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Catalase: A tetrameric enzyme with four tightly bound molecules of NADPH

(ultrafiltration/human erythrocytes/bovine liver)

HENRY N. KIRKMAN* AND GIAN F. GAETANI†

*Biological Sciences Research Center, University of North Carolina, Chapel Hill, NC 27514; and †Istituto Scientifico di Medicina Interna, University of Genoa, 16132 Genoa, Italy

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ABSTRACT **Catalases (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) from many species are known to be tetramers of 60,000-dalton subunits, with four heme groups per tetramer. Previous authors have determined the amino acid sequence and three-dimensional structure of bovine liver catalase. Studies of the regulation of the pentose phosphate pathway led the present authors to a search for proteins that bind $NADP^+$ and NADPH in human erythrocytes. An unexpected result of that search was the finding that a major reservoir of bound NADPH in human erythrocytes is catalase. Each tetrameric molecule of human or bovine catalase contains four molecules of tightly bound NADPH. The binding sites have the relative affinities NADPH > NADH > $NADP^+$ > NAD^+. NADPH does not seem to be essential for the enzymic conversion of H_2O_2 to O_2 and water but does provide protection of catalase against inactivation by H_2O_2.**

In the presence of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) hydrogen peroxide is rapidly converted to oxygen and water. Catalase was the source of some of the earliest information about the nature of enzymes. Noting the inhibition of catalase by cyanide, Warburg suggested in 1923 that catalase contains iron (1). Chance obtained evidence for an enzyme–substrate complex from studies of the absorption spectrum of catalase under conditions of rapid flow (2). In 1937, Sumner and Dounce crystallized catalase from bovine liver, achieving one of the first successful crystallizations of an intracellular enzyme (3). The complete amino acid sequence of bovine liver catalase is now known (4), and the three-dimensional structure has been determined at resolutions of 2.5 Å for the enzyme from bovine liver (5) and 3.5 Å for catalase from *Penicillium vitale* (6). Catalases from different sources exhibit similarities in molecular weight, number of subunits, and types of prosthetic groups (7, 8). The enzyme is a tetramer with a total molecular weight of approximately 240,000. Each tetrameric molecule contains four heme groups in which the iron is in the ferric state.

We now report that bovine and human catalase also contain four tightly bound molecules of NADPH. This reduced dinucleotide is not essential for activity of catalase. Instead, NADPH decreases the susceptibility of catalase to inactivation when the enzyme is exposed to low concentrations of its toxic substate, H_2O_2. Purified samples of human and bovine catalase were found to bind and release NADPH in a manner suggesting that catalase may also function as a regulatory protein, releasing $NADP^+$ when the cell is under peroxidative stress. This release would augment removal of H_2O_2 by the glutathione reductase–glutathione peroxidase mechanisms.

MATERIALS AND METHODS

Assays. Activity of catalase was expressed as the first-order kinetic constant for disappearance of H_2O_2, as determined at a wavelength of 240 nm with a recording spectrophotometer (9). Activities of catalase and NADPH diaphorase (10) (NADPH:methylene blue oxidoreductase, EC 1.6.99.1) were measured at 25°C. Assays for NADP and NADPH were by the cycling method of Lowry and Passonneau (ref. 11, p. 130) as described previously (12). Binding of dinucleotides by catalase was quantitated by addition of 0.2–0.4 mg of purified catalase to 6.5 ml of Krebs–Ringer/2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) buffer, pH 7.4 (12) containing dinucleotide at a final concentration of 5 μM. The resulting solutions contained 20–40 molecules of dinucleotide per molecule of catalase. After being allowed to stand on ice for 1 hr, the solution was poured into a CF 25 ultrafiltration cone on a supporting cone (Amicon) over a 45-ml centrifuge tube. The solution in this assembly was centrifuged for 10–15 min in a swinging-cup rotor at 1000 × *g* until reduced to a volume of 0.05–0.1 ml. The volume of concentrate was measured during transfer to a microcentrifuge tube. The cone was washed, by Vortex mixing, with two portions of the solution that had passed through the cone, the volumes of the washings being such as to bring the final volume of the concentrate to 0.2 ml when washings were added to the concentrate. The difference in concentration of NADP between ultrafiltrate and the 0.2-ml sample was regarded as the concentration of bound NADP. The protein assay was with Folin reagent (13). Molecular weights of bovine and human catalase were considered to be 240,000 (7, 14).

Sources of Catalase. Crystalline catalase from bovine liver was a product of Boehringer Mannheim. Human catalase was purified by the method of Mörikofer-Zwez *et al.* (15) but with the following precautions for minimizing contamination with NADPase from erythrocyte stroma and leukocytes. Hospital bank blood (500–1000 ml) was filtered through cellulose (16) for removal of leukocytes and platelets. Washed, packed erythrocytes were lysed by addition to 9 vol of 5 μM $NADP^+$. The preparation was allowed to stand at 0°C for 10 min with occasional mixing. After centrifugation at 16,000 × *g* for 20 min, supernatant fluid was collected for the two steps (15) for ion-exchange chromatography. Catalase was further purified by chromatography in Krebs–Ringer/Tes buffer/1 mM EDTA/5 μM $NADP^+$ on a 2.6 × 96 cm column of Sephadex G-200 (Pharmacia). The catalase fraction from the Sephadex column was washed free of excess $NADP^+$ by several ultrafiltrations and dilutions in 0.01 M sodium phosphate buffer, pH 6.0, adjusted to a protein concentration of 5 mg/ml in the buffer, then passed through a 1 × 5 cm column of type 3 agarose-hexane-$NADP^+$ affinity resin from P-L Biochemicals. After a wash with 6 ml of the phosphate buffer, catalase activity was eluted with 5 ml of 0.01 M sodium phosphate buffer/100 μM $NADP^+$. The catalase had a specific activity (3.8×10^7 M^{-1} sec^{-1}), matching that reported for pure preparations (15). Human catalase from both labora-

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Abbreviations: NADP, nicotinamide adenine dinucleotide phosphate in the oxidized form ($NADP^+$) or reduced form (NADPH); Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid.

tories moved essentially as a single band on electrophoresis in $NaDodSO_4$/7.5% acrylamide gels.

Labeled NAD and NADP. Nicotinamide [U-^{14}C]adenine dinucleotide ([^{14}C]NAD^+) was obtained as an aqueous solution from Amersham. NAD^+ kinase (Sigma) with an enzymic specific activity of 10–20 nmol min^{-1} mg^{-1} was dissolved in 0.1 M Tris·HCl buffer, pH 7.5. After being brought to dryness with a stream of air at 25°C, 10 μCi (35 nmol; 1 Ci = 37 GBq) of [^{14}C]NAD^+ was dissolved with 173 μl of water and mixed with 5 μl of 0.5 M $MgCl_2$, 30 μl of 10 mM ATP at pH 7.5, 16.5 μl of 10 mM NAD^+, and 25 μl of a 10 mg/ml solution of NAD^+ kinase. Enzymic determination (ref. 11, p. 17) of NAD^+ and $NADP^+$ at the end of a 36-hr incubation at 37°C revealed that all NAD^+ had become $NADP^+$. After the addition of 1.0 ml of ethanol, the mixture was brought to dryness with a stream of air at 25°C, then dissolved in 1.0 ml of water. The preparation was centrifuged at 1000 × g for 10 min; the supernatant fluid was evaporated to dryness; and the powder was dissolved in 0.25 ml of water. For studies of binding of [^{14}C]NAD^+ or [^{14}C]$NADP^+$ by purified catalase, labeled and unlabeled dinucleotide were added to Krebs–Ringer/Tes buffer to a final concentration of 5 μM with a specific activity of 0.5 μCi/μmol.

[^{14}C]NADH was generated on the day of use by the addition of 45 μg (15 units) of crystalline alcohol dehydrogenase to 3 ml of Krebs–Ringer/Tes/1 M ethanol/80 μM [^{14}C]NAD^+ (0.5 μCi/μmol), followed by incubation at 25°C for 30 min, removal of dehydrogenase by ultrafiltration on a CF 25 cone, then dilution of the 3-ml sample with 45 ml of Krebs–Ringer/Tes buffer. The corresponding generation of [^{14}C]NADPH was with 50 μg (7 units) of glucose-6-phosphate dehydrogenase and 3 ml of Krebs–Ringer/Tes/120 μM glucose 6-phosphate/80 μM [^{14}C]$NADP^+$. Both dehydrogenases were yeast preparations from Boehringer Mannheim.

RESULTS

NADP Binding by Proteins in Hemolysates. Earlier studies indicated that most of the NADP of human erythrocytes remains with the proteins during ultrafiltration (17, 18). Information about the molecular sizes of the NADP-binding proteins was obtained in the present study by equilibration of hemolysate with [^{14}C]$NADP^+$ and column chromatography of the hemolysate on Sephadex G-200 (Fig. 1). Most of the [^{14}C]NADP moved as two peaks: peak A coincided with activity of catalase, peak B with activity of NADPH diaphorase (Fig. 1). From earlier calibration of the column with eight proteins of known molecular mass, peak A corresponded to a protein of 239,000 daltons, peak B to a protein of 19,000 daltons.

Chromatography of hemolysate on the Sephadex G-200 column was repeated but with the substitution of unlabeled $NADP^+$ for [^{14}C]$NADP^+$. NADP-binding peaks were located by cycling assays of the 0.2-ml concentrates and were found to have the same elution volume as the two peaks of [^{14}C]NADP binding. In addition, the cycling assays revealed that the bound NADP of peak A consisted entirely of NADPH, that of peak B, of $NADP^+$. Peaks of activity of three NADP-utilizing enzymes were located in a region of lesser NADP binding (Fig. 1). Using cyclic assays, we obtained essentially identical results when a column of Sephadex G-200 superfine contained $NADP^+$ at a final concentration of 1 μM (Fig. 2 *Upper*). Determinations of hemoglobin confirmed that the protein peak consisted almost entirely of hemoglobin. In contrast to the result with normal erythrocytes, hemolysate from a man with Swiss-type acatalasemia (14) exhibited neither a peak A nor activity of catalase (Fig. 2 *Lower*).

Dinucleotide Binding by Purified Catalases. Cycling assays revealed that purified human catalase contained both NADPH and $NADP^+$ (Table 1). The catalase had been extensively purified in the presence of $NADP^+$ (5 μM) and had been washed free of excess $NADP^+$ by ultrafiltration/dilution after the final step of purification. Exposure to 5 μM [^{14}C]NAD^+ resulted in very little uptake of NAD^+ or alteration of amount of bound $NADP^+$ or NADPH (Table 1). Some uptake of $NADP^+$ or NADH could be observed after expo-

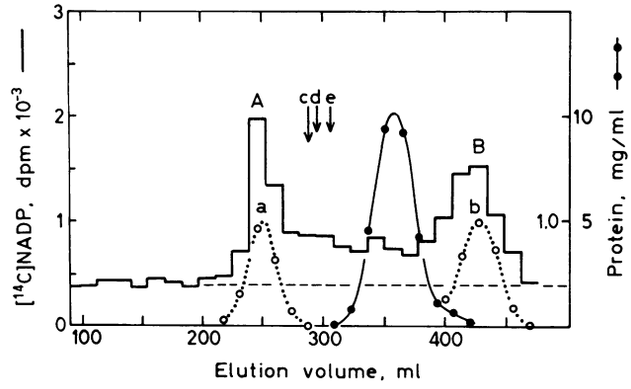

FIG. 1. Distribution of [^{14}C]NADP after chromatography of a hemolysate of normal erythrocytes in Sephadex G-200. Through a 2.6 × 96 cm column of the Sephadex was passed one bed volume of Krebs–Ringer/Tes buffer containing, in the following final concentrations: 5 mM 2-mercaptoethanol, 1 mM EDTA, and 5 μM [^{14}C]-$NADP^+$ (0.4 μCi/μmol). The addition of 0.017 μCi (0.25 nmol) of [^{14}C]$NADP^+$ to 12 ml of stroma-free hemolysate brought the specific activity of the NADP in the hemolysate also to 0.4 μCi/μmol. Incubation of the hemolysate for 30 min at 0°C allowed the conversion of [^{14}C]$NADP^+$ to [^{14}C]NADPH by the intrinsic dehydrogenases and substrates of the pentose phosphate pathway. The 12 ml of hemolysate was mixed with 3 ml of 0.75 M NaCl, then passed down the column at a rate of 8 ml/hr. Fractions (7 ml) were collected, and 6.5-ml portions from alternate tubes were concentrated on ultrafiltration cones to a final volume of 0.2 ml. Scintillation vials received 0.1 ml of each concentrate, 0.9 ml of water, and 10 ml of scintillation fluid (19). ^{14}C counts were for 10 min. Corrections were made for quenching of ^{14}C emission in vials containing hemoglobin (elution volume 336–392 ml). Curves represent concentrations determined before ultrafiltration. Dotted curves: enzymic activities expressed as fractions of the following peak concentrations: a, catalase activity (0.14 sec^{-1} ml^{-1}); and b, NADPH diaphorase activity (5.7 nmol min^{-1} ml^{-1}). Arrows denote centers of activity of: c, glucose-6-phosphate dehydrogenase; d, glutathione reductase; and e, 6-phosphogluconate dehydrogenase. Broken line, 5 μM baseline of $NADP^+$.

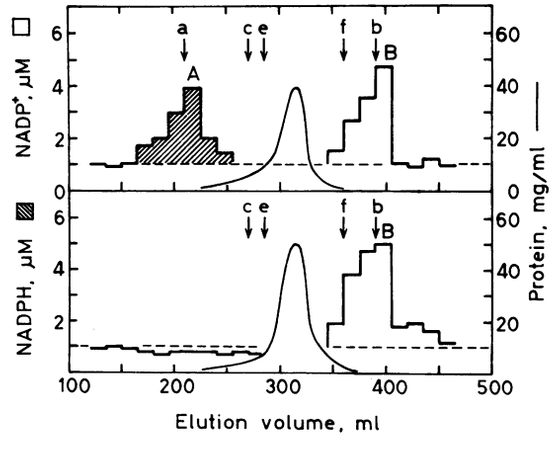

FIG. 2. Distribution of NADPH and $NADP^+$ after chromatography of hemolysate of normal (*Upper*) and acatalasemic (*Lower*) erythrocytes on a 2.5 × 96 cm column of Sephadex G-200 superfine. Each assay was performed on a concentrate obtained by ultrafiltration of the 15-ml fractions to a volume of 0.6 ml. Letter code as in Fig. 1 and f, NADH methemoglobin reductase. Broken line, 1 μM baseline of $NADP^+$.

Table 1. Binding of NADP and NAD by catalase from human erythrocytes

Dinucleotide added	Dinucleotide bound, nmol/nmol of catalase			
	$NADP^+$	NADPH	^{14}C-labeled dinucleotide	Total
None	0.6	2.1	—	2.7
[^{14}C]NAD^+	0.7	2.1	0.0	2.8
[^{14}C]$NADP^+$	1.7	1.9	1.2	3.6
[^{14}C]NADH	0.2	1.8	2.2	4.2
[^{14}C]NADPH	0.0	3.8	2.2	3.8
NADPH + [^{14}C]NADH	0.0	3.8	0.0	3.8
$NADP^+$ + [^{14}C]NADH	0.3	1.8	1.9	4.0

sure to either dinucleotide, although neither was effective in displacing bound NADPH. Exposure to 5 μM NADPH caused the human catalase to lose essentially all $NADP^+$ and to bind 3.8 molecules of NADPH per molecule of catalase (Table 1). In both of our laboratories, a binding ratio of 3.8 to 4.1 was found for a total of four preparations. Once exposed to NADPH, the preparations retained four molecules of NADPH per molecule of catalase despite dilution and reconcentration in the absence of NADPH. On exposure of the human catalase to equimolar (5 μM) concentrations of two dinucleotides, the enzyme bound NADPH in preference to NADH but NADH in preference to $NADP^+$ (Table 1). The results in Table 1 therefore indicate that the affinity of human catalase for dinucleotides has the order NADPH > NADH > $NADP^+$ > NAD^+.

A similar order of affinities could be demonstrated for bovine catalase (Table 2). In contrast to the human enzyme, bovine catalase was found to be saturated with NADPH even without exposure to dinucleotides after the commercial purification. A ratio of four molecules of NADPH per molecule of catalase was observed for preparations from both Boehringer Mannheim (Table 2) and Worthington. In contrast, no presence or uptake of NADPH or $NADP^+$ was detected with catalase of *Aspergillus niger* from either Calbiochem–Behring or Sigma.

After 1 hr of exposure to [^{14}C]NADPH, only 24% of the NADPH of human catalase had been displaced by [^{14}C]-NADPH (Table 1). Even less exchange occurred on the bovine enzyme (Table 2). An additional 23–47 hr of exposure to [^{14}C]NADPH did not alter the result for either enzyme. When the solutions of catalase contained 2-mercaptoethanol at a final concentration of 5 mM, however, the [^{14}C]NADPH after 36 hr represented 91% of the bound NADPH for the human enzyme and 44% for the bovine catalase. Exposure to 5 mM 2-mercaptoethanol for several days at 0–4°C caused the catalase to lose most of its activity. As reported for porcine catalase (20), part of the activity reappeared after removal of the 2-mercaptoethanol.

Table 2. Binding of NADP and NAD by catalase from bovine liver

Dinucleotide added	Dinucleotide bound, nmol/nmol of catalase			
	$NADP^+$	NADPH	^{14}C-labeled dinucleotide	Total
None	0.0	4.1	—	4.1
[^{14}C]NAD^+	0.0	3.8	0.0	3.8
[^{14}C]$NADP^+$	0.3	3.8	0.3	4.1
[^{14}C]NADH	0.0	3.4	0.5	3.9
[^{14}C]NADPH	0.0	3.9	0.6	3.9
NADPH + [^{14}C]NADH	0.0	3.8	0.0	3.8
None*	2.8	0.7	—	3.5
$NADP^+$*	3.7	0.5	—	4.2
$NADP^+$ + [^{14}C]NADH*	0.4	0.5	3.1	4.0

*Added to H_2O_2-treated catalase.

Repeat dilution/ultrafiltration of the bovine and human catalase, without $NADP^+$ in the diluting solution, resulted in no appreciable removal of bound NADP. The concentration of NADP in the buffer passing through the cone was less than 10 nM even with the solution being allowed to stand overnight before the last ultrafiltration. Comparison of the relative affinities of the bovine catalase for $NADP^+$ and NADH (Table 2) was possible after conversion of the bound NADPH to $NADP^+$ by exposure of the catalase to H_2O_2 under conditions that result in low steady-state concentrations of H_2O_2 (21). Those conditions are described in the following paragraph.

Effects of H_2O_2. A center well containing 1 mg of bovine catalase in 0.5 ml of Krebs–Ringer/Tes buffer was fitted into the top of a 20-ml vial after addition of 1.0 ml of 10 M H_2O_2 to the bottom of the vial. The vial was stoppered and placed in a metabolic shaker at 37°C and at 90 cycles per min. Preparation of replicate vials allowed the opening of vials at predetermined intervals. At 24 hr, 43% of the NADPH of the catalase had become $NADP^+$; at 48 hr, 83%. Gram staining revealed only occasional bacteria in the 48-hr sample. The catalase exposed for 48 hr to an atmosphere of H_2O_2 had 60% of the initial activity. Aliquots of the 48-hr samples were added to 9 vol of 0.01 M phosphate buffer, pH 7.0, with dinucleotide present at a final concentration of 10 μM. Nine replicate incubations with each dinucleotide at 25°C for 1 hr led to the following activities and standard errors of the mean, expressed as a percentage of the activity for the catalase incubated without added dinucleotide: NADPH 120.1 ± 4.0; NADH, 115.2 ± 2.7; $NADP^+$, 108.1 ± 3.4; and NAD^+, 103.1 ± 4.1. These results with NADPH and NADH represent a slight, but significant ($P < 0.005$), reversal of the loss in activity.

Incubation of the bovine catalase in an atmosphere of H_2O_2 for 12–24 hr beyond 48 hr caused total loss of cycling-detectable $NADP^+$ or NADPH, yet catalase activity remained at 30–50% of the initial activity. The presence of 1 unit of yeast glucose-6-phosphate dehydrogenase and 250 nmol of glucose 6-phosphate in the center well served to maintain the NADPH in the reduced form during the exposure to an atmosphere of H_2O_2. These additions also prevented inactivation of the enzyme. When placed in a boiling water bath for 90 sec or exposed to 8 M urea/0.1 M 2-mercaptoethanol, the human and bovine catalases released their bound NADP.

DISCUSSION

Because catalase is not considered to be an NADPH-utilizing enzyme, investigators have not been prompted to look for the bound dinucleotide over the nearly half-century that the highly purified enzyme has been available for study. Our finding that catalase binds NADPH came as an unexpected result of a search (17, 18) for proteins that seem to regulate the pentose phosphate pathway by tying up most of the NADP of the cell. Normal erythrocytes contain 1.31–2.71 μg of catalase per mg of hemoglobin (22). These values correspond to an expected concentration of 6.6 to 13.7 μM for catalase-bound NADPH in human erythrocytes. The areas of peaks A above the baselines (Figs. 1 and 2) correspond to observed values of 11 to 12 μM for normal erythrocytes. The dissociation constants of human and bovine catalase for NADPH were found to be immeasurably low ($<1 \times 10^{-8}$ M). [^{14}C]NADPH only partly exchanged with the bound NADPH (Tables 1 and 2). These findings prompt the ques-

tion of whether some of the NADPH molecules are covalently bound to catalase. The binding is disrupted, however, by brief exposure to 0.04 M NaOH/0.5 mM cysteine at 0°C during the cycling assay, by low concentration of H_2O_2, by 5 mM 2-mercaptoethanol, or by exposure to 8 M urea/0.1 M 2-mercaptoethanol. The more likely explanation is that the NADPH is tightly but noncovalently bound. In the process, NADPH is strongly protected from oxidation by atmospheric oxygen. In aqueous solutions exposed to air for many hours, NADPH undergoes nonenzymic oxidation to $NADP^+$ (ref. 11, p. 13). By contrast, NADPH remains bound and reduced during extensive commercial purification of bovine liver catalase and during storage of solutions of human catalase at 0–4°C for many months. The catalase of *Aspergillus niger* does not share these properties with the two mammalian enzymes, but the catalase from this fungus differs in several ways from catalases of other species (23). Of interest, therefore, is whether NADPH-binding of catalase is an early or recent development in evolution.

The function of the bound NADPH is not fully apparent, but three hypotheses can be formulated:

(*i*) NADPH is a substrate or cofactor in some enzymic action of catalase other than the action shown in the upper portion of Fig. 3. Cohen and Hochstein demonstrated that the glutathione reductase/peroxidase mechanism (Fig. 3 *Lower*), not catalase, is the principle means by which H_2O_2 is destroyed in human erythrocytes (24). Keilin and Hartree suggested that the physiological function of catalase may be that of a peroxidase (25). Genetic deficiencies of catalase appear to be relatively harmless in several species of mammals (14). Thus, the enzymic function of catalase *in vivo* is unclear.

(*ii*) NADPH serves to prevent and partially reverse the well-recognized (7) inactivation of catalase by its own toxic substrate. Whether NADPH is one of the "endogenous donors" involved in the recovery of activity (7) or is stabilizing the enzyme by having an allosteric effect on the configuration of catalase remains to be determined. Eaton *et al.*, studying activity of catalase in hemolysates, observed that NADPH prevented and partially reversed the inactivation of catalase that occurred when human erythrocytes and hemolysates were incubated with sodium ascorbate (26). H_2O_2 is generated during spontaneous oxidation of ascorbate in solutions exposed to air. McMahon and Stern noticed protective and partially restorative effects of NADPH and NADH on purified catalase exposed to a vitamin K analog (27).

(*iii*) Catalase has evolved into a regulatory protein, allowing the glutathione reductase/peroxidase mechanism (Fig. 3 *Lower*) to operate more efficiently when the cell is under peroxidative stress. The initial step of that mechanism is catalyzed by glucose-6-phosphate dehydrogenase and is rate limiting in human erythrocytes (12) because the concentration of unbound $NADP^+$ is lower than the Michaelis constant of the dehydrogenase for $NADP^+$ (17, 18). Prolonged generation of H_2O_2 at low concentrations seems to occur during certain drug-induced peroxidative stresses on erythrocytes (28). Studies of intact erythrocytes *in vitro* indicate that such stress leads to a moderate decrease of NADH and a severe decrease of NADPH (29, 30), the latter resulting from action of the glutathione reductase/peroxidase mechanism (Fig. 3 *Lower*). Under these conditions, the unbound and catalase-bound NADPH would be oxidized to $NADP^+$, the catalase-bound $NADP^+$ would be displaced by NADH, and the intracellular concentration of $NADP^+$ would rise. Catalase represents a reservoir of 11–12 μM NADP. Although this represents only one-third of the total NADP of the erythrocyte (12), these concentrations exceed the 0–5 μM concentation of unbound $NADP^+$ in normal erythrocytes (17, 18).

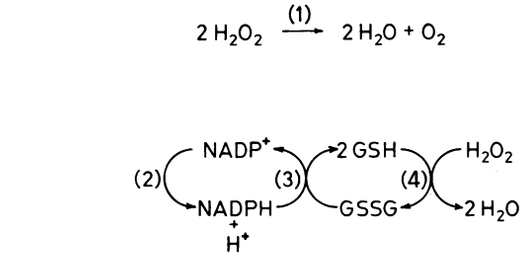

FIG. 3. Mechanisms for destruction of hydrogen peroxide. (*Upper*) Reaction 1 is catalyzed by catalase. (*Lower*) The glutathione reductase/peroxidase mechanism. Reaction 2 is catalyzed by the tandem dehydrogenases of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Reaction 3, glutathione reductase. Reaction 4, glutathione peroxidase. GSH, glutathione, GSSG, oxidized glutathione.

Results of the present study support mechanisms (*ii*) and (*iii*). Possibility (*i*) justifies a search for reactions requiring both catalase and NADPH. Studies of the absorption spectrum (7) and fluorescence spectrum of catalase should reveal whether the conversion of inactive forms of catalase (Compounds II and III) (7, 8) to active forms is accompanied by the oxidation of bound NADPH. Hypothesis (*iii*) would be strengthened by the demonstration of NADH·catalase complexes and of increased concentrations of unbound $NADP^+$ in human erythrocytes undergoing peroxidative stress. Current methods of biochemical investigation therefore would allow a test of each of the three hypotheses concerning the function of NADPH bound to bovine and human catalase.

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