Reduction of the metallochromic indicators arsenazo III and antipyrylazo III to their free radical metabolites by cytoplasmic enzymes

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At a concentration much lower than that usually employed for measuring cytosolic ionized Ca^{2+} concentrations, arsenazo III underwent a one-electron reduction by rat liver cytosolic fraction or a hypoxanthine–xanthine oxidase system to produce an azo anion radical metabolite. NADH, NADPH, N^1-methylnicotinamide, hypoxanthine, and xanthine, in that order, could serve as a source of reducing equivalents for the production of this free radical by the cytosolic fraction. The steady-state concentration of the azo anion radical and the arsenazo III-stimulated O_2 consumption were enhanced by calcium and magnesium. Antipyrylazo III was ineffective in increasing O_2 consumption by rat liver cytosolic fraction and gave a much weaker ESR signal of an azo anion radical with both the liver cytosolic fraction, in the presence of NADH, and the hypoxanthine–xanthine oxidase system.

Arsenazo III *Antipyrylazo III* *Free radical* *Cytoplasmic enzyme*

1. INTRODUCTION

Metallochromic Ca^{2+} indicators are substances which change color when they bind Ca^{2+} [1]. Two such dyes, arsenazo III and antipyrylazo III have the necessary properties to provide a method for measuring free Ca^{2+} which has the specificity and sensitivity for studies in intact cells [2]. Arsenazo III was the first of these dyes to be used in living cells [3–6]. Antipyrylazo III has only recently been applied to the measurements of Ca^{2+} transport within single cells [7–9]. Recent studies have shown that these dyes are reduced by rat liver microsomes to free radical metabolites whose autoxidation generates superoxide anion [10]. This study provides biochemical and ESR spectroscopy evidence that reduction of these dyes also occurs in the presence of xanthine oxidase and other cytoplasmic enzymes.

2. MATERIALS AND METHODS

CD male rats (150–200 g, Charles River Inc.) were used in the experiments. They were fed standard rat chow and water ad libitum and were not fasted prior to use. The animals were killed and their livers were rapidly removed and processed. The cytosolic fraction was prepared by homogenizing the livers in 4 vols cold sucrose–Tris–EDTA solution (250 mM sucrose, 5 mM Tris–HCl buffer, pH 7.4, 1 mM EDTA) and centrifuging the homogenate at $15000 \times g$ for 10 min. The $15000 \times g$ supernatant fraction was centrifuged for 1 h at $105000 \times g$. The supernatant fraction was dialyzed overnight in a cold room against 200 vols of the same buffer. The protein concentration was determined by the biuret assay in the presence of 0.2% deoxycholate [11].

Xanthine oxidase NADH, NADPH, hypoxanthine, xanthine, N^1-methylnicotinamide, dicumarol, arsenazo III (98% pure) and antipyrylazo III

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were obtained from Sigma.

ESR observations were made at room temperature (24°C), with a Varian E-104 spectrometer equipped with a TM_{110} cavity as in [12].

Oxygen uptake was determined with a Clark electrode (YS-5331, Yellow Springs Instrument Co.) in a water-jacketed glass vessel at 30°C.

3. RESULTS

An unresolved single line ESR spectrum corresponding to the arsenazo III anion radical [10] was obtained in incubations containing arsenazo III (100 μM), hypoxanthine and xanthine oxidase under anaerobic conditions. No antipyrylazo III radical could be detected under similar conditions. However, a weak signal was evident at 2 mM antipyrylazo III (fig.1B). These signals depended on all the components of the system and were not observable under aerobic conditions. Similar results were obtained using xanthine instead of hypoxanthine (not shown).

The arsenazo III anion radical signal intensity, but not the antipyrylazo III radical signal intensity, increased dramatically after addition of Ca^{2+} to the incubation medium and reached a plateau at approx. 100 μM Ca^{2+} (fig.2). The addition of Mg^{2+} to the incubation medium was much more effective than Ca^{2+} in increasing the azo anion radical steady-state concentration. In contrast to the effect of Ca^{2+}, Mg^{2+} increased the azo anion radical steady state concentration up to a concentration of 1 mM (fig.2).

The results presented in table 1 demonstrate that the liver cytosolic fraction was able to reduce arsenazo III to its free radical metabolite, using

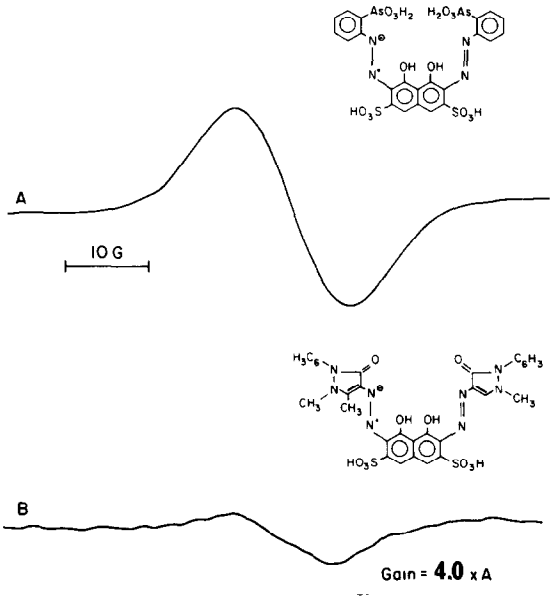

Fig.1. ESR spectra of hypoxanthine–xanthine oxidase incubations in the presence of arsenazo III and antipyrylazo III. (A) The ESR spectrum of arsenazo III anion radical observed after an anaerobic incubation of 100 μM arsenazo III with 0.5 mM hypoxanthine and 0.18 units/ml xanthine oxidase in 100 mM Tris–acetate buffer (pH 7.6) and 1 mM $MgCl_2$. The nominal microwave power was 20 mW and the modulation amplitude was 6.7 G. (B) The ESR spectrum of antipyrylazo III anion radical observed in an incubation similar to that in A but in the presence of 2 mM antipyrylazo III instead of arsenazo III.

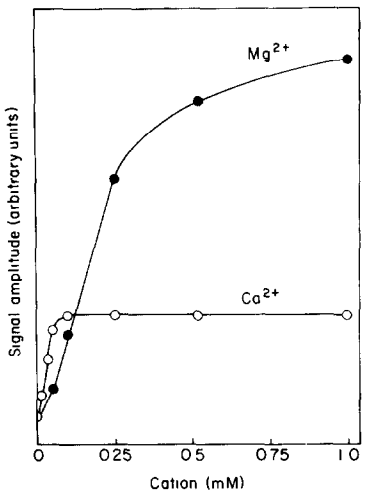

Fig.2. Effect of different concentrations of Ca^{2+} and Mg^{2+} on the steady-state ESR signal of arsenazo III anion radical. The same cell remained undisturbed in the cavity throughout the experiment to minimize any artifact due to differences in cell position. The nominal microwave power was 20 mW and the modulation amplitude was 6.7 G. The incubation contained 0.18 units/ml xanthine oxidase, 0.5 mM hypoxanthine, 100 μM arsenazo III in 100 mM Tris–acetate buffer (pH 7.6) and the concentrations of $CaCl_2$ or $MgCl_2$ noted on the figure. The oxygen was displaced by purging with nitrogen for 2 min and the reaction was initiated with hypoxanthine.

Table 1

Effect of different substrates on arsenazo III anion radical ESR signal and arsenazo III-stimulated oxygen consumption in incubations of rat liver supernatant

Addition (1 mM)	Arsenazo III anion radical (relative amplitude)[a]	Arsenazo III-stimulated oxygen consumption over (basal levels)[b]
NADH	100.0 ± 8.0[c]	1.27 ± 0.02 (0.40 ± 0.10)[c]
NADPH	66.0 ± 6.0	0.63 ± 0.03 (0.46 ± 0.17)
Xanthine	6.8 ± 0.6	0.13 ± 0.02 (0.30 ± 0.09)
Hypoxanthine	9.3 ± 0.5	0.16 ± 0.02 (0.54 ± 0.07)
N^1-methylnicotinamide	15.9 ± 1.3	0.14 ± 0.05 (0.43 ± 0.07)

[a] The same ESR cell remained undisturbed in the cavity throughout the experiment to minimize any artifact due to differences in cell position. To maximize the signal-to-noise ratio, the instrument settings were: 20 mW power, and 6.7 G of modulation amplitude. The incubation contained 4.8 mg/ml supernatant protein, 100 μM arsenazo III, 1 mM $MgCl_2$ in 100 mM Tris–acetate buffer (pH 7.6), and the concentration of substrate indicated. The oxygen was displaced by purging with nitrogen, and the reaction was initiated with the substrate

[b] The reaction mixture contained supernatant (2 mg/ml) and 100 μM arsenazo III in the same buffer used for the ESR experiments. The values are nmol O_2/min per mg protein

[c] The values are the averages ± SD of 3 incubations

either NADH, NADPH, hypoxanthine, xanthine or N^1-methylnicotinamide as substrates. The reactions depended on all the components of the system and were not observable under aerobic conditions or after the thermal denaturation of the fraction. At variance with the results obtained with rat liver microsomal incubations [10], NADH was more effective than NADPH as electron donor for arsenazo III reduction. Neither of these activities was inhibited by 0.1 mM dicumarol. In addition, xanthine and hypoxanthine (substrates for the xanthine oxidase) and N^1-methylnicotinamide (substrate for the aldehyde oxidase) were much less effective as electron donors for arsenazo III reduction in fresh liver cytosolic fraction preparations (table 1). However, the effectiveness of xanthine and hypoxanthine as electron donors for arsenazo III reduction increased 30% after freezing the cytosolic fraction at −20°C for 48 h. This effect may be attributed to the conversion of the xanthine dehydrogenase of liver cytosol to xanthine oxidase, as has been observed previously [13]. When the incubations reported in table 1 were done in the absence of 1 mM $MgCl_2$, both the arsenazo III anion radical signal and the arsenazo III-stimulated oxygen consumption decreased dramatically to undetectable levels (not shown). $CaCl_2$ (1 mM) could replace $MgCl_2$ but was 50% less effective in stimulating both the formation of the azo anion radical and the O_2 consumption.

Only the addition of a very high concentration of antipyrylazo III (3 mM), in the presence of NADH, resulted in the appearance of a weak signal of the azo anion radical similar to that observed with the hypoxanthine–xanthine oxidase system (fig.1B). No signals could be detected using the other substrates listed in table 1, and no significant stimulation of O_2 consumption by the cytosolic fraction was observed even with a concentration as high as 3 mM.

4. DISCUSSION

This study has shown that liver cytosolic enzymes are able to reduce arsenazo III to an anion-free radical metabolite. In the presence of air, the arsenazo III redox cycling results in an increased oxygen consumption by the same fraction. In this regard, a cytosolic enzyme, presumably DT diaphorase, has previously been shown to reduce a

limited number of azo compounds [14,15], and xanthine oxidase was postulated [16] to catalyze azo reduction. However, the lack of effect of dicumarol on the NAD(P)H reduction of arsenazo III and the different activity observed with each reduced pyridine nucleotide rule out a major role for cytosolic DT-diaphorase [17] as the arsenazo III reductase.

At variance with the results obtained with rat liver microsomal incubations [10], addition of cations increased the arsenazo III-stimulated O_2 consumption by liver cytosolic fraction, thus indicating a stimulation of arsenazo III reduction by liver cytosol in addition to the radical-stabilization of the arsenazo III anion radical by the cations [10]. In addition, NADH was more effective than NADPH as the electron donor for arsenazo III reduction. This rules out that the NAD(P)H reduction of arsenazo III by our cytosolic fraction was due to microsomal contamination.

Arsenazo III reduction by the liver cytosolic fraction in the presence of substrates for xanthine oxidase (hypoxanthine, xanthine) or aldehyde oxidase (N^1-methylnicotinamide) was much lower than in the presence of reduced pyridine nucleotides. However, with a purified xanthine oxidase preparation, the arsenazo III anion radical was easily detected. As has been shown previously with rat liver microsomal incubations [10], Mg^{2+} was much more effective than Ca^{2+} in increasing the arsenazo III anion radical steady-state concentration.

In spite of the related structures of arsenazo III and antipyrylazo III, antipyrylazo III did not significantly increase the oxygen consumption by the rat liver cytosolic fraction, even at higher concentrations than those which were used to demonstrate Ca^{2+} transport in intact cells [7–9]. In addition, only weak ESR spectra of the azo anion radical could be detected with hypoxanthine-xanthine oxidase or NADH-liver cytosolic fraction incubations in the presence of very high concentrations of antipyrylazo III (3 mM).

Arsenazo III has been used for measuring cytosolic ionized Ca^{2+} concentrations in single cells in situ, as well as changes of cytosolic Ca^{2+} in response to metabolic, contractile, and electrical events [1]. The technique is based upon the introduction of arsenazo III into the cytosol of a single large cell through microinjection or into the cytosol of smaller cells by liposomes or microcapsule entrapment, and upon the kinetic measurements of the dye absorbance at 675–685 nm [1]. Usually, this indicator is used at 0.3–1 mM concentration [3–6, 18–21]. Our finding that, at a concentration much lower than that usually employed in intact cells, arsenazo III undergoes a one-electron reduction by cytosolic enzymes to produce an azo anion radical whose autoxidation generates $O_2^{\cdot-}$, raises questions as to the utility of this indicator for those assays. Since antipyrylazo III does not elicit such side reactions, its use should be preferred. In addition, experiments in which the effect of xanthine-xanthine oxidase on Ca^{2+} transport by intact mitochondria was measured by changes in arsenazo III absorbance [22] should be re-evaluated in view of the reduction of this dye by that system.

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REFERENCES

[1] Scarpa, A. (1979) Methods Enzymol. 56, 301–338.
[2] Campbell, A.K. (1983) Intracellular Calcium, Wiley, New York.
[3] Di Polo, R., Requena, J., Brinley, F.J. jr, Mullins, L.S., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433–467.
[4] Thomas, M.V. and Gorman, A.L.F. (1977) Science 196, 531–533.
[5] Brinley, F.J. jr, Tiffert, T., Scarpa, A. and Mullins, L.S. (1977) J. Gen. Physiol. 70, 355–384.
[6] Miledi, R., Parker, I. and Schalow, G. (1977) Nature 268, 750–752.
[7] Baylor, S.M., Chandler, W.K. and Marshall, M.W. (1982) J. Physiol. 331, 139–177.
[8] Palade, P. and Vergara, J. (1982) J. Gen. Physiol. 79, 679–707.
[9] Baylor, S.M., Quinta-Ferreira, M.E. and Hui, C.S. (1983) Biophys. J. 44, 107–112.
[10] Docampo, R., Moreno, S.N.J. and Mason, R.P. (1983) J. Biol. Chem. 258, 14920–14925.
[11] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–756.
[12] Mason, R.P. (1984) Methods Enzymol. 105, 416–422.

[13] Della Corte, E. and Stirpe, F. (1968) Biochem. J. 108, 349–351.
[14] Autrup, H. and Warwick, G.P. (1975) Chem.-Biol. Interact. 11, 329–342.
[15] Huang, M.T., Miwa, G.T., Cronheim, M. and Lu, A.Y.H. (1979) J. Biol. Chem. 254, 11223–11227.
[16] Fujita, S. and Peisach, J. (1982) Biochim. Biophys. Acta 719, 178–189.
[17] Lind, C., Hochstein, P. and Ernster, L. (1982) Arch. Biochem. Biophys. 224, 568–578.
[18] Hockeberger, P. and Connor, J.A. (1983) Science 219, 869–871.
[19] Akerman, K.E.O. and Heinonen, E. (1983) Biochim. Biophys. Acta 732, 117–121.
[20] Williamson, J.R., Williams, R.J., Coll, K.E. and Thomas, A.P. (1983) J. Biol. Chem. 258, 13411–13414.
[21] Harary, H.H. and Brown, J.E. (1984) Science 224, 292–294.
[22] Harris, E.J., Booth, R. and Cooper, M.B. (1982) FEBS Lett. 146, 267–272.