OXIDATIVE STRESS INDUCES IMPAIRMENT OF HUMAN ERYTHROCYTE ENERGY METABOLISM THROUGH THE OXYGEN RADICAL-MEDIATED DIRECT ACTIVATION OF AMP-DEAMINASE*

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RUNNING TITLE: Oxidative stress-mediated activation of erythrocyte AMP-deaminase.
SUMMARY

The effect of oxidative stress on human red blood cell AMP-deaminase activity was studied by incubating either fresh erythrocytes or hemolysates with H$_2$O$_2$ (0.5, 1, 2, 4, 6, 8 and 10 mM) or NaNO$_2$ (1, 5, 10, 20 and 50 mM), for 15 min at 37 °C. AMP-deaminase assay revealed a tremendous increase of activity by increasing H$_2$O$_2$ or NaNO$_2$ at up to 4 mM and 20 mM, respectively (maximal effect for both oxidants = 9.5 and 6.5 times higher enzymatic activity than control erythrocytes or hemolysates, respectively). Higher H$_2$O$_2$ or NaNO$_2$ concentrations provoked a reduction of the phenomenon. The incubation of hemolysates with iodoacetate (5-100 mM), N-ethylmaleimide (0.1-10 mM) or $p$-hydroxymercuribenzoate (0.1-5 mM) mimicked the effect of oxidative stress on AMP-deaminase, indicating that sulfhydryl group modification is involved in the enzyme activation. In comparison with control hemolysates, changes of the kinetic properties of AMP-deaminase (decrease of AMP concentration necessary for half maximal activation, increase of $V_{\text{max}}$, modification of the curve shape of $V_o$ versus [S], Hill plots and coefficients) were recorded with 4 mM H$_2$O$_2$- and 1 mM N-ethylmaleimide-treated hemolysates. Data obtained using 90% purified enzyme, incubated with Fenton reagents (Fe$^{2+}$ + H$_2$O$_2$) or -SH-modifying compounds, demonstrated that: i) reactive oxygen species are directly responsible for AMP-deaminase activation; ii) this phenomenon occurs through sulfhydryl group modification; iii) the activation does not involve the loss of the tetrameric protein structure. Results of experiments conducted with glucose-6-phosphate dehydrogenase-deficient erythrocytes, challenged with increasing doses of the anti-malarial drug quinine hydrochloride and showing dramatic AMP-deaminase activation, suggest relevant physiopathological implications of this enzymatic activation in conditions of increased oxidative stress.
To the best of our knowledge, this is the first example of an enzyme, fundamental for the maintenance of the correct red blood cell energy metabolism, which is activated (rather than inhibited) by the interaction with reactive oxygen species.
INTRODUCTION

Reactive oxygen species (ROS)\(^1\) are dangerous molecules generated physiologically and pathologically from various intracellular and extracellular sources (1). Several ROS-induced irreversible modifications of biologically fundamental macromolecules have been described, including oxidation of protein -SH groups (2), oxidation of purine nucleotides of nucleic acids (3), initiation of lipid peroxidation reaction chain (4-5). Due to their very high ferrous iron concentration, human erythrocytes might be exposed to risks of increased oxidative stress, mainly through the formation of ferrylhemoglobin (6) and, in part, through the Fenton reaction of hydrogen peroxide with Fe\(^{2+}\) of hemoglobin which generates the powerful oxidant hydroxyl radical (7). Although provided of efficient enzymatic systems for H\(_2\)O\(_2\) removal (catalase, glutathione peroxidase) several ROS-mediated erythrocyte damages have been reported to occur in different in vitro experimental conditions, including challenge with hydrogen peroxide (8, 9), incubation with redox-cycling drugs (10) and during the aging process (11). Nevertheless, data available in literature give very little information on alteration of erythrocyte energy metabolism (in terms either of metabolites or of enzymatic activities) following oxidative stress, except for results reporting only modest effects of iron-ascorbate (as a ROS-generating system) on hexokinase (12), thus suggesting that enzymes involved in red blood cell energy production are scarcely affected by oxidative stress.

Very recently, we have viceversa shown that oxidative stress, induced by increasing the addition of H\(_2\)O\(_2\) concentrations to intact red blood cells, provoked a progressive ATP depletion which was unexpectedly paralleled by a marked IMP (but neither AMP nor ADP) increase. Such a phenomenon was very probably linked to AMP-deaminase activation (13). This enzyme plays a critical role in energy metabolism, catalyzing the deamination of AMP into IMP in the purine nucleotide cycle, and it is present in mammals in three different isoforms: AMP-deaminase 1 is
the predominant form in skeletal muscle; AMP-deaminase 2 is present in smooth muscle, non-muscle tissue, embryonic muscle and undifferentiated myoblasts; AMP-deaminase 3 is found in erythrocytes. The erythrocyte isoform is a homotetramer whose monomers are composed by 767 amino acids; each protein subunit contains nine cysteine residues. AMP-deaminase 3 is regulated by several positive (Ca^{2+}) and negative (2,3-BPG, P_i) effectors and it is physiologically maintained in a state with low catalytic activity, mainly because of erythrocyte low Ca^{2+} and high 2,3-BPG.

On the basis of these observations, we were prompted to study in detail the effect of increasing oxidative stress on AMP-deaminase activity of intact erythrocytes, of hemolysates, and of the purified AMP-deaminase from human red blood cells. Results, corroborated by data of experiments effected on quinine-treated erythrocytes and hemolysates and obtained from patients suffering from glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency, demonstrate that AMP-deaminase is activated by oxidative stress through the modification of its accessible -SH groups and it is responsible for a profound and probably irreversible derangement of erythrocyte energy metabolism.

1The abbreviations used are: ROS, reactive oxygen species; P_i, inorganic phosphorus; G-6-PDH, glucose-6-phosphate dehydrogenase; NEM, N-ethylmaleimide; PMB, p-hydroxymercuribenzoate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

2Enzymes: AMP-deaminase (EC 3.5.4.4); aspartate carbamoyltransferase (EC 2.1.3.2); catalase (EC 1.11.1.6); creatine phosphokinase (EC 2.7.3.2); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); glutathione peroxidase (EC 1.11.1.9); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); hexokinase (EC 2.7.1.1); phosphofructokinase (EC 2.7.1.11); pyruvate kinase (EC 2.7.1.40).
EXPERIMENTAL PROCEDURES

Experiments with intact human erythrocytes and hemolysates

Peripheral venous blood was obtained from healthy volunteers, collected into heparinized tubes and centrifuged for 10 min at 1,853 g at 4 °C, within 15 min from withdrawal. The buffy coat was discarded and erythrocytes were washed twice with large volumes of 10 mM glucose-supplemented phosphate buffered saline (PBS-glucose). Packed erythrocytes were then gently resuspended with PBS-glucose to a final 5% hematocrit and preincubated at 37 °C for 10 min with 1 mM NaN₃, for inhibiting catalase activity. Subsequently, aliquots were incubated with increasing concentrations either of H₂O₂ (0.5, 1, 2, 4, 6, 8 and 10 mM) or of NaNO₂ (1, 5, 10, 20 and 50 mM). Control was represented by erythrocytes incubated with buffer only. After 15 min at 37 °C, cells were immersed for 60 s in an ice-bath and then centrifuged at 1,853 g for 10 min, at 4 °C. Supernatants were discarded, packed erythrocytes were hemolyzed by adding ice-cold double-distilled H₂O (1:5; w:w) and an aliquot of each hemolyzed erythrocyte sample (200 µl) was used for determining AMP-deaminase activity.

Alternatively, washed and packed fresh erythrocytes were hemolyzed by adding ice-cold double-distilled H₂O (1:5; w:w). After 10 minutes at 37 °C in presence of 1 mM NaN₃ for inhibiting catalase activity, the hemolysate was divided into different aliquots and challenged with increasing H₂O₂ (0.5, 1, 2, 4, 6, 8 and 10 mM) or NaNO₂ concentrations (1, 5, 10, 20 and 50 mM). Hemolysate incubated with buffer only was used as control; incubations were carried out for 15 min at 37 °C (unless otherwise indicated). To test the involvement of sulfhydryl group modification in the mechanism of AMP-deaminase activation, hemolysates were allowed to react for 60 min at 37 °C either with iodoacetate (5-100 mM), N-ethylmaleimide (NEM) (0.1-10 mM) or p-hydroxymercuribenzoate (PMB) (0.1-5 mM), as -SH-blocking compounds. At the end of all
incubations, 200 µl were withdrawn from each sample and used for measuring AMP-deaminase activity.

In a different set of experiments, NaN₃-pretreated hemolysates were incubated with H₂O₂ (0.5, 2, 4 and 10 mM) or NEM (0.1, 1 and 10 mM) to measure eventual variations of GSH concentration and AMP-deaminase activity. Control hemolysates were incubated with buffer only. For GSH determination, at the end of incubations aliquots of all samples were deproteinized by the addition of a precipitating solution (1:1.5; v:v) composed by 1.67% m-phosphoric acid, 5 mM EDTA, 5 M NaCl.

Experiments with the purified AMP-deaminase

Purification of red blood cell AMP-deaminase was performed according to Nathans et al. (14), with minor modifications. Briefly, 5 ml of washed and packed erythrocytes, were hemolyzed with 5 ml of ice-cold double-distilled H₂O and kept for 30 min under stirring, in the cold. Membranes were sedimented by centrifugation at 35,000 g for 30 min at 4 °C and supernatant was mixed with an equal volume of a DEAE-cellulose slurry, previously equilibrated at pH 7.0. After 30 min of gentle stirring at 4 °C, the resin was filtered and washed with 20 mM tris-HCl + 20 mM KCl, pH 7.5, until the filtrate was colorless. The dried resin was resuspended with 20 ml of 20 mM tris-HCl + 500 mM KCl, pH 7.0, and kept overnight under continuous gentle mixing at 4 °C. DEAE-cellulose was again filtered and the filtrate was precipitated by adding solid ammonium sulfate at up to 65% saturation. After centrifugation at 12,900 g for 10 min at 4 °C, the supernatant was discarded and the pellet was dissolved in 300 mM KCl + 1 mM ammonium acetate, pH 7.0, and dialyzed with the same buffer for 24 h at 4 °C. At the end of dialysis, the sample was centrifuged at 41,700 g for 15 min at 4 °C and 3 ml of the supernatant were loaded onto a 8-(6-aminohexyl)-amino-AMP cross-linked 4% beaded agarose column (20 x 0.5 cm), previously equilibrated with 3 mM KCl + 2 mM ammonium acetate, pH 7.0. After having
washed the column with 20 ml of the same buffer, AMP-deaminase was eluted with 15 ml of 20 mM KCl + 150 mM KH₂PO₄, pH 6.5 at a flow rate of 1 ml/min. An approximate 90% purity of this enzyme preparation was determined by native polyacrylamide gel-electrophoresis (see below and Figure 6). Before any further use, the purified enzyme was extensively dialyzed against 10 mM KCl + 2 mM ammonium acetate, pH 6.5, to remove P₁ excess.

To evaluate the effect of a ROS-generating system and of -SH-blocking treatment, aliquots of the purified AMP-deaminase were incubated with: increasing H₂O₂ (5, 10, 50, 100, 200 and 500 µM) and fixed Fe²⁺ (5 µM) concentrations; increasing Fe²⁺ (0.5, 1, 2, 5, 10 and 50 µM) and fixed H₂O₂ (100 µM) concentrations; increasing iodoacetate concentrations (1, 2, 5, 10, 20, 50 mM); increasing concentrations of NEM (0.05, 0.1, 0.5, 1, 2, 5 mM). Incubations lasted 20 min at 37 °C, at the end of which the assay for AMP-deaminase activity was performed. In addition, aliquots of the purified enzyme were incubated in presence of the highest concentrations of hydrogen peroxide (500 µM) or ferrous iron (50 µM) only. In order to establish whether AMP-deaminase subjected to oxidative stress or -SH-blocking reagent treatment underwent to dissociation into subunits, aliquots of the purified enzyme were first incubated with the most effective doses of ROS-generating system (5 µM Fe²⁺ + 100 µM H₂O₂) or with -SH-modifying reagents (0.5 mM NEM and 5 mM iodoacetate) and then submitted to native polyacrylamide gel-electrophoresis. A 7-12.5% polyacrylamide gradient gel, containing 0.2% N,N'-methylen-bis-acrylamide, was run using 25 mM tris-HCl + 192 mM glycine buffer, pH 8.5, and stained with 0.2% Coomassie Brilliant-Blue dissolved in methanol:water:acetic acid (50:42.5:7.5; v:v:v).

To measure accessible -SH groups before and after oxidative stress or treatment with -SH-modifying reagents, a 3 mg/ml suspension of the 90% purified AMP-deaminase was prepared in 20 mM ammonium acetate buffer, pH 7.0, divided into different aliquots and treated as follows: 1) incubation with increasing Fe²⁺ (1, 5 and 50 µM) and fixed H₂O₂ (100 µM) for 20 min at 37 °C; 2) incubation with NEM (0.1, 0.5 and 5 mM) for 20 min at 37 °C, followed by extensive
dialysis to remove unreacted NEM; 3) incubation for 20 min at 37 °C in presence of buffer only. Quantification of free -SH groups of each aliquot was then effected by the reaction with 5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), for 10 min at 37 °C. Intensity of color development was measured spectrophotometrically at 412 nm and calculation was performed by using a molar extinction coefficient for DTNB-derivatized -SH groups of 14,290 M⁻¹ × cm⁻¹.

**Experiments with blood from G-6-PDH-deficient patients**

Heparinized blood from three selected G-6-PDH-deficient patients was withdrawn by the Department of Biomedical Sciences and Biotechnology, Laboratory of Hematology I, University of Cagliari, Italy, and kindly supplied by Prof. R. Galanello. After extensive washings, erythrocytes and hemolysates were prepared as above described and incubated for 30 min at 37 °C in presence of increasing concentrations of quinine hydrochloride (0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10 mM). At the end of incubations, all samples were processed for the determination of AMP-deaminase activity. The experiments with each blood sample were repeated in duplicate.

**Assay of AMP-deaminase and GSH determination**

For determining AMP-deaminase activity, aliquots from hemolyzed erythrocytes, hemolysates and purified enzyme were withdrawn and incubated for 30 min at 37 °C in presence of 2 mM AMP, 10 mM KCl, 10 mM MES, pH 6.5. $V_{max}$ and half maximal activation of control, 4 mM H₂O₂- and 1 mM NEM-treated hemolysates were determined by performing the AMP-deaminase assay with increasing AMP concentrations (0.5, 1, 2, 4, 6, 8, 10, 12 and 15 mM) and for an incubation time of 5 min. Influence of different concentrations of positive and negative AMP-deaminase effectors (Ca²⁺ and Pi, respectively) was evaluated in hemolysates treated with H₂O₂ or with -SH-modifying reagents, as well as in control hemolysates, by adding different
concentrations of CaCl$_2$ or KH$_2$PO$_4$, directly in the reaction mixture used for the assay of the enzymatic activity.

At the end of each incubation, mixtures were deproteinized by adding 70% HClO$_4$ (1:10; v:v), centrifuged at 20,690 g for 10 min at 4 °C and neutralized with 5 M K$_2$CO$_3$. After a second centrifugation to remove the potassium perchlorate debris, samples were filtered through a 0.45 μm Millipore-HV filters and then used (10 μl) for IMP determination by an ion-pairing HPLC method (15) using a Kromasil 250 x 4.6 mm, 5 μm particle size column (Eka Chemicals AB, Bohus, Sweden), provided of its own guard column. IMP was separated with a slight modification of the original method (15) using two buffers composed of: 10 mM tetrabutylammonium hydroxide, 10 mM KH$_2$PO$_4$, 0.25% methanol, pH 7.00 (buffer A); 2.8 mM tetrabutylammonium hydroxide, 100 mM KH$_2$PO$_4$, 30% methanol, pH 5.50 (buffer B). A step gradient was obtained as follows: 10 min 100% buffer A; 3 min at up to 90% buffer A; 10 min at up to 70% buffer A; 12 min at up to 55% buffer A; 15 min at up to 45% buffer A; 10 min at up to 25% buffer A; 5 min at up to 0% buffer A. The flow rate of chromatographic runs was 1.2 ml/min and the column temperature was constantly kept at 23 °C. The HPLC apparatus was a Constametric 3500 dual pump system connected with a SpectraSystem UV6000LP diode array detector (ThermoQuest Italia, Rodano, Milane, Italy) set up between 200 and 300 nm wavelength. Acquisition and analysis of data were performed by a PC provided of the software package (ChromQuest) supplied by HPLC manufacturer. IMP concentration was calculated at 250 nm wavelength (the maximum of IMP absorption) by comparing IMP peak areas of sample runs with those of chromatographic runs of freshly prepared ultrapure IMP standard with known concentrations. AMP-deaminase activity was calculated on the basis of the amount of IMP produced in the reaction mixture during the incubation with AMP.
GSH in control, H$_2$O$_2$- and NEM-treated hemolysates was determined spectrophotometrically by measuring at 412 nm the intensity of color development after the reaction of appropriately deproteinized hemolysates with DTNB, as described in detail elsewhere (16). All spectrophotometric measurements were effected with a Beckman 640-DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Ca, USA).

**Statistical analysis**

Data were analyzed for statistical differences by the 2-way and 1-way analysis of variance, as well as by the two-tailed Student’s t-test for paired and unpaired samples; a p value of less than 0.05 was considered significant.
RESULTS

Effect of ROS on AMP-deaminase activity of intact erythrocytes and hemolysates

In Figure 1 are reported the dose-response curves of AMP-deaminase activity of intact fresh human erythrocytes as a function of increasing concentrations of H$_2$O$_2$ (Panel A) or NaNO$_2$ (Panel B) added to red blood cell suspending medium, as well as those of AMP-deaminase activity of hemolysates challenged with the same H$_2$O$_2$ (Panel C) or NaNO$_2$ (Panel D) concentrations. Both oxidants produced a similar augmentation of AMP-deaminase activity (9-10 fold higher than that of control erythrocytes; 5-6 times higher than that recorded in control hemolysates); the maximal H$_2$O$_2$- and NaNO$_2$-mediated activations were obtained with concentrations of 4 and 20 mM, respectively. At higher doses, both compounds induced a progressive AMP-deaminase reduction, although with the maximal concentrations used 4-6 times higher enzymatic activity with respect to control red blood cells or control hemolysates still persisted. Experiments in which the challenge with oxidants (at the same doses already indicated) was shortened (5 min) or prolonged (45 min) did not show different extent of AMP-deaminase activation than incubations with oxidants lasting 15 min (data not shown); this suggests that the enzyme activation is mediated by compounds having a half-life shorter than 5 min (ROS) and which originate in the very fast reaction of Fe$^{2+}$-Hb with H$_2$O$_2$ and NaNO$_2$. Therefore, it is impossible to increase or decrease the extent of AMP-deaminase activation by increasing or decreasing the incubation time of erythrocytes or hemolysates with oxidants (at least in the time range used, i.e. 5-45 min) but it is viceversa possible to obtain this result by increasing oxidant concentrations.

Figure 2 reports the effect on AMP-deaminase activity of hemolysates previously incubated (for 1 hour at 37 °C) with increasing concentrations of iodoacetate (Panel A), NEM (Panel B) or PMB (Panel C). All these -SH-blocking reagents induced a bell-shaped curve of AMP-deaminase activation, comparable even in magnitude to that obtained with the oxidants H$_2$O$_2$ and NaNO$_2$.
(increase of AMP-deaminase activity with respect to control erythrocytes = 4.5 fold with 20 mM iodoacetate, 5.4 fold with 1 mM NEM and 3.4 fold with PMB). Residual activation of 4 mM H$_2$O$_2$-treated AMP-deaminase by sulfhydryl agents and residual activation of iodoacetate- or NEM-treated enzyme by 4 mM H$_2$O$_2$, is shown in Table I. The absence of synergism between the two treatments reinforces the hypothesis of a key role of sulfhydryl group modification in the enzyme activation. Table II shows the effects on GSH concentration and AMP-deaminase activity of hemolysates incubated with increasing concentrations of H$_2$O$_2$ or NEM. It is worth underlining that the lowest concentrations of both stimuli (0.5 mM and 0.1 mM for H$_2$O$_2$ and NEM, respectively) induced a GSH depletion by 56% and 30.6% and a concomitant increase of AMP-deaminase activity by 180% and 218%, i.e. the enzyme was markedly activated in spite of relevant GSH concentrations still present in the incubation mixtures.

To characterize changes of the kinetic parameters of ROS- and -SH-activated AMP-deaminase, incubations of control, 4 mM H$_2$O$_2$- and 1 mM NEM-treated hemolysates were carried out in presence of increasing AMP concentrations (0.5, 1, 2, 4, 6, 8, 10, 12 and 15 mM). The curves of $V_0$ as a function of increasing [S], illustrated in Figure 3, showed that AMP-deaminase of control hemolysates had a different slope with respect to those of 4 mM H$_2$O$_2$- and 1 mM NEM-treated hemolysates. Consequently, control hemolysates had a half maximal activation obtained with 7.25 mM AMP and a $V_{max}$ of 2.32 $\mu$mol of IMP produced/min/l rbc, whilst 4 mM H$_2$O$_2$-treated hemolysates had a half maximal activation reached with 3.30 mM AMP and a $V_{max}$ of 7.77 $\mu$mol of IMP produced/min/l rbc, and 1 mM NEM-treated hemolysates had a half maximal activation recorded with 3.22 mM AMP and a $V_{max}$ of 7.95 $\mu$mol of IMP produced/min/l rbc. Hill plots of the data shown in Figure 3 gave $n$ values of 2.24, 1.76, and 1.80 for the enzyme of control, 4 mM H$_2$O$_2$- and 1 mM NEM-treated hemolysates, respectively (Figure 4). It is worth mentioning that Hill plots evidenced the absence of the lower asymptote in the curves of both H$_2$O$_2$- and NEM-
treated hemolysates, suggesting the presence of AMP-deaminase in the R state only, in these samples.

In order to study AMP-deaminase responsiveness towards some of its natural positive (Ca\(^{2+}\)) and negative (P\(_i\)) effectors, we tested either various CaCl\(_2\) (1, 2, 5 and 10 mM) or KH\(_2\)PO\(_4\) (1, 5, 10, 50 and 100 mM) concentrations on control, 4 mM H\(_2\)O\(_2\)- and 1 mM NEM-treated hemolysates. Data reported in Table III showed that Ca\(^{2+}\) did not further increase AMP-deaminase activity of hemolysates stimulated with both compounds. It should be underlined that Ca\(^{2+}\)-activated AMP-deaminase was comparable with 4 mM H\(_2\)O\(_2\)- and 1 mM NEM-activated AMP-deaminase when Ca\(^{2+}\) in the incubation mixture was at least 5 mM. Data referring to effects of P\(_i\) (Table IV) indicated evident differences among AMP-deaminase activity of control and treated hemolysates. In particular, IC\(_{50}\) of P\(_i\) were 7.56, 3.24 and 3.07 mM, in control, 4 mM H\(_2\)O\(_2\)- and 1 mM NEM-treated hemolysates, respectively. Even if the activated enzyme was responsive to P\(_i\) inhibition, AMP-deaminase activity of 4 mM H\(_2\)O\(_2\)- and 1 mM NEM-treated hemolysates was comparable to that of control hemolysates only when P\(_i\) in the medium was higher than 10 mM.

Effect of ROS and -SH-blocking reagents on the activity, the quaternary structure and the free -SH groups of the purified AMP-deaminase

To reproduce a ROS-generating system in part similar to that present in H\(_2\)O\(_2\)-treated erythrocytes and hemolysates, we incubated the 90% purified AMP-deaminase (obtained from fresh human erythrocytes) in presence of: different H\(_2\)O\(_2\) (5, 10, 50, 100 and 200 µM) and fixed Fe\(^{2+}\) (5 µM) concentrations (Figure 5, Panel A); increasing Fe\(^{2+}\) (0.5, 1, 2, 5, 10 and 50 µM) and fixed H\(_2\)O\(_2\) (100 µM) concentrations (Figure 5, Panel B); various iodoacetate (Figure 5, Panel C) or NEM doses (Figure 5, Panel D). A bell-shaped curves of AMP-deaminase activity as a function of ROS generated in the medium through the Fenton reaction, as well as a function of the increase of -SH reagents, were recorded. Maximal effects were observed with 5 µM Fe\(^{2+}\) +
100 µM H₂O₂ or 0.5 µM NEM and produced 2.2 and 2.5 times increase of AMP-deaminase activity, respectively, in comparison with AMP-deaminase incubated in presence of buffer only (p < 0.001). In the case of the ferrous iron + hydrogen peroxide-mediated AMP-deaminase activation, it is important to observe that it was independent on the order of the Fenton reagent addition (data not shown), i.e. it seems that the formation of an eventual Fe²⁺-enzyme complex is not a prerequisite for the efficacy of this type of activation. It should also be underlined that AMP-deaminase incubation with maximal concentrations of H₂O₂ (500 µM) or Fe²⁺ (50 µM) only did not induce any significant change of the enzymatic activity. Since ROS- and -SH-induced AMP-deaminase activation might have occurred in consequence of a dissociation process of the tetrameric form of the enzyme, we subjected 90% purified AMP-deaminase to native polyacrylamide gel-electrophoresis either after the challenge with 5 µM Fe²⁺ + 100 µM H₂O₂, 5 mM iodoacetate or 0.5 µM NEM, i.e. with the doses of stimuli inducing the maximal enzymatic activation, or after the incubation with buffer only (control enzyme). The result of the electrophoretic run, illustrated in Figure 6 and showing no differences among control and treated enzymes, clearly demonstrates that ROS and -SH-modifying reagents do not induce the transition tetramer-dimer-monomer, at least under the experimental conditions used.

Data concerning the determination of accessible -SH groups of AMP-deaminase, before and after treatment with increasing concentrations of Fenton reagents (H₂O₂ + Fe²⁺) or -SH modifying compound (NEM), are summarized in Table V. Results indicate that either oxidative stress or NEM induced a dose-dependent decrease of accessible AMP-deaminase -SH groups, thus suggesting profound structural changes of the enzymatic tridimensional conformation produced by both treatments. The number of accessible -SH groups of treated AMP-deaminase was similar in the case of the most effective concentrations of both H₂O₂ + Fe²⁺ and NEM (6.3 and 5.7, respectively) and it was different from the number determined in the control enzyme (10 accessible -SH groups).
Effect of anti-malarial drug on AMP-deaminase activity of G-6-PDH deficient erythrocytes and hemolysates

Figure 7 reports results of AMP-deaminase activity of erythrocytes (Panel A) and hemolysates (Panel B) from G-6-PDH-deficient patients after the incubation with increasing concentrations of the anti-malarial drug quinine hydrochloride. Also this type of oxidative stress produced a bell-shaped activation curve of the enzyme, with a maximal effect recorded with 5 mM quinine (respectively, erythrocytes and hemolysates showed 3.8 and 3.2 times higher activity than corresponding controls; p < 0.001). It is important to underline that even the lowest quinine concentration tested (0.01 mM) produced an increase, although not significant, of AMP-deaminase activity (+13%); statistically significant differences from control G-6-PDH-deficient erythrocytes and hemolysates were reached with 0.1 mM quinine (+41% and +38% for erythrocytes and hemolysates, respectively; p < 0.05).
DISCUSSION

The relative metabolic simplicity of red blood cells is one of their peculiarities but, of course, it also represents one of the main limitations for their survival. In this light, any change of enzymatic activities, particularly those involved in the maintenance of the correct energy state, might signify such a drastic problem to produce even the erythrocyte death. Among the various possible causes affecting erythrocyte energy metabolism, oxidative stress might certainly represent a negative factor whose relevance, unlike to other tissues (5, 17) and cell types (18, 19) has not been fully evaluated. Previous investigation, using iron-ascorbate as a ROS-generating system, apparently showed a poor capacity of oxidative stress to alter enzymes and metabolites of human red blood cell energy metabolism (12). Viceversa, we very recently demonstrated that incubation of fresh human erythrocytes with increasing hydrogen peroxide concentrations (i.e., with a free radical-generating system producing ferrylhemoglobin and possibly OH·), provoked a progressive ATP depletion which was paralleled by a dramatic IMP and a modest AMP or ADP increase. Furthermore, such a metabolic modification was attributed to AMP-deaminase activation (13). This enzyme plays a critical role in energy metabolism, catalyzing the deamination of AMP into IMP in the purine nucleotide cycle, particularly in erythrocytes because they lack of the salvage pathway of purine nucleotides. Therefore, IMP production represents for red blood cell a remarkable waste of energy.

The experiments reported in the present study, in which we evaluated in detail (on intact erythrocytes, on hemolysates and on the 90% purified AMP-deaminase) the activity of the type 3 erythrocytic isoenzyme under different experimental conditions, showed that the extent of AMP-deaminase activation depended on the severity of oxidative stress at up to a certain limit, beyond which an inhibitory effect was observed. We found that in intact erythrocytes and hemolysates (Figure 1), as well as in the 90% purified enzyme (Figure 5, Panels A and B), even a modest
ROS production, unable to produce GSH depletion higher than 50% (Table II), caused a significant increase of AMP-deaminase activity, thus indicating a high reactivity of specific amino acid residues of the protein towards oxidative stress. Furthermore, the -SH-modifying reagents iodoacetate, NEM and PMB (Figure 2; Figure 5, Panels C and D) mimicked the same enzymatic activation obtainable with the ROS-generating systems tested, supporting the hypothesis that oxidative stress-mediated AMP-deaminase activation occurs via irreversible modification of cystein(s) crucial for controlling enzymatic activity. In experiments with hemolysates, this indication was reinforced by the lack of synergism between ROS and reagents for -SH (Table I) and, furthermore, by changes of the kinetic properties of AMP-deaminase of hemolysates treated with either 4 mM H₂O₂ or 1 mM NEM (Figure 3). With respect to controls, the enzyme of stimulated hemolysates doubled its affinity for AMP and increased about 3.5 fold the reaction velocity. As evidenced by the Hill plots showing the absence of the lower asymptote in the curves of both H₂O₂- and NEM-treated hemolysates (Figure 4), oxidative stress or challenge with -SH-blocking reagents were accompanied by the AMP-deaminase T → R transition, therefore producing a conformational state of the enzyme with high affinity for the substrate (AMP). In addition, treated hemolysates underwent to modest change of AMP-deaminase \( n \) values (1.76 and 1.80 for 4 mM H₂O₂- or 1 mM NEM-treated hemolysates, respectively) in comparison with that of control hemolysates (\( n = 2.24 \)). This strongly suggests that AMP-deaminase of the oxidatively-stressed and -SH-treated hemolysates is cooperative in the AMP binding and, consequently, the enzyme has the tetrameric or dimeric structure. The result of the native polyacrylamide gel-electrophoresis (Figure 6) clearly evidenced that treatment of the 90% purified AMP-deaminase with 5 □M Fe²⁺ + 100 □M H₂O₂ or 0.5 □M NEM (both treatments producing remarkable increase of the enzymatic activity) did not induce enzyme dissociation. Therefore, this demonstrates that activated AMP-deaminase of oxidatively-stressed
and -SH-treated erythrocytes, hemolysates and 90% purified enzyme is in its native tetrameric structure.

On the basis of the results obtained by titrating free -SH groups of the 90% purified AMP-deaminase (Table V), it is possible to sustain that the mechanism of oxidative stress-induced enzymatic activation involves, among the 10 freely accessible -SH groups, the modification of 2-4 cystein residues/AMP-deaminase tetramer. The minimal and maximal numbers of modified -SH groups determine the corresponding minimal and maximal extent of AMP-deaminase activation. Such an -SH group modification, mimicked by the -SH-blocking compound NEM, should induce conformational changes of the tetrameric protein structure leading to AMP-deaminase stabilization in the R state with high affinity for AMP, and consequent enzyme activation. It is also presumable that the bell-shaped dose response curve of the enzyme activity, as a function of increasing oxidants or -SH-modifying reagents, might occur either because of the reaction with a specific cystein residue not easily accessible by reagents and directly involved in the AMP-deaminase catalytic mechanism, or because of the possible unspecific modification of amino acids other than cysteins (lysines, tyrosines, methionines). In both cases, this occurrence was encountered at definitely high concentrations of both stimuli. It is worth underlining that the H₂O₂ and NaNO₂ AMP-deaminase activation was not reverted by reducing reagents, such as GSH or β-mercaptoethanol (data not shown), thereby indicating an irreversible oxidative cystein modification, as it occurs in the reaction of ROS with -SH groups (20).

The evident demonstration of ROS-activated AMP-deaminase, obtained by the experiments conducted with the 90% purified enzyme challenged with a ROS-generating system based on the Fenton reaction, allowed to hypothesize that hydroxyl radicals are directly responsible for AMP-deaminase activation. Under these experimental conditions, the purified enzyme represented one of the main macromolecular targets present in the incubation mixture and no possible interferences by eventual low molecular weight modulators might have occurred. Since OH⁻ are
produced in part during the reaction of hydrogen peroxide with the ferrous iron of hemoglobin (7), it is conceivable to speculate that hydroxyl radicals are involved in the AMP-deaminase modification of intact erythrocytes and hemolysates.

The importance of the results reported in the present study lies in the key role played by AMP-deaminase in determining the correct energy state of human erythrocytes. Previous studies showed that red blood cells underwent to ATP depletion, through AMP-deaminase activation, after exposure to various Ca^{2+} concentrations (21-23). Similar results were obtained upon exposure of erythrocytes to alkalinization and deoxyadenosine addition (24), as well as after incubation with different nucleoside analogues (25). It was supposed that change of AMP-deaminase activity is involved in the erythrocyte aging process (26), albeit this original hypothesis was subsequently contradicted by other studies (27, 28). Results of the present study certainly demonstrated that ROS can now be added to the list of the most potent activators of erythrocyte AMP-deaminase. From the data reported in literature, this phenomenon should be peculiar of the red blood cell isoform. In fact, it was shown a significant AMP-deaminase inhibition of yeast cells subjected to oxidative stress (29) and, furthermore, it was demonstrated a similar inhibitory pattern of the rabbit cardiac isoenzyme (30). In this last study, AMP-deaminase inactivation was correlated to an altered thiol status of the enzyme, quickly occurring (5-10 min) upon submission to oxidative stress, that should be responsible for irreversible modifications of AMP-deaminase conformation altering its catalytic activity (30). Our data showed that the reaction of ROS with the erythrocyte isoenzyme was similarly fast, but the final result of oxidative stress was the striking enzyme activation rather than the enzyme inhibition. Such a totally opposite effect might be attributable to the amino acid sequence differences of the various isoforms that should give rise to different conformational structures and thus to different sensitivities towards ROS effects; in addition, it is evident from our experiments performed with iodoacetate, NEM and PMB, in which modification of -SH groups mimicked the activity of 4
mM H$_2$O$_2$, that the change of erythrocyte AMP-deaminase thiol state do stimulate (instead of inhibiting) the enzymatic activity; at present, the only AMP-deaminase capable to be markedly activated by ROS seems, therefore, the erythrocyte isoform.

Our results obtained by challenging erythrocytes and hemolysates from G-6-PDH-deficient patients with the anti-malarial drug quinine hydrochloride (Figure 7), showed the same patterns of AMP-deaminase activation than those occurring to erythrocytes and hemolysates obtained from blood of healthy subjects and then subjected to oxidative stress. From the physiopathological point of view, this finding implies that, through ROS-induced activation of AMP-deaminase, oxidative stress might represent one of the main negative event leading to profound impairment of the erythrocyte energy state in physiological (aging) and, moreover, pathological conditions. In fact, all the situations in which erythrocytes may be subjected to oxidative stress by different causes, such as those induced by redox cycling xenobiotics (anthracycline drugs, paraquat, etc.), by hemolytic crisis (G-6-PDH deficiency), by hemoglobinopathies (hemoglobin S), by increase of met-hemoglobin and free radicals (smokers), by hemodyalisis (nephropathies), should be reconsidered in light of the negative contributions for energy metabolism that occur when AMP-deaminase is activated (13).

In conclusion, although further studies will be necessary for elucidating the mechanism of ROS-mediated activation of human erythrocyte AMP-deaminase, as well as the specific amino acid residues implicated and the structural changes driving this activation, we demonstrated, for the first time to the best of our knowledge, that direct interaction of ROS with an enzyme of energy metabolism is able to activate rather than inhibiting the catalytic activity of the enzyme itself. At present, only examples of enzymatic inactivation by oxidative stress can be found in literature, such as those occurring to glyceraldehyde-3-phosphate dehydrogenase (31, 32), glucose-6-phosphate dehydrogenase (31), creatine phosphokinase (33), pyruvate kinase (34) etc., even though the increase of the biological activity of a protein molecule, such as the conversion of the
inactive form of glycosylation inhibiting factor into its bioactive derivatives, has been reported to occur upon -SH modification (35). Since AMP-deaminase regulation is crucial for the maintenance of the correct red blood cell energy state, we believe that our results represent a new and important improvement either of the knowledge of ROS damaging activity towards biologically fundamental molecules, or of the understanding of erythrocyte energy metabolism functioning in physiopathological conditions of increased oxidative stress.
REFERENCES


FOOTNOTES

*This work was supported in part by a grant from the Italian National Research Council (C.N.R.) Target Project on Biotechnology (no. 115.32764), and by research funds of Catania University.
Table I

Residual activation of ROS activated AMP-deaminase by sulphydryl agents and residual activation of sulphydryl agent-treated AMP-deaminase by ROS.

Hydrogen peroxide-treated hemolysates (incubation with 1 mM NaN₃ + 4 mM H₂O₂ for 15 min at 37 °C) were challenged for 60 min at 37 °C with 20 mM iodoacetate or 1 mM NEM. Iodoacetate (20 mM) or 1 mM NEM-treated hemolysates (60 min of incubation at 37 °C) were allowed to react with 1 mM NaN₃ + 4 mM H₂O₂ for 15 min at 37 °C. Control hemolysates were incubated with buffer only. Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 µl of each hemolysate after the incubation at 37 °C for 45 min with 2 mM AMP. Each point is the mean ± S.D. of 4 different blood samples and is expressed as % of AMP-deaminase activity (control hemolysates = 100% of activity).

<table>
<thead>
<tr>
<th>AMP-deaminase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>4 mM H₂O₂</td>
</tr>
<tr>
<td>4 mM H₂O₂ + 20 mM iodoacetate</td>
</tr>
<tr>
<td>4 mM H₂O₂ + 1 mM NEM</td>
</tr>
<tr>
<td>20 mM iodoacetate</td>
</tr>
<tr>
<td>20 mM iodoacetate + 4 mM H₂O₂</td>
</tr>
<tr>
<td>1 mM NEM</td>
</tr>
<tr>
<td>1 mM NEM + 4 mM H₂O₂</td>
</tr>
</tbody>
</table>

Values of treated hemolysates were significantly different from control (p < 0.001).
Table II

*Effect of oxidative stress and -SH reagents on GSH and AMP-deaminase activity of human red blood cell hemolysates*

Erythrocytes were hemolyzed with double-distilled water (1:5; w:w) and incubated for 30 min at 37 °C with increasing H$_2$O$_2$ or NEM concentrations. Control hemolysates were incubated with buffer only. GSH was determined spectrophotometrically by the reaction with DTNB and enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 µl of each hemolysate after the incubation at 37 °C for 45 min with 2 mM AMP. Each point is the mean ± S.D. of 4 different blood samples. AMP-deaminase activity and is expressed as % of control (control hemolysates = 100% of activity).

<table>
<thead>
<tr>
<th></th>
<th>GSH (mmol/l 20% hemolysate)</th>
<th>AMP-deaminase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.418 ± 0.066</td>
<td>100 ± 19</td>
</tr>
<tr>
<td>0.5 mM H$_2$O$_2$</td>
<td>0.184 ± 0.042$^a$</td>
<td>180 ± 38$^a$</td>
</tr>
<tr>
<td>2 mM H$_2$O$_2$</td>
<td>0.065 ± 0.018$^a$</td>
<td>389 ± 65$^a$</td>
</tr>
<tr>
<td>4 mM H$_2$O$_2$</td>
<td>0$^a$</td>
<td>623 ± 90$^a$</td>
</tr>
<tr>
<td>10 mM H$_2$O$_2$</td>
<td>0$^a$</td>
<td>316 ± 42$^a$</td>
</tr>
<tr>
<td>0.1 mM NEM</td>
<td>0.290 ± 0.043$^a$</td>
<td>218 ± 54$^a$</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>0$^a$</td>
<td>638 ± 103$^a$</td>
</tr>
<tr>
<td>10 mM NEM</td>
<td>0$^a$</td>
<td>111 ± 26</td>
</tr>
</tbody>
</table>

$^a$significantly different from control (p < 0.001).
Table III

Effect of Ca\(^{2+}\) on the AMP-deaminase activity of human red blood cell hemolysates.

Erythrocytes were hemolyzed with double-distilled water (1:5; w:w) and incubated for 30 min at 37 °C with 4 mM H\(_2\)O\(_2\) or 1 mM NEM. Control hemolysates were incubated with buffer only. Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 µl of each hemolysate after the incubation at 37 °C for 45 min with 2 mM AMP and various CaCl\(_2\) concentrations. Each point is the mean ± S.D. of 6 different blood samples and is expressed as % of AMP-deaminase activity (control hemolysates = 100% of activity).

<table>
<thead>
<tr>
<th>CaCl(_2) (mM)</th>
<th>Control hemolysates</th>
<th>4 mM H(_2)O(_2)-treated hemolysates</th>
<th>1 mM NEM-treated hemolysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 15</td>
<td>554 ± 33(^{b})</td>
<td>611 ± 40(^{b})</td>
</tr>
<tr>
<td>1</td>
<td>410 ± 36(^{a})</td>
<td>642 ± 45(^{b})</td>
<td>687 ± 39(^{b})</td>
</tr>
<tr>
<td>2</td>
<td>478 ± 41(^{a})</td>
<td>692 ± 48(^{b})</td>
<td>703 ± 52(^{b})</td>
</tr>
<tr>
<td>5</td>
<td>774 ± 66(^{a})</td>
<td>663 ± 58</td>
<td>685 ± 61</td>
</tr>
<tr>
<td>10</td>
<td>865 ± 70(^{a})</td>
<td>792 ± 65</td>
<td>834 ± 77</td>
</tr>
</tbody>
</table>

\(^{a}\)significantly different from 0 mM CaCl\(_2\) (p < 0.001).

\(^{b}\)significantly different from corresponding value of control hemolysates (p < 0.001).
Effect of Pi on the AMP-deaminase activity of human red blood cell hemolysates.

Erythrocytes were hemolyzed with double-distilled water (1:5; w:w) and incubated for 30 min at 37 °C with 4 mM H₂O₂ or 1 mM NEM. Control hemolysates were incubated with buffer only. Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 μl of each hemolysate after the incubation at 37 °C for 45 min with 2 mM AMP and various KH₂PO₄ concentrations. Each point is the mean ± S.D. of 6 different blood samples and is expressed as % of AMP-deaminase activity (control hemolysates = 100% of activity).

<table>
<thead>
<tr>
<th>KH₂PO₄ (mM)</th>
<th>Control hemolysates</th>
<th>4 mM H₂O₂-treated hemolysates</th>
<th>1 mM NEM-treated hemolysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 11</td>
<td>596 ± 48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>645 ± 54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>88 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>376 ± 31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>401 ± 43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>61 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>234 ± 33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250 ± 36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>41 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>9 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13 ± 3</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>100</td>
<td>8 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 3</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup>significantly different from 0 mM KH₂PO₄ (p < 0.001).

<sup>b</sup>significantly different from corresponding value of control hemolysates (p < 0.001).
Effect of oxidative stress and NEM on accessible -SH groups of AMP-deaminase

AMP-deaminase was purified at the 90% level from fresh human erythrocytes. Aliquots of the partially purified enzyme were then incubated with 100 µM H₂O₂ and different FeCl₂ concentrations or with increasing NEM concentrations. Procedure of AMP-deaminase purification and determination of its accessible -SH groups (carried out by the reaction with DTNB) are fully described in Experimental Procedures. Control enzyme was incubated with buffer only. Each point is the mean ± S.D. of 3 different experiments.

<table>
<thead>
<tr>
<th></th>
<th>Number of accessible -SH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>10 ± 1</td>
</tr>
<tr>
<td>1 µM Fe²⁺ + 100 µM H₂O₂</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>5 µM Fe²⁺ + 100 µM H₂O₂</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>50 µM Fe²⁺ + 100 µM H₂O₂</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>0.1 mM NEM</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>0.5 mM NEM</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>5 mM NEM</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Effect of increasing oxidative stress on AMP-deaminase activity of intact human erythrocytes and hemolysates. Red blood cell suspensions at a 5% hematocrit (Panels A and B) or 20% hemolysates (Panels C and D), pre-treated with 1 mM NaN₃, were incubated for 15 min at 37 °C with different H₂O₂ (Panels A and C) or NaNO₂ (Panels B and D) concentrations. Control erythrocytes and hemolysates were incubated with buffer only. Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced after incubation at 37 °C for 45 min with 2 mM AMP by appropriate aliquots of each sample. Each point is the mean of 6 different blood samples and is expressed as % of AMP-deaminase activity (control erythrocytes and hemolysates = 100% of activity). Standard deviations are represented by vertical bars. All values of H₂O₂- and NaNO₂-treated erythrocytes and hemolysates were significantly different from controls (p < 0.001).

Figure 2. Effect of -SH modifying reagents on AMP-deaminase activity of human red blood cell hemolysates. Erythrocytes were hemolyzed with double-distilled water (1:5; w:w) and incubated for 30 min at 37 °C with different iodoacetate (Panel A), NEM (Panel B) or PMB concentrations (Panel C). Control hemolysates were incubated with buffer only. Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 µl of each hemolysate after the incubation at 37 °C for 45 min with 2 mM AMP. Each point is the mean of 6 different blood samples and is expressed as % of activity (control hemolysates = 100% of activity). Standard deviations are represented by vertical bars. All values of iodoacetate-, NEM- and PMB-treated hemolysates were significantly different from controls (p < 0.001).
Figure 3. **Velocity of AMP-deaminase reaction of control, 4 mM H$_2$O$_2$- and 1 mM NEM-treated hemolysates as a function of increasing AMP concentrations.** Red blood cells were hemolyzed with double-distilled water (1:5; w:w) and incubated for 30 min at 37 °C with 4 mM H$_2$O$_2$ (●) or 1 mM NEM (♦). Control hemolysates were incubated with buffer only (○). Enzymatic activity was evaluated by determining (by HPLC) the amount of IMP produced by 200 µl of each hemolysate after the incubation at 37 °C for 5 min with various AMP concentrations. Each point is the mean of 6 different blood sample and is expressed as µmol IMP produced/min/l rbc. Standard deviations are represented by vertical bars. All values of H$_2$O$_2$- and NEM-treated hemolysates were significantly different from controls (p < 0.001).

Figure 4. **Hill plots of the velocity of AMP-deaminase reactions of control, 4 mM H$_2$O$_2$- and 1 mM NEM-treated hemolysates.** Curves of affinity of 4 mM H$_2$O$_2$-treated hemolysates (●), 1 mM NEM-treated hemolysates (♦) and control hemolysates (○) were obtained from data illustrated in Figure 3. The linear portion of each plot was used for calculating the respective $n$ values.
Figure 5. **Effect of increasing oxidative stress and of -SH-blocking reagents on the activity of the purified AMP-deaminase from human erythrocytes.** AMP-deaminase was purified at the 90% level from fresh human erythrocytes, as described in detail in Experimental Procedures. Aliquots of the partially purified enzyme were then incubated with: 5 µM FeCl₂ and different H₂O₂ concentrations (Panel A); 100 µM H₂O₂ and different FeCl₂ concentrations (Panel B); increasing iodoacetate concentrations (Panel C); increasing NEM concentrations (Panel D). Control enzyme was incubated with buffer only. Each point is the mean of 6 different experiments and is expressed as % of activity (control enzyme = 100% of activity). Standard deviations are represented by vertical bars. All values of the purified AMP-deaminase subjected to oxidative stress or to reaction with -SH-modifying reagents were significantly different from control (p < 0.01). H₂O₂ (500 µM) or Fe²⁺ (50 µM) only did not induce any significant change of the enzymatic activity.

Figure 6. **Native polyacrylamide gel-electrophoresis of purified AMP-deaminase from human erythrocytes after treatment with ROS and -SH-blocking reagents.** Aliquots of the 90% purified enzyme were incubated with: 5 µM Fe³⁺ + 100 µM H₂O₂ (lane 1), as a ROS-generating system; 5 mM iodoacetate (lane 2), as a -SH-modifying reagents; 0.5 mM NEM (lane 3), as a -SH-modifying reagents; buffer only (lane 4), as control. After incubation, aliquots of each sample were submitted to native polyacrylamide gel-electrophoresis. Lane 5 was loaded with a standard protein mixture with known molecular weights.
Figure 7. AMP-deaminase activity of erythrocytes and hemolysates form G-6-PDH-deficient patients incubated with increasing quinine hydrochloride addition. Erythrocytes (Panel A) and hemolysates (Panel B) from G-6-PDH-deficient patients were incubated for 30 min at 37 °C with different concentrations of quinine hydrochloride and then assayed for AMP-deaminase activity. Controls were represented by G-6-PDH-deficient erythrocytes and hemolysates incubated with buffer only. Each point is the mean of 3 different blood samples (challenged in duplicate with quinine) and is expressed as % of activity (control G-6-PDH-deficient erythrocytes and hemolysates = 100% of activity). Standard deviations are represented by vertical bars.

* significantly different from controls (p < 0.01).
FIGURE 1.

Panel A

Panel B

Panel C

Panel D
FIGURE 2

Panel A

AMP-deaminase activity (%)

Iodoacetate (mM)

Panel B

AMP-deaminase activity (%)

NEM (mM)

Panel C

AMP-deaminase activity (%)

PMB (mM)
FIGURE 3

[Graph showing the relationship between AMP (mM) and µmol IMP produced/min/l rbc. The x-axis represents AMP concentrations ranging from 0 to 16 mM, while the y-axis represents µmol IMP produced/min/l rbc ranging from 0 to 9.]
$\log \left( \frac{V_o}{V_{max}} - V_o \right)$ vs. $\log [\text{AMP}]$
FIGURE 5

Panel A

Panel B

Panel C

Panel D
FIGURE 7

Panel A

Panel B
Additions and Corrections


Oxidative stress induces impairment of human erythrocyte energy metabolism through the oxygen radical-mediated direct activation of AMP-deaminase.

Barbara Tavazzi, Angela Maria Amorini, Giovanna Fazzina, Donato Di Pierro, Michele Tuttobene, Bruno Giardina, and Giuseppe Lazzarino

Page 48084, left column, line 16: The word “biphosphoglycerate” should be deleted from this sentence. The correct sentence is: “Results, corroborated by data of experiments effected on quinine-treated erythrocytes and hemolysates and obtained from patients suffering from glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency, demonstrate that AMP-deaminase is activated by oxidative stress through the modification of its accessible –SH groups and is responsible for a profound and probably irreversible derangement of erythrocyte energy metabolism.”


Reelin is a serine protease of the extracellular matrix.

Carlo C. Quattrocchi, Francesca Wannenes, Antonio M. Persico, Silvia Anna Ciafre, Gabriella D’Arcangelo, Maria G. Farace, and Flavio Keller

Page 303: The affiliation for Dr. Farace was incorrect. The correct affiliation is: “Department of Experimental Medicine and Biochemical Sciences, Universita di Tor Vergata, Via di Tor Vergata 135, 00133 Roma, Italia.”

Also, the grant footnote should read: “This work was supported by Consiglio Nazionale delle Ricerche, Programma “Biomolecole per la salute umana,” Grant 99.00555.PF33 (to F. K.) and by grants from the Consiglio Nazionale delle Ricerche and Ministero dell’Istruzione, dell’Università e della Ricerca (to M. G. F. and S. A. C.).

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Oxidative stress induces impairment of human erythrocyte energy metabolism through the oxygen radical-mediated direct activation of AMP-deaminase
Barbara Tavazzi, Angela Maria Amorini, Giovanna Fazzina, Donato Di Pierro, Michele Tutiobene, Bruno Giardina and Giuseppe Lazzarino

*J. Biol. Chem.* published online October 23, 2001

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