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THE NITRIC OXIDE-MEDIATED DEGRADATION OF ACTIVE CENTER IN AN IRON-SULPHUR PROTEIN ADRENODOXIN

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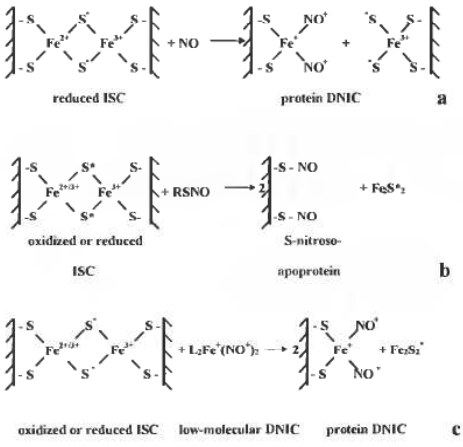
No effects of gaseous NO (700 mkm) or S-nitrosoglutathione (15-20 mkm) on the stability of active center of oxidized iron sulfur protein adrenodoxin (0.2 mkm) have been observed by using EPR method. However, dinitrosyl iron complexes (DNIC) with cysteine, thiosulphate or phosphate (1.8 mkm) were capable to destroy efficiently this center in both oxidized and pre-reduced adrenodoxin. This degradation was suggested to be due to the attack of $Fe^{+}(NO^{+})_2$ group from low-molecular DNIC to the thiol groups in active centers of adrenodoxin.

INTRODUCTION

At present many investiugators believe that one of the manifestations of nitric oxide cytotoxicity is the NO ability to destroy active centers of iron-sulphur proteins (ISP) (Reddy, Lancaster & Cornforth, 1983; Butler, Glidewell & Li, 1988; Drapier & Hibbs, 1988; Hibbs, Taintor, Vavrin, Granger, Drapier, Amber & Lancaster, 1990; Lancaster & Hibbs, 1990; Drapier, Pellat & Henry, 1991; Henry, Ducrocq, Drapier, Pellat & Giussani, 1991; Stadler, Bergonia, DiSilvio, Sweetland, Billiar, Simmons, & Lancaster, 1993; Drapier, Hirling, Wietzerbin, Kaldy & Kühn, 1993; Drapier, 1997). Since ISP is present as an important component of the mitochondrial respiratory chain, this impact results in the blockade of electron transport and coupled phosphorylation. Cells and tissues become depleted of energy supply provided by mitochondria and as a result die (Drapier & Hibbs, 1988, Hibbs *et al.*, 1990; Drapier *et al.*, 1993; Stadler *et al.*, 1993). Results of many investigations are in agreement with this hypothesis: nitric oxide completely suppresses mitochondial function at the level of Green's complexes I-II due to degradation of the complex constituent ISP (irreversible inhibition) (Drapier & Hibbs, 1988; Stadler *et al.*, 1993; Welter, Yu & Yu, 1996). Studies on the effect of nitric oxide on the inhibition of ISP have shown that degradation of active centers of these proteins (iron sulphur centers - ISC) are accompanied by formation of paramagnetic dinitrosyl iron complexes (DNIC) with thiol-containing protein ligands characterized by an EPR signal with axially symmetric g-tensor with $g_{\perp} = 2.04$ and $g_{\parallel} = 2.014$ (Reddy *et al.*, 1983; Lancaster & Hibbs, 1990; Drapier *et al.*, 1991; Henry *et al.*, 1991; Stadler *et al.*, 1993; Drapier *et al.*, 1993; Geng, Petersson, Wennmalm & Hansson, 1994; Welter *et al.*, 1996; Drapier, 1997). However, these investigations did not involve quantification of the DNIC formation and the ISC degradation. Our group did perform such investigations but on more complex systems (macrophage culture and animal tissues) (Vanin, 1987; Vanin, Men'shikov, Moroz, Mordvintcev, Serezhenkov & Burbaev, 1992). It was demonstrated that the DNIC formation was not coupled with degradation of ISC. Nitric oxide did not affect the intactness of ISC in these subjects. Based on these facts and on previous studies of the DNIC formation mechanism it was concluded that the complexes result from a reaction of nitric oxide with so-called "free" iron (loosely bound form of non-heme iron). These results are in agreement with data obtained by Fridovich's and Radi's groups that nitric oxide does not affect the activity of ISP aconitase (mitochondrial or cytosolic) (Hausladen & Fridovich, 1994; Castro, Rodriguez & Radi, 1994). Nevertheless, Kennedy, Antholine and Beinert (1997) showed using the EPR method that the contact of aconitase with nitric oxide induced both aconitase activity inhibition and DNIC formation. However, kinetic investigations have demonstrated a very slow rate of this inhibition. The biological activity twice decreased in 20 minutes while DNIC amount achieved 50% from maximum value in 40 minutes. Therefore, results of investigations by different authors provide rather controversial data on the interaction between nitric oxide and ISP and the question on the ability



Scheme 1

of NO to mediate the ISC degradation remains open.

In the present study we attempted to solve this question using adrenodoxin, an ISP isolated from bovine adrenal glands. The active center of this protein is characterized by a simpler structure than that of aconitase. As distinct from aconitase which contains a four-nuclear ISC, the adrenodoxin active center contains two iron atoms. The initial oxidized species of adrenodoxin is characterized by the trivalent state of both iron atoms. Upon reduction, one iron atom transforms into the bivalent state and the total electron spin is equal to 1/2 (Orme-Johnson & Beinert, 1969; Mukhin, 1969). In initial isolated aconitase, two iron atoms are in the bivalent state while the other two are in the trivalent state, with the total spin $S = 0$. Upon aconitase reduction, three iron atoms are in the bivalent state with the total electron spin $S = 1/2$ (Beinert, Kennedy & Stout, 1996).

DNIC emerge during the interaction between nitric oxide and bivalent iron Fe^{2+} (McDonald, Phllips & Mower, 1965; Vanin, 1967). Therefore, incubation of oxidized adrenodoxin in the NO atmosphere should not in principle lead to the formation of DNIC because, in this instance, iron atoms are in the trivalent state. These complexes can in principle appear only in the reaction of NO with reduced adrenodoxin. In this instance, the DNIC formation suggests replacement of inorganic bridge sulphur (S*) by NO (Scheme 1a). There are also other possible NO-mediated mechanisms of ISC degradation in adrenodoxin (Scheme 1 b,c). According to Castro *et al.* (1994), who observed the aconitase inactivation in the presence of S-nitrosoglutathione, this degradation could be initiated by S-nitrosation of ISC upon their contact with S-nitrosothiols. For example, as a result of S-nitrosation reaction, NO^+ can transfer from S-nitrosothiol to thiol groups of adrenodoxin to form S-nitroso-apoadrenodoxin and release an iron complex with inorganic sulphur (Scheme 1b) In this process, DNIC is not formed. Another mec -anism of ISP degradation in the presence of NO is possible: DNIC with low-molecular ligands (L) can transfer their $Fe^+(NO^+)_2$ groups to thiol ligands of ISP to displace Fe_2S_2* complexes (Scheme 1c).

In the present work, it was elucidated which of the proposed mechanisms can provide the NO - mediated degradation of adrenodoxin ISC.

MATERIALS AND METHODS

Materials

L-cysteine, sodium phosphate (Sigma, USA), sodium dithionite (Merck, Germany) and ferrous sulfate (Fluka, UK) were used in the experiments. Gaseous NO was synthesized in the reaction of $FeSO_4$ with $NaNO_2$ in 0.1 M HCl with subsequent purification by the method of fractional low-temperature sublimation in an evacuated system.

Synthesis of dinitrosyl-iron complexes

DNIC with cysteine, or phosphate were synthesized as described elsewhere (Vanin, Malenkova & Serezhenkov, 1997).

Experiments on adrenodoxin

Adrenodoxin was isolated from bovine adrenal glands by the method described elsewhere (Orme-Johnson & Beinert, 1969). The concentrations of isolated protein were evaluated using the optical method with the intensity of absorption band at 414 nm. The concentrations estimated were from 0.2 to 0.23 mM. The purity index of adrenodoxin preparation that was evaluated with the ratio of the intensities of absorption bands at 414 nm to 280 nm was equal to 0.8 that indicated high purity of adrenodoxin preparations . The reduced species of adrenodoxin were obtained by treatment of initial oxidized preparations with sodium dithionite dry powder (final concentration 10 mM) + methyl viologen as a redox mediator (final concentration 0.1 mM). The treatment of adrenodoxin preparations with NO was carried out in a Thunberg vial pre-evacuated before the administration of gaseous NO at the pressure of 100 mm Hg following NO evacuation. In some experiments 1.8 mM Fe^{2+} - citrate complex ($FeSO_4$: sodium citrate = 1:5) was added to the solutions before the NO treatment. In all experiments adrenodoxin preparations were treated with NO, NO + Fe^{2+} - citrate, or low-molecular DNIC for 10 min. ISC decomposition in

adrenodoxin preparations induced by these agents was monitored with downfall in the intensity of the EPR signal at g 1.94 characteristic for reduced species of adrenodoxin.

EPR measurments.

EPR spectra were recorded at 90K or ambient (room) temperature using an EPR radiospectrometer, either a modified Radiopan (Poland) or an ESC-106 (Bruker, Germany) in *X*-diapason, respectively. The concentration of various paramagnetic centers was evaluated by the method of double integration of EPR signals using DNIC-cysteine with known concentration as a reference sample.

RESULTS AND DISCUSSION

The contact of oxidized adrenodoxin (0.2 mM) with gaseous NO (at the pressure of 100 mm Hg) did not result in degradation of the protein active center: after subsequent reduction of the preparation by dithionite (Fig. 1b), the intensity of EPR signal at g 1.94 was the same as in the original reduced adrenodoxin (Fig. 1). The broad signal recorded in the g-factor range 2.07 - 1.98 (Fig. 1) with a triplet hyperfine structure (HFS) derived from nitrosyl complexes of admixtured heme-containing proteins. The concentrations of the latters did not excced 10 mkM. A similar result was obtained in the treatment of oxidized adrenodoxin with S-nitrosoglutathione (20 mM) (not shown). The evaluation of the amount of NO in Thunberg flask (100 ml) made by according to Avogadro`s low led to the value of 700 mkm of NO that was 300 times more than the amount of adrenodoxin in the solution (0.2 mkm).

Another effect was observed after NO treatment of oxidized adrenodoxin in the presence of Fe^{2+}-citrate complex (1.8 mM) in the solution. The DNIC formation in this preparation (0.2-0.3 mM)

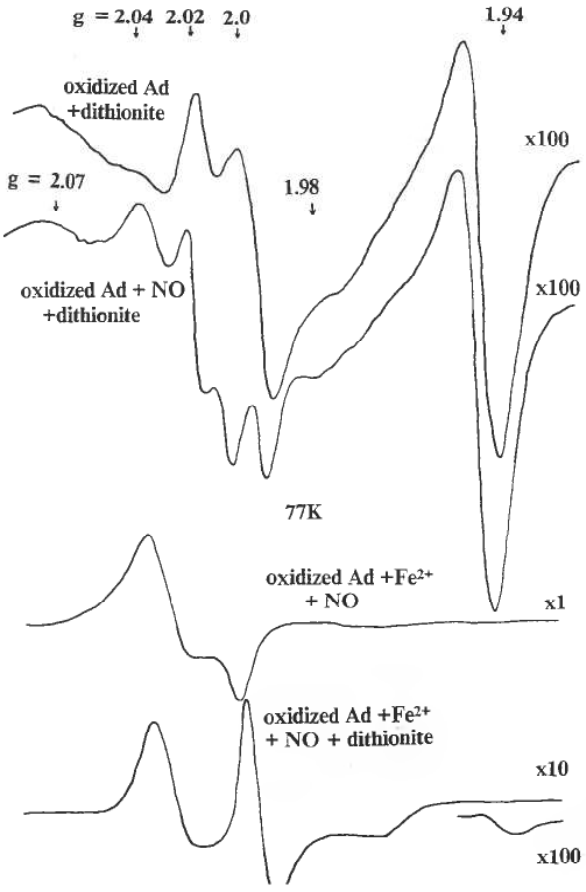


Fig. 1 EPR spectra from 0.2 mM solutions of oxidized adrenodoxin treated with dithionite, NO + dithionite, Fe^{2+} (1.8 mM)+ NO, or Fe^{2+} + NO + dithionite. Recordings were made at 77K, microwave power 5 mW, and modulation amplitude 0.5 mT. To the right of the spectra, relative amplification of radiospectrometer.

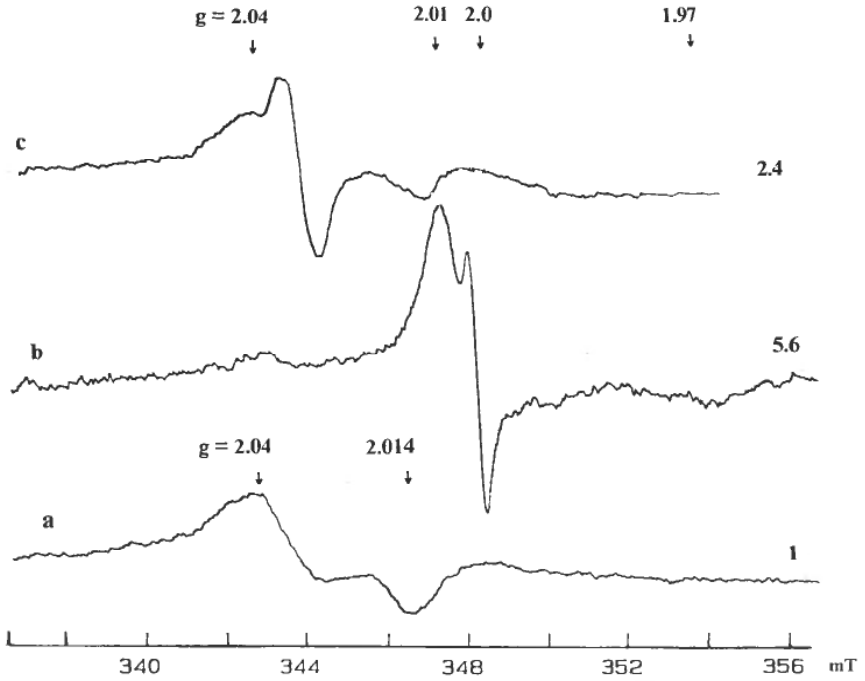


Fig. 2 EPR spectra from 0.2 mM solution of oxidized adrenodoxin treated with $FeSO_4$ (1.8 mM) + NO (a) followed with dithionite (b) and then exhibited in air for 10 min (c). The spectra were recorded at ambient temperature at a microwave power of 20 mW and modulation amplitude 0.5 mT. At right, relative amplification of radiospectrometer.

was accompanied with complete ISC degradation in adrenodoxin (Fig. 1). The shape of the EPR signal from DNIC resulting from the g-factor anisotropy remained unchanged in increasing the registration temperature from 77K (Fig. 1) to ambient (Fig. 2a). This unambiguously indicated a connection of the complexes with the protein globule: the low mobility of protein DNIC was not sufficient for averaging the forementioned anisotropy. The treatment of protein DNIC with dithionite resulted in a sharp decrease of the intensity of this EPR signal and the registration of transitory EPR signal with $g_{\perp} = 2.01$ and $g_{||} = 1.97$(Fig. 1, Fig. 2b). This transformation was due to the capability of DNIC to accept two electrons by one-electron mechanism. In the process DNIC changes from the state with d^7 electronic configuration of iron to the states that may be formally (if suggesting localization of both arriving electrons on the iron atom) described as d^8 (diamagnetic state) and d^9 (paramagnetic state) (Burbaev & Vanin, 1973). The effect of dithionite treatment was reversible: being exhibited in air this solution gave again the EPR signal with $g_{\perp} = 2.04$ and $g_{II} = 2.014$ (d^7 electronic configuration of the iron atom) (Fig. 2c). The registration of the narrow symmetric EPR signal at $g = 2.03$ overlapping the anisotropic signal from protein DNIC was due to the formation of low-molecular DNIC with thiosulphate ligands. The latters were originated from dithionite.

The addition of pre-prepared low-molecular DNIC with cysteine or phosphate at the concentrations 1.8 mM or 0.4 mM respectively to the solutions of oxidized adrenodoxin (0.2 mM) resulted also in efficient degradation of ISC (Fig. 3). The effect of less stable DNIC with phosphate was more expressed. Similar results were obtained in addition of Fe^{2+} ions (1.8 mM) in the presence of NO or "ready-to-use" low-molecular DNIC with phosphate to the solutions of adrenodoxin pre-reduced with dithionite (Fig. 4). Thus, these results demonstrated that DNIC with various anionic ligands are capable to destroy efficiently ISC in adrenodoxin apparently by transfer of their $Fe^{+}(NO^{\bullet})_2$ groups to thiol components of ISC according to Scheme 1c.

As soon as 1-2 minutes after gaseous NO treatment of adrenodoxin preparation pre-reduced with dithionite, the signal at g 1.94 characteristic of this species dissapeared (Fig. 4). Furthermore an EPR signal at 2.04 appeared which is typical for DNIC. The following dithionite treatment of these prepa-

rations resulted in emergence of a signal from reduced adrenodoxin species. However, the signal intensity was 2 times less than that of initial reduced adrenodoxin (Fig. 4). It means that NO alone can destroy reduced ISC in adrenodoxin and form simultaneously DNIC obviously through the mechanism shown in Scheme 1a. However the amount of formed DNIC was too small (2-4 mkm) as compared to the amount of degraded ISC (100 mkm). These data are not in line with the mechanism of ISC decomposition induced with NO alone. The formation of high amount of DNIC including thiol groups and Fe^{2+} from degraded ISC should be expected in according to this mechanism (Scheme 1a). The low level of DNIC experimentally observed might be due to destabilizing effect of nitrogen dioxide (NO_2) admixed to gaseous NO on protein DNIC described elsewhere (Vanin *et al.*, 1997). The presence of NO_2 characterized by strong oxidative properties is indicated by the fact that the signal from reduced adrenodoxin rapidly dissapears on the contact with gaseous NO (Fig. 4). The occurrence of NO_2 in gaseous NO has been shown to be ensured by interaction of NO molecules with formation of both NO_2 and N_2O (Bonner & Stedman, 1996), The interaction takes place continuously in gaseous NO so that NO_2 is always present in gaseous NO in some or another amount.

The decomposition of protein DNIC with NO_2 could also mobilize $Fe^{+}(NO^{+})_2$ groups by including these groups into unstable low-molecular DNIC. The formation of the latters facilitate the transfer of $Fe^{+}(NO^{+})_2$ groups to thiol components of ISC thereby inducing ISC decomposition according to the mechanism shown in Scheme 1c. These two possible mechanisms of ISC degradation in pre-reduced adrenodoxin treated with gaseous NO are under study now.

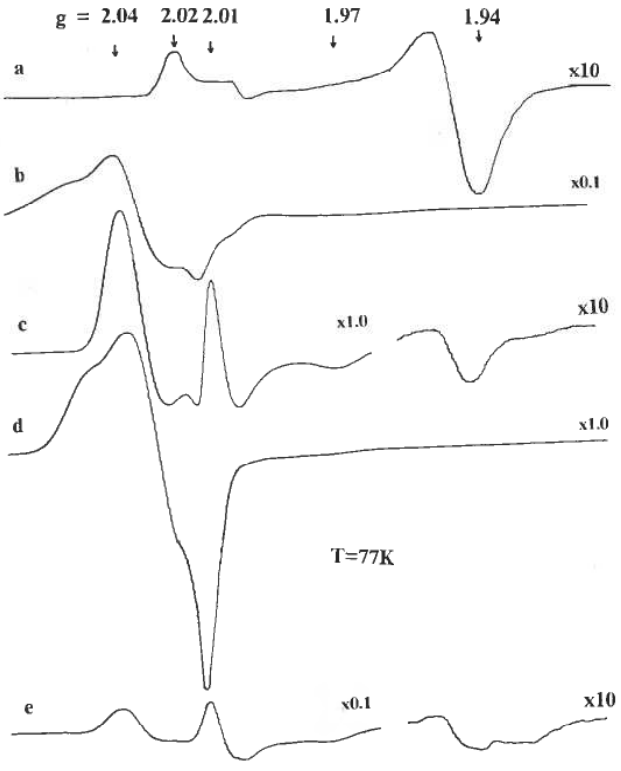


Fig. 3 EPR spectra from 0.2 mM solution of reduced adrenodoxin (a), 0.2 mM solution of oxidized adrenodoxin treated with DNIC- cysteine (1.8 mM) followed with dithionite (b and c, respectively), 0.2 mM solution of oxidized adrenodoxin treated with DNIC-phosphate (0.4 mM) followed with dithionite (d and e, respectively). Conditions of spectrum recording are the same as those in Fig. 1. At right, relative amplification of radiospectrometer.

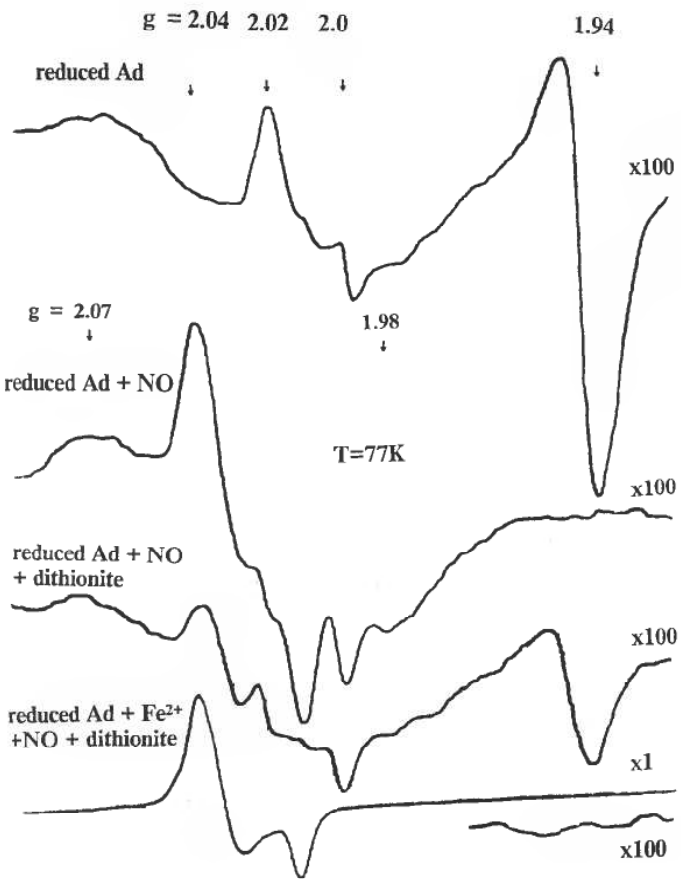


Fig. 4 EPR spectra of reduced adrenodoxin untreated, or treated with NO, NO + dithionite, or Fe^{2+} (1.8 mM) + NO + dithionite. Conditions of spectrum recordings are the same as those in Fig. 1. At right, relative amplification of radiospectrometer.

GENERAL CONCLUSIONS

Therefore, the NO-mediated ISC decomposition in adrenodoxin can be catalyzed by Fe^{2+} ions added to the medium or released from degraded ISC. Bivalent iron and NO form DNIC which are capable to transfer their $Fe^{+}(NO^{+})_2$ groups to thiol groups in ISC that results in ISC degradation and protein DNIC formation.

Acknowledgments

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