflydryl groups tested altered the arsenazo III absorbance spectrum. However in the presence of any of the eight divalent cations tested (Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Sr^{2+}, Cu^{2+}, Cd^{2+}), the sulfhydryl compounds were able to reduce arsenazo III. The order of effectiveness of the divalent cations (1 mM) in promoting the reduction of arsenazo III by 20 mM dithiothreitol was $Ni^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Co^{2+}$.

Oxidation of sulfhydryl groups by arsenazo III

The rate of oxidation of 30 mM 2-aminoethanethiol by 0.05 mM to 2.0 mM arsenazo III under aerobic and anaerobic conditions was measured using 5,5′-dithiobis(2-nitrobenzoic acid) to assay the sulfhydryl concentration (Fig. 3). Under aerobic conditions, the amount of sulfhydryl groups oxidized far exceeds the concentration of arsenazo III, indicating that arsenazo

III acts as a catalyst for sulfhydryl oxidation driven by oxygen. For example, 30 mM 2-aminoethanethiol was completely oxidized in the presence of 2.0 mM arsenazo III with a first order rate constant of 0.013 min^{-1}.

Although oxygen is not required for sulflydryl compounds to alter the spectrum of arsenazo III (Fig. 1), oxygen is required for arsenazo III to catalyze the oxidation of nonstoichiometric amounts of sulfhydryl compounds (Fig. 3). Without oxygen, reduced arsenazo III is not reoxidized so that it can not catalyze the oxidation of more sulfhydryl groups. The oxidation of sulfhydryl groups stops once all the arsenazo III is converted into the reduced form. It is the reoxidation of arsenazo III by oxygen permits the further oxidation of other sulfhydryl groups. This process can continue until all sulfhydryl groups are oxidized. Under aerobic conditions, the steady-state oxidation rate of sulfhydryl groups is more than 100 times faster in the presence of 1 mM arsenazo III as compared with that measured without arsenazo III added.

Effect of arsenazo III and sulfhydryl compounds on the activity of the (Ca^{2+} + Mg^{2+})-ATPase

The reduction of arsenazo III by sulfhydryl groups results in the formation of an azo anion radical. The reoxidation of reduced arsenazo III by oxygen is expected to generate active oxygen radicals [3]. It is possible that either the azo anion radical or oxygen radicals react with proteins to inhibit enzyme activity. We have investigated the effect of reduced arsenazo III on the activity of the (Ca^{2+} + Mg^{2+})-ATPase (Fig. 4). The (Ca^{2+} + Mg^{2+})-ATPase was rapidly inactivated in the presence of 1–5 mM arsenazo III and 40 mM 2-aminoethanethiol. Similar inactivation was observed when sarcoplasmic reticulum vesicles were incubated with 1 mM arsenazo III and 20 mM 2-mercaptoethanol. In the absence of either 2-aminoethanethiol or arsenazo III there was no inactivation of the (Ca^{2+} + Mg^{2+})-ATPase indicating that the reduction of arsenazo III by 2-aminoethanethiol is required for the inactivation process. The (Ca^{2+} + Mg^{2+})-ATPase was inactivated by 2-aminoethanethiol and arsenazo III even under anaerobic conditions indicating that the azo anion radical or other reduction products such as the hydrazine derivative can inactivate the (Ca^{2+} + Mg^{2+})-ATPase directly without the formation of oxygen radicals.

It should be noted that under the conditions in which arsenazo III is normally used to measure the in vitro ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum vesicles (low arsenazo III concentration (10–100 μM, and low sulfhydryl concentration) there is no significant inactivation of the (Ca^{2+} + Mg^{2+})-ATPase. The measurement of the in situ cytoplasmic Ca^{2+} concentration however could pose a problem since much higher arsenazo III concentrations are used. Under these

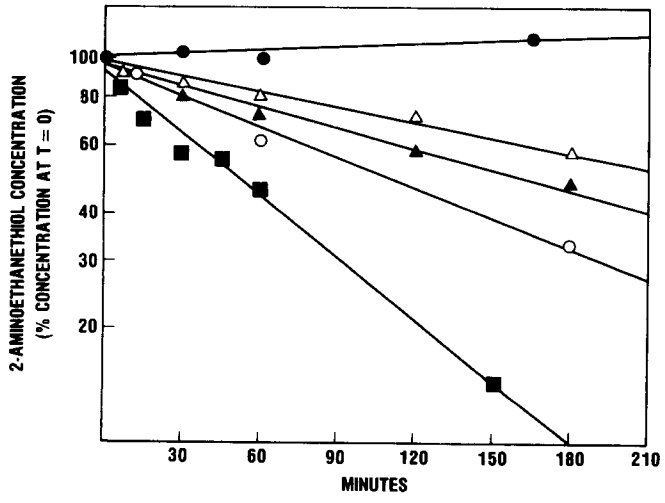

Fig. 3. Rate of oxidation of aminoethanethiol by arsenazo III and oxygen. At time = 0, 30 mM 2-aminoethanethiol was added to a solution containing 10 mM $CaCl_2$, 10 mM Hepes (pH 7.0) and 0.05 (△), 0.25 (▲), 0.5 (●, ○) and 2.0 (■) mM arsenazo III. At various times, 5 μl aliquots were removed and the concentration of sulfhydryl groups assayed by the method described by Ellman [6]. One sample was kept anaerobic under nitrogen (●); The other samples were exposed to air by constant stirring.

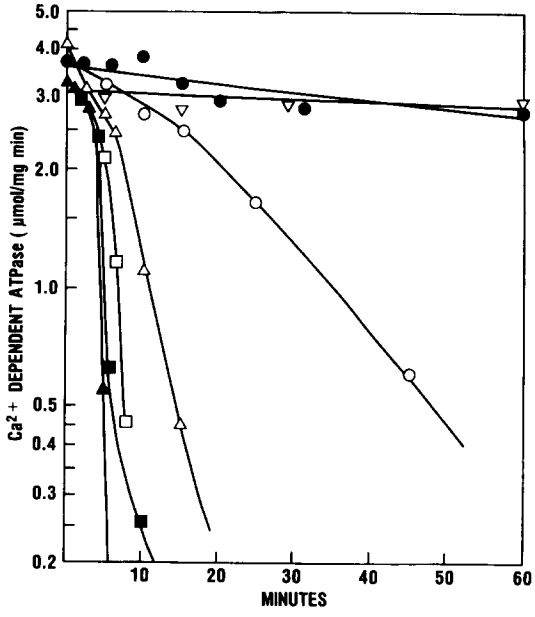

Fig. 4. Inhibition of the (Ca^{2+} + Mg^{2+})-ATPase by arsenazo III and aminoethanethiol. Sarcoplasmic reticulum vesicles (20 mg/ml protein) were added to a solution containing 0.15 M KCl, 10 mM 3-(N-Morpholino)propanesulfonic acid (Mops) (pH 6.8), 5 mM $MgSO_4$, 40 mM 2-aminoethanethiol, 20 μM $CaCl_2$ and either 1 mM (○), 2 mM (△), 3 mM (□), 4 mM (■) or 5 mM (▲) arsenazo III at 37 °C. In one control (●), arsenazo III was omitted from the incubation solution. In another control, 5 mM arsenazo III was added but the 2-aminoethanethiol was omitted (▽). At various times after the addition of the sarcoplasmic reticulum vesicles, 5 μl aliquots were removed and added to a solution containing 0.15 M KCl, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$, 0.5 mM phospho(enol)pyruvate, 16 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 0.15 mM NADH, and 1 mM ATP. The rate of ATP hydrolysis was monitored by following the rate of NADH oxidation by measuring the change in the absorbance at 340 nm.

conditions, the addition of sulfhydryl compounds should probably be avoided.

Discussion

Arsenazo III is reduced by a variety of sulfhydryl compounds such as cysteine, dithiothreitol, 2-mercaptoethanol, 2-aminoethanethiol and 3-mercaptopropionate (Fig. 1). Therefore, functionally important sulfhydryl groups of proteins may also be oxidized by arsenazo III. Beeler et al. [7] have previously demonstrated that many proteins found in muscle cells bind arsenazo III. It is likely that some of these binding sites have sulfhydryl groups that can react with arsenazo III. In addition, the reduction of arsenazo III leads to the formation of an azo anion radical which may react with neighboring proteins, or with oxygen to regenerate arsenazo III and form a reactive superoxide anion [3]. The $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum is rapidly inactivated by 1–5 mM arsenazo III in the presence of 2-aminoethanethiol (Fig. 4) or 2-mercaptoethanol.

The change in the absorbance spectrum of the arsenazo III-Ca complex caused by the reduction of arsenazo III with sulfhydryl compounds can interfere with the determination of the Ca^{2+} concentration since the reduced arsenazo III absorbance spectrum is different than that of the arsenazo III-Ca complex (Fig. 2). Reduction of arsenazo III by sulfhydryl compounds causes a decrease in the absorbance between 580 nm and 680 nm. This reduction could be misinterpreted as a decrease in the Ca^{2+} concentration since a decrease in the Ca^{2+} concentration leads to a similar change in the absorbance in this region.

The data presented here does not exclude the use of arsenazo III as a Ca^{2+} indicator in biological systems. In the absence of added sulfhydryl reagents arsenazo III did not inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase, indicating that sulfhydryl groups on the pump protein that are required for activity does not react with arsenazo III under our experimental conditions. In addition the rate of inactivation at low arsenazo III concentrations (0.01–0.05 mM) in the presence of sulfhydryl compounds was so slow that it would not affect the results of most experiments. The reduction of arsenazo III by sulfhydryl compounds also required the presence of divalent cations such as Mg^{2+} so that the amount of azo anion radical formed would depend on the Mg^{2+} concentration. In the absence of divalent ions, arsenazo III and 2-aminoethanethiol did not inactivate the $(Ca^{2+} + Mg^{2+})$-ATPase.

In conclusion, we have demonstrated that arsenazo III, in the presence of divalent cations can be reduced by sulfhydryl groups to form an azo anion radical which has a different absorption spectrum than the nonreduced dye. The formation of the azo anion radical is toxic to the $(Ca^{2+} + Mg^{2+})$-ATPase. Therefore caution should be used when using arsenazo III in the presence of sulfhydryl compounds to monitor changes in the Ca^{2+} concentration of biological systems.

Acknowledgements

This research was funded by grants form the National Institutes of Health (GM 29300), and the Uniformed Services University of the Health Sciences (R07197) The opinions or assertions contained herein are private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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