JOURNAL OF BACTERIOLOGY, June 2009, p. 3804–3810 Vol. 191, No. 12
0021-9193/09/$08.00+0 doi:10.1128/JB.00046-09

α-Ketoglutarate Dehydrogenase and Glutamate Dehydrogenase Work in Tandem To Modulate the Antioxidant α-Ketoglutarate during Oxidative Stress in *Pseudomonas fluorescens*▽

Ryan J. Mailloux, Ranji Singh, Guy Brewer, Christopher Auger, Joseph Lemire, and Vasu D. Appanna*

Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada

Received 14 January 2009/Accepted 8 April 2009

α-Ketoglutarate (KG) is a crucial metabolite in all living organisms, as it participates in a variety of biochemical processes. We have previously shown that this keto acid is an antioxidant and plays a key role in the detoxification of reactive oxygen species (ROS). In an effort to further confirm this intriguing phenomenon, *Pseudomonas fluorescens* was exposed to menadione-containing media, with various amino acids as the sources of nitrogen. Here, we demonstrate that KG dehydrogenase (KGDH) and NAD-dependent glutamate dehydrogenase (GDH) work in tandem to modulate KG homeostasis. While KGDH was sharply decreased in cells challenged with menadione, GDH was markedly increased in cultures containing arginine (Arg), glutamate (Glu), and proline (Pro). When ammonium (NH_4) was utilized as the nitrogen source, both KGDH and GDH levels were diminished. These enzymatic profiles were reversed when control cells were incubated in menadione media. ^{13}C nuclear magnetic resonance and high-performance liquid chromatography studies revealed how KG was utilized to eliminate ROS with the concomitant formation of succinate. The accumulation of KG in the menadione-treated cells was dependent on the redox status of the lipoic acid residue in KGDH. Indeed, the treatment of cellular extracts from the menadione-exposed cells with dithiothreitol, a reducing agent, partially restored the activity of KGDH. Taken together, these data reveal that KG is pivotal to the antioxidative defense strategy of *P. fluorescens* and also point to the ROS-sensing role for KGDH.

All aerobic organisms have to contend with the dangers associated with reactive oxygen species (ROS), toxic moieties that are routinely generated as a consequence of ATP production via oxidative phosphorylation (34). The transfer of electrons from NADH and reduced flavin adenine dinucleotide to oxygen is mediated by the respiratory complexes, the major sites of intracellular ROS generation (1). These by-products of oxidative phosphorylation are very harmful and have to be nullified if organisms are to survive in an aerobic environment (24). If left unchecked, ROS can damage biological macromolecules, leading to the demise of the cell. Hence, it is not surprising that all aerobic organisms have devised intricate antioxidative defense strategies in an effort to proliferate in the presence of oxygen.

Enzymes such as superoxide dismutase and catalase are uniquely bestowed with the task of eliminating superoxide and hydrogen peroxide, two important ROS (11, 12, 21). Glutathione (GSH), a tripeptide, also plays a pivotal role in the detoxification of ROS (22). However, to be effective, all these ROS disposal processes have to be regenerated with the aid of NADPH. This nicotinamide dinucleotide is the main power behind all antioxidative defense strategies, as it provides the reducing fuel necessary to recharge all effectors involved in combating ROS (32). Thus, various enzyme systems and metabolic networks that orchestrate the biogenesis of NADPH have to be activated if an organism is to acquire ATP via the reduction of oxygen (23). We have recently shown the crucial role played by NADK, an enzyme that mediates the formation of NADP, a key ingredient known to tilt cellular metabolism toward the synthesis of NADPH and away from the formation of NADH, a prooxidant (28). Hence, aerobic respiration, ROS production, and antioxidative defense strategies have to be intricately modulated.

Although the elimination of ROS is critical to the survival of all organisms, it is also important to appreciate the role that adaptative mechanisms play to lower the production of ROS. Our laboratory has recently identified an intriguing role of the tricarboxylic acid (TCA) cycle in this regard (15, 27). By modulating the production of NADH and NADPH, this metabolic network appears to be instrumental in striking the proper balance between the generation of ROS and the aerobic formation of ATP. As part of our study to delineate the molecular mechanisms that allow cellular systems to adapt to oxidative stress, we have identified how α-ketoglutarate dehydrogenase (KGDH) and glutamate dehydrogenase (GDH) play a critical role in modulating α-ketoglutarate (KG) homeostasis in *Pseudomonas fluorescens* challenged with menadione. This keto acid can readily nullify these ROS with the concomitant formation of succinate, a moiety that may signal anaerobic metabolism. The roles of KGDH in sensing ROS and limiting NADH production are also discussed.

MATERIALS AND METHODS

Microbial growth conditions and cellular fractionation. *Pseudomonas fluorescens* (ATCC 13525) was grown in a mineral medium containing 20 mM citrate as the sole carbon source, as described previously (28). Media were supple-

* Corresponding author. Mailing address: Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada. Phone: (705) 675-1151, ext. 2112. Fax: (705) 675-4844. E-mail: vappanna@laurentian.ca.

▽ Published ahead of print on 17 April 2009.

mented with various nitrogen sources (15 mM NH_4, Glu, Arg, or Pro) in order to study the contribution of nitrogen metabolism toward KG production. Prior to inoculation, the media were dispensed in 200-ml aliquots and autoclaved. Menadione (100 μM) was added to the media following sterilization. Cells from citrate (control) and menadione-containing cultures were isolated at similar growth phases for analysis (25 h for control cells and 30 h for menadione-stressed cells). Bacterial cells were harvested and suspended in a cell storage buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride [pH 7.3]), as described previously (28). Cells were disrupted by sonication and subjected to centrifugation at 3,000 × *g* to remove any intact cells. The cell extract (CFE) was then centrifuged for 3 h at 180,000 × *g* to afford a soluble CFE fraction and a membranous CFE fraction. The purity of the fractions was verified by monitoring glucose-6-phosphate dehydrogenase and complex I activity in the soluble and membrane CFE, respectively. The protein contents of both fractions were determined using the Bradford assay (5). Bovine serum albumin served as the protein standard.

Regulation and time profile experiments. Regulation experiments were performed as described previously (28). Briefly, 10 mg of protein equivalent from menadione-stressed cells was transferred to control media, and 10 mg of protein equivalent from control cells was transferred to menadione-supplemented (100 μM) media. Following an 8-h incubation, cells were harvested, and the cellular fractions were isolated and assayed for enzymatic activities. To afford a proper comparison, control cells grown for 25 h and menadione cells grown for 30 h were used in the regulation experiments.

Time profile experiments were carried out as described by Singh et al. (27). Cells were grown in control or menadione-containing medium supplemented with Glu and isolated at various time intervals. Cells were then treated accordingly for enzymatic activity and expression.

Measurement of cellular KG and succinate levels. Levels of KG and succinate were assessed by high-performance liquid chromatography (HPLC). The soluble fraction was diluted to 2 mg/ml in double-distilled water and then treated with 200 μl of 0.5% (vol/vol) perchloric acid. Following the removal of the precipitate, the supernatant was injected into an Alliance HPLC (Waters) equipped with a C_{18} reverse-phase column (4-μm Synergi Hydro-RP column [250 by 4.6 mm]; Phenomenex) operating at a flow rate of 0.7 ml/min (16). KG and succinate were detected using a dual wavelength absorbance detector operating at 210 nm for organic acids. The mobile phase used was 20 mM KH_2PO_4 (pH 2.9). Metabolites were identified by injecting known standards, and the peaks were quantified using Empower software (Waters Corporation). The HPLC was standardized using a five-point calibration prior to each injection protocol.

BN-PAGE and electroblotting. Blue native polyacrylamide gel electrophoresis (BN-PAGE) and in-gel activity staining were performed as described previously (17). Membrane proteins were prepared in a native buffer (50 mM bis-Tris, 500 mM ε-aminocaproic acid [pH 7.0]) containing 1% maltoside at a final concentration of 4 mg/ml. The in-gel activities of KGDH and GDH were tested using phenazine methosulfate and iodonitrotetrazolium chloride, as described by Singh et al. (28). KGDH was visualized using the reaction buffer containing KG, NAD, and coenzyme A (15). GDH was made apparent using glutamate and NAD (28). The reactions were quenched using a destaining solution (40% methanol, 10% acetic acid) once the bands reached their desired intensity. Band intensity was assessed using Scion imaging software. All values were expressed as the percent intensity of the control. The specificity of the activity bands was confirmed by running known standards and by performing the reactions in the absence of substrates. Proper loading was assured by Coomassie staining for total proteins. Bovine serum albumin was used as a molecular mass marker. The oxidation state of the lipoic acid residue in KGDH was tested by incubating 60 μg of protein equivalent to the membrane fraction prepared for BN-PAGE in 5 mM dithiothreitol (DTT) for 24 h. Incubation was performed at 4°C to avoid protein denaturation. Following incubation, the in-gel activity of KGDH was probed.

Sodium dodecyl sulfate-PAGE and immunoblotting were performed as previously described (10, 16, 27). Upon completion of electroblotting, the nonspecific binding sites on the membrane were blocked using 5% (wt/vol) nonfat skim milk in TTBS (20 mM Tris-HCl, 0.8% NaCl, 1% Tween 20 [pH 7.6]) for 1 h. Polyclonal antibodies directed against GDH (Abcam, Cambridge, MA) were used to probe the membrane. The secondary antibody consisted of an infrared-tagged rabbit anti-mouse antibody (LI-COR Biosciences, NE). Detection and quantification of the desired protein were achieved using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, NE). Band specificity was assured by loading a GDH standard (Sigma).

In vitro H_2O_2 scavenging by KG. Analysis of H_2O_2 consumption by KG was determined using the *p*-anisidine assay, as previously described (33). KG (1 to 3 mM) was incubated in a reaction buffer (25 mM Tris, 5 mM $MgCl_2$ [pH 7.4]) containing 0.5 mM H_2O_2 and *p*-anisidine for 1 h. Reactions were monitored at 458 nm ($\varepsilon = 1.173\ M^{-1}\ cm^{-1}$). The in vitro sequestration of H_2O_2 by KG was confirmed using ^{13}C nuclear magnetic resonance (^{13}C-NMR). Following 24 h of incubation of 10 mM KG with 10 mM H_2O_2, the ^{13}C spectra were recorded. Reactions performed with 10 units of catalase served as the control. D_2O was utilized as the solvent. Analyses were performed using a Varian Gemini 2000 spectrometer, operating at 50.38 MHz for ^{13}C. Experiments were executed with a 5-mm dual probe (35-degree pulse, 1-s relaxation delay, 8 kilobytes of data, and 2,000 scans). Chemical shifts were referenced to KG and succinate standards treated under the same conditions.

Susceptibility of cells to oxidative stress: measurement of oxidized lipids and proteins. To evaluate the ability of adapted *P. fluorescens* cells to tolerate oxidative stress, the microbe was grown in Glu and NH_4, as the nitrogen source, in the presence or absence (control) of menadione. Following the attainment of stationary growth phase, the spent media were removed and supplemented with fresh media containing 500 μM H_2O_2 but devoid of any nitrogen source. After 4 h of incubation, the cells were harvested and analyzed for oxidized lipids and proteins. The thiobarbituric acid reactive species assay was performed to assess the level of oxidized lipids in the membrane, as previously described (3). To quantify oxidized protein levels, protein carbonyl content was measured with the dinitrophenyl hydrazine assay (8).

Statistical analysis. Data were expressed as means ± standard deviations (SD). Statistical correlations of data were checked for significance using the Student *t* test. All experiments were performed twice and in triplicate.

RESULTS

Amino acids are a source of KG formation in menadione-treated cells. HPLC studies revealed that the cells subjected to menadione stress had more KG and succinate than the control cells (Fig. 1). However, these two metabolites were found at higher levels in the cells grown in media containing amino acids than in those grown with NH_4 as the nitrogen source. Indeed, the menadione-treated cells grown with Arg or Glu had severalfold more KG and succinate than the controls. The accumulation of KG in the menadione-treated cells prompted us to test the activity of KGDH. Menadione-exposed cells grown in media containing NH_4, Glu, Arg, or Pro displayed a marked decrease in KGDH activity (Fig. 2A). Regulation experiments confirmed the observed decrease in KGDH activity. KGDH activity was diminished in control cells exposed to menadione for 8 h (Fig. 2B). In contrast, the exposure of the menadione-treated cells to control conditions for 8 h recovered KGDH activity.

To probe the contribution of amino acid metabolism to KG production, the activity of GDH was tested. GDH activity was diminished in menadione cells grown in NH_4. In contrast, the activity of GDH was enhanced in the menadione-exposed cells grown in Glu, Arg, and Pro, amino acids which are catabolized to KG (Fig. 3A). The increase in GDH activity in the amino acid-supplemented cultures containing menadione was further investigated at various growth intervals. A steady increase in GDH activity was observed in the Glu medium exposed to oxidative stress (Fig. 3B). This phenomenon was associated with the increased level of the enzyme (Fig. 3C). To further probe the influence of the nitrogen source on GDH in the menadione-exposed cells, regulation experiments were performed. The exposure of control cells grown in NH_4 to menadione for 8 h diminished GDH activity (Fig. 3D). In comparison, the trend was reversed when menadione-treated cells in NH_4 media were exposed to control growth conditions (i.e., GDH activity was increased). However, when control cells grown in Glu were exposed to menadione for 8 h, a reverse trend was observed. Exposure of control cells to menadione

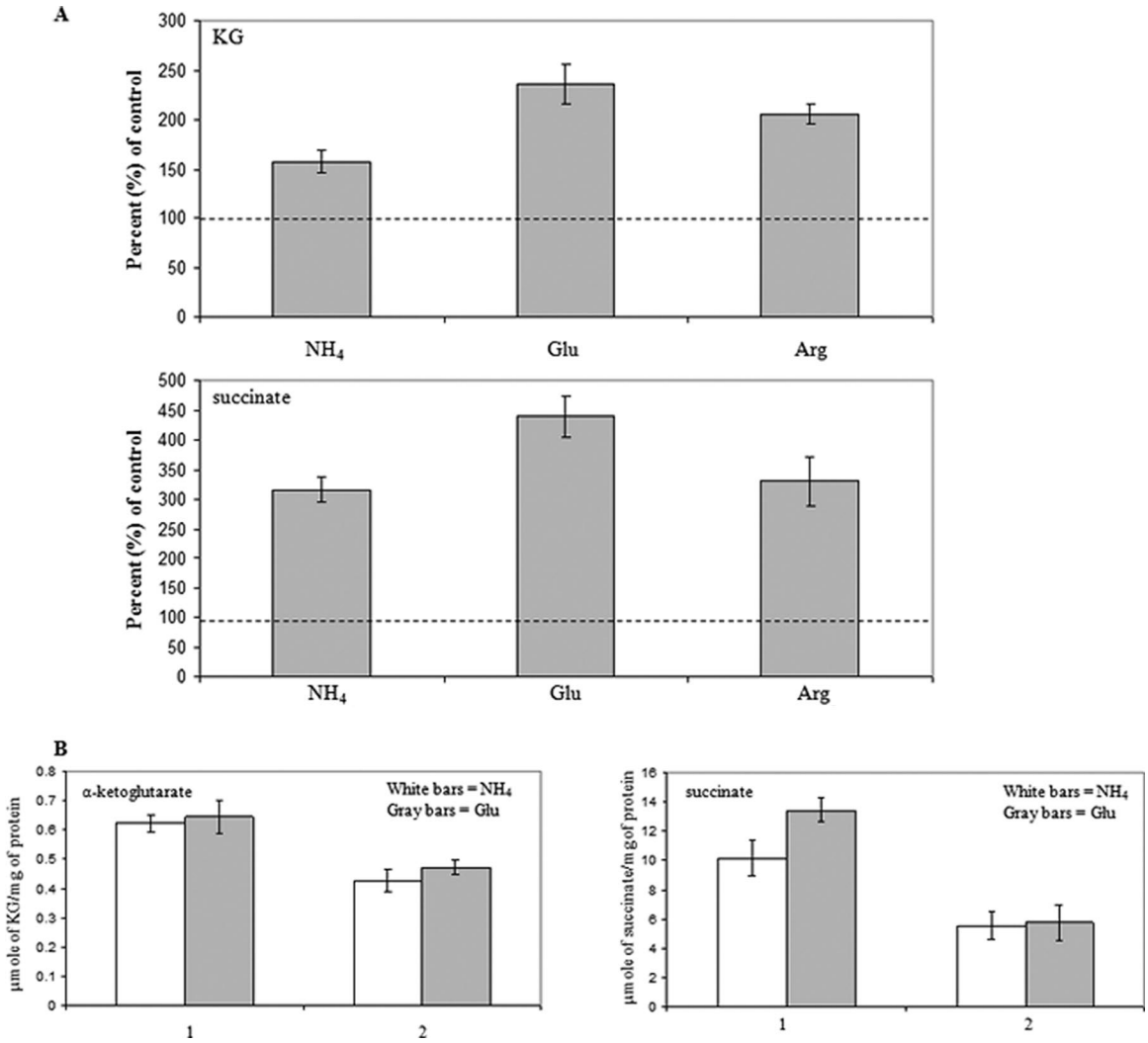

FIG. 1. (A) HPLC analysis of the cellular levels of KG and succinate in *P. fluorescens* exposed to control and menadione-stressed conditions. Following growth in media containing NH_4, Glu, or Arg, a 2-mg/ml equivalent of CFE was treated with 0.5% perchloric acid and then injected into the HPLC. The levels of KG and succinate were estimated using Empower software. Values of 100% for KG in controls were considered to be 0.461 ± 0.046, 0.418 ± 0.039, and 0.465 ± 0.051 μmol of KG/mg of protein equivalent of cells grown in NH_4, Glu, and Arg, respectively. Values of 100% for succinate in controls were considered to be 2.35 ± 0.45, 3.15 ± 0.32, and 4.01 ± 0.89 μmol of succinate/mg of protein equivalent of cells grown in NH_4, Glu, and Arg, respectively ($n = 3$, $P \leq 0.05$, means ± SD). Cells were grown for 25 h under control conditions and 30 h under menadione conditions. (B) Antioxidative role of KG and the redox state of KGDH. Cells were grown in media containing either NH_4 or Glu as nitrogen sources. Cellular levels of KG and succinate are modulated by menadione. The HPLC detection of KG and succinate levels in control cells incubated in menadione-containing medium for 8 h (1) and menadione-stressed cells incubated in control medium for 8 h (2). Cells were grown in media containing either NH_4 (white bars) or Glu (gray bars). Levels of KG and succinate were estimated using Empower software and by injecting known standards ($n = 3$, $P \leq 0.05$, means ± SD).

enhanced GDH activity, while the incubation of menadione-exposed cells in a control medium reduced GDH activity (Fig. 3D).

Redox state of KGDH is central to the antioxidative role of the TCA cycle. The activity of KGDH is dependent on a lipoic acid residue (26). ROS are known to oxidize the thiol groups in KGDH, leading to the inactivation of this enzyme complex. However, the role of KGDH in sensing oxidative stress and prompting antioxidant defense has never been shown. Menadione-exposed cells incubated in a control medium contained smaller amounts of KG than control cells exposed to menadione. If oxidative stress inhibits KGDH by deactivating the lipoic acid residue, then a reducing agent like DTT would potentially help restore this moiety. DTT is an agent commonly utilized to maintain the reduced state of thiol residues in cellular systems. Incubation of membrane extracts from the menadione-treated cells in 5 mM DTT partially restored the activity of KGDH (Fig. 3E). Treatment with DTT led to a significant recovery of the activity of KGDH in the menadione-exposed cells in either Glu or NH_4. We have previously demonstrated that under oxidative stress, the expression of the E_2 subunit of KGDH, a lipoic-acid rich moiety, is decreased (15). To further establish that the cells exposed to menadione can tolerate oxidative stress more readily while the control cells are

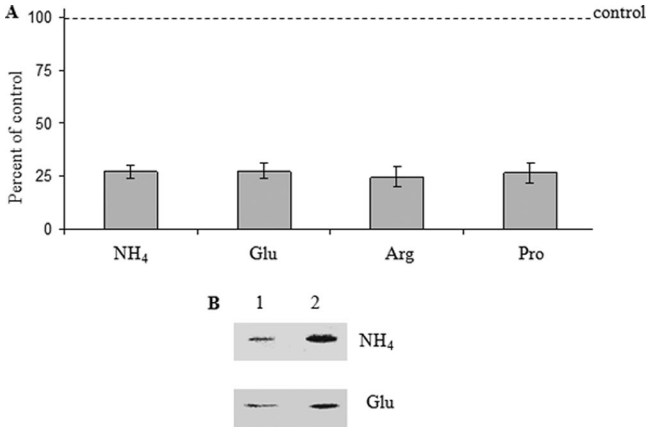

FIG. 2. (A) In-gel detection of KGDH in control and menadione-treated cells grown in NH_4, Glu, Arg, and Pro as nitrogen sources. Cells were grown for 25 h under control conditions and 30 h under menadione conditions. Bands were quantified using Scion imaging software. Values of 100% in control cells were considered to be 10,136, 9,947, 10,442, and 9,996 arbitrary units for control cells grown in NH_4, Glu, Arg, and Pro, respectively ($n = 3$, $P \leq 0.05$, means ± SD). (B) Regulation of KGDH activity by ROS. In-gel detection of KGDH in control cells exposed to menadione for 8 h (lane 1) and in menadione-treated cells exposed to the control for 8 h (lane 2). Control and menadione-treated cells were grown for 25 h and 30 h, respectively, prior to the regulation experiment.

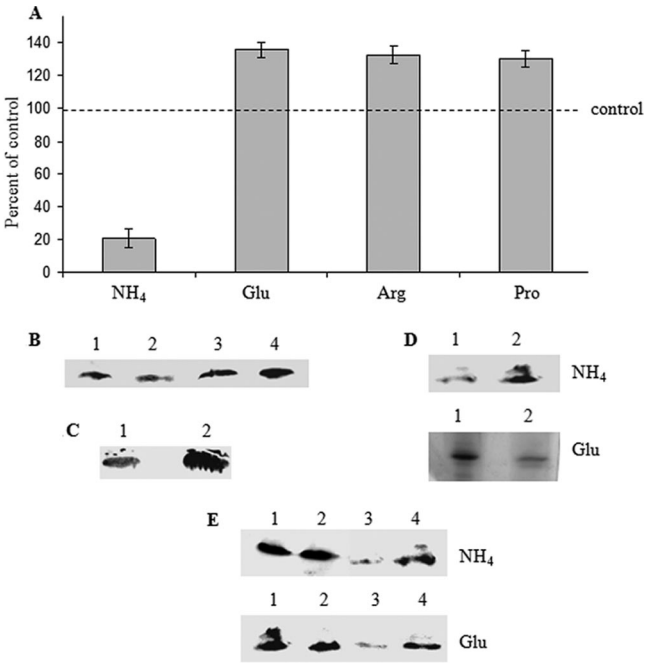

FIG. 3. (A) In-gel detection of GDH in control and menadione-treated cells grown with NH_4, Glu, Arg, and Pro as nitrogen sources. Cells were grown for 25 h under control conditions and 30 h under menadione conditions. Bands were quantified using Scion imaging software. Values of 100% in control cells were taken as 7,071, 7,345, 7,134, and 6,875 arbitrary units for control cells grown in NH_4, Glu, Arg, and Pro, respectively ($n = 3$, $P \leq 0.05$, means ± SD). (B) Time profile analysis of GDH activity in *P. fluorescens* grown in control and menadione media containing Glu. In-gel activity was assessed following the isolation of cells at different time intervals. Lane 1, 15-h control; lane 2, 24-h control; lane 3, 25-h menadione-treated cells; and lane 4, 30-h menadione-treated cells. (C) Immunoblot analysis of GDH levels in the control (lane 1) and menadione-stressed cells (lane 2) grown in a medium containing Glu. (D) Regulation of GDH activity by menadione. In-gel detection of GDH in control cells exposed to menadione for 8 h (lane 1) and in menadione-treated cells exposed to the control for 8 h (lane 2). Control and menadione-treated cells were grown for 25 h and 30 h, respectively, prior to the regulation experiment, unless indicated otherwise. (E) The activity of KGDH in the menadione-treated cells is restored by DTT. In-gel activity of KGDH in membrane CFE from control cells (lane 1), membrane CFE from control cells incubated in 5 mM DTT (lane 2), membrane CFE from menadione-treated cells (lane 3), and membrane CFE from menadione-treated cells (lane 4) incubated in 5 mM DTT.

more susceptible to oxidative insult, the control and menadione-stressed cells were incubated in 500 μM H_2O_2 for 4 h. There was a six- to sevenfold increase in oxidized lipids in the control cells exposed to the 500 μM H_2O_2, while lower levels of oxidized lipids were evident in the stressed cells (Fig. 4A). A similar trend was observed with oxidized protein levels (data not shown).

KG scavenges ROS in vitro. To confirm that KG is capable of scavenging ROS, in vitro studies were performed. When KG was incubated with H_2O_2, this ROS was readily consumed (Fig. 4B). The sequestration of H_2O_2 by KG was confirmed by ^{13}C-NMR (Fig. 4C). KG was nonenzymatically decarboxylated to succinate in the presence of H_2O_2. This process was sharply inhibited in the presence of catalase.

DISCUSSION

The data presented in this report point to the importance of KG and KGDH in the detoxification of ROS in *Pseudomonas fluorescens*. KGDH and GDH work in tandem to modulate the concentration of this keto acid. This strategy to eliminate ROS appears to be very effective, as it does not necessitate the direct or indirect utilization of NADPH, a moiety that is known to power most if not all of the detoxification stratagems involved in combating oxidative stress (29). The present approach in nullifying ROS may be more advantageous than the utilization of GSH. This tripeptide has to be regenerated, a process that is mediated by NADPH and GSH reductase (13). GSH synthesis requires three amino acids and ATP, factors which may become limiting during oxidative stress. Furthermore, unlike KG, GSH is metabolized only via a few pathways. It appears to be involved only in dedicated functions, including the diminution of oxidative moieties (2).

On the other hand, KG is known to contribute to a variety of metabolic processes, including the TCA cycle, the biogenesis of numerous amino acids, carnitine biosynthesis, and as a cofactor in numerous dioxygenases (4, 9, 31). The latter function may have an important sensing role. Hence, it can be readily diverted to cellular functions that are in high demand at a given time. In this instance, combating ROS may be the priority. Indeed, the ability of the cell to channel moiety toward ROS detoxification may signal an ineffective oxidative metabolism and may promote anaerobiosis. In fact, the activity of HIF-1α, a transcription factor known for its O_2-sensing attribute, is hydroxylated with the aid of KG (14, 18). The hydroxylated HIF-1α is subsequently earmarked for degradation. However, when the level of KG is insufficient, HIF-1α is in its unhydroxylated forms and helps with the activation of the

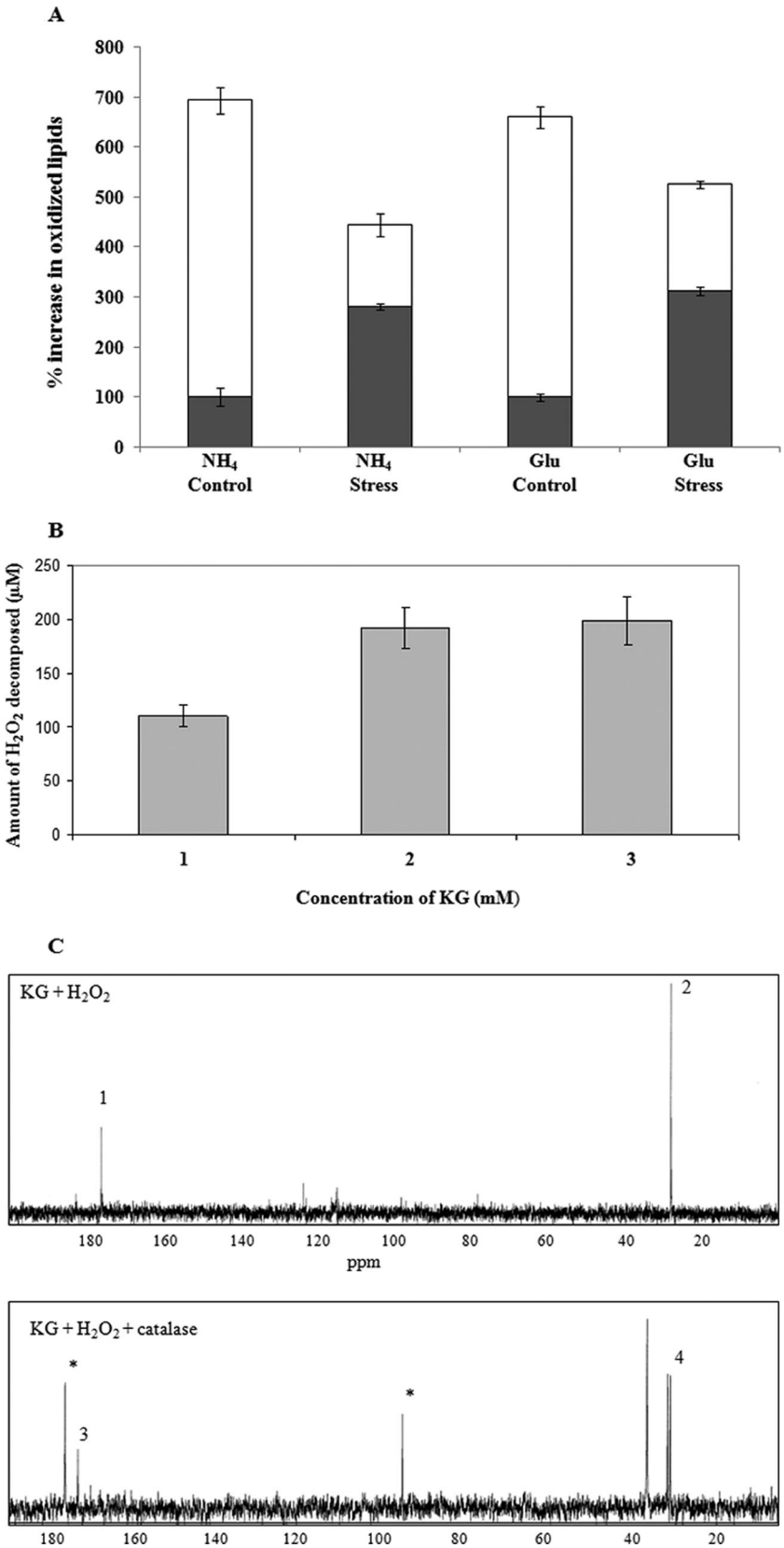

FIG. 4. (A) Susceptibility of control and menadione-stressed *P. fluorescens* cells to oxidative insult. Control and menadione-stressed cells were grown in either Glu or NH_4 medium and harvested at the same growth phase. The cells were then exposed to 500 μM H_2O_2 for 4 h, and oxidized lipids were measured as malondialdehyde equivalents at time zero (prior to introduction of H_2O_2) and after exposure. Values are given as percent

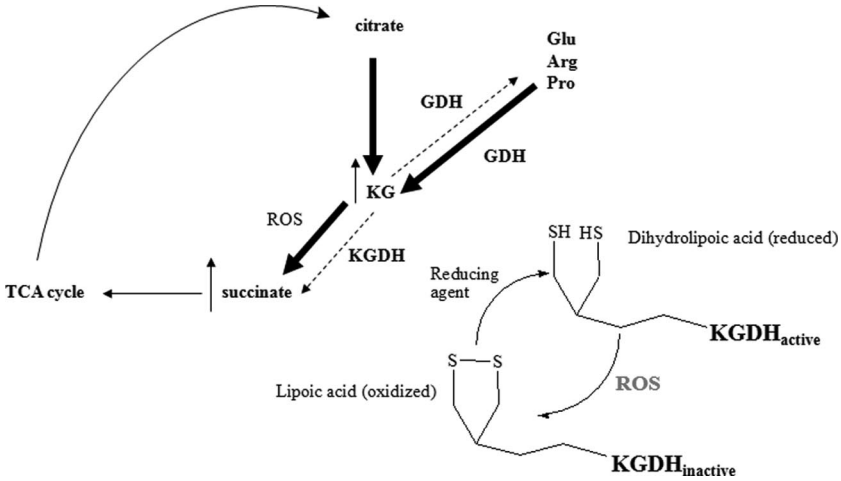

FIG. 5. KG homeostasis and the sensing role of lipoic acid. While KGDH is deactivated by ROS, the activity of GDH is modulated to ensure KG is supplied for ROS scavenging. Thick arrows represent increases, and dashed arrows represent decreases.

anaerobic program, a process that apparently does contribute to the reduction of the ROS burden of a cell. Hence, it is within the realm of possibilities that KG is predominantly utilized in the detoxification of ROS during oxidative stress (7, 19, 25). The elevated levels of KG and succinate in the cells exposed to menadione would point to such a possibility. KG may have a dual role of directly eliminating ROS and diminishing ROS production by promoting anaerobiosis. The in vitro studies clearly demonstrate the ability of KG to eliminate H_2O_2. We have recently demonstrated the reversal of anaerobiosis in hepatocytes by KG (18).

KGDH is a multienzyme complex consisting of the following three components: KGDH (E_1), dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3). It is located at a critical junction in the TCA cycle, as it is an important link between carbohydrate and protein metabolisms. In addition, its ability to generate NADH makes it a major participant in aerobic metabolism. The NADH is eventually oxidized via the electron transport chain which produces ATP. It has been postulated that this decarboxylation of KG and the formation of NADH contribute to the oxidative burden of the cell. Hence, the downregulation of KGDH would help alleviate oxidative stress. Thus, in this study, as the organism was challenged with oxidative stress, it is not surprising that this enzyme was markedly decreased. This situation would create a pool of KG that may be utilized to scavenge the ROS. KGDH may be an important tool against ROS. Its inactivation would lead to diminished production of NADH, a prooxidant, and would supply KG for the neutralization of ROS. It is also tempting to postulate that lipoic acid, an important component of the E_2 subunit, can be utilized as a ROS sensor. Hence, the oxidation of the sulfdryl group in lipoic acid may signal oxidative stress, a situation that would inactivate KGDH. This phenomenon would divert KG toward ROS detoxification, with the concomitant reduction in ROS formation. It has been shown that lipoic acid is oxidized under oxidative stress (30). In this study, it appears that lipoic acid was oxidized during menadione treatment. This situation was partially reversed by treatment with DTT. Hence, KGDH appears to be pivotal in combating and sensing oxidative stress. This important component of the TCA cycle and other enzymes of this crucial metabolic network appear to be involved in combating a variety of other stressful situations (6, 20).

To further establish the significance of KG in ROS detoxification, amino acids that are readily metabolized to KG with the aid of GDH were utilized. It is evident that GDH was more active in the menadione-exposed cells grown in either Glu, Arg, or Pro. This signifies that there is a dedicated effort by the cells to generate KG. On the other hand, when NH_4 was the source of nitrogen, the GDH activity in the stressed cells was markedly diminished. The fixation of NH_4 would necessitate the utilization of KG, a feature that would divert this essential participant in ROS detoxification. Hence, the activity of GDH was markedly diminished in NH_4 medium subjected to oxidative stress. However, in the media where amino acids were the source of nitrogen, the expression of GDH was increased. Hence, it is quite likely that GDH and KGDH work in tandem to ensure that KG is diverted toward ROS detoxification during oxidative stress.

In conclusion, it appears that KG is an important tool utilized by *P. fluorescens* to combat oxidative stress. KGDH and GDH are instrumental in modulating this keto acid. This strat-

increase compared to control cultures (100% is 0.384 ± 0.07 nmol of malondialdehyde equivalent/mg of protein for NH_4 and 0.349 ± 0.02 nmol of malondialdehyde equivalent/mg of protein for Glu) Time zero, closed bars; 4-h exposure to H_2O_2, open bars. Values are means ± SD. $n = 3$; $P \leq 0.05$. (B) KG is utilized as an antioxidant in *P. fluorescens* exposed to menadione. A total of 1 to 3 mM KG was incubated in a reaction buffer for 1 h containing 0.5 mM H_2O_2 and *p*-anisidine. (C) Proton-decoupled ^{13}C-NMR spectra obtained from the incubation of KG with H_2O_2. Reactions performed in the presence of 10 units of catalase served as the control. (1) COO^- succinate; (2) CH_2 succinate; (3) COO^- KG; (4) CH_2 KG. ∗, unidentified peaks.

egy affords numerous advantages, as it is a non-NADPH consuming system and does generate a readily usable metabolite. Furthermore, the downregulation of KGDH limits the entry of KG into the TCA cycle, thus impeding the generation of ROS. GDH is an important participant, as it mediates the production of KG in media containing amino acids that readily generate this keto acid. Thus, KG, KGDH, and GDH appear to work in unison to mitigate oxidative stress (Fig. 5). Hence, this strategy may provide a more effective defense against ROS than other known molecular stratagems that are virtually restricted to the detoxification of ROS without significantly attenuating their formation.

ACKNOWLEDGMENT

This study was funded by a grant from the Northern Ontario Heritage Fund.

REFERENCES

1. **Adam-Vizi, V., and C. Chinopoulos.** 2006. Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol. Sci. **27:**639–645.
2. **Apel, K., and H. Hirt.** 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55:**373–399.
3. **Aydin, S., P. Yargicoglu, N. Derin, Y. Aliciguzel, I. Abidin, and A. Agar.** 2005. The effect of chronic restraint stress and sulfite on visual evoked potentials (VEPs): relation to lipid peroxidation. Food Chem. Toxicol. **43:**1093–1101.
4. **Bott, M.** 2007. Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. Trends Microbiol. **15:**417–425.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Denayer, S., S. Matthijs, and P. Cornelis.** 2006. Resistance to vanadium in *Pseudomonas fluorescens* ATCC 17400 caused by mutations in TCA cycle enzymes. FEMS Microbiol. Lett. **264:**59–64.
7. **Fedotcheva, N. I., A. P. Sokolov, and M. N. Kondrashova.** 2006. Nonezymatic formation of succinate in mitochondria under oxidative stress. Free Radic. Biol. Med. **41:**56–64.
8. **Frank, J., A. Pompella, and H. K. Biesalski.** 2000. Histochemical visualization of oxidant stress. Free Radic. Biol. Med. **29:**1096–1105.
9. **Koivunen, P., M. Hirsila, A. M. Remes, I. E. Hassinen, K. I. Kivirikko, and J. Myllyharju.** 2007. Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. J. Biol. Chem. **282:**4524–4532.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
11. **Latifi, A., M. Ruiz, and C. C. Zhang.** 2009. Oxidative stress in cyanobacteria. FEMS Microbiol. Rev. **33:**258–278.
12. **Limauro, D., E. Pedone, L. Pirone, and S. Bartolucci.** 2006. Identification and characterization of 1-Cys peroxiredoxin from *Sulfolobus solfataricus* and its involvement in the response to oxidative stress. FEBS J. **273:**721–731.
13. **Lu, S. C.** 1999. Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J. **13:**1169–1183.
14. **MacKenzie, E. D., M. A. Selak, D. A. Tennant, L. J. Payne, S. Crosby, C. M. Frederiksen, D. G. Watson, and E. Gottlieb.** 2007. Cell-permeating α-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. Mol. Cell. Biol. **27:**3282–3289.
15. **Mailloux, R. J., R. Beriault, J. Lemire, R. Singh, D. R. Chenier, R. D. Hamel, and V. D. Appanna.** 2007. The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. PLoS One **2:**e690.
16. **Mailloux, R. J., R. Hamel, and V. D. Appanna.** 2006. Aluminum toxicity elicits a dysfunctional TCA cycle and succinate accumulation in hepatocytes. J. Biochem. Mol. Toxicol. **20:**198–208.
17. **Mailloux, R. J., J. Lemire, S. Kalyuzhnyi, and V. Appanna.** 2008. A novel metabolic network leads to enhanced citrate biogenesis in *Pseudomonas fluorescens* exposed to aluminum toxicity. Extremophiles **12:**451–459.
18. **Mailloux, R. J., S. Puiseux-Dao, and V. D. Appanna.** 2009. Alpha-ketoglutarate abrogates the nuclear localization of HIF-1alpha in aluminum-exposed hepatocytes. Biochimie **91:**408–415.
19. **Mallet, R. T., and J. Sun.** 2003. Antioxidant properties of myocardial fuels. Mol. Cell. Biochem. **253:**103–111.
20. **Middaugh, J., R. Hamel, G. Jean-Baptiste, R. Beriault, D. Chenier, and V. D. Appanna.** 2005. Aluminum triggers decreased aconitase activity via Fe-S cluster disruption and the overexpression of isocitrate dehydrogenase and isocitrate lyase: a metabolic network mediating cellular survival. J. Biol. Chem. **280:**3159–3165.
21. **Mongkolsuk, S., and J. D. Helmann.** 2002. Regulation of inducible peroxide stress responses. Mol. Microbiol. **45:**9–15.
22. **Perelman, A., A. Uzan, D. Hacohen, and R. Schwarz.** 2003. Oxidative stress in *Synechococcus* sp. strain PCC 7942: various mechanisms for H_2O_2 detoxification with different physiological roles. J. Bacteriol. **185:**3654–3660.
23. **Pollak, N., C. Dolle, and M. Ziegler.** 2007. The power to reduce: pyridine nucleotides—small molecules with a multitude of functions. Biochem. J. **402:**205–218.
24. **Sanz, A., R. Pamplona, and G. Barja.** 2006. Is the mitochondrial free radical theory of aging intact? Antioxid. Redox Signal. **8:**582–599.
25. **Satpute, R. M., J. Hariharakrishnan, and R. Bhattacharya.** 2008. Alpha-ketoglutarate and N-acetyl cysteine protect PC12 cells from cyanide-induced cytotoxicity and altered energy metabolism. Neurotoxicology **29:**170–178.
26. **Shi, Q., H. Xu, W. A. Kleinman, and G. E. Gibson.** 2008. Novel functions of the alpha-ketoglutarate dehydrogenase complex may mediate diverse oxidant-induced changes in mitochondrial enzymes associated with Alzheimer's disease. Biochim. Biophys. Acta **1782:**229–238.
27. **Singh, R., J. Lemire, R. J. Mailloux, and V. D. Appanna.** 2008. A novel strategy involved anti-oxidative defense: the conversion of NADH into NADPH by a metabolic network. PLoS One **3:**e2682.
28. **Singh, R., R. J. Mailloux, S. Puiseux-Dao, and V. D. Appanna.** 2007. Oxidative stress evokes a metabolic adaptation that favors increased NADPH synthesis and decreased NADH production in *Pseudomonas fluorescens*. J. Bacteriol. **189:**6665–6675.
29. **Temple, M. D., G. G. Perrone, and I. W. Dawes.** 2005. Complex cellular responses to reactive oxygen species. Trends Cell Biol. **15:**319–326.
30. **Tretter, L., and V. Adam-Vizi.** 2005. Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. Philos. Trans. R. Soc. Lond. B **360:**2335–2345.
31. **Vaz, F. M., and R. J. Wanders.** 2002. Carnitine biosynthesis in mammals. Biochem. J. **361:**417–429.
32. **Ying, W.** 2008. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid. Redox Signal. **10:**179–206.
33. **Yumoto, I., D. Ichihashi, H. Iwata, A. Istokovics, N. Ichise, H. Matsuyama, H. Okuyama, and K. Kawasaki.** 2000. Purification and characterization of a catalase from the facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1^T exhibiting high catalase activity. J. Bacteriol. **182:**1903–1909.
34. **Zuin, A., N. Gabrielli, I. A. Calvo, S. Garcia-Santamarina, K. L. Hoe, D. U. Kim, H. O. Park, J. Hayles, J. Ayte, and E. Hidalgo.** 2008. Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. PLoS One **3:**e2842.