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NADPH-dependent flavoenzymes catalyze one electron reduction of metal ions and molecular oxygen and generate hydroxyl radicals

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This study reports a new property of the important NAD(P)H-dependent flavoenzymes, glutathione reductase, lipoyl dehydrogenase and ferredoxin-$NADP^+$ oxidoreductase, that can catalyze a one electron reduction of metal ions such as chromium(VI) and vanadium(V). During the enzymatic reduction process, molecular oxygen is reduced to H_2O_2, which reacts with the reduced metal complexes to generate hydroxyl radicals. Since the hydroxyl radicals have been suggested to play an important role in Cr(VI) toxicity, this study provides a basis for a recent observation that Cr(VI) mutagenesis is strongly oxygen dependent. These results also point to an enzymatic pathway for the metabolism of some metal ions and concomitant generation of hydroxyl radicals.

Flavoenzyme; Reduction of metal ions and molecular oxygen; ESR; Spin trapping; Hydroxyl radical

1. INTRODUCTION

The purpose of this communication is to report that some important NAD(P)H-dependent flavoenzymes (glutathione reductase, lipoyl dehydrogenase and ferredoxin-$NADP^+$ oxidoreductase) can catalyze one electron reduction of metal ions such as chromium(VI) and vanadium(V) and simultaneously reduce molecular oxygen to generate H_2O_2 and, eventually, the $^{\cdot}OH$ radical. This finding is significant because (i) the known substrates for the flavoenzymes are organic species and not metal ions, and (ii) the report on the enzymatic formation of the $^{\cdot}OH$ radical provides a new pathway for understanding the mechanism of genotoxicity of some non-ferrous metal ions. For example, it has been suggested that $^{\cdot}OH$ radical is likely to be the major species responsible for Cr(VI) genotoxicity [1-5]. The $^{\cdot}OH$ radical is generated through the reaction of Cr(V) complexes with cellular H_2O_2 [4,5]. Cr(V) itself is known to be generated from Cr(VI) in various biological systems, in particular, microsomes [6], mitochondria [7] and mitochondrial electron transfer chain complexes [8]. However, no specific enzyme responsible for the Cr(V) generation has been identified except possibly glutathione reductase [4]. In this study we have used ESR and spin trapping techniques to show that the flavoenzymes can generate Cr(V) and provide a catalytic redox cycle for $^{\cdot}OH$ formation in such reactions. Herein we present our results on Cr(VI) and summarize those on other systems.

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2. EXPERIMENTAL PROCEDURES

Ethanol, hydrogen peroxide, phosphate buffer solution of pH 7.2 and potassium dichromate were purchased from Fisher. Glutathione reductase (GSSG-R) from bovine intestinal mucosa, lipoyl hydrogenase from Clostridium, ferredoxin-$NADP^+$ oxidoreductase from spinach leaves, catalase from bovine liver, NADPH and sodium formate were purchased from Sigma. The spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was purchased from Aldrich and purified by charcoal decolorization [9].

ESR measurements were made using a Bruker ER 200D ESR spectrometer. This spectrometer operates at X-band (~9.7 GHz) frequencies and uses a rectangular TE_{102} resonant cavity equipped with a flat quartz cell. For accurate measurements of g-values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, model ER035A) and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, model 5340A). An ASPECT 2000 computer was used for data acquisition and analysis. The concentrations given in the figure legends were final concentrations. All experiments were carried out at room temperature.

3. RESULTS AND DISCUSSION

An aqueous Cr(VI) solution containing NADPH, lipoyl dehydrogenase, and the spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), at physiological pH (7.2) exhibits an ESR spectrum consisting of a 1:2:2:1 quartet with splittings of $a_H = a_N = 14.9$ G (Fig. 1a). Based on these splitting constants, the 1:2:2:1 quartet was assigned to a DMPO-OH adduct [10,11]. The peak at $g = 1.9792$ was assigned to a Cr(V) species as will be discussed later. Omission of any one component results in a strong decrease in the overall spectral intensity (Fig. 1b-d). A confirmational piece of evidence for the $^{\cdot}OH$ radical trapping was obtained through the standard $^{\cdot}OH$ scavenging competition ex-

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periments involving formate or ethanol [10,11]. As shown in Fig. 1e, addition of formate causes a significant decrease in the intensity of DMPO-OH spin adduct signal and leads to the appearance of a new spin adduct signal with measured hyperfine splittings of $a_H = 18.7$ G and $a_N = 15.7$ G. These splittings are typical of those of the DMPO-COO^- adduct [10]. Similar, confirmational, results were obtained with ethanol (Fig. 1f), showing that the DMPO-OH spin adduct is indeed the result of trapping of the $^{\cdot}OH$ radical formed during the reaction. ESR measurements showed also that the $^{\cdot}OH$ adduct signal decreases under anaerobic conditions (Fig. 1g), confirming the participation of molecular oxygen in $^{\cdot}OH$ generation. Furthermore, addition of catalase, whose function is to remove H_2O_2, essentially eliminated the DMPO-OH signal and substantially enhanced the Cr(V) peak (Fig. 1h). This observation is consistent with the result of the anaerobic measurement (Fig. 1g) wherein the Cr(V) signal is stronger than in Fig. 1a but smaller than that in Fig. 1h. Additional measurements involving controlled amounts of added H_2O_2 showed that the signal from the DMPO-OH adduct increases but that from Cr(V) decreases in proportion to the amount of H_2O_2 added (Fig. 1i, 1j). These results clearly indicate that molecular oxygen is reduced to H_2O_2 during the enzymatic Cr(VI) reduction, and this H_2O_2 subsequently reacts with Cr(V) to generate the $^{\cdot}OH$ radical.

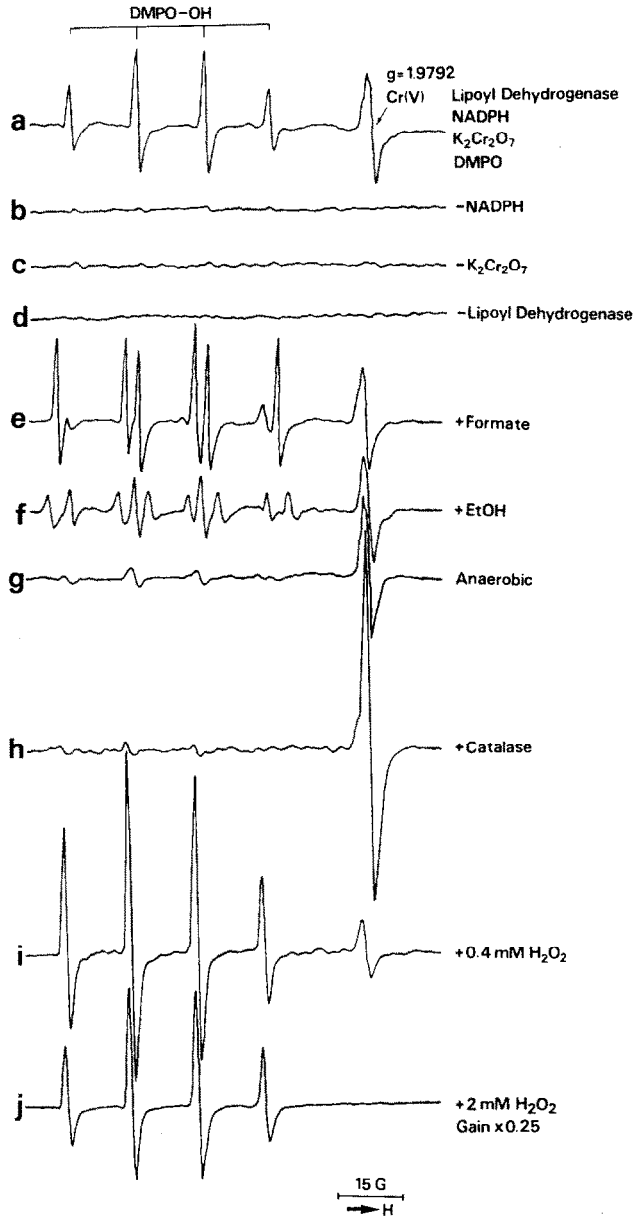


Fig. 1.(a) ESR spectrum recorded after mixing in a phosphate buffer solution (pH 7.2) of 60 mM DMPO, 2 mM NADPH, 2 mM $K_2Cr_2O_7$, and 40 units/ml lipoyl dehydrogenase. (b) Same as (a) but without NADPH. (c) Same as (a) but without $K_2Cr_2O_7$. (d) Same as (a) but without lipoyl dehydrogenase. (e) Same as (a) but with 0.2 M sodium formate added. (f) Same as (a) but with 0.2 M ethanol added. (g) Same as (a) but experiment was carried out in a nitrogen atmosphere. (h) Same as (a) but with 5000 units/ml catalase added. (i) Same as (a) but with 0.4 mM H_2O_2 added. (j) Same as (a) but with 2 mM H_2O_2 added.

For identification of the enzymatically generated Cr(V) intermediates and their role in $^{\cdot}OH$ generation, ESR measurements were made on a reaction mixture containing lipoyl dehydrogenase, NADPH and Cr(VI) under a narrower magnetic scan field (Fig. 2a). The spectrum obtained is centered at $g = 1.9792$ and exhibits five partially resolved principal components with 0.84 G spacing. This spectrum is reminiscent of that of a Cr(V)-NADPH complex reported earlier [5]. When the spectrum of Fig. 2a was recorded at a higher modulation amplitude and a wider scan width, it exhibited four satellite signals due to ^{53}Cr (9.55%, abundance, $I = 3/2$) hyperfine structure (Fig. 1b). The observed ^{53}Cr hyperfine splitting of 17.8 G is nearly the same as that for the earlier reported Cr(V)-NADPH complex and related

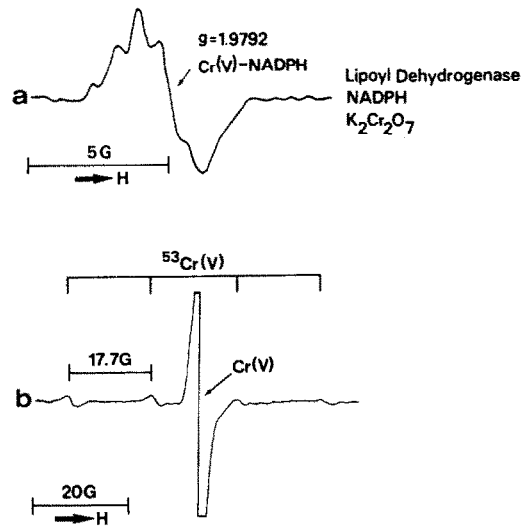
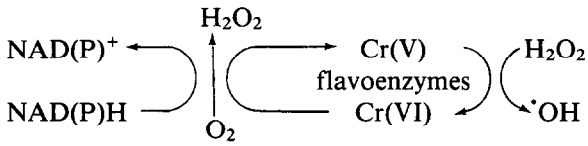


Fig. 2. (a) ESR spectrum recorded after mixing in a phosphate buffer solution (pH 7.2) of 2 mM NADPH, 2 mM $K_2Cr_2O_7$, and 40 units/ml lipoyl dehydrogenase. (b) Same as (a) but spectrum recorded at high spectrometer gain and wider scan width (indicated).

Cr(V) complexes with oxygen ligands [5,12–14]. Thus the spectra in Fig. 1a and 1b are assigned to a Cr(V)-NADPH complex.

Essentially similar results were obtained using glutathione reductase or ferredoxin-$NADPH^+$ oxidoreductase in place of lipoyl dehydrogenase, and NADH instead of NADPH. These results suggest the following pathway for the Cr(VI) reduction and $^{\cdot}OH$ generation:



This reaction scheme implies that during the Cr(VI)-mediated enzymatic $^{\cdot}OH$ generation, only NAD(P)H and molecular oxygen are consumed. Thus, a trace amount of Cr(VI) could catalytically generate the highly toxic $^{\cdot}OH$ radical and act as a radiomimetic $^{\cdot}OH$ generating system. The relatively long life-time (longer than minutes) of the Cr(V) complexes could enable them to reach cellular targets and cause a site-specific $^{\cdot}OH$ radical attack [15].

Using the same methodology, we detected vanadate(IV) formation in the NAD(P)H-dependent reduction of vanadate(V) by lipoyl dehydrogenase, as well as the production of $^{\cdot}OH$ radical (data not shown). It is possible that the above-mentioned flavoenzymes may also catalyze the reduction of other metal ions; such studies are currently in progress.

In conclusion, while earlier preliminary studies [4,16] had indicated that glutathione reductase can act as a reductase for chromate Cr(VI) and vanadate(V), the present study suggests that this is likely to be a general property of the NAD(P)H-dependent flavoenzymes. Since metal ions and molecular oxygen have no obvious similarity to the currently known (i.e. organic) substrates of these flavoenzymes, further investigation of the mechanism of the reduction of metal ions and molecular oxygen by such enzymes should be of fundamental importance from the general view point of enzymology and metal ion metabolism. Another new finding is that the reduction process uses molecular oxygen to generate H_2O_2, which in turn participates in the generation of the $^{\cdot}OH$ radical. The involvement of oxygen reduction in the Cr(VI)-mediated $^{\cdot}OH$ radical generation provides a possible basis for a recent observation that Cr(VI) mutagenesis is strongly oxygen dependent [17]. Further investigations on the mechanism of their H_2O_2 generation might reveal new insight on the biochemical, and, in particular, the metal toxicity aspects of these enzymes.

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REFERENCES

[1] Aiyar, J., Borges, K.M., Floyed, R.A. and Wetterhahn, K.E. (1989) Toxicol. Environ. Chem. 22, 135–148.
[2] Kawanishi, S., Inoue, S. and Sano, S. (1986) J. Biol. Chem. 261, 5952–5958.
[3] Kortenkamp, A., Ozolins, Z., Beyersmann, D. and O'Brein, P. (1989) Mut. Res. 216, 19–26.
[4] Shi, X. and Dalal, N.S. (1989) Biochem. Biophys. Res. Commun. 277, 342–350.
[5] Shi, X. and Dalal, N.S. (1990) Arch. Biochem. Biophys. 277, 342–350.
[6] Jennette, K.W. (1982) J. Am. Chem. Soc. 104, 874–875.
[7] Rossi, S.C., Gorman, N. and Wetterhahn, K.E. (1989) Chem. Res. Toxicol. 1, 101–107.
[8] Rossi, S.C. and Wetterhahn, K.E. (1989) Carcinogenesis 10, 913–920.
[9] Buettner, G.R. and Oberley, L.W. (1978) Biochem. Biophys. Res. Commun. 83, 69–74.
[10] Buettner, G.R. (1987) Free Radical Biol. Med. 3, 259–303.
[11] Morehouse, K.M. and Mason, R.P. (1988) J. Biol. Chem. 263, 1204–1211.
[12] Derouane, E.G. and Ouhadi, T. (1975) Chem. Phys. Lett. 31, 70–71.
[13] Gould, E.S. (1986) Acc. Chem. Res. 19, 66–72.
[14] Mitewa, M., Malinovski, A., Bontchev, P.R. and Kabassanov, K. (1984) Inorg. Chim. Acta 8, 17–23.
[15] Pryor, W.A. (1988) Free Radical Biol. Med. 4, 219–223.
[16] Shi, X. and Dalal, N.S. (1990) Arch. Biochem. Biophys. 278, 288–290.
[17] Sugden, K.D., Burris, R.B. and Rogers, S.J. (1990) Mut. Res. 244, 239–244.