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NON-PYRIDINE NUCLEOTIDE DEPENDENT L-(+)-GLUTAMATE OXIDOREDUCTASE IN *AZOTOBACTER VINELANDII*

PETER JURTSHUK and LINDA McMANUS

Department of Biology, University of Houston, Houston, Texas 77004 (U.S.A.)

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SUMMARY

L-(+)-Glutamate oxidation that is non-pyridine nucleotide dependent is readily carried out by a membrane-bound enzyme in *Azotobacter vinelandii* strain O. Enzyme activity concentrates in a membranous fraction that is associated with the *Azotobacter* electron transport system. This L-glutamate oxidation is not dependent on externally added NAD^+, $NADP^+$, FAD, or FMN for activity. O_2, phenazine methosulfate and ferricyanide all served as relatively good electron acceptors for this reaction; while cytochrome *c* and nitrotetrazolium blue function poorly in this capacity. Paper chromatographic analyses revealed that the 2,4-dinitrophenylhydrazine derivative formed from the enzymatic oxidation of L-glutamate was α-ketoglutarate, while microdiffusion studies indicated that ammonia was also a key end product. These findings suggest that the overall reaction is an oxidative deamination. Ammonia formation was found to be stoichiometric with the amount of oxygen consumed (2 : 1 respectively, on a molar basis). The oxidation of glutamate was limited to the L-(+)-enantiomer indicating that this reaction is not the generalized type carried out by the L-amino acid oxidase. This oxidoreductase is functionally related to the *Azotobacter* electron transport system: (a) the activity concentrates almost exclusively in the electron transport fraction; (b) the L-glutamate oxidase activity is markedly sensitive to electron transport inhibitors, i.e. 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, cyanide, and 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione; and (c) spectral studies on the *Azotobacter* R_3 fraction revealed that a substantial amount of the flavoprotein (non-heme iron) and cytochrome (a_2, a_1, b_1, c_4 and c_5) are reduced by the addition of L-glutamate.

INTRODUCTION

In most biological systems, the oxidation of L-glutamic acid is usually carried out by the soluble and highly specific pyridine nucleotide dependent dehydrogenases which require NAD^+ and/or $NADP^+$. The end result of this reaction is the formation

Abbreviations: DCIP, dichlorophenolindophenol; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TTFB, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butadione; NBT, (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′dimethoxy-4,4′-biphenylene) ditetrazolium chloride); PMS, phenazine methosulfate.

of α-ketoglutarate and ammonia. The reverse reaction, or the reductive amination of α-ketoglutarate, occurs only in the presence of reduced pyridine nucleotide. The glutamate dehydrogenases characterized to date are: (a) the NAD^+-dependent L-glutamate dehydrogenase (EC 1.4.1.2) found in higher plants and most animal tissues; (b) the non-specific NAD^+- or $NADP^+$-dependent L-glutamate dehydrogenase (EC 1.4.1.3) found in most animal livers; and finally, (c) the L-glutamate dehydrogenase (EC 1.4.1.4) that requires only $NADP^+$ and is found in most yeasts, molds, and bacteria [1].

In some bacteria, an alternative biosynthesis of glutamate involves a new enzyme, the glutamine (amide): 2-oxoglutarate aminotransferase [2–10] or glutamate synthase. This $NADP^+$-dependent enzyme synthesizes glutamate by an amino transfer reaction from glutamine to α-ketoglutarate and, in *Escherichia coli* is an iron–sulfide flavoprotein [5, 9]. This novel enzyme system has only been found in bacteria and appears to synthesize glutamate when environmental ammonia levels are low. Recently, this enzyme was also characterized in the eukaryote, *Saccharomyces cerevisiae* [10], however, unlike the bacterial enzyme it required NAD^+ instead of $NADP^+$.

Glutamate can be oxidatively deaminated in the absence of pyridine nucleotides by another class of enzymes, the amino acid oxidases. These oxidases are relatively unspecific with regard to the type of amino acid oxidized, but do possess an absolute stereospecificity, oxidizing only the L-enantiomer or the D-enantiomer. This class of enzyme possess flavoprotein and use O_2 (as well as other artificial electron carriers) as electron acceptors. The L-amino acid oxidase (EC 1.4.3.2) is specific for the L-enantiomers and, depending upon its source, may require either FAD or FMN as a coenzyme; while the D-amino acid oxidase (EC 1.4.3.3) oxidizes only the D-enantiomer and FAD generally serves as the prosthetic group. L-Glutamate usually serves as a poor substrate for the L-amino acid oxidase [1].

In the study to be presented here, we will show that *Azotobacter vinelandii* possesses a new type of L-(+)-glutamate oxidoreductase. This enzyme is membrane-bound and does not require the addition of any pyridine nucleotides for activity. It appears to be a flavoprotein-type enzyme which carries out the oxidative deamination of L-glutamate to α-ketoglutarate and ammonia using O_2 as an electron acceptor. Furthermore, it will be shown that this enzyme is not a type of L-amino acid oxidase. The L-(+)-glutamate oxidase rate is sensitive to electron transport inhibitors and L-glutamate is capable of reducing the flavoprotein (non-heme iron) and cytochrome components in the *Azotobacter* electron transport chain. The enzyme is apparently unique in that, to date, it has not been listed by the Enzyme Commission in the I.U.B. classification of enzymes.

MATERIALS AND METHODS

Chemical methods

α-Ketoglutarate was identified as a major end product of L-(+)-glutamate oxidation by forming [11] and chromatographically characterizing the 2,4-dinitrophenylhydrazine derivative [12]. Ammonia was determined by the Conway microdiffusion technique [13] which employed Nesslerization [14] for quantitative colorimetric analyses.

Solutions of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), 4,4,4-trifluoro-

1-(2-thienyl)-1,3-butanedione (TTFB) and menadione (Vitamin K_3) were prepared in 95% ethanol while known concentrations of dichlorophenolindophenol (DCIP), $K_3Fe(CN)_6$, methylene blue, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (NBT), and KCN were prepared in deionized water. Enzymatically oxidized cytochrome *c* (10 mg/ml) was prepared in batch quantities and stored at −20 °C. The concentration of protein was determined by the biuret method of Gornall et al. [15].

Preparation of cell-free extracts

The preparation of the *Azotobacter* R_3 fraction and its characterization as an electron transport fraction has been described previously [16–20].

Enzyme assays

All assays were performed as previously described by Jurtshuk et al. [19] with the exception that L-(+)-glutamate was used as substrate rather than succinate; all manometric assays were performed at 30 °C rather than 37 °C. The final concentration of *Azotobacter* R_3 fraction used in the manometric assays ranged from 1.0 to 1.5 mg protein/ml.

Spectral studies

The difference spectrum of the *Azotobacter* R_3 electron transport fraction was carried out in a Beckman DK-2A recording spectrophotometer. The *Azotobacter* R_3 fraction was suspended in 0.02 M phosphate buffer (pH 7.5). Steady-state reduction was attained by adding L-(+)-glutamate (final concn of 0.25 M). Chemical reduction was achieved by adding a few crystals of dithionite to the sample cuvette. The final concentration of cyanide used in the spectral studies was 5 mM.

RESULTS

Stoichiometry of the reaction

The oxidation of L-(+)-glutamate by the *Azotobacter* R_3 fraction is carried out according to the reaction equation given in Fig. 1. Paper chromatographic analyses on the 2,4-dinitrophenylhydrazones verified that only one keto acid, i.e. α-ketoglutarate, was formed as the major end product of this reaction. Ammonia also was detected as a major end product. Assuming the reaction stoichiometry shown in Fig. 1, 2 μmoles of glutamate would be oxidized by 1 μmole of O_2 to form 2 μmoles each of α-ketoglutarate and ammonia. As shown in Table I, it was possible to establish this exact stoichiometric relationship. The amount of O_2 consumed was stoichiometric to the amount of ammonia liberated during L-glutamate oxidation (1 : 2 on a molar basis), confirming the reaction equation postulated in Fig. 1.

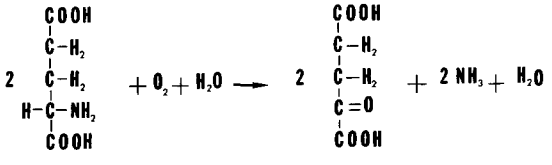

Fig. 1. Reaction equation.

TABLE I

REACTION STOICHIOMETRY CORRELATING AMMONIA PRODUCTION WITH O_2 CONSUMPTION DURING L-GLUTAMATE OXIDATION BY THE *AZOTOBACTER* R_3 ELECTRON TRANSPORT FRACTION

The final protein concentration of the *Azotobacter* R_3 fraction used in the assay was 1.43 mg/ml. In theory, it was assumed that 2 μmoles of NH_3 would be liberated when 2 μmoles of glutamate were oxidized by 1 μmole of O_2.

Reaction time (min)	O_2 consumed (μmoles)	NH_3 produced (μmoles)		Theoretical (%)
		Actual*	Theoretical	
0	0	0	0	0
30	1.9	3.5	3.8	92
60	3.6	6.7	7.2	93
90	4.5	9.2	9.0	102
120	5.5	11.1	11.0	101
180	6.8	13.8	13.6	101

* All values were corrected for the endogenous NH_3 formed.

Distribution of enzyme activity units for L-*glutamate oxidation in cell-free extracts*

A representative fractionation scheme showing the distribution of activity units for L-glutamate oxidation in *Azotobacter* cell-free fractions is shown in Table II. These cell-free extracts were obtained by differential centrifugation from turbidimetrically standardized resting cells of *A. vinelandii* that were disrupted by sonic oscillation [16–20]. The residue fractions are designated "R" and the supernatant fractions are designated "S". L-Glutamate oxidation by phenazine methosulfate (L-glutamate–PMS reductase) was employed for the assay and specific activities were determined for each of the fractions. The S_1 fraction was obtained after centrifugation of the "sonicated" homogenate (10 min at $4300 \times g$) which removed all unbroken

TABLE II

DISTRIBUTION OF ACTIVITY UNITS FOR L-(+)-GLUTAMATE OXIDATION IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF SONICALLY DISRUPTED RESTING CELLS OF *A. VINELANDII*

The spectrophotometric PMS-mediated DCIP reductase assay was used for this study. Spec. act. expressed as moles of DCIP reduced per min per mg protein at 25 °C.

Azotobacter fraction	Total protein (mg)	Protein recovery (%)	Spec. act.	Total activity units*	Total activity units (%)
S_1	701	100	0.016	11.2	100
S_2	528	75	0.013	6.9	62
R_2	77	11	0.025	1.9	17
S_2	528	100	0.013	6.9	100
S_3	284	54	0.002	0.6	9
R_3	106	20	0.044	4.7	68

* Total activity units are calculated by multiplying total protein content by spec. act.

cells and large cellular debris. The activity and protein concentration of this fraction was assumed to be 100 %. Centrifugation of the S_1 fraction at 37 000 $\times g$ for 20 min gave two fractions, the S_2 and R_2; and further centrifugation of the S_2 fraction at 144 000 $\times g$ for 2 h separated the S_3 from the R_3. In these studies, the highest specific activity (and total activity units) for L-glutamate oxidation were always found in the particulate R_3 fraction regardless of the assay employed. This study shows that a 2.8-fold purification was achieved by this differential centrifugation procedure and that all fractions that contained membrane fragments (all except the S_3 fraction) were active for L-glutamate oxidation. The S_3 fraction, which contained all the soluble enzymes (among which would be the pyridine-nucleotide-dependent L-glutamate dehydrogenase), exhibited little activity for L-glutamate oxidation, regardless of the electron acceptor used. The total recovery of enzyme activity units in the R_3 (from that of the S_1 fraction) was always approx. 33 % for the L-glutamate oxidase assay and 50–86 % for the L-glutamate phenazine methosulfate reductase assay.

Oxidation of other amino acids

The membrane-bound L-(+)-glutamate oxidoreductase in the *Azotobacter* R_3 fraction was examined for its ability to oxidize other amino acids. Table III shows the results of this study. The specific activity for L-(+)-glutamate oxidation is expressed

TABLE III

RELATIVE RATES OF AMINO ACID OXIDATION BY THE MEMBRANE-BOUND R_3 ELECTRON TRANSPORT FRACTION OF *A. VINELANDII*

The following amino acids were found to be completely inert to oxidative attack by the *Azotobacter* R_3 fraction: L-aspartate, L-arginine, DL-serine, L-tryptophane, hydroxy-L-lysine, DL-cystine, L-alanine, glycine, DL-valine, L-tyrosine, DL-isoleucine, DL-homocystine, L-α-amino-*n*-butyrate, L-histidine, DL-leucine and L-(+)-lysine.

Substrate (16.7 mM)	O_2	PMS
L-(+)-Glutamate	100*	100**
DL-Methionine	18	22
DL-Asparagine	15	17
DL-Phenylalanine	13	13
DL-α-Aminoadipate	11	2
L-Methionine	0	—
L-Asparagine	0	—
L-Phenylalanine	0	—
L-Proline	4.0	—
D-(−)-Glutamate	2.3	—
L-Ornithine	1.6	—
L-Glutamine	1.0	—
DL-Threonine	0.1	—

* L-(+)-Glutamate oxidase rate, non-pyridine nucleotide dependent whose spec. act. is arbitrarily assigned a value of 100 %.

** L-(+)-Glutamate phenazine methosulfate reductase (PMS) rate, non-pyridine nucleotide dependent, whose spec. act. is arbitrarily assigned a value of 100 %.

as 100 % and the activities obtained for the other amino acids are compared on a relative basis. As noted, the D-(−)-isomer of glutamate was relatively inactive, exhibiting only 2.3 % of the activity observed for the L-isomer. This small amount of activity could be due to the presence of contaminating amounts of the L-isomer. The oxidase activities noted for the DL-isomers of methionine, asparagine, and phenylalanine undoubtedly represent the oxidation of the D-isomers since the L-isomers were completely inactive. Activity rates observed for the oxidation of the L-isomers of proline, ornithine, glutamine, and DL-threonine were measurable by our assay system, but the rates obtained were very low when compared to the oxidation rate obtained for L-(+)-glutamate. Also shown in Table III are the PMS reductase rates for the more active amino acids. These values correspond to those given for the oxidase rates with the noted exception of DL-aminoadipate. This compound reacted chemically with PMS in the assay system.

These substrate specificity studies indicate that the L-(+)-glutamate oxidase (oxidoreductase) of the *Azotobacter* R_3 electron transport fraction is highly specific in its ability to oxidize the L-enantiomer of glutamate. Further, the R_3 fraction cannot appreciably oxidize any other L-amino acid to the extent noted for L-glutamate. This pattern of substrate specificity implies that the enzyme in the *Azotobacter* R_3 fraction is not similar to the known amino acid oxidases which have the ability to oxidize a variety of D- or L-amino acids many at fairly high rates. More will be made of this point in the Discussion.

Effects of NAD^+, $NADP^+$, FAD and FMN on L-glutamate oxidation

Since other known glutamate oxidizing enzymes use either NAD^+ and/or $NADP^+$ as functional components [1, 21, 22], a study was undertaken to determine the effects of these compounds on L-glutamate oxidation by the *Azotobacter* R_3 fraction. As shown in Fig. 2 neither externally added NAD^+, FAD, or FMN stimulated this oxidation rate, while $NADP^+$ was found to be slightly inhibitory. If a pyridine-nucleotide-dependent oxidoreductase was present in the R_3 fraction, a marked stimulation of O_2 consumption would have occurred since the *Azotobacter* R_3 fraction has a very active NADH and NADPH oxidase [16, 23]. In fact, a marked stimulation does occur when both NAD^+ and the NAD^+-dependent L-glutamate dehydrogenase are added to the L-glutamate oxidoreductase assay system [24].

Reduction of cytochrome and flavoprotein components in the Azotobacter R_3 fraction by L-glutamate

Steady-state reduction studies affecting the redox states of the membrane-bound components in the *Azotobacter* R_3 fraction are shown in Fig. 3. These were readily accomplished by the addition of L-glutamate, cyanide, and dithionite. The time-dependent reduction of the R_3 electron transport components by L-glutamate have been previously demonstrated [24]. Spectral changes occur only after 5 min incubation with L-glutamate. Only minor steady-state changes occurred in the R_3 fraction after the addition of cyanide (5 mM), which is a very potent inhibitor of the *Azotobacter* terminal oxidase reaction [16]. Significant oxidation–reduction changes resulting from cyanide addition occurred in the visible or 552–553-nm region while only minimal reductive changes are noted in the Soret or 428-nm region. The subsequent addition of L-glutamate to this same cuvette caused marked steady-state changes

particularly in the 561-nm region which would represent the cytochrome b_1 and o components and a significantly greater reduction in the Soret peak (428 nm). Finally, the maximum reduction for the cytochrome and flavoprotein (non-heme iron) components is achieved by the addition of $Na_2S_2O_4$. Similar type steady-state reduction changes have already been shown for the *Azotobacter* electron transport system with succinate [19], D-lactate [18], L-malate [17], and tetramethyl-*p*-phenylenediamine [19]. The components reduced in the R_3 fraction by L-glutamate were cytochrome a_1+a_2 (590 and 629 nm, respectively), cytochrome b_1 (561 and 531 nm) and cytochromes c_4+c_5 (553 and 523 nm, respectively). The bleaching represented by the trough at 457 nm is characteristic of flavoprotein and non-heme iron reduction.

Inhibition of the L-(+)-*glutamate oxidase*

Further data which strongly indicate that L-glutamate oxidation in *Azotobacter* involves electron transport function are the inhibitor studies. The effects of the various electron transport inhibitors on L-(+)-glutamate oxidase activity in the *Azotobacter* R_3 fraction are shown in Fig. 4. HQNO proved to be the most potent inhibitor of the L-(+)-glutamate oxidase activity, 50 and 100 % inhibition occurring at concentration levels of $1.6 \cdot 10^{-5}$ M and $1.0 \cdot 10^{-4}$ M, respectively. This inhibitor is known to inhibit mammalian electron transport oxidations somewhere between cytochrome

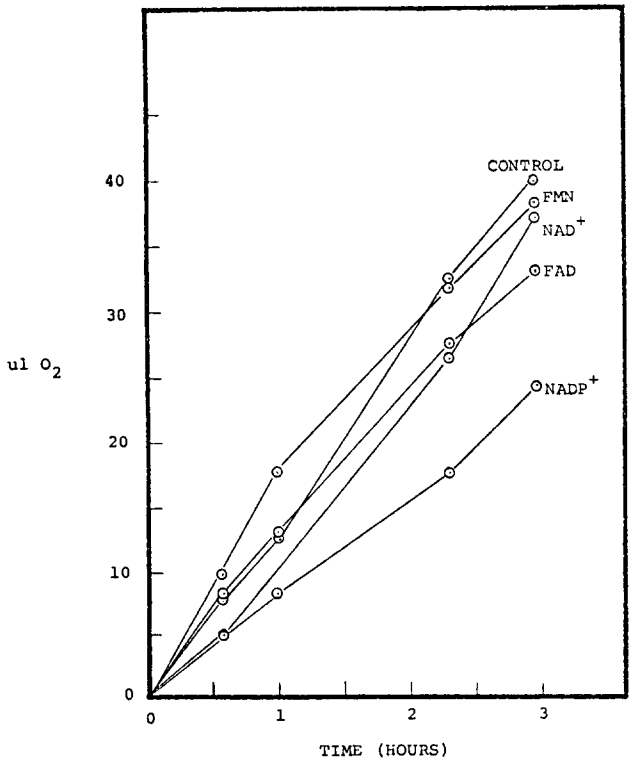

Fig. 2. The effect of the coenzymes FMN, NAD^+, FAD and $NADP^+$ on L-(+)-glutamate oxidase activity of the *Azotobacter* R_3 electron transport fraction. The final concentration of all coenzymes in the assay was 1.0 mM.

b and c_1 [25], and readily inhibits other known electron transport reaction in *A. vinelandii* [17–19]. Similar type inhibition patterns for the L-glutamate oxidase activity also was noted with cyanide and TTFB (Fig. 4). Cyanide is known to inhibit electron transport systems at the terminal cytochrome oxidase site while TTFB is an inhibitor which acts somewhere beyond the succinoxidase flavoprotein site [25]. An interesting aspect of this study was that TTFB inhibited the transport fraction at the same concentration level at which it inhibited D-lactate oxidase and succinoxidase activity [18, 19], suggesting that it might be a generalized inhibitor for the *Azotobacter* electron transport chain, somewhere beyond the flavoprotein site.

The relationship of the L-*glutamate oxidoreductase to other known membrane-bound electron transport enzymes*

L-(+)-Glutamate oxidation was compared to other known electron transport enzymes in the *Azotobacter* R_3 fraction [17–20] in its capability to use different artificial and natural electron acceptors. The data summarizing these studies are presented in Fig. 5 in which manometric assays were employed. As shown in Fig. 5A,

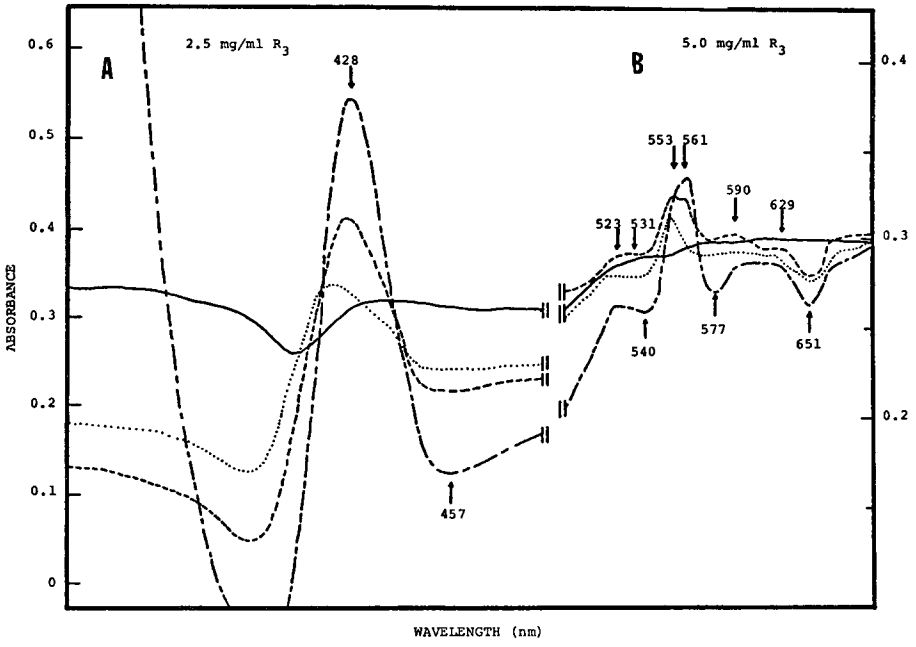

Fig. 3. Difference spectra (reduced minus oxidized) exhibiting steady state reduction changes in the oxidation-reduction components of the *Azotobacter* R_3 electron transport fraction. In A, the reduced minus oxidized changes are shown for the Soret region 400–470 mm using the R_3 fraction at concentration of 2.5 mg protein/ml. In B, the redox changes are shown for the visible region 500–700 mm for an R_3 protein concentration of 5.0 mg/ml. The straight line represents the control or oxidized minus oxidized R_3 fraction; the dotted line (· · ·) represents the changes caused by the addition of CN^- after 10 min incubation time (final concn 5.0 mM); the dashed line (- - -) represents the changes caused by the addition of L-(+)-glutamate after 10 min incubation time (final concn 0.25 M) or glutamate reduced minus oxidized; and finally, the broken line (– – –) which shows the changes result ing in the complete reduction of all the cytochrome, flavoprotein and non-heme iron components by the addition of a few crystals of sodium dithionite or a dithionite reduced minus oxidized spectrum.

PMS served as the best electron acceptor for L-glutamate oxidation giving rates comparable to that observed for succinate and D-lactate oxidation, although it was substantially less active than the high rate noted for the L-malate PMS reductase. Fig. 5B shows that O_2 can also serve as a good acceptor for L-glutamate oxidation, although the rate was much lower than that obtained when PMS was used as the acceptor. The L-glutamate oxidase rate was significantly lower than that observed for the oxidation of L-malate, succinate, and D-lactate. Methylene blue (Fig. 5C) can also serve as electron acceptor for L-glutamate oxidation, but the rate of reaction was very poor when compared to that observed for succinate, D-lactate, and L-malate. Vitamin K_3 (menadione) could not serve as an electron acceptor for either L-glutamate or succinate oxidation although the vitamin K_3 reductase rates for D-lactate and L-malate were substantial, confirming the findings previously published [17, 18].

The data presented in Fig. 5 indicate that the enzyme responsible for L-(+)-glutamate oxidation in the *Azotobacter* R_3 fraction is somewhat similar to the other known membrane-bound electron transport enzyme systems found there. It appears to utilize those electron acceptors that allow for succinate oxidation, and to a lesser extent, those acceptors involved in the oxidation of L-malate and D-lactate.

L-Glutamate oxidation in the *Azotobacter* R_3 fraction was examined also with various other artificial electron acceptors using spectrophotometric assays. The PMS-mediated L-glutamate DCIP reductase was found to yield the highest specific activity,

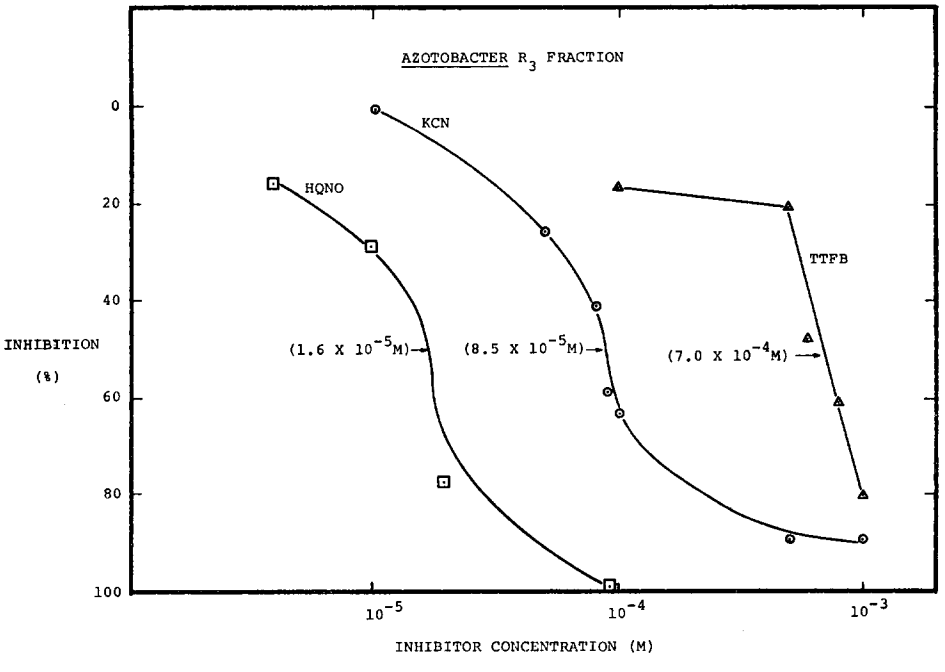

Fig. 4. The effect of various electron transport inhibitors on the L-(+)-glutamate oxidase rate using the *Azotobacter* R_3 electron transport fraction. All assays were performed manometrically, the inhibitor was added in the main compartment with the R_3 fraction; the reaction being initiated by the addition of L-glutamate after a 7-min incubation interval. The values in parentheses indicate the molar concentration at which 50 % inhibition occurred.

i.e., 0.056 mole L-(+)-glutamate oxidized $min^{-1} \cdot mg^{-1}$ protein at 25 °C. On a comparable 2 e^- basis, the specific activity for the L-glutamate $K_3Fe(CN)_6$ reductase was 0.030 and the activity for L-glutamate oxidation with DCIP as the sole acceptor was 0.011. NBT and cytochrome *c* served as the poorest acceptors and gave specific activities (on a 2 e^- basis) of 0.002 and 0.001, respectively.

DISCUSSION

Glutamate as an intracellular metabolite has long been known to be of primary

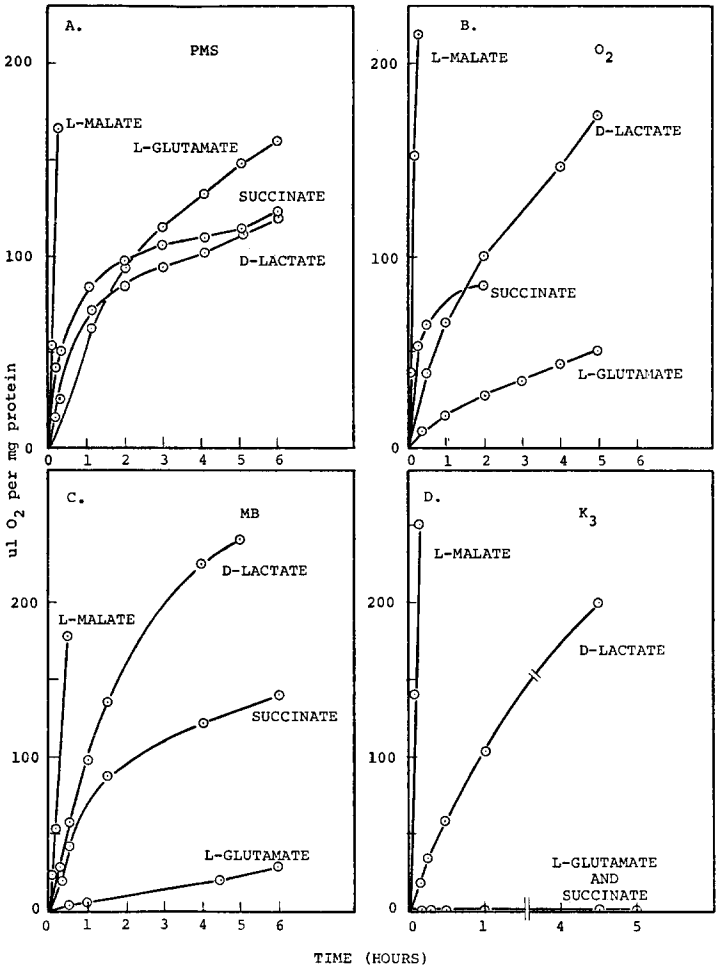

Fig. 5. Comparison of the rate of L-(+)-glutamate oxidation to the other known membrane-bound electron transport reactions carried out by the *Azotobacter* R_3 fraction. The electron acceptors used in this study were (A) PMS, (B) O_2, (C) methylene blue, and (D) vitamin K_3 or menadione. The other membrane-bound enzymes present in the *Azotobacter* R_3 fraction that were used for this study were the non-pyridine nucleotide/dependent L-malate and D-lactate oxidoreductases as well as the succinate oxidoreductase. Previous studies have shown that all of these activities concentrate almost exclusively in the *Azotobacter* R_3 electron transport fraction [17–20].

importance in *Azotobacter* metabolism. Physiologically, this genera of bacteria represent a unique class of free-living, nitrogen-fixing aerobes in which the pyridine-nucleotide-dependent L-glutamate dehydrogenases are considered to be important in the conversion of NH_3 (derived from nitrogen fixation) to L-glutamate via the reductive amination of α-ketoglutarate [26]. The reversal of this reaction is also readily carried out in which case the pyridine-nucleotide-dependent L-glutamate dehydrogenase deaminates L-glutamate forming α-ketoglutarate as well as reduced pyridine nucleotides. Studies on the purified mammalian L-glutamate dehydrogenase (EC 1.4.1.3), isolated from rat liver, indicate that the reductive amination of α-ketoglutarate occurs approx. 7 times faster than the forward reaction [27], i.e. the oxidative deamination of L-glutamate. Studies on the bacterial L-glutamate dehydrogenases suggest that the metabolic role of the non-regulated NAD^+-specific enzyme appears to be in generating α-ketoglutarate; while those organisms which possess a predominantly non-regulative $NADP^+$-specific enzyme appear to utilize the L-glutamate dehydrogenase for biosynthetic nitrogen incorporation [21].

In *Azotobacter* spp., however, the role of the pyridine-nucleotide-dependent L-glutamate dehydrogenase has yet to be experimentally established, although numerous studies [26, 28–31] indicate that the NH_3 formed from nitrogen fixation is incorporated into L-glutamate via this type of pyridine-nucleotide-dependent enzyme. Glutamate is then used in amino acid-protein synthesis via the transamination mechanism. The pertinent findings concerning L-glutamate metabolism in *Azotobacter* are: (a) It was the first organic nitrogen containing compound accumulated in cells grown under nitrogen-fixing conditions [26]; (b) an NAD^+-dependent L-glutamate dehydrogenase is found in cells grown under nitrogen-fixing conditions which suggests that the enzyme plays a role in the incorporation of ammonia into the cell protein [31]; and (c) Kotelnikova and Ivanova [29] studying oxidative phosphorylation in an *Azotobacter* subcellular particle, described an NAD^+-dependent L-glutamate oxidizing reaction capable of concomitant phosphorylation. This latter study represents the first report on the existence of a bioenergetically important glutamate oxidizing electron transport reaction in *Azotobacter*. This same type of particulate NAD^+-dependent glutamate oxidation (in *Azotobacter vinelandii*) was subsequently studied by Lenchenko et al. [30]. In both Russian studies [29, 30], NAD^+ was employed routinely in assays measuring L-glutamate oxidation. No mention is made of L-glutamate oxidation in the absence of pyridine nucleotides. Gupta and Das [28], working with cell-free extracts of *Azotobacter chroococcum*, reported the presence of an NAD^+-dependent glutamate dehydrogenase which was stimulated by the addition of PMS. This finding is difficult to interpret since NADH generated from L-glutamate oxidation would chemically reduce PMS and in the presence of air this would cause an auto-oxidation reaction. No mention was made of any significant L-glutamate oxidation occurring in the absence of NAD^+ in this latter study [28].

This report is the first which describes a L-glutamate oxidizing enzyme that is non-pyridine nucleotide dependent, in bacteria. An enzyme similar to the one described here has already been partially purified from spinach leaves [32]. Two enzymes were isolated that were shown to be specific for L-glutamate oxidation, but unlike the *Azotobacter* enzyme, they were soluble and both were able to oxidize L-aspartate approx. 4 and 9 %, respectively, of the rate observed for L-glutamate oxidation. PMS was readily used as an electron acceptor and it was reported that the mechanism of

TABLE IV

COMPARATIVE SUBSTRATE SPECIFICITIES OF THE *AZOTOBACTER* L-GLUTAMATE OXIDOREDUCTASE TO SEVERAL AMINO ACID OXIDASES

Except for the oxidation rates given for *Azotobacter* R_3 fraction, the data presented for the amino acid oxidase rates is cited in [1] p. 298.

Amino acid	*Azotobacter* R_3 fraction*	L-Amino acid oxidase			D-Amino acid oxidase	
		N. crassa	Rattlesnake venom	Rat kidney	Sheep kidney	Octopus liver
Glutamate	100**	12	<0.1	0	0	57
Methionine	18***	51	100	81	42	70
Asparagine	15***	—	—	—	—	—
Phenylalanine	13***	53	76	45	14	—
α-Aminoadipate	11	78	7	—	0	—
Proline	4	0	0	77	78	46
Ornithine	1.6	65	0.1	0	2	—
Threonine	0.1	3	0	0	1	—
Aspartate	0	6	0.1	0	0.5	25
Arginine	0	—	7	—	—	—
Serine	0	10	0	0	22	23
Tryptophan	0	35	82	40	19	16
Cystine	0	72	26	15	1	—
Alanine	0	53	0.4	—	34	53
Valine	0	8	4	28	18	34
Tyrosine	0	31	76	20	100	—
Isoleucine	0	42	29	71	12	37
α-Amino-*n*-butyrate	0	82	20	3	16	100
Histidine	0	47	14	9	3	62
Leucine	0	100	92	100	7	53
Lysine	0	18	0.2	0	0.3	—

* Sonically prepared *A. vinelandii* R_3 electron transport fraction.

** Rate reflects solely the oxidation of the L-(+)-isomer; the D-(−)-isomer was essentially inactive.

*** Rate reflects primarily the oxidation of the D-isomer; the L-isomer was completely inactive.

oxidation was similar to the amino acid oxidase reaction [32]. Substrate specificity of the two plant enzymes were limited solely to glutamate and aspartate. The enzymes were flavoprotein in nature as suggested by the fact that ferricyanide could serve as an electron acceptor [32]. This report appears to be the only one which described an enzyme similar in nature to the one described herein for *Azotobacter*, although it differs in the fact that the *Azotobacter* L-glutamate oxidoreductase is: (a) membrane-bound (Table II); (b) incapable of oxidizing aspartate (Table III); and finally (c) is probably integrated into the *Azotobacter* electron transport system (Figs 3–5).

Although the reaction mechanism of the *Azotobacter* enzyme might be similar to that of the L-amino acid oxidase reaction, it cannot be considered as such primarily because of substrate specificity studies. Table IV summarizes the substrate specificities exhibited by the *Azotobacter* L-glutamate oxidoreductase in the R_3 fraction and compares it to the substrate specificity oxidation pattern reported for both the L- and D-amino acid oxidases found in other organisms and tissues. The differences noted are most striking, particularly with the inability of the enzyme in the R_3 fraction to

oxidize leucine, tyrosine, α-amino-*n*-butyrate and L-methionine. Only the D-(−)-enantiomers of methionine, asparagine and phenylalanine were oxidized to any extent by the R_3 fraction and the significance of this is still not understood. Since the oxidation of these latter amino acids appears to be restricted to the D-(−)-enantiomers they are thus unrelated to L-glutamate oxidation described herein.

Detailed studies have not as yet been performed to ascertain the nature of the oxidative mechanism involved in this L-glutamate oxidation in *Azotobacter*. Although the enzyme involved is not an L-amino acid oxidase, it does not preclude that its mechanism for L-glutamate oxidation will be different than that described for the L-amino acid oxidase reaction. Preliminary studies suggest that the *Azotobacter* L-glutamate oxidoreductase will probably be a flavoprotein-containing enzyme since both PMS and $K_3Fe(CN)_6$ serve as good electron acceptors; and since it is characteristically similar in nature to the other known oxidoreductase-type enzymes already associated with membrane-bound electron transport systems. Flavoprotein reduction by L-glutamate is also indicated by the difference spectra studies by the bleaching noted in the 457–458-nm regions. This region would also show the oxidation–reduction changes that can be attributed to the non-heme iron component which is present in the *Azotobacter* R_3 fraction [33, 34].

Major attempts were also made to implicate NAD^+ and or $NADP^+$ as an actual coenzyme that is required for L-(+)-glutamate oxidation by the *Azotobacter* R_3 fraction. All such attempts failed and neither NAD^+ nor $NADP^+$ could serve as electron acceptors under the numerous conditions employed. However, it is also possible that a type of membrane-bound NAD^+ (or $NADP^+$) might function as the actual oxidation–reduction coenzyme. This coenzyme would be of an unusual nature because it does not react with externally added NAD^+ (or $NADP^+$). This possibility appears unlikely as the only known case of a bound NAD^+ is that associated with the soluble (and crystalline) D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and it can react with externally added NAD^+ for catalytic purposes. Should such a bound NAD^+ be present in the *Azotobacter* R_3 fraction it would be the first instance of a bound NAD^+ being present and functional in an electron transport system that does not require externally added NAD^+ as a coenzyme for a specific catalytic reaction.

The last significant feature of the enzyme described may be its involvement in ammonia fixation, or the reductive amination of α-ketoglutarate for the synthesis of L-glutamate. Since *Azotobacter* species are free living, aerobic, nitrogen-fixing bacteria, the reverse reaction of this enzyme could function in a novel way for glutamate synthesis. In essence, this would represent the reversal of the reaction described in this communication. The ammonia formed from nitrogen fixation would react immediately with α-ketoglutarate to form L-glutamate. This reaction could be the primary one as it is unlikely that L-glutamate would be oxidized by the *Azotobacter* electron transport system as an energy source, although this could occur if the need arose. The α-ketoglutarate would be generated from the unusually active isocitric dehydrogenase (EC 1.1.1.42) that is present in this organism [35]. Other studies in our laboratory indicate that the specific activity for the pyridine-nucleotide-dependent L-glutamate dehydrogenase (EC 1.4.1.2 or EC 1.4.1.3) is extremely low and if measurable, is found exclusively in the S_3 fraction indicating that it is a soluble enzyme in *A. vinelandii*. This would indicate that there are at least two types of L-(+)-gluta-

mate oxidizing enzymes in *Azotobacter* and that both are capable of playing a role in glutamate metabolism in this microorganism. The role of both these enzymes is now being examined by our laboratory and studies are now in progress to establish whether the enzyme characterized in this communication can carry out the reverse reaction, i.e. the reductive amination of α-ketoglutarate. What is of major interest in the reverse reaction is that electron transport reversal may be required for L-glutamate synthesis. Some reductant (either in the presence or absence of ATP) also would be needed in order to serve as NADH does for the reversal of the conventional glutamate dehydrogenase reaction. The natural reductant undoubtedly will be one that could equilibrate with the *Azotobacter* electron transport system and serve as a suitable energy source for the reductive amination process.

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