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Interaction of Oxoferrylmyoglobin and Dinitrosyl-Iron Complexes

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Abstract—It is shown that dinitrosyl-iron complexes (DNIC) with glutathione can reduce oxoferrylmyoglobin forming on interaction of *tert*-butyl hydroperoxide and metmyoglobin. A rapid decrease in the DNIC concentration was observed under the conditions of production of *tert*-butyl free radicals; however, destruction of DNIC in the presence of oxoferrylmyoglobin alone was negligible. It is demonstrated that DNIC reduces oxoferrylmyoglobin more than an order more efficiently than S-nitrosoglutathione and glutathione. DNIC also inhibits formation of the thiyl radicals of glutathione in a medium containing metmyoglobin and *tert*-butyl hydroperoxide. A mechanism of the antioxidant action of DNIC based on regeneration of the nitrosyl complexes from the products of their interaction with oxoferrylheme is proposed.

Key words: nitric oxide, dinitrosyl-iron complexes, oxoferrylmyoglobin, free radicals, hydroperoxides

It is known that myoglobin, hemoglobin, and other heme proteins play a role in destructive processes developing in the course of oxidative stress [1-8]. Reactions between heme proteins and organic hydroperoxides give alkoxyl and alkyl peroxyl radicals—intermediates of free-radical oxidation of lipids [2-4]. However, on interaction of hydrogen peroxide or organic hydroperoxides with heme proteins, oxoferrylheme (porphyrin-Fe^{IV}=O) is formed; the latter can also cause an oxidative modification of biologically important molecules [1-8]. It has been found that many antioxidants including ascorbate, α-tocopherol, ubiquinone (coenzyme Q), glutathione, and other thiols reduce oxoferrylheme proteins [5-7]. It has been shown that nitric oxide (NO) decreases prooxidant action of oxoferrylheme proteins and reduces them to ferri form (porphyrin-Fe^{III}) [2, 3, 9, 10]. However, interaction of NO with free lipid radicals is considered to be the main mechanism of antioxidant action of nitric oxide; as a consequence chain reactions of free-radical oxidation are terminated, and nitro- and nitroso- peroxiderivatives of lipids are formed [11-13]. Earlier we demonstrated that oxidative destruction of β-carotene initiated by metmyoglobin and hydroperoxides of polyunsaturated fatty acids is efficiently inhibited by dinitrosyl-iron complexes with glutathione (DNIC) [14]. Dinitrosyl-iron complexes are one of the physiological forms of NO transport and deposition, the nitrosyl and thiol ligands (glutathione, cysteine) being DNIC constituents. These data suggest that DNIC can react with oxoferrylheme and free organic radicals. In this work, we have attempted to study the effect of DNIC on formation and prooxidant action of the oxoferryl form of myoglobin.

MATERIALS AND METHODS

Reagents. In this study, we used reduced glutathione from Calbiochem (USA); *tert*-butyl hydroperoxide from Merck (Germany); 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) from OXIS (USA); and metmyoglobin from Sigma (USA).

DNIC, *S*-nitrosoglutathione, and oxoferrylmyoglobin preparations. *S*-Nitrosoglutathione (GSNO) and dinitrosyl-iron complexes (DNIC) with glutathione in diamagnetic dimeric form were obtained as described earlier [15]: 300 mM $NaNO_2$ and 200 mM reduced glutathione in acidic medium, or $FeSO_4$ and glutathione solutions in

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the molar ratio 1 : 2, respectively, were treated with gaseous NO in a Thunberg vessel. GSNO and DNIC preparations were stored at –20°C. Ferrylmyoglobin was obtained by addition of 0.4 mM *tert*-butyl hydroperoxide or 1 mM H_2O_2 to 0.1 mM metmyoglobin in 0.1 M phosphate buffer, pH 7.4. On incubation of DNIC with oxoferrylmyoglobin and *tert*-butyl hydroperoxide, 0.1-1.0 mM diethylenetriaminopentaacetic acid (DTPA), an iron chelator, was added to the reaction mixture. DNIC concentration was determined by EPR spectroscopy. Since dimeric form of DNIC used in this study is not EPR-detectable, it was converted into the monomeric EPR-detectable form. For this, cysteine was added to the studied solutions in the molar ratio cysteine/DNIC = 25 : 1, and this resulted in formation of the monomeric EPR-detectable DNIC with cysteine. GSNO (ε_{338} = 930 $M^{-1}\cdot cm^{-1}$) [14], metmyoglobin (ε_{630} = 3.5 $mM^{-1}\cdot cm^{-1}$) and oxymyoglobin (ε_{580} = 14.4 $mM^{-1}\cdot cm^{-1}$) [5] concentrations were determined spectrophotometrically using the given molar extinction coefficients. Oxoferrylmyoglobin concentration was determined by optical absorption at 421 nm (ε_{421} = 111 $mM^{-1}\cdot cm^{-1}$) and 556 nm [5, 9]. For GSNO, oxymyoglobin, metmyoglobin, and oxoferrylmyoglobin, optical absorption spectra were recorded using a Beckman Coulter Δ 650 spectrometer (USA) in the wavelength range from 410 to 700 nm.

EPR spectra. Sample aliquots (80 μl) were placed in glass capillaries and EPR spectra were recorded using a E-109E spectrometer from Varian (USA) under the following conditions: 25°C, microwave power 10 mW, microwave frequency 9.15 GHz, and modulation amplitudes 0.1 or 0.4 mT for DEPMPO spin-adducts or DNIC, respectively.

RESULTS AND DISCUSSION

Earlier we showed that DNIC can function as typical antioxidants in a system metmyoglobin–arachidonic acid–β-carotene, that is they are able of capturing alkoxyl (RO$^\cdot$) and alkyl peroxyl (ROO$^\cdot$) radicals formed in reactions (1) and (2):

$$\text{porphyrin-Fe}^{III} + \text{ROOH} \rightarrow$$

$$\rightarrow \text{porphyrin-Fe}^{II} + \text{ROO}^\cdot + \text{H}^+, \qquad (1)$$

$$\text{porphyrin-Fe}^{II} + \text{ROOH} \rightarrow$$

$$\rightarrow \text{porphyrin-Fe}^{III} + \text{RO}^\cdot + \text{OH}^-. \qquad (2)$$

Nonetheless, it cannot be excluded that DNIC inhibit destruction of β-carotene due to their interaction with oxoferrylmyoglobin forming in reaction (3) and also capable of oxidizing β-carotene [7]:

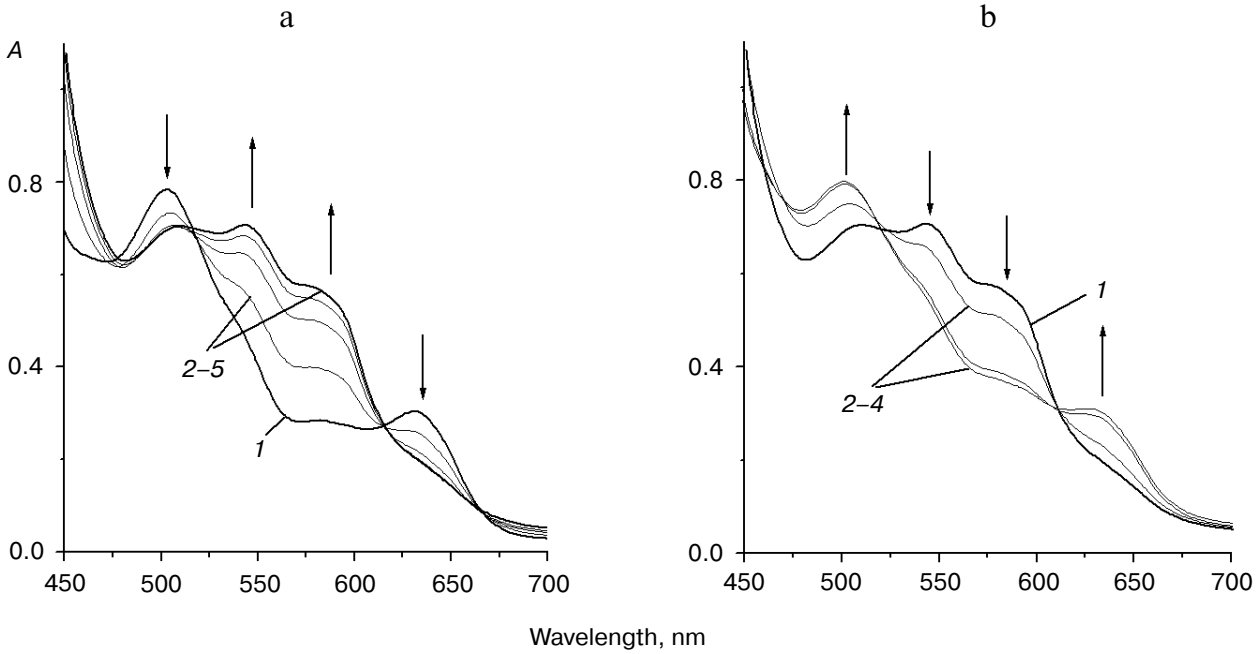

Fig. 1. a) Kinetics of formation of oxoferrylmyoglobin on incubation of *tert*-butyl hydroperoxide with metmyoglobin: *1*) 0.1 mM metmyoglobin in 0.1 M Na,K-phosphate buffer, pH 7.4; *2-5*) the same solution with addition of 0.4 mM *tert*-butyl hydroperoxide after 1, 2, 4, and 6 min incubation, respectively. b) Kinetics of reduction of oxoferrylmyoglobin by DNIC: *1*) oxoferrylmyoglobin formed after 6 min incubation of metmyoglobin with *tert*-butyl hydroperoxide (the same as *5* in Fig. 1a); *2-4*) the same solution with addition of 0.1 mM DNIC after 1, 3, and 5 min incubation, respectively. Decrease and increase in characteristic maxima of metmyoglobin and oxoferrylmyoglobin are marked by arrows.

$$\text{porphyrin-Fe}^{III} + \text{ROOH} \rightarrow$$

$$\rightarrow \text{porphyrin-Fe}^{IV}\text{=O} + \text{RO}^{\cdot} + \text{H}^{+}. \quad (3)$$

In this study, oxoferrylmyoglobin was formed in a system containing metmyoglobin and *tert*-butyl hydroperoxide; this was proved by characteristic changes in optical absorption spectra (absorption increase at 548 and 582 nm) [7]. The kinetics of this process is presented in Fig. 1a. Addition of DNIC initiated a reverse reaction — reduction of oxoferrylmyoglobin to metmyoglobin in accordance with the kinetics presented in Fig. 1b. Under these conditions we could expect formation of the ferro form of myoglobin (reaction (1)) and further, of oxymyoglobin in reaction (4):

$$\text{porphyrin-Fe}^{II} + \text{O}_2 \rightarrow \text{porphyrin-Fe}^{II}\text{–O}_2. \quad (4)$$

As shown in Fig. 1b, in our experiments significant formation of oxymyoglobin was not observed; this seems to be due to interaction of ferromyoglobin with tert-butyl hydroperoxide (reaction (2)) or autooxidation of oxymyoglobin catalyzed by nitric oxide according to reaction (5) [16]:

$$\text{porphyrin-Fe}^{II}\text{–O}_2 + \text{NO} \rightarrow$$

$$\rightarrow \text{porphyrin-Fe}^{III} + \text{NO}_3^{-}. \quad (5)$$

Reduction of oxoferrylmyoglobin was accompanied by rapid decrease in the DNIC concentration, which was monitored by the EPR signal typical of dinitrosyl-iron complexes (Fig. 2). This is consistent with our suggestion that DNIC are capable of interaction with intermediates arising in reactions (1)-(3), that is with the free organic radicals and oxoferrylmyoglobin. Within the model scheme including *tert*-butyl hydroperoxide and metmyoglobin, it was rather difficult to determine the contributions of free radicals and oxoferrylmyoglobin to the destruction of DNIC. To determine this contribution, we studied only the effect of oxoferrylmyoglobin on DNIC. The former was obtained in the reaction of metmyoglobin and H_2O_2; excess H_2O_2 was removed with catalase. Interaction of DNIC with oxoferrylmyoglobin really caused destruction of the studied complexes, and this process was accompanied by reduction of oxoferrylheme to its met-form (Figs. 3 and 4). It should be noted that significant decrease in the DNIC concentration was observed only at high molar ratios oxoferrylmyoglobin/DNIC. Thus, 50% decrease in the DNIC concentration was observed at $[Mb^{IV}=O]/[DNIC]_0 = 8$ (Fig. 3). However, essentially complete reduction of oxoferrylmyoglobin took place at $[Mb^{IV}=O]/[DNIC]_0 = 3$ and lower (Fig. 4).

So, on reduction of oxoferrylmyoglobin there was no significant decrease in the DNIC concentration compa-

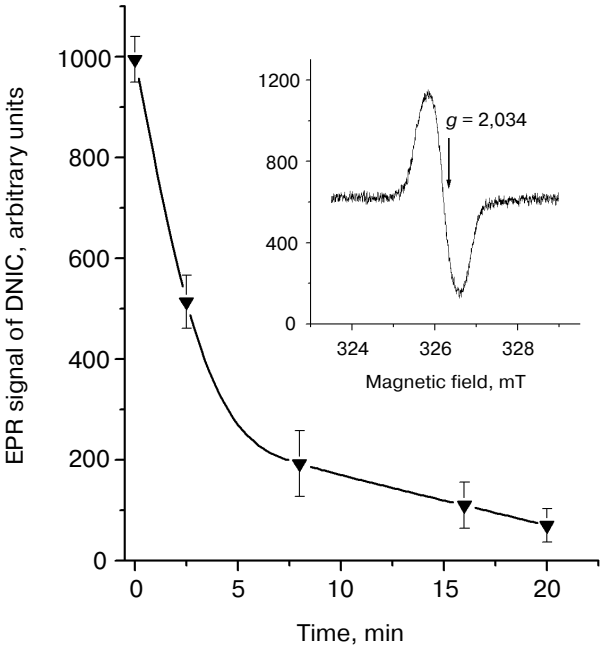

Fig. 2. Kinetics of destruction of 0.15 mM DNIC (monitored by change in the EPR signal) incubated with 0.1 mM metmyoglobin in the presence of 0.4 mM *tert*-butyl hydroperoxide. Before the EPR spectra were recorded, 4 mM cysteine was added to the samples. The upper inset presents the EPR spectrum of 0.15 mM DNIC in the presence of 0.1 mM metmyoglobin.

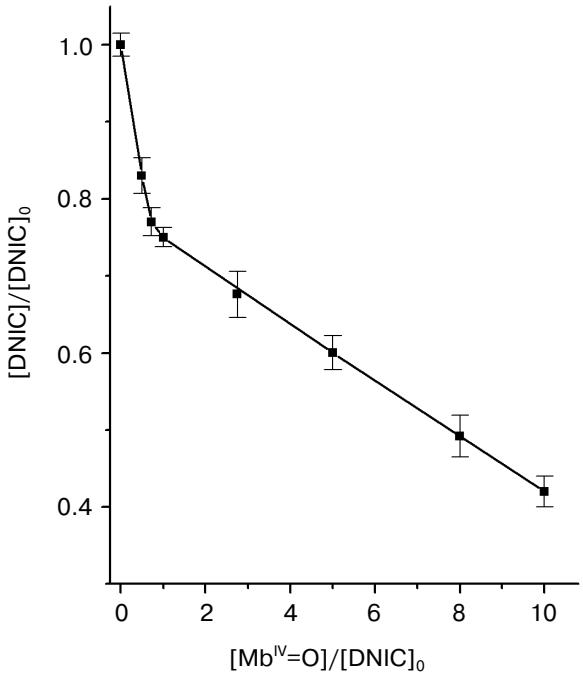

Fig. 3. Destruction of DNIC versus oxoferrylmyoglobin (Mb^{IV}=O) concentration. Oxoferrylmyoglobin (0.075-0.8 mM) was obtained by incubation of 1 mM H_2O_2, after 1 min 0.1 mM DTPA, 400 U/ml catalase, and 0.08-0.15 mM DNIC were added. After 5-min incubation, 2-4 mM cysteine (depending on the DNIC concentration) was added, and the EPR spectra were recorded.

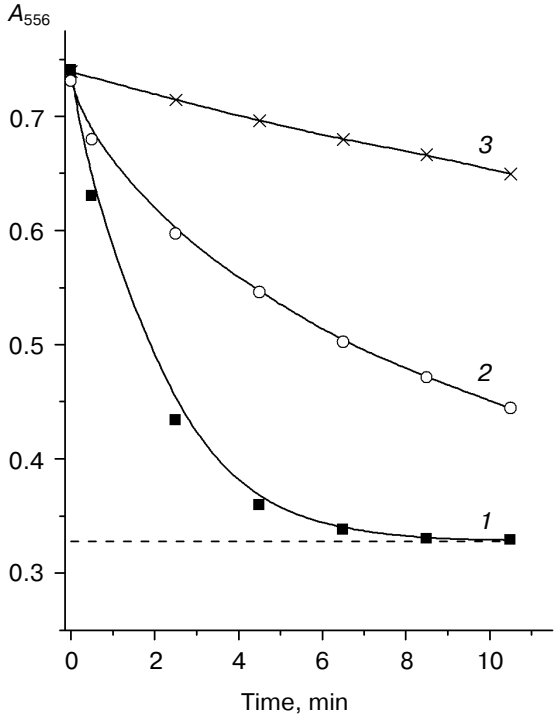

Fig. 4. Kinetics of reduction of oxoferrylmyoglobin by the action of DNIC. Oxoferrylmyoglobin (75 μM) was obtained as described in Fig. 3 caption. Optical absorption of metmyoglobin at 556 nm is shown by the dotted line. DNIC concentrations (μM): *1*) 25; *2*) 12.5; *3*) 0.

rable with analogous decrease in the system containing additionally *tert*-butyl hydroperoxide, that is, when alkoxyl and alkyl peroxyl radicals are generated (Figs. 2 and 3). These results indicate that there are free *tert*-butyl radicals, which are mainly responsible for decrease in concentration of the EPR-detectable DNIC in our experiments.

In principle, all components of DNIC being in the state of chemical equilibrium with these complexes can participate in reduction of oxoferrylmyoglobin and capture of free *tert*-butyl radicals:

$$(RS^-)_2{-}Fe{-}(NO^+)_2 \Leftrightarrow$$

$$\Leftrightarrow Fe^{2+} + NO + RSNO + RS^-. \qquad (6)$$

The molecules of NO and thiols can play a special role in this process. According to [5], reduction of oxoferrylmyoglobin by various thiols is accompanied by formation of the thiyl radicals. In fact, we observed formation of a typical adduct of thiyl radical with a DEPMPO spin trap in the reaction medium containing metmyoglobin, *tert*-butyl hydroperoxide, and GSH (Fig. 5, spectrum *1*) [17, 18]. Nonetheless, thiyl radicals were not formed on addition of DNIC instead of GSH into the reaction mixture (Fig. 5, spectrum *2*). Moreover, under these conditions DNIC acted as the typical antioxidants, significantly inhibiting formation of the thiyl radical of free GSH (Fig. 5, spectrum *3*). It should be noted that DNIC also inhibited formation of the DEPMPO adduct with *tert*-butyl radicals (Fig. 5, spectra *2* and *4*).

Reducing action of DNIC on oxoferrylmyoglobin also manifests itself in the quantitative inhibition of its accumulation in the system containing *tert*-butyl hydroperoxide and metmyoglobin (Fig. 6, curves *4-7*). It should be noted that GSH and GSNO added to the reaction mixture instead of DNIC inhibit this process by more than an order weaker than these complexes (Fig. 6, curves *1-4*). Consequently, reduction of oxoferrylmyoglobin by DNIC is mainly caused by the NO molecules from these complexes (reaction (6)).

According to the latest data, in the course of reaction of NO with oxoferryl forms of hemoglobin and myoglobin a porphyrin-Fe^{III}-ONO complex is formed, which is later decomposed to nitrite and the ferri-forms of heme proteins [9, 10]:

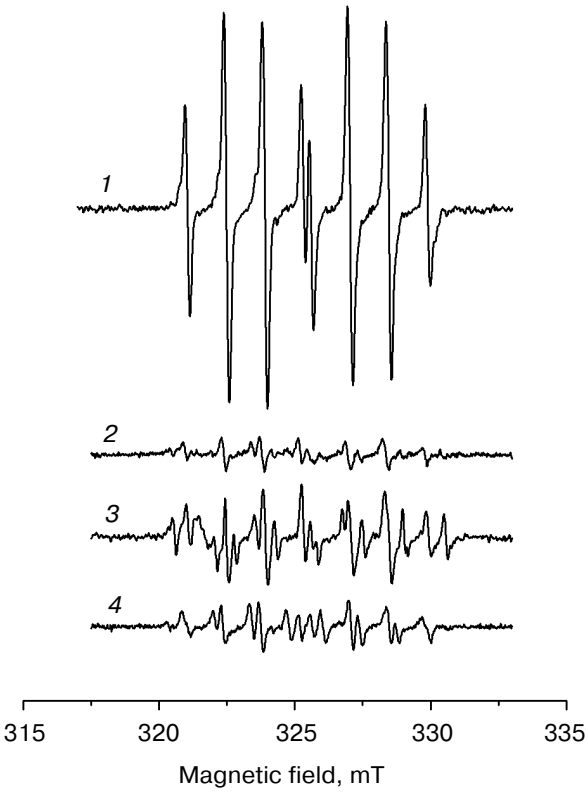

Fig. 5. EPR spectra of DEPMPO spin-adducts with the thiyl radical obtained after addition to the reaction mixture of 1.6 mM GSH (*1*), 0.8 mM DNIC (*2*), 0.8 mM DNIC and 1.6 mM GSH (*3*) and without additions (superposition of the spectra of DEPMPO spin-adducts and *tert*-butyl radicals) (*4*). The reaction mixture contained 0.1 M Na,K-phosphate buffer, pH 7.4, 20 mM DEPMPO, 0.1 mM metmyoglobin, 1.6 mM *tert*-butyl hydroperoxide, and 1 mM DTPA.

porphyrin-Fe^{IV}=O$^{\cdot}$ + NO →

→ porphyrin-Fe^{III}–ONO →

→ porphyrin-Fe^{III} + NO_2^-. (7)

An analogous mechanism can be suggested for interaction of oxoferrylmyoglobin with DNIC:

porphyrin-Fe^{IV}=O + $(RS^-)_2$–Fe–$(NO^+)_2$ →

→ porphyrin-Fe^{III} +

+ $(RS^-)_2$–Fe^{II}–(NO^+)ONO. (8)

It cannot be excluded that, on interaction with oxoferrylmyoglobin, DNIC themselves form Fe-ONO-containing intermediates, which are probably again reduced to the dinitrosyl-iron complexes in the presence of cysteine or GSH (reaction (9)):

$(RS^-)_2$–Fe^{II}–(NO^+)ONO + RSH →

→ RSOH + $(RS^-)_2$–Fe–$(NO^+)_2$. (9)

Interaction of the porphyrin-Fe^{III}-ONO complex and thiols with formation of *S*-nitrosothiols (reaction (10)) converting into DNIC in the reaction with iron can be one more mechanism of the regeneration of DNIC [15]:

porphyrin-Fe^{III}–ONO + RSH →

→ porphyrin-Fe^{III}–OH + RSNO. (10)

Biphasic dependence of DNIC destruction on concentration of oxoferrylmyoglobin seems to be related with formation of various intermediate complexes (Fig. 3). So, DNIC can probably catalyze reduction of oxoferrylheme by thiols; thus, it can possibly explain a low level of DNIC destruction while interaction with oxoferrylmyoglobin. The data indicate that under our experimental conditions DNIC can be regenerated like the low-molecular-weight phenol antioxidants such as ubiquinone and α-tocopherol [13, 19, 20].

However, in the system containing metmyoglobin and *tert*-butyl hydroperoxide, interaction of DNIC with free organic radicals can affect catalytic function of the former in the reduction of oxoferrylmyoglobin by thiols, and such interaction results in the irreversible destruction of DNIC (Fig. 2). It is known that the rate constant of interaction of NO with oxoferrylmyoglobin ($1.8\cdot10^7$ $M^{-1}\cdot sec^{-1}$) [9] is almost two orders of magnitude lower than the rate constant of its interaction with alkyl peroxyl radicals ($1.3\cdot10^9$ $M^{-1}\cdot sec^{-1}$) [11]. Consequently, as we suggested, destruction of DNIC in the presence of metmyoglobin and *tert*-butyl hydroperoxide occurs mainly as a result of the reaction between NO and free *tert*-butyl radicals. Nonetheless, kinetic ratio of the reactions of DNIC with oxoferrylmyoglobin and free *tert*-butyl radicals provides an efficient reduction of oxoferrylheme by these complexes (Figs. 1 and 6).

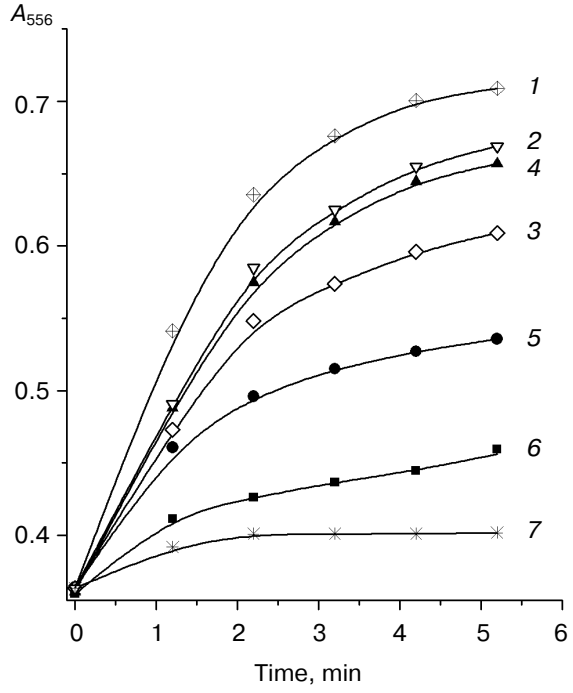

Fig. 6. Kinetics of formation of ferrylmyoglobin on interaction of 0.4 mM *tert*-butyl hydroperoxide and 0.1 mM metmyoglobin (*1*) and kinetic curves of ferrylmyoglobin accumulation in the presence of 360 μM GSNO (*2*), 360 μM glutathione (*3*), or 25 (*4*), 60 (*5*), 120 (*6*), and 200 μM DNIC (*7*).

The suggested mechanism of the mediatory action of DNIC in reduction of oxoferrylheme by thiols can be the basis for the antioxidant properties of NO and *S*-nitrosothiols in a free-radical oxidation induced by heme proteins [4, 14, 21]. In this case, nitric oxide and *S*-nitrosothiols can be precursors of the nitrosyl-iron complexes able to reduce efficiently the oxoferryl forms of heme proteins. It has been shown that on incubation of erythroleukemic cell culture with *tert*-butyl hydroperoxide and hemoglobin, the antioxidant action of NO is accompanied by formation of the nitrosyl-iron complexes [4, 21]. In humans and animals, analogous conditions arise in ischemia and subsequent reperfusion, which is accompanied by enhanced generation of the active forms of oxygen and stimulation of the synthesis of nitric oxide [22]. Based on this, we suggest that detoxication of the oxoferryl forms of heme proteins forming in oxidative stress is one of important functions of the nitrosyl-iron complexes *in vivo* [1, 6, 8].

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