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Oxidative Stress Induces Impairment of Human Erythrocyte Energy Metabolism through the Oxygen Radical-mediated Direct Activation of AMP-deaminase*

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The effect of oxidative stress on human red blood cell AMP-deaminase activity was studied by incubating either fresh erythrocytes or hemolysates with H_2O_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 tremendously increased by increasing H_2O_2 or $NaNO_2$ at up to 4 and 20 mM, respectively (maximal effect for both oxidants was 9.5 and 6.5 times higher enzymatic activity than control erythrocytes or hemolysates, respectively). 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_{max}, modification of the curve shape of V_o *versus* [S], Hill plots, and coefficients) were recorded with 4 mM H_2O_2- and 1 mM *N*-ethylmaleimide-treated hemolysates. Data obtained using 90% purified enzyme, incubated with Fenton reagents (Fe^{2+} + H_2O_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; and (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, that is activated (rather than inhibited) by the interaction with reactive oxygen species.

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), and 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 with efficient enzymatic systems for H_2O_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 the 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 shown, on the contrary, that oxidative stress, induced by increasing the addition of H_2O_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[2] 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.

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[1] The abbreviations used are: ROS, reactive oxygen species; G-6-PDH, glucose-6-phosphate dehydrogenase; NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MES, 4-morpholineethanesulfonic acid; HPLC, high-performance liquid chromatography.

[2] The following enzymes were used or were mentioned in the text: 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); and pyruvate kinase (EC 2.7.1.40).

AMP-deaminase 1 is the predominant form in skeletal muscle; AMP-deaminase 2 is present in smooth muscle, nonmuscle tissue, embryonic muscle, and undifferentiated myoblasts; and 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-biphosphoglycerate, 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-biphosphoglycerate.

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 biphosphoglycerate is responsible for a profound and probably irreversible derangement of erythrocyte energy metabolism.

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. Packed erythrocytes were then gently resuspended with glucose-supplemented phosphate-buffered saline to a final 5% hematocrit and preincubated at 37 °C for 10 min with 1 mM NaN_3, for inhibiting catalase activity. Subsequently, aliquots were incubated with increasing concentrations either of H_2O_2 (0.5, 1, 2, 4, 6, 8, and 10 mM) or of $NaNO_2$ (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 H_2O (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 H_2O (1:5; w/w). After 10 min at 37 °C in the presence of 1 mM NaN_3 for inhibiting catalase activity, the hemolysate was divided into different aliquots and challenged with increasing H_2O_2 (0.5, 1, 2, 4, 6, 8, and 10 mM) or $NaNO_2$ 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, NaN_3-pretreated hemolysates were incubated with H_2O_2 (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 of 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 H_2O 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 plus 20 mM KCl, pH 7.5, until the filtrate was colorless. The dried resin was resuspended with 20 ml of 20 mM Tris-HCl plus 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 plus 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 an 8-(6-aminohexyl)-amino-AMP-cross-linked 4% beaded agarose column (20 × 0.5 cm), previously equilibrated with 3 mM KCl plus 2 mM ammonium acetate, pH 7.0. After the column was washed with 20 ml of the same buffer, AMP-deaminase was eluted with 15 ml of 20 mM KCl plus 150 mM KH_2PO_4, 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 Fig. 6). Before any further use, the purified enzyme was extensively dialyzed against 10 mM KCl plus 2 mM ammonium acetate, pH 6.5, to remove P_i 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 H_2O_2 (5, 10, 50, 100, 200, and 500 μM) and fixed Fe^{2+} (5 μM) concentrations; increasing Fe^{2+} (0.5, 1, 2, 5, 10, and 50 μM) and fixed H_2O_2 (100 μM) concentrations; increasing iodoacetate concentrations (1, 2, 5, 10, 20, and 50 mM); and increasing concentrations of NEM (0.05, 0.1, 0.5, 1, 2, and 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 the presence of the highest concentrations of hydrogen peroxide (500 μM) or ferrous iron (50 μM) only. To establish whether AMP-deaminase subjected to oxidative stress or –SH-blocking reagent treatment underwent dissociation into subunits, aliquots of the purified enzyme were first incubated with the most effective doses of the ROS-generating system (5 μM Fe^{2+} plus 100 μM H_2O_2) 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*′-methylenebisacrylamide, was run using 25 mM Tris-HCl plus 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: (i) incubation with increasing Fe^{2+} (1, 5, and 50 μM) and fixed H_2O_2 (100 μM) for 20 min at 37 °C; (ii) incubation with NEM (0.1, 0.5, and 5 mM) for 20 min at 37 °C, followed by extensive dialysis to remove unreacted NEM; and (iii) incubation for 20 min at 37 °C in the 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. The 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 $M^{-1} \times cm^{-1}$.

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 described above and incubated for 30 min at 37 °C in the presence of increasing concentrations of quinine hydrochloride (0.01, 0.05, 0.1, 0.5, 1, 2, 5, and 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 45 min at 37 °C in the presence of 2 mM AMP, 10 mM KCl, 10 mM MES, pH 6.5. V_{max} and half-maximal activation of control, 4 mM H_2O_2- 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. The influence of different concentrations of positive and negative AMP-deaminase effectors (Ca^{2+} and P_i, respectively) was evaluated in hemolysates treated with H_2O_2 or with –SH-modifying reagents, as well as in control hemolysates, by adding different concentrations of $CaCl_2$ or KH_2PO_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

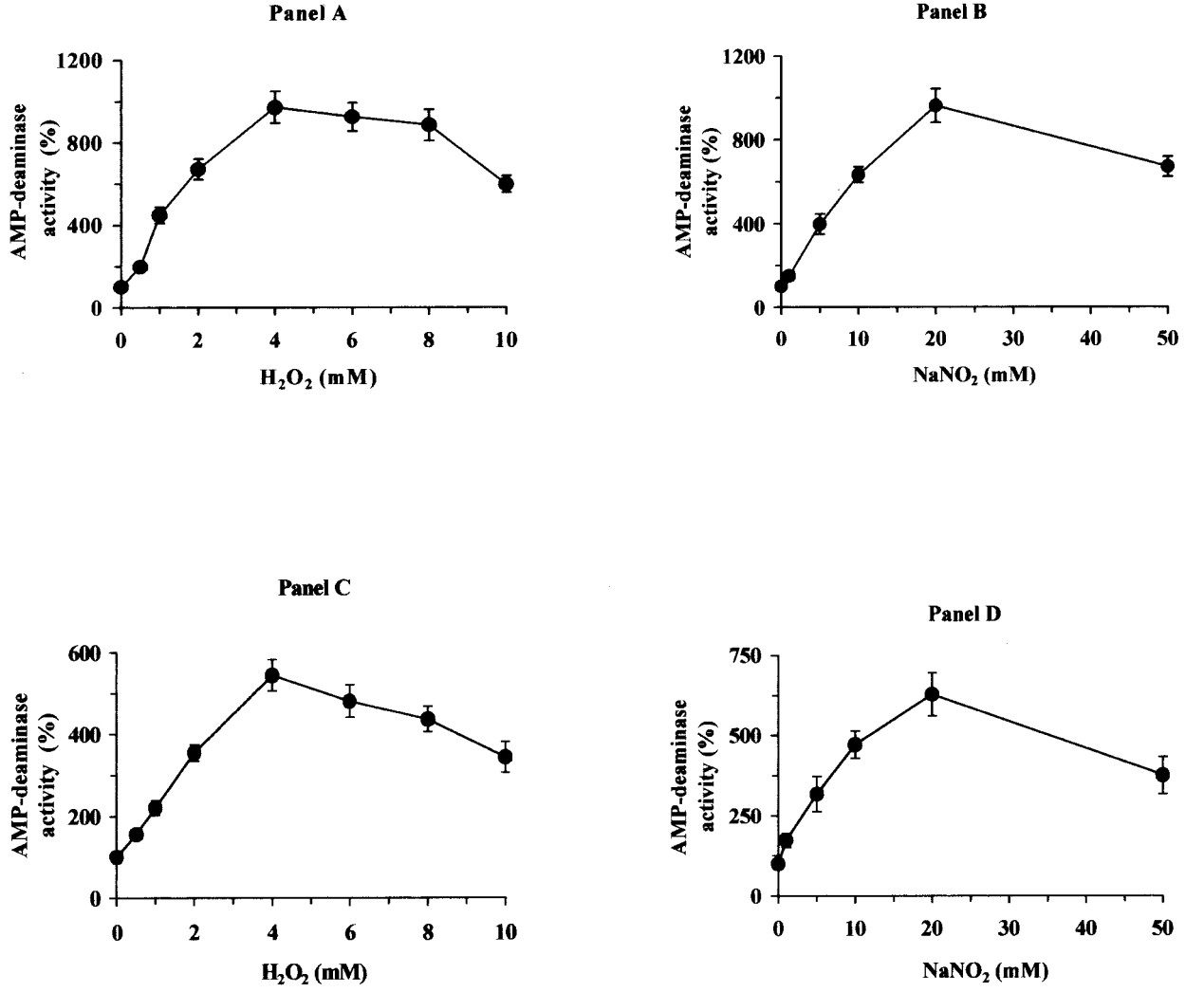

FIG. 1. **Effect of increasing oxidative stress on AMP-deaminase activity of intact human erythrocytes and hemolysates.** Red blood cell suspensions at a 5% hematocrit (*A* and *B*) or 20% hemolysates (*C* and *D*), pretreated with 1 mM NaN_3, were incubated for 15 min at 37 °C with different H_2O_2 (*A* and *C*) or $NaNO_2$ (*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 six different blood samples and is expressed as percentage of AMP-deaminase activity (control erythrocytes and hemolysates = 100% of activity). S.D. values are represented by *vertical bars*. All values of H_2O_2- and $NaNO_2$-treated erythrocytes and hemolysates were significantly different from controls ($p < 0.001$).

70% $HClO_4$ (1:10; v/v), centrifuged at 20,690 × *g* for 10 min at 4 °C, and neutralized with 5 M K_2CO_3. After a second centrifugation to remove the potassium perchlorate debris, samples were filtered through a 0.45-μm Millipore-HV filter and then used (10 μl) for IMP determination by an ion-pairing HPLC method (15) using a Kromasil 250 × 4.6 mm, 5-μm particle size column (Eka Chemicals AB, Bohus, Sweden), provided with 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_2PO_4, 0.25% methanol, pH 7.00 (buffer A); 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_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 with the software package (ChromQuest) supplied by the HPLC manufacturer. The IMP concentration was calculated at 250-nm wavelength (the maximum of IMP absorption) by comparing the 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 and H_2O_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.).

Statistical Analysis—Data were analyzed for statistical differences by the two-way and one-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—Fig. 1 shows the dose-response curves of AMP-deaminase activity of intact fresh human erythrocytes as a function of increasing concentrations of H_2O_2 (*A*) or $NaNO_2$ (*B*) added to red blood cell-suspending medium, as well as those of AMP-deaminase activity of hemolysates challenged with the same H_2O_2 (*C*) or $NaNO_2$ (*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_2O_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 a different extent of AMP-deaminase activation from incubations with oxidants lasting 15 min (data not shown); this suggests that the enzyme activation is mediated by compounds that have a half-life shorter than 5 min (ROS) and that originate in the very fast reaction of Fe^{2+}-Hb with H_2O_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 possible, on the contrary, to obtain this result by increasing oxidant concentrations.

Fig. 2 reports the effect on AMP-deaminase activity of hemolysates previously incubated (for 1 h at 37 °C) with increasing concentrations of iodoacetate (*A*), NEM (*B*), or PMB (*C*). All of these –SH-blocking reagents induced a bell-shaped curve of AMP-deaminase activation, comparable even in magnitude with that obtained with the oxidants H_2O_2 and $NaNO_2$ (increase of AMP-deaminase activity with respect to control erythrocytes was 4.5-fold with 20 mM iodoacetate, 5.4-fold with 1 mM NEM, and 3.4-fold with 1 mM PMB). Residual activation of 4 mM H_2O_2-treated AMP-deaminase by sulfhydryl agents and residual activation of iodoacetate- or NEM-treated enzyme by 4 mM H_2O_2 are 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_2O_2 or NEM. It is worth emphasizing that the lowest concentrations of both stimuli (0.5 and 0.1 mM for H_2O_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 despite 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 and 4 mM H_2O_2- and 1 mM NEM-treated hemolysates were carried out in the 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 Fig. 3, showed that AMP-deaminase of control hemolysates had a different slope with respect to those of 4 mM H_2O_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 μmol of IMP produced/min/liter of red blood cells, while 4 mM H_2O_2-treated hemolysates had a half-maximal activation reached with 3.30 mM AMP and a V_{max} of 7.77 μmol of IMP produced/min/liter of red blood cells, and 1 mM NEM-treated hemolysates had a half-maximal activation recorded with 3.22 mM AMP and a V_{max} of 7.95 μmol of IMP produced/min/liter of red blood cells. Hill plots of the data shown in Fig. 3 gave n values of 2.24, 1.76, and 1.80 for the enzyme of control and 4 mM H_2O_2- and 1 mM NEM-treated hemolysates, respectively (Fig. 4). It is worth mentioning that Hill plots evidenced the absence of the lower asymptote in the curves of both H_2O_2- and NEM-treated hemolysates, suggesting the presence of AMP-deaminase in the R state only, in these samples.

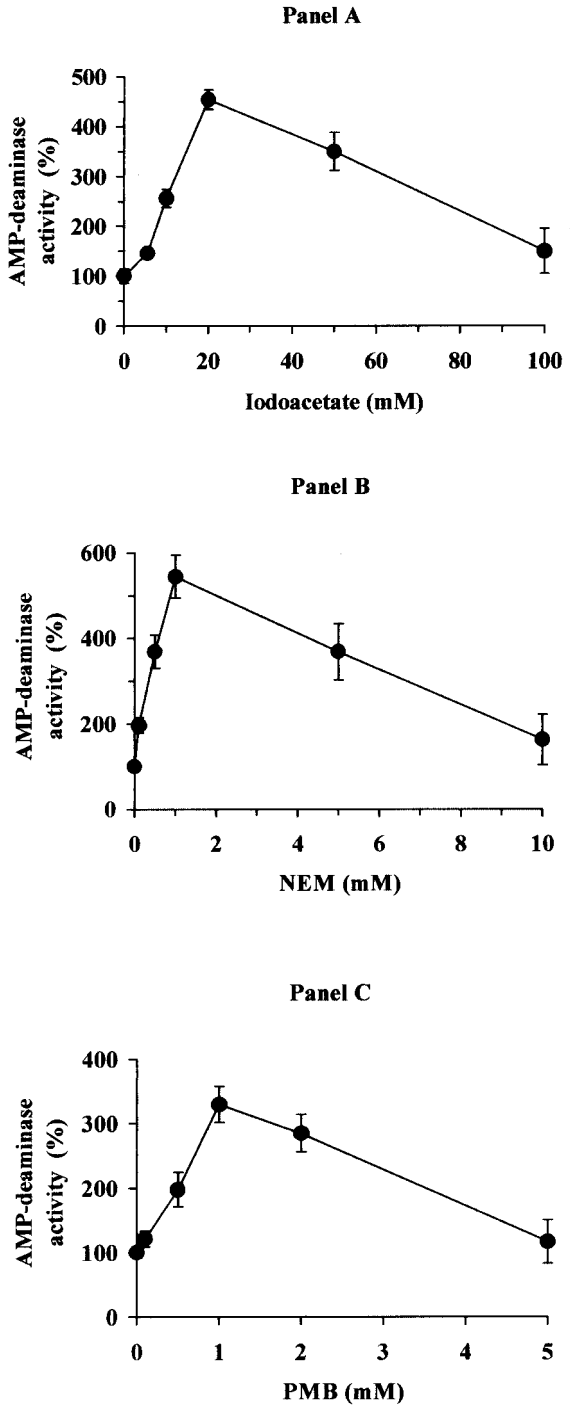

FIG. 2. **Effect of –SH-modifying reagents on AMP-deaminase activity of human red blood cell hemolysates.** Erythrocytes were hemolyzed with double-distilled water (1:5; w/w) and incubated for 30 min at 37 °C with different iodoacetate (*A*), NEM (*B*), or PMB concentrations (*C*). Control hemolysates 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 six different blood samples and is expressed as percentage of activity (control hemolysates = 100% of activity). S.D. values are represented by *vertical bars*. All values of iodoacetate-, NEM-, and PMB-treated hemolysates were significantly different from controls ($p < 0.001$).

To study AMP-deaminase responsiveness toward 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_2PO_4 (1, 5, 10, 50, and 100 mM) concentrations on control and 4 mM H_2O_2- and 1 mM NEM-treated hemolysates. Data reported in Table III

TABLE I
Residual activation of ROS-activated AMP-deaminase by sulfhydryl agents and residual activation of sulfhydryl agent-treated AMP-deaminase by ROS

Hydrogen peroxide-treated hemolysates (incubation with 1 mM NaN_3 + 4 mM H_2O_2 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 NaN_3 plus 4 mM H_2O_2 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 four different blood samples and is expressed as percentage of AMP-deaminase activity (control hemolysates = 100% of activity). Values of treated hemolysates were significantly different from control ($p < 0.001$).

	AMP-deaminase activity
	%
Control	100 ± 19
4 mM H_2O_2	580 ± 46
4 mM H_2O_2 + 20 mM iodoacetate	528 ± 75
4 mM H_2O_2 + 1 mM NEM	546 ± 57
20 mM iodoacetate	539 ± 61
20 mM iodoacetate + 4 mM H_2O_2	518 ± 49
1 mM NEM	574 ± 82
1 mM NEM + 4 mM H_2O_2	610 ± 87

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_2O_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 four different blood samples. AMP-deaminase activity is expressed as percentage of control (control hemolysates = 100% of activity).

	GSH	AMP-deaminase activity
	mmol/liter 20% hemolysate	%
Control	0.418 ± 0.066	100 ± 19
0.5 mM H_2O_2	0.184 ± 0.042[a]	180 ± 38[a]
2 mM H_2O_2	0.065 ± 0.018[a]	389 ± 65[a]
4 mM H_2O_2	0[a]	623 ± 90[a]
10 mM H_2O_2	0[a]	316 ± 42[a]
0.1 mM NEM	0.290 ± 0.043[a]	218 ± 54[a]
1 mM NEM	0[a]	638 ± 103[a]
10 mM NEM	0[a]	111 ± 26

[a] Significantly different from control ($p < 0.001$).

showed that Ca^{2+} did not further increase the AMP-deaminase activity of hemolysates stimulated with both compounds. It should be emphasized that Ca^{2+}-activated AMP-deaminase was comparable with 4 mM H_2O_2- and 1 mM NEM-activated AMP-deaminase when Ca^{2+} in the incubation mixture was at least 5 mM. Data referring to the effects of P_i (Table IV) indicated evident differences among AMP-deaminase activity of control and treated hemolysates. In particular, IC_{50} values of P_i were 7.56, 3.24, and 3.07 mM in control and 4 mM H_2O_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_2O_2- and 1 mM NEM-treated hemolysates was comparable with 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_2O_2-treated erythrocytes and hemolysates, we incubated the 90% purified AMP-deaminase (obtained from fresh human erythrocytes) in the presence of different H_2O_2 (5, 10, 50, 100, and 200 μM) and fixed Fe^{2+} (5 μM) concentrations (Fig. 5*A*); increasing Fe^{2+} (0.5, 1, 2, 5, 10, and 50 μM) and fixed H_2O_2 (100 μM) concentrations (Fig. 5*B*); and various iodoacetate (Fig. 5*C*) or NEM doses (Fig. 5*D*). 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+} plus 100 μM H_2O_2 or 0.5 μM NEM and produced a 2.2- and 2.5-fold increase of AMP-deaminase activity, respectively, in comparison with AMP-deaminase incubated in the presence of buffer only ($p < 0.001$). In the case of the ferrous iron plus hydrogen peroxide-mediated AMP-deaminase activation, it is important to observe that it was independent of the order of the Fenton reagent addition (data not shown) (*i.e.* it seems that the formation of an eventual Fe^{2+}-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 H_2O_2 (500 μM) or Fe^{2+} (50 μM) only did not induce any significant change of the enzymatic activity. Since ROS- and –SH-induced AMP-deaminase activation might have occurred as the 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 Fe^{2+} plus 100 μM H_2O_2, 5 mM iodoacetate, or 0.5 μM NEM (*i.e.* with the doses of stimuli inducing the maximal enzymatic activation) or after incubation with buffer only (control enzyme). The result of the electrophoretic run, illustrated in Fig. 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.

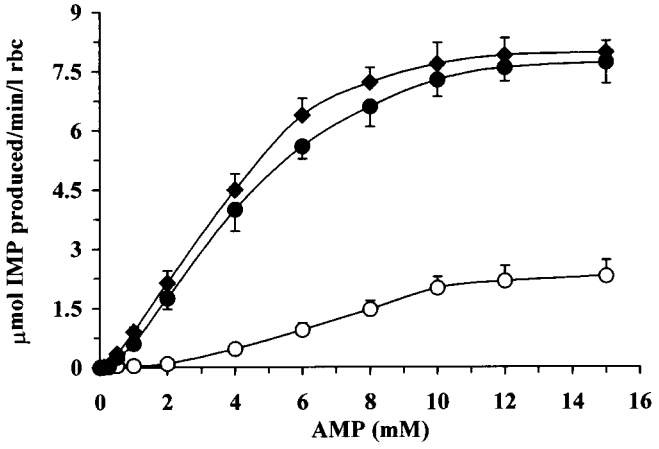

FIG. 3. **Velocity of AMP-deaminase reaction of control and 4 mM H_2O_2- and 1 mM NEM-treated hemolysates as a function of increasing AMP concentrations.** Red blood cells were hemolyzed with double-distilled water (1:5; w/w) and incubated for 30 min at 37 °C with 4 mM H_2O_2 (●) or 1 mM NEM (♦). Control 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 six different blood samples and is expressed as μmol of IMP produced/min/liter of red blood cells. S.D. values are represented by *vertical bars*. All values of H_2O_2- and NEM-treated hemolysates were significantly different from controls ($p < 0.001$).

Data concerning the determination of accessible –SH groups of AMP-deaminase, before and after treatment with increasing concentrations of Fenton reagents (H_2O_2 + Fe^{2+}) or –SH-modifying compound (NEM), are summarized in Table V. The 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

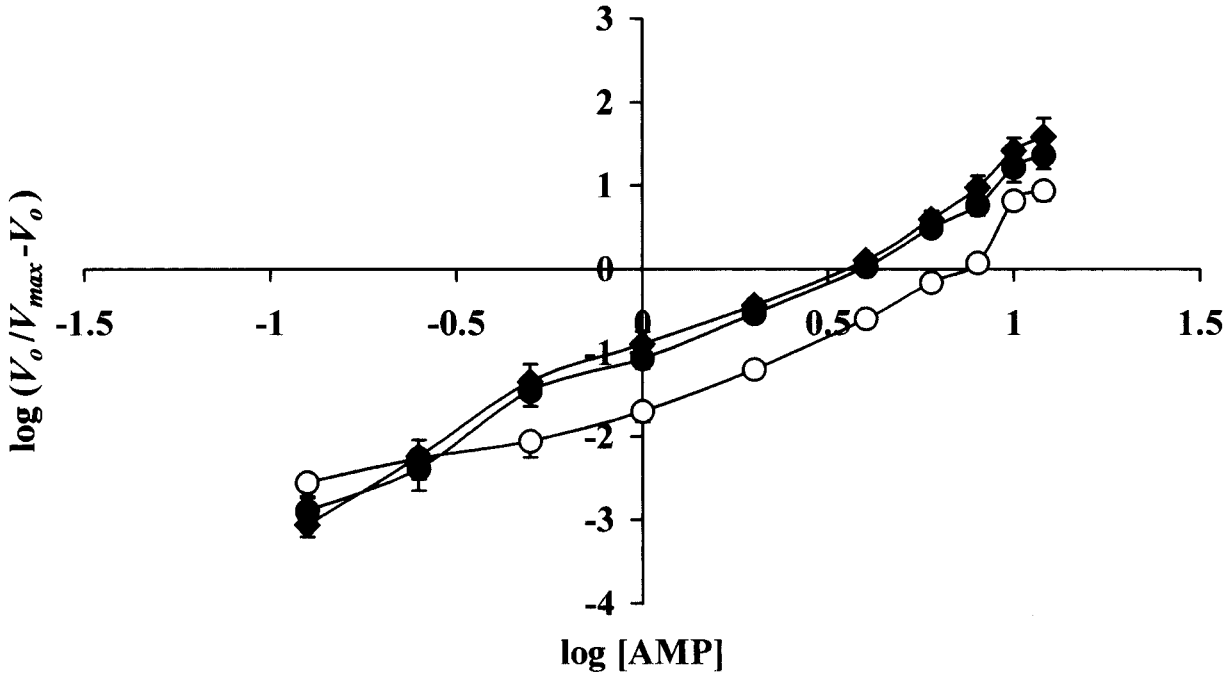

FIG. 4. **Hill plots of the velocity of AMP-deaminase reactions of control and 4 mM H_2O_2- and 1 mM NEM-treated hemolysates.** Curves of affinity of 4 mM H_2O_2-treated hemolysates (●), 1 mM NEM-treated hemolysates (♦), and control hemolysates (○) were obtained from data illustrated in Fig. 3. The linear portion of each plot was used for calculating the respective *n* values.

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_2O_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 six different blood samples and is expressed as percentage of AMP-deaminase activity (control hemolysates = 100% of activity).

$CaCl_2$	Control hemolysates	4 mM H_2O_2-treated hemolysates	1 mM NEM-treated hemolysates
mM	%	%	%
0	100 ± 15	554 ± 33[a]	611 ± 40[a]
1	410 ± 36[b]	642 ± 45[a]	687 ± 39[a]
2	478 ± 41[b]	692 ± 48[a]	703 ± 52[a]
5	774 ± 66[b]	663 ± 58	685 ± 61
10	865 ± 70[b]	792 ± 65	834 ± 77

[a] Significantly different from corresponding value of control hemolysates ($p < 0.001$).
[b] Significantly different from corresponding 0 mM $CaCl_2$ ($p < 0.001$).

TABLE IV
Effect of P_i 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_2O_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 KH_2PO_4 concentrations. Each point is the mean ± S.D. of six different blood samples and is expressed as percentage of AMP-deaminase activity (control hemolysates = 100% of activity).

KH_2PO_4	Control hemolysates	4 mM H_2O_2-treated hemolysates	1 mM NEM-treated hemolysates
mM	%	%	%
0	100 ± 11	596 ± 48[a]	645 ± 54[a]
1	88 ± 10[b]	376 ± 31[a]	401 ± 43[a]
5	61 ± 7[b]	234 ± 33[a]	250 ± 36[a]
10	41 ± 5[b]	88 ± 18[a]	95 ± 14[a]
50	9 ± 2[b]	13 ± 3	15 ± 2
100	8 ± 1[b]	12 ± 3	11 ± 2

[a] Significantly different from corresponding value of control hemolysates ($p < 0.001$).
[b] Significantly different from corresponding 0 mM KH_2PO_4 ($p < 0.001$).

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 H_2O_2 plus Fe^{2+} 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—Fig. 7 reports results of AMP-deaminase activity of erythrocytes (*A*) and hemolysates (*B*) from G-6-PDH-deficient patients after incubation with increasing concentrations of the anti-malarial drug quinine hydrochloride. Even this type of oxidative stress produced a bell-shaped activation curve of the enzyme, with a maximal effect recorded with 5 mM quinine (erythrocytes and hemolysates showed 3.8 and 3.2 times higher activity, respectively, 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; $p < 0.01$).

DISCUSSION

The relative metabolic simplicity of red blood cells is one of their peculiarity, but, of course, it also represents one of the main limitations for their survival. In this light, any change of enzymatic activities, particularly of those involved in the maintenance of the correct energy state, might present such a drastic problem that it would even result in erythrocyte death. Among the various possible causes affecting erythrocyte energy

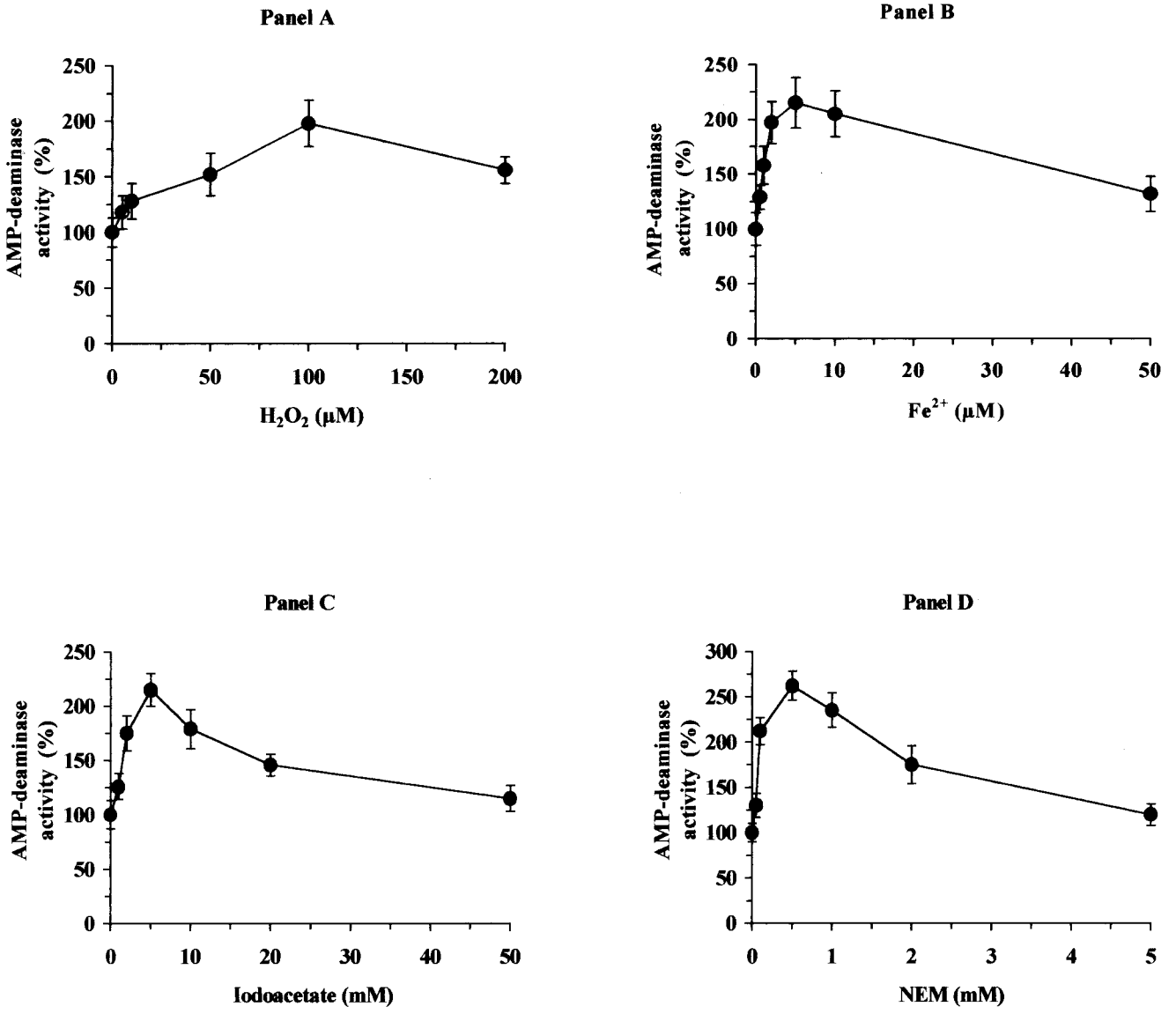

FIG. 5. **Effect of increasing oxidative stress and of –SH-blocking reagents on the activity of the purified AMP-deaminase from human erythrocytes.** AMP-deaminase was purified at the 90% level from fresh human erythrocytes, as described in detail under "Experimental Procedures." Aliquots of the partially purified enzyme were then incubated with 5 μM $FeCl_2$ and different H_2O_2 concentrations (*A*), 100 μM H_2O_2 and different $FeCl_2$ concentrations (*B*), increasing iodoacetate concentrations (*C*), or increasing NEM concentrations (*D*). Control enzyme was incubated with buffer only. Each point is the mean of six different experiments and is expressed as percentage of activity (control enzyme = 100% of activity). S.D. values 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 ($p < 0.01$). H_2O_2 (500 μM) or Fe^{2+} (50 μM) only did not induce any significant change of the enzymatic activity.

metabolism, oxidative stress might certainly represent a negative factor whose relevance, unlike that of 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). On the contrary, 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 $OH^{\cdot}$), 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 the salvage pathway of purine nucleotides. Therefore, IMP production represents for red blood cells 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 up to a certain limit, beyond which an inhibitory effect was observed. We found that in intact erythrocytes and hemolysates (Fig. 1), as well as in the 90% purified enzyme (Fig. 5, *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 toward oxidative stress. Furthermore, the –SH-modifying reagents iodoacetate, NEM, and PMB (Figs. 2 and 5, *C* and *D*) mimicked the same

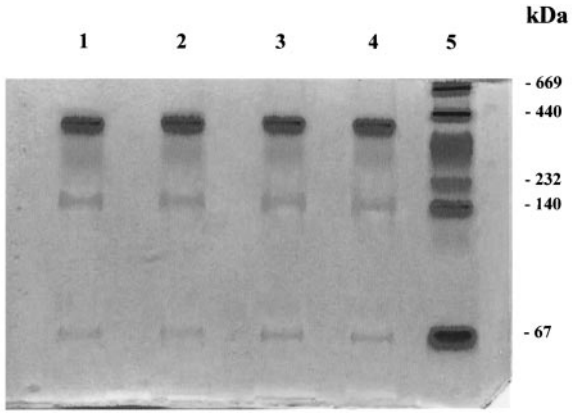

FIG. 6. **Native polyacrylamide gel electrophoresis of purified AMP-deaminase from human erythrocytes after treatment with ROS and –SH-blocking reagents.** Aliquots of the 90% purified enzyme were incubated with 5 μM Fe^{2+} plus 100 μM H_2O_2 (*lane 1*), as a ROS-generating system; 5 mM iodoacetate (*lane 2*), as –SH-modifying reagents; 0.5 mM NEM (*lane 3*), as –SH-modifying reagents; or 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.

TABLE V
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 H_2O_2 and different $FeCl_2$ concentrations or with increasing NEM concentrations. Procedures of AMP-deaminase purification and determination of its accessible –SH groups (carried out by the reaction with DTNB) are fully described under "Experimental Procedures." Control enzyme was incubated with buffer only. Each point is the mean ± S.D. of three different experiments.

	Number of accessible –SH
Control	10 ± 1
1 μM Fe^{2+} + 100 μM H_2O_2	8.3 ± 0.6
5 μM Fe^{2+} + 100 μM H_2O_2	6.3 ± 0.6
50 μM Fe^{2+} + 100 μM H_2O_2	2.7 ± 0.6
0.1 mM NEM	6.7 ± 0.6
0.5 mM NEM	5.7 ± 0.6
5 mM NEM	0

enzymatic activation obtainable with the ROS-generating systems tested, supporting the hypothesis that oxidative stress-mediated AMP-deaminase activation occurs via irreversible modification of cysteine(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 H_2O_2 or 1 mM NEM (Fig. 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 H_2O_2- and NEM-treated hemolysates (Fig. 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 modest change of AMP-deaminase n values (1.76 and 1.80 for 4 mM H_2O_2- 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, that the enzyme has the tetrameric or dimeric structure. The result of the native polyacrylamide gel electrophoresis (Fig. 6) provided clear evidence that treatment of the 90% purified AMP-deaminase with 5 μM Fe^{2+} plus 100 μM H_2O_2 or 0.5 μM NEM (both treatments producing a 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 can be affirmed that the mechanism of oxidative stress-induced enzymatic activation involves, among the 10 freely accessible –SH groups, the modification of 2–4 cysteine 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. We can also presume 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 cysteine residue not easily accessible by reagents and directly involved in the AMP-deaminase catalytic mechanism or because of the possible nonspecific modification of amino acids other than cysteines (lysines, tyrosines, or methionines). In both cases, this occurrence was encountered at definitely high concentrations of both stimuli. It is worth emphasizing that the H_2O_2 and $NaNO_2$ AMP-deaminase activation was not reverted by reducing reagents, such as GSH or β-mercaptoethanol (data not shown), thereby indicating an irreversible oxidative cysteine 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 us 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 interference by eventual low molecular weight modulators might have occurred. Since $OH^{\cdot}$ are produced in part during the reaction of hydrogen peroxide with the ferrous iron of hemoglobin (7), it is conceivable 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), although this original hypothesis was subsequently contradicted by other studies (27, 28). The results of the present study certainly demonstrate that ROS can now be added to the list of the most potent activators of erythrocyte AMP-deaminase. From the data reported in the literature, this phenomenon should be peculiar to the red blood cell isoform. In fact, there is a significant AMP-deaminase inhibition of yeast cells subjected to oxidative stress (29), and, furthermore, it has

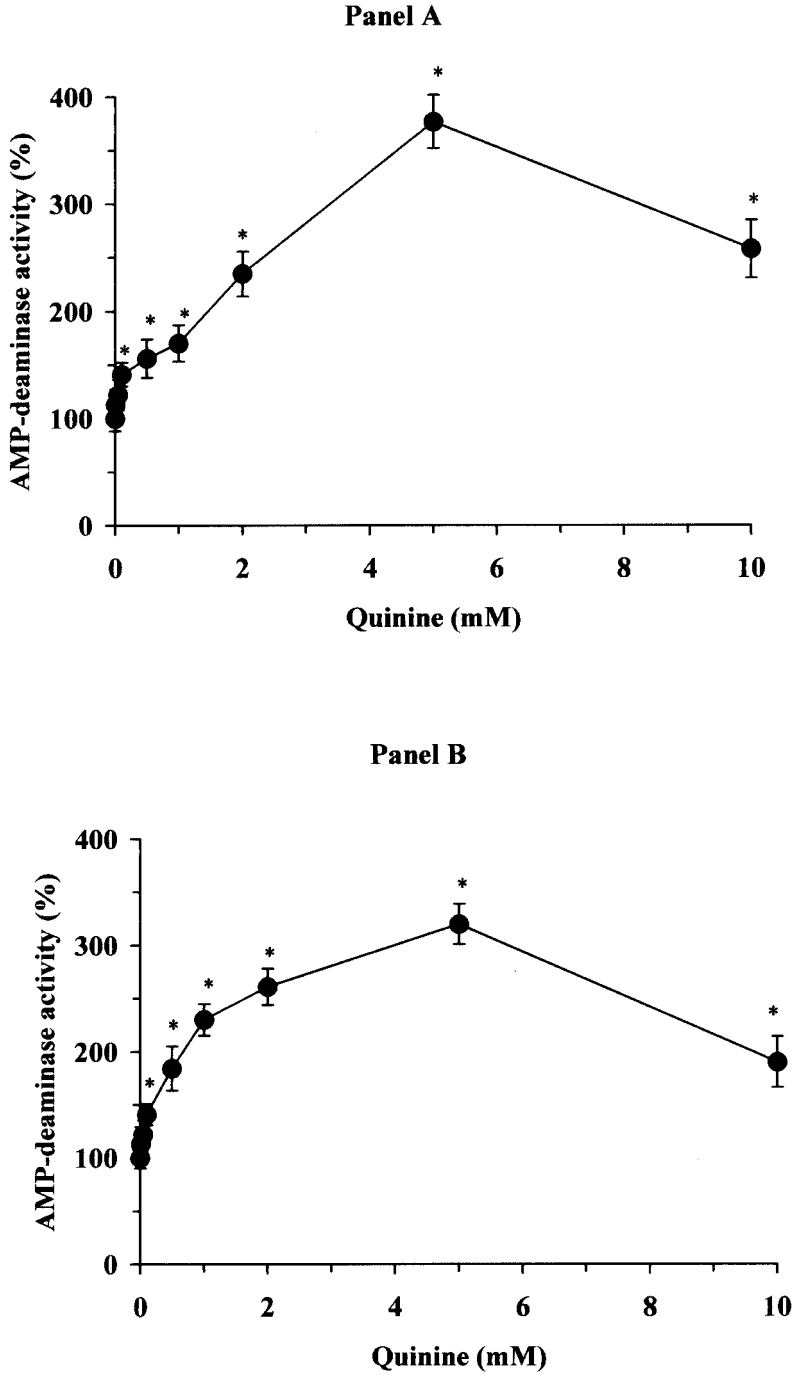

FIG. 7. **AMP-deaminase activity of erythrocytes and hemolysates from G-6-PDH-deficient patients incubated with increasing quinine hydrochloride addition.** Erythrocytes (*A*) and hemolysates (*B*) from G-6-PDH-deficient patients were incubated for 30 min at 37 °C with different concentrations of quinine 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 three different blood samples (challenged in duplicate with quinine) and is expressed as percentage of activity (control G-6-PDH-deficient erythrocytes and hemolysates = 100% of activity). S.D. values are represented by *vertical bars*. *, significantly different from controls ($p < 0.01$).

been demonstrated that a similar inhibitory pattern exists in 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 toward 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_2O_2, that the change of erythrocyte AMP-deaminase thiol state does stimulate (instead of inhibiting) the enzymatic activity. At present, the only AMP-deaminase capable of being markedly activated by ROS, therefore, seems to be the erythrocyte isoform.

Our results obtained by challenging erythrocytes and hemolysates from G-6-PDH-deficient patients with the anti-malarial drug quinine hydrochloride (Fig. 7) showed the same patterns of AMP-deaminase activation as 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 events leading to profound impairment of the erythrocyte energy state in physiological (aging) and, moreover, pathological conditions. In fact, all of

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), and by hemodialysis (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 inhibit the catalytic activity of the enzyme itself. At present, only examples of enzymatic inactivation by oxidative stress can be found in the literature, such as those occurring to glyceraldehyde-3-phosphate dehydrogenase (31, 32), glucose-6-phosphate dehydrogenase (31), creatine phosphokinase (33), pyruvate kinase (34), etc., although 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 toward biologically fundamental molecules or of the understanding of erythrocyte energy metabolism functioning in physiopathological conditions of increased oxidative stress.

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