Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1521–1525, March 1983
Biochemistry

Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing

(cytochrome P-450/NADH oxidase/enzymic inactivation)

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Contributed by E. R. Stadtman, December 8, 1982

ABSTRACT **Several mixed-function oxidation systems catalyze the inactivation of *Escherichia coli* glutamine synthetase. Inactivation involves modification of a single histidine residue in each enzyme subunit and makes the enzyme susceptible to proteolytic degradation. We show here that 10 key enzymes in metabolism are inactivated by a bacterial NADH oxidase and by an oxidase system comprised of NADPH, cytochrome P-450 reductase, and cytochrome P-450 isozyme 2 from rabbit liver microsomes. Most of the inactivatable enzymes require a divalent cation for activity and all but one (enolase) possess a nucleotide binding site. Glutamine synthetase, pyruvate kinase, and phosphoglycerate kinase are protected from inactivation by their substrates; substrate protection of other enzymes was not tested. We propose that inactivation involves mixed-function oxidization system-catalyzed synthesis of H_2O_2 and reduction of Fe(III) to Fe(II) followed by oxidation of enzyme-bound Fe(II) by H_2O_2 to generate oxygen radicals that attack a histidine (or other oxidizable amino acid) at the metal binding site of the enzyme. This is supported by the following: (*i*) most of the inactivation reactions are inhibited by EDTA and by catalase, (*ii*) both mixed-function oxidation systems reduce Fe(III), and (*iii*) H_2O_2 together with Fe(II) catalyzes nonenzymic inactivation of glutamine synthetase. In view of the fact that inactivation of glutamine synthetase makes it susceptible to proteolytic degradation, it is possible that mixed-function oxidation system-catalyzed inactivation of enzymes is a regulatory step in enzyme turnover. In addition, the implication of oxidative inactivation reactions in ageing is suggested by the fact that many of the enzymes inactivated by mixed-function oxidation systems are known to accumulate as inactive forms during ageing.**

The inactivation of *Escherichia coli* glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] is catalyzed by a variety of mixed-function oxidation systems, including nonenzymic systems comprised of either ascorbate, O_2 and Fe(III), or Fe(II) and O_2 and enzymic systems comprised as follows: (*i*) rabbit liver microsomal cytochrome P-450 reductase together with cytochrome P-450 isozyme 2 [P-450(LM2)], (*ii*) microbial NADH oxidase, (*iii*) putidaredoxin reductase together with putidaredoxin with or without cytochrome P-450, (*iv*) xanthine oxidase together with ferredoxin or putidaredoxin, and (*v*) partially purified enzymes (NADH oxidase) from *Klebsiella aerogenes* or *E. coli* (1–3). Inactivation of glutamine synthetase by all enzyme systems is dependent on O_2 and NAD(P)H (except in the case of xanthine oxidase, for which hypoxanthine serves as an electron donor). All systems are stimulated by Fe(III) and inhibited by catalase, Mn(II), EDTA, *o*-phenanthroline, and histidine (1–3). Inactivation of glutamine synthetase by either the ascorbate system or the NADH oxidase system (other systems not tested) is associated with the modification of a single histidine residue in each glutamine synthetase subunit (4). This inactivation renders the glutamine synthetase more susceptible to proteolytic degradation by subtilisin (5) as well as by proteases in *E. coli* (1, 2). Thus, the inactivation reaction appears to "mark" glutamine synthetase for proteolytic digestion.

We report here that, in addition to the glutamine synthetase from *E. coli*, the glutamine synthetase from rat liver and a number of other key metabolic enzymes, many of which possess a histidine or another easily oxidizable residue at the catalytic site, are inactivated *in vitro* by the NADH oxidase system and also by the rabbit liver microsomal cytochrome P-450 system.

During ageing, inactive or less active forms of several enzymes accumulate in some cells (6, 7). The possibility that these inactive forms are generated by mixed-function oxidation reactions is supported by the present study, showing that many of the enzymes found by others to be inactivated during ageing are also inactivated by the mixed-function oxidation systems.

MATERIALS AND METHODS

Enzymes and Chemicals. Glutamine synthetase from *E. coli* was prepared by the zinc precipitation method as described (8). Rabbit liver microsomal P-450(LM2) was purified according to Coon *et al.* (9) and NADPH cytochrome P-450 reductase was purified by using the procedure of French and Coon (10). Microbial diaphorase (NADH oxidase), NADH, and NADPH were obtained from Boehringer Mannheim. Catalase from bovine liver, superoxide dismutase (SOD) from bovine erythrocytes, and dilauroylglyceryl-3-phosphorylcholine were obtained from Sigma. *E. coli* acetate kinase and aspartokinase III were gifts of T. C. Stadtman and M. Maurizi, respectively. Rat liver glutamine synthetase was provided by R. Miller.

Enzyme Assays. Cytochrome P-450 was assayed by the method of Omura and Sato (11), and NADPH cytochrome P-450 reductase was assayed by the method of Phillips and Langdon (12). All other enzyme activities were determined according to standard published procedures as referenced in Tables 1 and 2 (13–20).

Inactivation Assays. For inactivation by the NADH oxidase system, reaction mixtures (200 μl, 37°C) contained 50 mM Tris·HCl, pH 7.4, 1 mM NADH, 100 μM $FeCl_3$, and either 100 mM KCl/10 mM $MgCl_2$ or 10 mM KCl/1 mM $MgCl_2$. Both levels of KCl and $MgCl_2$ were usually tested because the inactivation of some enzymes (e.g., phosphoglycerate kinase and pyruvate kinase) is dependent on the concentrations of these reagents. Inactivation reaction mixtures of *E. coli* glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase

Abbreviations: P-450(LM2), cytochrome P-450 isozyme 2; SOD, superoxide dismutase.

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contained 0.08 and 0.03 unit of NADH oxidase, respectively; all other inactivations were carried out with 0.32 unit of NADH oxidase.

The inactivation reactions were initiated by the addition of NADH. With the exception of glutamine synthetase, aliquots of inactivation reaction mixtures were removed at various times and assayed immediately for enzyme activity. For glutamine synthetase, aliquots were first diluted with 10 mM $MnCl_2$/50 mM Tris·HCl, pH 7.4, and then assayed. Conditions were the same for inactivation by cytochrome P-450 except that 1 mM NADPH was used in place of NADH and 0.35 μM NADPH cytochrome P-450 reductase/0.15 μM cytochrome P-450 containing 20 μg of dilauroylglyceryl-3-phosphorylcholine was used in place of NADH oxidase. The concentrations of enzymes used are given in Tables 1 and 2.

Anaerobic Studies. For anaerobic experiments, all reagents were prepared in freshly boiled water through which a stream of argon was bubbled during cooling. Incubations were in small test tubes (12 × 75 mm) fitted with rubber caps into which two hyperdermic needles were inserted to serve as inlet and outlet ports for the introduction of argon. After argon was bubbled through the incubation mixture for 2 min, the reactions were started by the addition of either NADH or H_2O_2 by means of a hyperdermic syringe.

Table 2. Enzymes not inactivated by NADH oxidase system

Enzyme	Supplier	Conc., μM	Source	Assay ref.
Acetate kinase		1.4	*E. coli*	17
Aldolase	Sigma	0.5	Rabbit muscle	13
Alkaline phosphatase A	Sigma	0.1	*E. coli*	13
α-Amylase	Sigma	5.6	Porcine pancreas	13
Carboxy-peptidase A	Sigma	1.4	Bovine pancreas	13
Fructose-1,6-di-phosphatase	Sigma	1.9	Rabbit muscle	15
β-Galactosidase	Boehringer Mannheim	0.91	*E. coli*	18
Glucose-6-phos-phate dehydro-genase	Sigma	0.05	Baker's yeast	13
		0.05	*L. mesenteroides*	13
β-Glucuroni-dase	Sigma	2.0	*E. coli*	19
Hexokinase	Sigma	0.27	Yeast	13
Lysozyme	Sigma	13.4	Hen egg white	20
Malate dehydrogenase	Sigma	0.06	Porcine heart	13
		0.06	Bovine heart	13

RESULTS AND DISCUSSION

Enzyme Inactivation by NADH Oxidase and Cytochrome P-450. Of 25 enzymes tested (Tables 1 and 2), 10 were readily inactivated by both mixed-function oxidation systems used in this study (Table 1). All of the inactivation reactions required NAD(P)H and were inhibited by catalase (data not shown). In no case was enzyme activity restored after dialysis against appropriate buffers, even in the presence of 5 mM dithiothreitol.

It is noteworthy that most of the susceptible enzymes are either synthetases, kinases, or NAD(P)-dependent dehydrogenases; i.e., they possess a nucleotide binding site at the catalytic center. In addition, where such information is available, they require divalent metal cations for activity and contain a histidine residue at or near the catalytic site (21–26).

Properties of the Inactivation Reactions of Glutamine Synthetase, Pyruvate Kinase, and Phosphoglycerate Kinase. Qualitative and quantitative differences were observed during the inactivation of various enzymes with the NADH oxidase and rabbit liver microsomal cytochrome P-450 inactivating systems. However, the properties of the inactivating reactions are remarkably similar. As shown in Fig. 1 and Table 3, the inactivation of glutamine synthetase, pyruvate kinase, and phosphoglycerate kinase by either oxidation system requires O_2 and NAD(P)H, is stimulated by $FeCl_3$, and inhibited by catalase, $MnCl_2$, EDTA, and *o*-phenanthroline. With all three enzymes, SOD and azide have little or no effect. The inhibitory effect of SOD, observed for the NADH oxidase-directed inactivation of glutamine synthetase was probably not due to SOD activity because boiled fully inactive SOD still inhibited NADH oxidase inactivation of glutamine synthetase.

Mechanism of Inactivation of Enzymes by Mixed-Function Oxidation Systems. From the evidence presented here and in earlier communications (1, 2), the inactivation of enzymes by mixed-function oxidation (MFO) systems could occur by the fol-

Table 1. Enzymes inactivated by mixed-function oxidation systems

Enzyme	Supplier	Source	Conc., μM	% inactivation: NADH oxidase	% inactivation: Cytochrome P-450	Assay ref.
Alcohol dehydrogenase	Boehringer Mannheim	*Leuconostoc mesenteroides*	0.1	55	59	13 (pp. 10–11)
		Yeast	0.1	55	50	13 (pp. 10–11)
Aspartokinase III		*E. coli*	0.24	52	NT	14 (p. 2034)
Creatine kinase	Sigma	Rabbit muscle	2.2	75	55	13 (pp. 55–56)
Enolase	Sigma	Yeast	0.88	55	NT	15 (p. 449)
Glutamine synthetase		*E. coli*	1.8	49	38	16
		Rat liver	0.15	75	49	16
Glyceraldehyde-3-phosphate dehydrogenase	Boehringer Mannheim	Rabbit muscle	0.85	63	90	15 (pp. 466–467)
Lactate dehydrogenase	Boehringer Mannheim	Rabbit muscle	0.06	46	49	13 (pp. 111–112)
Phosphoglycerate kinase	Sigma	Yeast	0.15	47	40	15 (pp. 502–503)
Pyruvate kinase	Boehringer Mannheim	Rabbit muscle	0.21	48	39	13 (pp. 178–179)

Incubation times (5–90 min) were chosen to obtain 40–75% inactivation. Conc., concentration; NT, not tested.

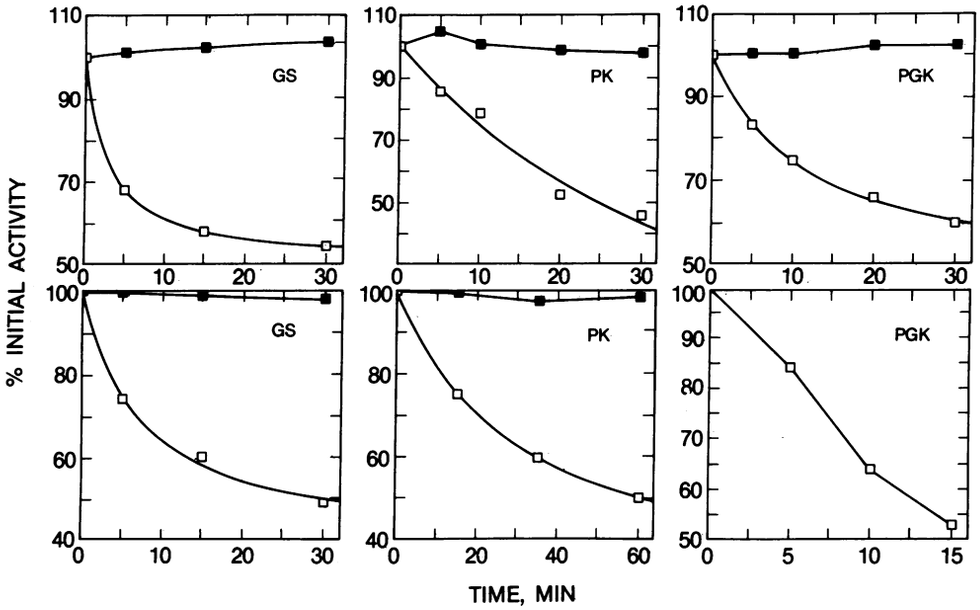

FIG. 1. Inactivation of glutamine synthetase (GS), pyruvate kinase (PK), and phosphoglycerate kinase (PGK) by cytochrome P-450 system (*Upper*) and NADH oxidase system (*Lower*). Inactivation reactions were carried out in the presence (□) or absence (■) of NAD(P)H.

lowing mechanism:

$$NAD(P)H + H^+ + O_2 \xrightarrow{MFO} H_2O_2 + NAD(P)^+ \quad [1]$$

$$NAD(P)H + 2\ Fe(III) \xrightarrow{MFO} 2\ Fe(II) + NAD(P)^+ + H^+ \quad [2]$$

$$Fe(II) + H_2O_2 \longrightarrow \text{activated oxygen} + Fe(III) \quad [3]$$

$$\text{Activated oxygen} + \text{enzyme} \longrightarrow \text{oxidized enzyme.} \quad [4]$$

Involvement of H_2O_2 is implied by the fact that the inactivation reactions are all inhibited by catalase. A role of Fe(III) is indicated by the fact that the inactivation reactions are inhibited by chelating agents and stimulated by the addition of Fe(III). Involvement of Fe(II) is supported by the fact that both mixed-function oxidation systems are capable of reducing Fe(III) to Fe(II) and also by the fact that Fe(II) alone in the presence of O_2 catalyzes inactivation of glutamine synthetase (1). To the contrary, Fe(III) by itself has no inactivation capacity under either anaerobic or aerobic conditions. The fact that, under strictly anaerobic conditions, H_2O_2 and Fe(II) together (Table 4), but neither one by itself, inactivates glutamine synthetase supports the involvement of reaction 3, which is known to generate activated oxygen species, (e.g., OH·, peroxyderivatives, singlet oxygen). However, the "activated oxygen" species presumed to be generated in reaction 3 has not been identified. Although a likely candidate, the participation of OH· is contraindicated by the failure of radical scavengers such as mannitol, dimethyl sulfoxide, and thiourea to inhibit the inactivation reaction (Table 5). An involvement of superoxide anion is also contraindicated by the failure of SOD to inhibit the inactivation reaction.

It is possible that reactions 3 and 2 occur at the catalytic site on the target enzyme. The site-specific nature of the inactivation reaction is implied from the finding that inactivation of glutamine synthetase is associated with the loss of just 1 of 16 histidine residues in each subunit (4). Preliminary results of amino acid analysis indicate that the inactivation of phosphoglycerate kinase is also associated with the loss of one histidine residue (unpublished data). A single histidine residue is also lost during the inactivation of mammalian SOD (27). In such a site-specific process, binding of Fe(II) at the metal binding site on the enzyme might be followed by peroxidation of the Fe(II) enzyme complex to generate an activated oxygen derivative (e.g., OH· or singlet oxygen) that attacks a histidine or another oxidizable amino acid in the catalytic site. In such a site-specific mecha-

Table 3. Properties of inactivation reactions

	Glutamine synthetase		Phospho-glycerate kinase		Pyruvate kinase	
Addition	P-450*	NADH oxidase	P-450*	NADH oxidase	P-450*	NADH oxidase
None	100	100	100	100	100	100
Inert gas	3	0	0	0	0	0
Catalase	0	0	0	0	0	0
SOD	106†	65	86	100	100	85
$FeCl_3$	135	143	227	308	140	306
$MnCl_2$	0	0	11	4	0	23
EDTA	0	0	16	0	24	15
o-Phenanthroline	7	0	0	0	24	15
NaN_3	100	96	81	100	100	100

Results represent pseudo-first-order rates of inactivation relative to rates in the absence of inhibition, which are set equal to 100.
*The P-450 system consisted of purified NADPH cytochrome P-450 reductase/P-450(LM2)/NADPH.
†This datum was provided by K. Nakamura of this laboratory.

Table 4. Glutamine synthetase inactivation by a nonenzymatic system [Fe(II)/H_2O_2] under anaerobic conditions

Addition	Conc., μM	% inactivation
None		0
Fe(II)	10	5
H_2O_2	100	2
Fe(II)/H_2O_2	10/100	50
Fe(II)/H_2O_2/catalase	10/100/1	0
Fe(II)/H_2O_2/SOD	10/100/4.7	53

Results represent percentage of inactivation after 2 min of incubation. Conc., concentration.

Table 5. Effect of scavengers on glutamine synthetase inactivation by enzymatic and nonenzymatic systems

Addition	NADH oxidase	Fe(II)/H_2O_2
None	100	100
Mannitol	83	87
Dimethyl sulfoxide	102	88
Thiourea	92	NT
Histidine	0	28

Results are expressed as in Table 3. NT, not tested. Additions were as follows: Fe(II), 5 μM; H_2O_2, 0.1 mM; mannitol, 100 mM; thiourea, 100 mM; histidine, 100 mM; dimethyl sulfoxide, 43 mM (NADH oxidase experiment) or 1 mM [Fe(II)/H_2O_2 experiment].

nism, the radical intermediate would be generated at the catalytic site and therefore might be shielded from attack by radical scavengers. A similar mechanism has been proposed to explain the inability of radical scavengers to affect the H_2O_2-catalyzed inactivation of mammalian SOD (28).

This interpretation is not consistent with observations that the inactivation of glutamine synthetase by other mixed-function oxidation systems is inhibited by radical scavengers. Inactivation by a system composed of putidaredoxin reductase, putidaredoxin, Fe(III), and NADH is partially inhibited by either SOD, dimethyl sulfoxide, or histidine (3). Curiously, when this oxidation system is supplemented with bacterial cytochrome P-450, the radical scavengers have little or no effect on the inactivation reaction (3). Similarly, the O_2-dependent inactivation of glutamine synthetase by a system comprised of xanthine oxidase, hypoxanthine, ferredoxin, and Fe(III) is inhibited by SOD and by either dimethyl sulfoxide, thiourea, mannitol, or histidine; moreover, the sensitivity of this system to these radical scavengers is also moderated by the presence of cytochrome P-450 (3). It is therefore likely that more than one kind of activated oxygen could be involved in the inactivation reaction. Whereas the inactivation of glutamine synthetase, phosphoglycerate kinase, and SOD (27) is accompanied by the loss of only one histidine residue, it is possible that modification of other amino acid residues (e.g., cysteine, methionine, tryptophan, tyrosine) is involved in the inactivation of some of the enzymes tested. In this regard, it is noteworthy that the generation of H_2O_2 (reaction 1) may of itself be sufficient to cause inactivation of some of the enzymes examined. Although glutamine synthetase and enolase were not inactivated by H_2O_2, most of the enzymes that were inactivated by the mixed-function oxidation systems were also inactivated when they were incubated with H_2O_2 alone. Even so, the possibility that reaction 3 or an analogous reaction is involved in the nonenzymic inactivation reaction is implied by the fact that, of eight enzymes tested, all were inhibited by EDTA except for alcohol dehydrogenase (*L. mesenteroides*) and glyceraldehyde-3-phosphate dehydrogenase (data not shown). Curiously, histidine inhibits glutamine synthetase inactivation by all inactivation systems tested. Histidine inhibition has been considered presumptive evidence for a role of singlet oxygen in oxidase-catalyzed reactions (29). However, the possibilities that it scavenges $OH^{\cdot}$ (30), serves merely to chelate metal ions, or exerts its effect through allosteric interactions with glutamine synthetase (31) must also be considered.

Physiological Significance. *Protein turnover.* The physiological significance of enzyme inactivation by mixed-function oxidation systems remains to be explored. In the case of *E. coli*, there is evidence (1, 2) that the oxidative inactivation of glutamine synthetase renders the enzyme susceptible to proteolytic degradation by endogenous proteases. This invites speculation that such inactivation marks enzymes for proteolytic degradation and is therefore a key step in the regulation of enzyme levels (i.e., in protein turnover). In this respect, it is noteworthy that the inactivation of glutamine synthetase is dependent on both the state of adenylylation of the enzyme and the concentrations of its substrates, ATP and glutamate (1, 2, 32). In the absence of substrates (i.e., where the enzyme is ineffectual), the unadenylylated enzyme is most susceptible to inactivation by any one of several mixed-function oxidation systems; however, in the presence of high concentrations of both ATP and glutamate, the unadenylylated enzyme is protected from inactivation whereas the inactivation of the adenylylated enzyme is enhanced. As shown here, ATP and phospho*enol*pyruvate decrease the susceptibility of pyruvate kinase to oxidative inactivation (Table 6) and both ATP and 3-phosphoglycerate protect phosphoglycerate kinase from inactivation. These results suggest that the concentration of substrates may play an important role in the regulation of enzyme inactivation and consequently in enzyme turnover.

Ageing. It appears well established that ageing of some cells is associated with the accumulation of modified catalytically inactive forms of several enzymes (6, 7), but the nature of the modification reaction is poorly understood. In view of the fact that mixed-function oxidation systems are ubiquitous among aerobic organisms, and the demonstration here that many key enzymes in metabolism are susceptible to oxidative inactivation, we suggest that the accumulation of inactive enzymes during ageing may be due in part to mixed-function oxidation system-catalyzed reactions of the type described here.

Indeed, 12 enzymes have been shown to undergo inactivation during ageing of one or another of the following cells: *Turbatrix aceti*, erythrocytes, liver, and cultured cells (for review, see refs. 6 and 7). Albeit from different sources, five of these same enzymes, SOD (27), pyruvate kinase, lactic dehydrohydrogenase, phosphoglycerate kinase, and enolase are readily inactivated by the oxidase systems. Other enzymes shown to undergo age-related inactivation are glutathione reductase, hypoxanthine phosphoribosyltransferase, isocitrate lyase, and elongation factor I; these have not been tested for inactivation in the oxidation systems. Thus far, two of the enzymes, fructose-1,6-diphosphatase and glucose-6-phosphate dehydrogenase, that undergo age-related loss of activity are not affected by the NADH oxidase system. It is noteworthy that variation in the specific activities of apparently homogeneous preparations of glutamine synthetase isolated from different batches of *E. coli* is accounted for by the presence of inactive enzyme with characteristics of the inactive form generated *in vitro* by the mixed-function oxidation type of reaction (R. Levine, personal communication).

Table 6. Effect of substrate on inactivation of glutamine synthetase, phosphoglycerate kinase, and pyruvate kinase reactions by the NADH oxidase system

Enzyme	Substrate	Conc., mM	% inactivation
Glutamine synthetase			100
	L-Glutamate	100	96
	ATP	1.65	62
Phosphoglycerate kinase			100
	ATP	1.65	22
	3-Phosphoglycerate	6.5	26
Pyruvate kinase			100
	ADP	4.5	23
	Phospho*enol*pyruvate	4.5	78

Conc., concentration.

If, as we propose, the inactivation reaction marks enzymes for proteolytic degradation, the accumulation of inactive enzyme forms during ageing could be caused by any one of the following deficiencies: (*i*) a decrease in the activity of specific proteases that attack the marked enzyme; (*ii*) a decrease in the intracellular levels of metabolites that protect the enzyme from inactivation; (*iii*) a loss in the ability of catalase, SOD, or glutathionine peroxidase to scavenge the reactive oxygen species generated by mixed-function oxidation systems; and (*iv*) an increase in the level of oxidases that catalyze the inactivation reaction.

Bacteriocidal activity of neutrophils. Finally, it is noteworthy that oxidative inactivation of proteins may play an important role in the protection of animal cells against bacterial infection. It has been established that, during a period of so-called oxidative burst that is characteristic of mammalian neutrophils, flavoproteins with NAD(P)H oxidase activities catalyze the generation of several activated oxygen species (33). Results of preliminary experiments in this laboratory indicate that, during this period of oxidative burst, *E. coli* glutamine synthetase is inactivated. Thus, the inactivation of this and other key enzymes in bacteria could be responsible for the bacteriocidal action of neutrophils.

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