Aspects of regulation of ketoglutarate dehydrogenase activity from ribbed mussel (Modiolus demissus) gills and cauliflower (Brassica oleracea)

George Antun Karam
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Biochemistry Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/9207

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700  800/521-0600
Aspects of regulation of ketoglutarate dehydrogenase activity from ribbed mussel (*Modiolus demissus*) gills and cauliflower (*Brassica oleracea*)

Karam, George Antun, Ph.D.

Iowa State University, 1989
Aspects of regulation of ketoglutarate dehydrogenase activity from ribbed mussel (*Modiolus demissus*) gills and cauliflower (*Brassica oleracea*)

by

George Antun Karam

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Zoology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1989
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>5</td>
</tr>
<tr>
<td><strong>PART I. KETOGLUTARATE DEHYDROGENASE FROM RIBBED MUSSEL GILL MITOCHONDRIA: MODULATION BY ADENINE NUCLEOTIDES AND CALCIUM IONS</strong></td>
<td>9</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>16</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>33</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>38</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>39</td>
</tr>
<tr>
<td><strong>PART II. KETOGLUTARATE DEHYDROGENASE FROM CAULIFLOWER (BRASSICA OLERACEA L.) MITOCHONDRIA: PREPARATION AND REACTIVITY WITH SUBSTRATES</strong></td>
<td>44</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>46</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>47</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>49</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>52</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>60</td>
</tr>
<tr>
<td><strong>PART III. ACTIVATION OF CAULIFLOWER KETOGLUTARATE DEHYDROGENASE BY CALCIUM IONS</strong></td>
<td>62</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>64</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>66</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>69</td>
</tr>
<tr>
<td>RESULTS</td>
<td>74</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>80</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>84</td>
</tr>
<tr>
<td><strong>PART IV. KETOGLUTARATE DEHYDROGENASE FROM THE CAULIFLOWER</strong></td>
<td>88</td>
</tr>
<tr>
<td><strong>BRASSICA OLERACEA: MODULATION BY ADENINE NUCLEOTIDES, CoA DERIVATIVES</strong>, AND NaCl</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>90</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>91</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>93</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>96</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>109</td>
</tr>
<tr>
<td>GENERAL DISCUSSION</td>
<td>111</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>115</td>
</tr>
</tbody>
</table>
DEDICATION

To my parents as a sign of gratitude and to those true, special and supporting friends who helped and cared when I needed someone: Susan Harlocker, Helen Man-Son Hing, Nicholas Marini, and Donald Reading.
GENERAL INTRODUCTION

All living organisms respond to environmental changes by regulating their metabolic pathways and the interconversion of their intracellular organic compounds, thus controlling their cell volume (Pierce, 1982). One of these regulatory processes is the accumulation of compatible osmolytes, specifically amino acids, in response to salt stress (Yancey et al., 1982). Insects, molluscs and plants are capable of accumulating a variety of free amino acids that play a key role in maintaining their cell volume and in regulating their energy metabolism (Aspinall and Paleg, 1981; Bishop et al., 1981, 1983; Bowlus and Somero, 1979; Bursell, 1981; Crowe et al., 1977; Somero and Bowlus, 1983). Some of the reactions involved in the regulation of amino acid accumulation include glycolysis, Krebs’ cycle, protein turnover, ammonia fixation, transamination reactions, and the malate and aspartate shuttling systems (Bishop, 1976). Studying the regulation of the catabolism of these amino acids and their role in the energy production involves studying the regulation of the enzymes that catabolize these amino acids and their carbon skeletons. A major control for the reactions involved in the regulation of amino acid accumulation occurs at the level of specific mitochondrial enzymes.

A major osmolyte that is accumulated in invertebrates and plants in response to osmotic stress and plays an important role in the
energy metabolism is proline (see references above). In the osmoconformer euryhaline bivalves, proline accumulates transiently before entering the Krebs' cycle through ketoglutarate (Bishop et al., 1981). In plants, on the other hand, the accumulation of proline due to its synthesis from glutamate is a continuous response that dramatically increases in response to salt stress (Buhl and Stewart, 1983; Stewart and Voetberg, 1985; Voetberg and Stewart, 1984). The role of ketoglutarate as a cosubstrate or product in the recycling transaminase reactions and in the glutamate dehydrogenase reactions and its function in interlocking amino acid metabolism and energy metabolism is of considerable importance. A major enzyme that regulates the catabolism of ketoglutarate and the accumulation of proline in invertebrates and plants is ketoglutarate dehydrogenase (KGDH). KGDH is an intramitochondrial multienzyme system of an approximate molecular weight of $2.5 \times 10^6$ (Reed and Cox, 1966). The nonequilibrium reaction of KGDH is the primary site of control for the flow of carbon atoms in the TCA cycle from ketoglutarate to malate and therefore, plays an important role in regulating the energy metabolism of the cell (Williamson 1979). The absence of the enzyme (P-5-C synthase) that controls the conversion of glutamate to P-5-C (an intermediate of proline synthesis) in the invertebrates (Bishop et al., 1981) and its presence in plants (Voetberg and Stewart, 1984), is of a major importance for the understanding of proline accumulation in these organisms. Understanding the
regulation of KGDH that plays the common role of allowing the entry of proline into the Krebs' cycle of both organisms will lead to a major understanding of the various ways by which amino acids are accumulated and metabolized and their contribution to cellular energy metabolism in general.

KGDH and the two other intramitochondrial enzymes that are rate-limiting or control points, Pyruvate dehydrogenase (PDH) and NAD-Isocitrate dehydrogenase (NAD-ICDH), play a major role in the control of the mitochondrial energy cycle. These enzymes are regulated by the pH and by other various effectors within the mitochondrion (Williamson and Cooper, 1980). The regulation of these three mitochondrial matrix enzymes (and specifically KGDH) in animal systems is well documented: (Hansford, 1972a,b, 1985; Hansford and Castro, 1981; Lawlis and Roche, 1980, 1981a, 1981b; Roche and Lawlis, 1982; McCormack and Denton, 1979, 1980, 1984, 1985, 1986; Denton et al., 1980; Randle et al., 1970; Karam et al., 1987). On the other hand, little is known about the regulation of plant matrix enzymes (Denton and McCormack, 1980; Dry and Wiskich, 1982, 1985, 1987; Moore and Akerman, 1984; Poulsen and Wedding, 1970; Randall et al., 1981; Wedding and Black, 1971).

In this study, the regulation of KGDH in the ribbed euryhaline bivalve Modiolus demissus and the cauliflower Brassica oleracea was investigated. The effect of the various modulators of enzyme activity on the regulation of KGDH was studied. In addition, the
role of PDH and ICDH and their contribution to the regulation of KGDH activity and to the energy metabolism in both organisms is discussed.


Lawlis, V. B. and T. E. Roche (1981a) Regulation of bovine kidney ketoglutarate dehydrogenase complex by calcium ion and adenine nucleotides. Effects on S0.5 for ketoglutarate. Biochemistry, 20, 2512-2518

Lawlis, V. B. and T. E. Roche (1981b) Inhibition of bovine kidney ketoglutarate dehydrogenase complex by reduced nicotinamide adenine dinucleotide in the presence or absence of calcium ion and effect of adenosine 5'-diphosphate on reduced nicotinamide adenine dinucleotide inhibition. Biochemistry, 20, 2519-2524


PART I. KETOGLUTARATE DEHYDROGENASE FROM RIBBED MUSSEL GILL MITOCHONDRIA: MODULATION BY ADENINE NUCLEOTIDES AND CALCIUM IONS
KETOGLUTARATE DEHYDROGENASE FROM RIBBED MUSSEL GILL MITOCHONDRIA: MODULATION BY ADENINE NUCLEOTIDES AND CALCIUM IONS

George A. Karam, M.Sc.
Stephen H. Bishop, Ph.D.

From the Department of Zoology, Iowa State University, Ames, IA 50011
ABSTRACT

The transient accumulation of proline in the gills of the osmotically stressed ribbed mussel (*Modiolus demissus*), may be controlled by a transient regulation of ketoglutarate dehydrogenase (KGDH) activity. In this study, KGDH was partially purified from lysed mitochondria of gill tissue by differential centrifugation techniques. Various modulators of enzyme activity assayed at a saturating concentration (2.5 mM) of ketoglutarate (KG), showed inhibition by high concentrations of Cl⁻ (10-100 mM) and a slight activation or inhibition by the other compounds (10 mM), when assayed in the absence of added Ca²⁺. Addition of Ca²⁺ at pH 7.2 caused no change in Km for KG and a 1.5-fold increase in V_max whereas at pH 7.8, no change in V_max but a 40-50% decrease in K_m and slight positive cooperative effects (1.4-1.7) were observed. At pH 7.8, addition of AMP, ADP and ATP (5 mM) had no effect on the V_max; however, ATP and ADP lowered the K_S0.5 by 3.5-4-fold. The CoA derivatives of short chain fatty acids were found to inhibit the enzyme by 30-60%. This inhibition was reversed by Ca²⁺ but only at higher KG concentrations (250 uM). The enzyme showed a major change in activity at an NAD/NADH ratio < 1, an effect that could not be modified by Ca²⁺. It appears therefore, that the mussel gill KGDH is mainly regulated by changes in mitochondrial pH and ketoglutarate or...
adenine nucleotide levels, rather than by changes in mitochondrial calcium levels.
INTRODUCTION

The metabolic regulation of the amino acids and organic acids that accumulate in hyperosmotic or anaerobic stressed estuarine bivalves involves changes in glycolysis, protein turnover, the TCA cycle reactions, the transaminases and metabolite shuttling between cellular compartments (Bishop et al., 1983; deZwaan, 1983, Somero and Bowlus, 1983). A most important aspect of these pathways is the flux of carbon through KG. These pathways include the glutamate dependent transaminases, glutamate dehydrogenase (GDH), proline-ornithine-arginine metabolism and the TCA cycle enzymes used in the metabolism of the five carbon intermediates (Reiss et al., 1977; Greenwalt and Bishop, 1980; Bishop et al., 1981; deZwaan et al., 1975, 1981, 1982, 1983; Paynter et al., 1984 a,b). The major TCA cycle enzyme involved in the catabolism rather than the production or recycling of KG is KGDH. KGDH catalyzes the formation of CO₂, NADH and succinyl-CoA from KG, NAD⁺ and CoA.

Calcium ions and adenine nucleotides and not protein phosphorylation-dephosphorylation have been shown to be the major factors regulating the KGDH activities in mitochondria from mammalian tissues (McCormack and Denton, 1979; Lawlis and Roche, 1980, 1981 a,b; Roche and Lawlis, 1982; McCormack, 1985a,b). Calcium ions and adenine nucleotides also play an important role in the coordinate regulation of both the membrane transport (Pierce, 1982) and the
metabolic (Ellis et al., 1985; Paynter et al., 1985) processes controlling amino acid accumulation in the tissues of osmotically stressed estuarine bivalves. Therefore, we initiated this study on the possible role of Ca^{2+}, salt and adenine nucleotides on the regulation of the KGDH activity in ribbed mussel tissues.

Previous studies (Burcham et al., 1984) have demonstrated that L-glutamate, proline and KG stimulated a rapid rate of ADP dependent oxygen consumption by ribbed mussel gill mitochondria, thereby providing indirect evidence for the existence of GDH, proline oxidase and KGDH in these mitochondria and direct evidence for the tight coupling of ATP synthesis to the oxidation of these five carbon substrates. Among other invertebrates, low levels of KGDH activity have been reported in mitochondrial extracts of sea mussel tissue (Addink and Veenhof, 1975), insect flight muscle mitochondria (Hansford, 1972 a,b; Norden and Matanganyidye, 1979; McCormack and Denton, 1981), and some helminth parasites (Barrett, 1976).

Recently, Paynter et al. (1985) have demonstrated KGDH activity in ribbed mussel gill mitochondria in a conclusive manner and have shown that the level of KGDH activity may greatly exceed (5X) the level of another important regulatory enzyme, pyruvate dehydrogenase (PDH). However, in preliminary studies, adenine nucleotide but not Ca^{2+} activation of insect flight muscle KGDH was demonstrated leading to the suggestion that Ca^{2+} may not be a regulator of KGDHs from invertebrates (Hansford, 1972a; McCormack and Denton, 1981).
The experiments described here indicate that the KGDH activity in ribbed mussel gill mitochondria may be regulated by changes in ADP, ATP, pH and KG levels at low mitochondrial KG concentrations (10-300 uM) rather than by changes in mitochondrial Ca$^{2+}$ levels.
MATERIALS AND METHODS

Enzyme Preparation

Ribbed mussels (*Modiolus demissus*) were purchased from Northeast Marine Environmental Institute (Monument Beach, MA). The animals were maintained as described by Greenwalt and Bishop (1980). Unless otherwise noted, all reagents were purchased from Sigma Chem. Co. (St. Louis, MO) or Fisher Sci. Co. (Pittsburgh, PA).

The enzyme complex was partially purified from a lysed mitochondrial preparation according to Paynter et al., (1985). Gills were homogenized in 10 volumes of mitochondria isolation buffer (MIB) consisting of 0.4M sucrose, 20 mM HEPES, 1 mM EGTA at pH 7.5. The homogenate was filtered through Miracloth (CalBiochem.), then centrifuged at 1500 x g for 10 min. The resulting supernatant was centrifuged at 9000 x g for 15 min, and the pellet resuspended in the above buffer containing 0.33 mM leupeptin, 0.1 mg/ml trypsin inhibitor, 1 mM dithiothreitol, (DTT) and 20 uM rotenone. The resuspended pellet preparation (mitochondria) was sonicated and centrifuged at 20,000 x g for 30 min. This supernatant was centrifuged at 150,000 x g for 90 min and the pellet resuspended in MIB containing 0.1 mM DTT. This preparation was cleared by centrifugation at 20,000 x g for one h and the supernatant used for all studies as a crude preparation of KGDH. EGTA was included in these buffer washes to remove Ca^{2+} (Lawlis and Roche, 1980).
Assay of KGDH activity

The enzyme activity was assayed spectrophotometrically in a 1 ml mixture which contained the following ingredients: 0.5 mM NAD⁺, 0.25 mM KG, 0.1 mM CoA, 0.1 mM TPP, 0.1 mM EGTA and 1.0 mM DTT, in 150 mM HEPES, (pH 7.5). These assays were performed at 340 nm and at room temperature (22°C) in a Beckman model 3600 recording spectrophotometer. The enzyme was preincubated for one minute with an assay medium which did not contain KG, then KG was added and the increase in absorbance at 340 nm resulting from NAD reduction was recorded. One unit of enzyme activity was defined as the amount of enzyme that would reduce a nmole of NAD per min. PDH was assayed as described previously (Paynter et al., 1985).

The kinetics of the enzyme activity were studied by assaying the enzyme as described above but in 50 mM Tris-acetate at pH 7.2 and 7.8 with increasing concentrations of KG (12.5-1500 μM). The velocity curves and the Hill plots of the enzyme were constructed as described by Segel (1975). Protein concentration was measured by the method of Lowry et al. (1951).

Modulators of enzyme activity

Various modulators of enzyme activity were tested for their effect on enzyme activity. The enzyme was assayed in HEPES buffer as described above except that the modulators were added two minutes
after the beginning of each assay and their effect on the activity was compared to the control samples with no modulator added.

**Loss of enzyme activity with storage**

Aliquots of the resuspended high speed pellet (see above) were stored for up to one month in various solutions consisting of one of the following ingredients: 60% sucrose, 10% glycerol or 10% polyethylene glycol with either 100 mM potassium phosphate or 100 mM HEPES at pH 6.8, 7.5, 8.0, or 8.5. To each, 5 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol and 1% Triton X-100 were added in the presence or absence of protease inhibitors (0.34 g/l benzamidine-HCl and 0.1 g/l phenylmethane sulfonyl fluoride (PMSF). The preparations were assayed for both PDH and KGDH then stored either at -10°C or at 5°C. At intervals of 3, 15, and 30 days, the stored fractions were assayed to determine the percentage of PDH and KGDH activity remaining.

**Effect of nucleotides on the activity of KGDH**

The effect of 5 mM AMP, ADP and ATP on the activity of KGDH was assayed as described above but in 50 mM Tris-acetate, pH 7.8. The enzyme was preincubated in the presence of each nucleotide for one minute and the assay started by the addition of KG.
Effect of CoA derivatives on the KGDH activity

The effect of acetyl-CoA, butyryl-CoA, propionyl-CoA and succinyl-CoA on the activity of the enzyme was measured at 250 uM and 100 uM KG in presence or absence of Ca\(^{2+}\). The enzyme was preincubated for 2 min with the CoA derivative at a final concentration of 0.1 mM and 0.5 mM, and the reaction started by the addition of KG. CaCl\(_2\) (2 mM) was added to the assay mixture 5 min later and the effect on KGDH activity monitored for an additional 5 min.
RESULTS

The technique used to prepare the enzyme from gill mitochondria yielded 1000-1500 units of KGDH activity from about 30 g of gill tissue with a specific activity of 40 units/mg protein.

Assay of the enzymatic activity in 50-150 mM HEPES at pH 7.5, showed activity values that were double those of enzyme assayed in 100 or 200 mM Tris-Cl, Tris-acetate or potassium phosphate buffers at the same pH. Activity in 50 mM Tris-acetate was equivalent to activity in HEPES. Rotenone (20 uM) was added to the mitochondrial lysate to inactivate an indigenous NADH oxidase activity that pelleted with the KGDH activity. Rotenone at 100 uM did not affect the activity of these KGDH preparations. This preparation was free of transaminase and glutamate dehydrogenase activities.

The enzyme was reasonably stable if stored under appropriate conditions. This KGDH preparation could be stored for up to 1 month in a buffer consisting of 10% glycerol, 5 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol, 1% Triton X-100, in 100 mM HEPES (pH 7.5) with a loss of only 30% activity. Enzyme stored at -10°C in buffers consisting of the above ingredients but containing 60% sucrose or 10% polyethylene glycol, in 100 mM HEPES or potassium phosphate, and enzyme stored in the glycerol plus phosphate buffer showed a significant (65-100%) decline in activity after one month. Fractions to which protease inhibitors (benzamidine-HCl or PMSF) were added
during storage showed no change in loss of activity as compared to those fractions to which no protease inhibitors were added. Fractions stored at 5°C and at pH values higher or lower than pH 7.5, showed greater loss of activity after one month (60% for sucrose-phosphate, 70% for sucrose-HEPES and 100% for all the others (data not shown) as compared to those stored at -10°C and pH 7.5 for the same amount of time. PDH could not be stored under the above conditions because of the inhibitory concentration of EDTA (5 mM) included in the storage buffer.

**Kinetics**

Lawlis and Roche (1981a, b) have shown that the Km for KG with the mammalian KGDH increased considerably between pH 7 and pH 8 in the presence and absence of Ca$^{2+}$. Kinetic studies of the gill KGDH at pH 7.2 and 7.8 in presence or absence of 2 mM Ca$^{2+}$ showed slightly sigmoidal, rectangular hyperbolic responses when the velocities were plotted against KG concentration (Fig 1A and 1B). Addition of EGTA to 1 mM or 5 mM in these reaction mixtures in the absence of CaCl$_2$ did not change this result (Fig. 1B). When assayed at pH 7.2, Ca$^{2+}$ addition caused only a 1.3-1.5-fold increase in $V_{\text{max}}$ and no change in the $K_{S0.5}$ (50 uM) for KG (Fig. 1A) On the other hand, at pH 7.8, $K_{S0.5}$ for KG was 250-300 uM and decreased slightly to 150 uM in the presence of Ca$^{2+}$ with no change in $V_{\text{max}}$ (Fig. 1B). A Hill plot of these values showed an "n$_{\text{app}}$" of 1.7 at pH 7.2 and 1.4 at pH 7.8 in
Fig. 1. Kinetic study of gill KGDH activity. Aliquots of 0.6 units of KGDH purified in the HEPES buffer system at pH 7.5 were preincubated for 1 min, in the absence of KG. Increasing concentrations of the substrate were added at 23°C in the presence (●) or absence (○) of Ca²⁺ and at pH 7.2 (IA) or 7.8 (IB); or KGDH purified in 3% Triton X-100 with 50 mM MOPS (pH 7.0) buffer system, assayed at pH 7.8 (IC) and the velocity recorded ($A_{340}$ min⁻¹).
the presence or absence of Ca^{2+}. None of the negative cooperative effects observed for the mammalian KGDH (see Roche and Lawlis, 1982) were observed with the gill KGDH.

Tissue extraction with non-ionic detergents (Triton X-100) has been used in the purification of the keto acid dehydrogenases from many mammalian tissues (Stanley and Perham, 1980). Therefore, we performed kinetic studies on gill KGDH prepared in a detergent containing buffer consisting of 50 mM MOPS at pH 7.0, 2.7 mM EDTA, 0.1 mM DTT, 3% Triton X-100, 1 mM benzamidine-HCl and 2 μM leupeptin. When activity was assayed in the Tris-acetate buffer at pH 7.8, there was a stronger positive cooperative type of behavior ("n_{app}" = 2.5) with a low K_{50.5} of 54 μM for KG and an "n_{app}" value of 2.5 (Fig. 1C). Higher concentrations of Triton X-100 (5%) did not change this effect on the activity of KGDH. Because of the possible modifying effect of Triton on the KGDH activity, KGDH prepared in the absence of Triton, was used in the studies reported here.

A number of other potential modulators of gill KGDH activity had only a modest effect on the KGDH activity when added at 10 mM under assay conditions of fixed, high (2.5 mM) KG concentrations (Table 1). At higher concentrations (100 mM), NaCl and KCl showed about 60% inhibition of activity whereas 100 mM sodium or potassium acetate were not inhibitory (data not shown). These results indicate that high Cl^{-} concentrations inhibited KGDH activity. Very high
Table 1. Effect of modulators on KGDH activity from mussel gill tissue. Mussel gill KGDH was assayed in HEPES at pH 7.5 in the presence and absence (control) of 10 mM of the indicated compounds and at 2.5 mM KG

<table>
<thead>
<tr>
<th>Compound (10mM)</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>97</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>100</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>50</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>95</td>
</tr>
<tr>
<td>EDTA</td>
<td>88</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>80</td>
</tr>
<tr>
<td>GTP</td>
<td>100</td>
</tr>
<tr>
<td>GDP</td>
<td>130</td>
</tr>
<tr>
<td>IDP</td>
<td>160</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>120</td>
</tr>
<tr>
<td>AMP-PCP</td>
<td>110</td>
</tr>
</tbody>
</table>
concentrations of CaCl₂, EGTA and MgCl₂ (100 mM) showed similar inhibitions of 67%, 72%, and 65%, respectively.

When KGDH from either mussel gills or bovine heart (Sigma Chem. Co.) was assayed at 2.5 mM KG at pH 7.8, neither showed inhibition in the presence of 1 mM EDTA (Fig. 2). This EDTA concentration was found to inhibit completely the mussel gill PDH (Paynter et al., 1985). Under these conditions, Ca²⁺ (2.5 mM) activated the mussel gill KGDH slightly (1.5-fold) and activated the bovine heart KGDH more profoundly (5-fold). The result was in general agreement with the results of McCormack and Denton, (1979, 1981) and Roche and Lawlis (1982) for mammalian KGDH. On the other hand, 2.5 mM Mg²⁺, which showed an activating effect similar to the Ca²⁺ activating effect on KGDH from bovine heart tissues, but had no effect on KGDH from mussel gills (Fig. 2).

The KGDH activities of insect flight muscle and pig heart KGDH (Hansford, 1972 a,b; McCormack and Denton, 1979, 1981) are modified by adenine nucleotides; ATP tended to inhibit and ADP or AMP tended to stimulate these enzymes by raising and lowering the Km for KG, respectively. With the gill KGDH at pH 7.8 and varying KG concentrations, 5 mM ATP, ADP and AMP had little effect on the Vₘₐₓ (Fig. 3). On the other hand at pH 7.8, ATP and ADP lowered the K₆0.5 for KG to 60 uM and 85 uM, respectively; AMP had no effect. The sigmoidicity or "n_app" with KG changed only slightly from 1.4 to 1.7, 1.5 and 1.6 for AMP, ADP and ATP, respectively (Fig. 3). Addition of
Fig. 2. Effect of Ca\(^{2+}\), Mg\(^{2+}\) and EDTA on gill and bovine KGDH activity. Aliquots of one unit of KGDH from mussel gills or bovine heart were assayed at 2.5 mM KG in the presence or absence of 2.5 mM Ca\(^{2+}\) or Mg\(^{2+}\) and 1 mM EDTA.
Fig. 3. Effect of nucleotides on gill KGDH activity. Aliquots of 0.5 mg of the enzyme extract were assayed in the absence (A) or the presence of 5 mM AMP (B), ADP (C) or ATP (D) at the indicated KG concentrations and the velocity recorded (A_340 \text{ min}^{-1} \text{mg}^{-1})$. The Hill plots, as \log V/V_{\text{max}} vs. [S] (\mu\text{M} \times 10^2)$, are shown as inserts.
10 mM or 2 mM MgCl₂ did not modify this ATP, ADP or AMP effect. The results of the adenine nucleotide and Ca²⁺ effects on the KGDH activity at pH 7.8 are summarized in Table 2.

CoA derivatives of short chain fatty acids have been shown to modify the activity of the KGDH and the other keto acid dehydrogenases (see Roche and Lawlis, 1982). The KGDH activity was assayed at pH 7.8 and at two low KG concentrations in order to evaluate any effect of Ca²⁺ on the modification of the activity by the CoA derivatives (Fig. 1B). With the gill KGDH, CoA derivatives (Fig. 4) at either 0.1 mM or 0.5 mM had similar effects on the activity when assayed in the presence and absence of 2 mM Ca²⁺. With 100 µM KG, Ca²⁺ slightly activated all preparations except those treated with butyryl CoA; all acyl-CoA derivatives but succinyl CoA were somewhat inhibitory in the absence of Ca²⁺. The slight activation by Ca²⁺ when assayed with 100 µM KG was essentially lost when the CoA derivatives were added. On the other hand, at 250 µM KG the activation by Ca²⁺ and reversal of this activation by the CoA derivatives was much less pronounced than at 100 µM KG.

The KGDH activity in mammalian tissues is inhibited by NADH accumulation and this inhibition is modified by Ca²⁺ (McCormack and Denton, 1981; Lawlis and Roche, 1980, 1981a,b). Because of the modest Ca²⁺ activating effect on the gill KGDH (Figs. 1B and 4), it was important to determine whether or not Ca²⁺ would modify any NADH inhibitory effect at a low (near Kₘ) KG concentrations. At NAD
Table 2. Effect of Ca\textsuperscript{2+}, AMP, ADP and ATP on the activity of mussel gill KGDH at pH 7.8\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CaCl\textsubscript{2}</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{50.5}$(uM)</td>
<td>250-300</td>
<td>150</td>
<td>250</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>$SA(V_{max})^{b}$</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>$n_{app}$</td>
<td>1.4</td>
<td>1.4</td>
<td>1.7</td>
<td>1.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are taken from Figures 2 and 4
\textsuperscript{b} $SA(V_{max}) = \text{units/mg protein at maximum velocity}$
Fig. 4. Effect of CoA derivatives on the activity of gill KGDH. Aliquots of 0.5 mg of the enzyme were assayed at a concentration of 100 μM (□) and 250 μM (■) ω-KG in the presence of 1 mM or 5 mM acetyl-CoA (A-CoA), butyryl-CoA (B-CoA), propionyl-CoA (P-CoA), and succinyl-CoA (S-CoA) and in the presence or absence of 2 mM Ca²⁺.
concentrations of 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM, the greatest change in the gill KGDH activity occurred at NAD/NADH ratios less than unity (Fig. 5) indicating that the KGDH complex could function maximally at NADH concentrations that would strongly inhibit the gill PDC (Paynter et al., 1985). Addition of 2 mM Ca$^{2+}$ at any of the NAD/NADH ratios studied did not modify this response (Fig. 5). It would appear that Ca$^{2+}$ did not modify the inhibitory effect of NADH at fixed, low KG concentrations.
Fig. 5. Effect of NADH ratios on the activity of gill KGDH. Aliquots of the enzyme extract were assayed in 250 µM KG in the presence or absence of CaCl$_2$ (2 mM) and at various NAD$^+$/NADH ratios. KGDH was assayed at the indicated NADH concentrations and at NAD concentrations of 1 mM (○), 0.5 mM (■), 0.1 mM (●), and 0.05 mM (not shown).
DISCUSSION

The ribbed mussel gill KGDH activity appears to be less responsive to regulation by Ca$^{2+}$ than the KGDH activity in mammalian tissues. With the mammalian KGDHs, Ca$^{2+}$ caused a decrease in the $K_m$ for KGDH from the mM range to about 30 μM (Roche and Lawlis, 1982) depending upon the pH. The response of the gill KGDH to Ca$^{2+}$ is similar to that reported by McCormack and Denton (1981) for the insect flight muscle KGDH. For instance, Ca$^{2+}$ addition resulted in only a small decrease (40-50%) in the $K_m$ for KG at pH 7.8 but no change in the $K_m$ for KG and only a slight increase in $V_{\text{max}}$ at pH 7.2 (Table 2). Added Ca$^{2+}$ had only a small effect on the inhibition of the gill KGDH by acyl CoA derivatives (Fig. 4) at low KG concentrations. Ca$^{2+}$ addition did not modify the inhibitory response of NADH with the gill KGDH. NADH was inhibitory only at NAD/NADH ratios below 1 or at relatively high NADH concentrations under fixed KG assay conditions (Fig. 5).

In studies by McCormack and Denton (1979) and Lawlis and Roche (1980, 1981b), Ca$^{2+}$ reduced the inhibitory response of the mammalian KGDH to added NADH. Because of this reduced response to NADH, the gill KGDH could probably operate in the forward direction at a reasonable rate even at the higher NADH concentrations that might occur during anaerobiosis. These results with the gill KGDH strongly support the suggestion by McCormack and Denton (1981) that KGDHs from
Invertebrate tissues are considerably less responsive to changes in mitochondrial Ca\(^{2+}\) levels than KGDHs from vertebrate tissues.

Recently, McCormack and Denton (1982) have suggested that Ca\(^{2+}\) may not be an important regulator of mitochondrial metabolism in invertebrates because of lack of KGDH Ca\(^{2+}\) sensitivity and the presence of a high K\(_T\), ruthenium red insensitive Ca\(^{2+}\) transporter in some invertebrate mitochondria. If this is so, then marine invertebrates, such as marine bivalves, with high intracellular Na\(^{2+}\) levels (see Pierce, 1982) would have an additional Ca\(^{2+}\) regulatory problem because the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) antiporter would act to override any regulatory action of the Ca\(^{2+}\) transporter. Although these limited data do not permit exclusion of Ca\(^{2+}\) as a regulator of metabolism in marine invertebrate mitochondria, it is evident that there are some profound differences between vertebrates and invertebrates in both the performance of some key regulatory enzymes and the ability to control mitochondrial Ca\(^{2+}\) levels.

The effect of lower pH on the lowering of the K\(_m\) for KG with gill KGDH (Figs. 1A and 1B) is similar to that reported for the mammalian KGDHs (Roche and Lawlis, 1982). Both ADP and ATP caused a marked activation of the gill KGDH activity by lowering the apparent K\(_m\) for KG from 250-300 \(\mu\)M to 60 \(\mu\)M and 85 \(\mu\)M, respectively, when assayed at pH 7.8 (Table 2). The ADP activating effect is similar to that reported for the mammalian KGDHs (Lawlis and Roche, 1981a; McCormack and Denton, 1979) and insect flight muscle KGDH (Hansford,
However, the activation rather than inhibition of the gill KGDH by ATP as reported for the mammalian and insect KGDHs means that the gill KGDH may differ somewhat from the other KGDHs. This ATP-ADP activating effect was Mg$^{2+}$ independent, thereby negating any complicating effect of adenylate kinase. It would appear that the gill KGDH is regulated by changing pH and adenine nucleotide (ADP and ATP) levels and by the ability of the mitochondrion to concentrate KG or to provide reasonable KG levels (10-300 uM) with the mitochondrion.

Studies on ADP dependent respiration by mitochondria from a number of bivalves (Zaba et al., 1978; Burcham et al., 1983, 1984; Ballantyne and Storey, 1983; Ballantyne and Moon, 1985; Moyes et al., 1985) indicate that the rate of O$_2$ consumption by glutamate and KG is essentially identical when supplied with substrate in the 1-10 mM range. Therefore, KG and glutamate can be transported into and possibly concentrated within respiring bivalve tissue mitochondria. Although the concentration of KG in bivalve tissue mitochondria is not known, the estimated KG concentration in mammalian heart mitochondria (Williamson et al., 1972; LaNoue and Schoolwerth, 1979) can vary between 30 and 400 uM depending upon the respiratory state and pumping abilities of the mitochondria. Bayne (1973) estimated that ketoglutarate concentrations in sea mussel tissues are in the 2-15 uMol/kg wet wt range. Because the performance of the bivalve tissue mitochondria and mammalian mitochondria are similar (see
references cited above), one can reasonably assume that abilities of gill mitochondria to concentrate KG should be similar to those of mammalian mitochondria. Given this assumption, the level of KG within the mitochondria should vary in a concentration range of 30-300 uM or below the $K_m$ for KG with the KGDH at pH 7.8 but far above or near the $K_m$ for KG at pH 7.2. Lowering the pH to 7.2 or increasing the ADP or ATP levels would provide a strong activating effect on the KGDH activity at KG concentrations between 10 and 300 uM. Therefore it would appear that the flux of metabolites through the gill KGDH reaction would be regulated by the respiratory state of the mitochondrion and the availability of substrate to the mitochondrion.

As a corollary, the isocitrate dehydrogenase (ICDH) from insect flight muscle differs from the mammalian ICDH by not being activated by Ca$^{2+}$ (McCormack and Denton, 1981). Both the NAD$^+$ and NADP$^+$ dependent ICDHs are present in bivalve tissues (Addink and Veenhof, 1975; Head and Gabbott, 1980; Kargbo and Swift, 1983; Ruiz Ruano et al., 1985b). Although Kargbo and Swift (1983) showed ADP activation of the oyster NAD$^+$ dependent mitochondrial ICDH, there is no data on the role of Ca$^{2+}$. These studies on ICDH and this report on KGDH combined with those on the activation of GDH by ADP (Addink and Veenhof, 1975; Reiss et al., 1977; Ruiz Ruano et al., 1985a) mean that the three mitochondrial dehydrogenases utilizing or producing KG in bivalves are probably regulated by varying H$^+$, adenine nucleotide,
and substrate levels rather than by varying $Ca^{2+}$ levels within the mitochondrion.
SUMMARY

The ketoglutarate dehydrogenase (E.C. 1.2.4.2) activity in ribbed mussel gill can be demonstrated in resuspended material from high speed (150,000 x g) centrifugation of lysed mitochondria (see Paynter et al., 1985). Kinetic plots of the reactivity at varying ketoglutarate (KG) concentrations indicate slight positive cooperativity, a reduction in the $K_m$ for KG from 250-300 uM to 50 uM with shift in assay pH from pH 7.8 to pH 7.2, a reduction in $K_m$ for KG with the addition of ATP or ADP at pH 7.8, and little or no change in $V_{\text{max}}$ or $K_m$ for KG with the addition of $\text{Ca}^{2+}$. Major factors regulating the mussel gill ketoglutarate dehydrogenase activity in this tissue are variations in pH, KG concentrations and adenine nucleotide concentrations within the mitochondria. Variations in $\text{Ca}^{2+}$ within the mitochondria would not appear to be important in the regulation of this activity in this animal.
LITERATURE CITED


Lawlis, V. B. and T. E. Roche (1981b) Inhibition of bovine kidney ketoglutarate dehydrogenase complex by reduced nicotinamide adenine dinucleotide in the presence or absence of calcium ion and effect of adenosine 5'-diphosphate on reduced nicotinamide adenine dinucleotide inhibition. Biochemistry, 20, 2519-2524


Norden, D. A. and C. Matanganyidye (1979) Activities of Krebs' cycle enzymes in the flight muscles of the tsetse fly (Glossina) and the fleshfly (Sarcophaga). Insect Biochem., 9, 85-87


PART II. KETOGLUTARATE DEHYDROGENASE FROM CAULIFLOWER
(BRASSICA OLERACEA L.) MITOCHONDRIA:
PREPARATION AND REACTIVITY WITH SUBSTRATES
KEToglutarate Dehydrogenase from Cauliflower (Brassica Oleracea L.) Mitochondria: Preparation and Reactivity with Substrates

George A. Karam, M.Sc.
Stephen H. Bishop, Ph.D.

From the Department of Zoology, Iowa State University, Ames, IA 50011
ABSTRACT

The enzyme system ketoglutarate dehydrogenase (KGDH) was partially purified from mitochondria of cauliflower (Brassica oleracea L.) florettes. After sonication in the presence of amphotericin B, KGDH was partially purified by differential centrifugation in buffered sucrose solutions to give a final activity of 4-7 units/mg of protein. The preparations had low levels of pyruvate dehydrogenase (20%) and occasionally contained a small amount (10% of KGDH activity) of the internal rotenone-insensitive NADH oxidase activity. KGDH showed optimal activity at pH 7.4. Analysis of the kinetics at pH 7.4 showed an increase in $V_{\text{max}}$ from 28.7 nmol.min.$^{-1}$ml$^{-1}$ at pH 7.0 to 97 nmol.min.$^{-1}$ml$^{-1}$ at pH 7.4 and a decrease in $K_m$ from 115.5 uM at pH 7.0 to 564 uM at pH 7.4. The $n_{\text{app}}$ was equal to "1" at both pH 7.0 and 7.4. Addition of NAD at pH 7.0 and 7.4, showed an increase in $K_m$ for NAD to 441 uM and to 981 uM, respectively. In the presence of CoA, $K_m$ showed a decrease to 15.74 at pH 7.0 and 12.23 at pH 7.4. Addition of thiamine pyrophosphate (TPP) decreased the $K_m$ to 105.7 uM at pH 7.0 and 87.47 at pH 7.4. The $V_{\text{max}}$ showed a decrease under all conditions except in the presence of NAD at pH 7.0. Analysis of the $n_{\text{app}}$ in response to added cofactors showed no cooperativity at pH 7.0 and a slightly positive cooperativity at pH 7.4. Studies on the response of KGDH to the NAD/NADH showed a decline in the enzyme activity at a ratio of 5.0.
INTRODUCTION

In the tissues of higher plants, considerable amounts of proline accumulate in response to osmotic stress (Buhl and Stewart, 1983). Proline accumulation is regulated by several mitochondrial enzymes. The Krebs' cycle intermediate ketoglutarate (KG) serves as both the precursor for proline biosynthesis and the product of proline catabolism. The regulation of the KG utilization in the Krebs' cycle is then an essential aspect in the control of carbon flow to and from proline. Utilization of KG by the cycle is controlled by ketoglutarate dehydrogenase (KGDH) in the presence of TPP, CoA, and NAD, to produce \( \text{CO}_2 \), succinyl-CoA, and NADH.

Plant mitochondria have low concentrations of endogenous cofactors and can accumulate high amounts of NAD (Douce, 1985; Tobin et al., 1980), TPP (Day and Hanson, 1977), and CoA (Neuburger et al., 1984) suggesting a strong role for these cofactors in the regulation of mitochondrial matrix enzymes. NAD and other nucleotides are considered as powerful regulators of mitochondrial respiration (Neuburger and Douce, 1983).

The absolute requirement of KGDH for TPP (Douce, 1985), the active uptake of TPP and CoA by isolated mitochondria (Douce and Neuburger, 1987; Neuburger et al., 1984) and the sharing of CoA and TPP by KGDH, pyruvate dehydrogenase (PDH) and other mitochondrial dehydrogenases (Dry and Wiskich, 1987), mean that the KGDH activity
must be modulated by the varying mitochondrial concentrations of these cofactors and cosubstrates. This necessitated the study of KGDH activity in the presence of varying amounts of these cofactors and cosubstrates using an intact KGDH preparation.

Earlier kinetic studies on the cauliflower KGDH by Poulsen and Wedding (1970) were performed on preparations of a salt-extracted, reassociated enzyme. In order to obtain activity with these preparations, pig heart lipoamide dehydrogenase (Lip-DH) was added to the preparations. The endogenous Lip-DH and enzyme-bound TPP were dissociated from the complex during purification. There have been no studies of the optimal requirement of the plant enzyme for the various cofactors at various pH conditions that match the internal pH of plant mitochondria (7.3-7.5) (Martin et al., 1982; Roberts et al., 1982).

In this study, we report the partial purification of an intact KGDH preparation that was free from other competing ketoglutarate-utilizing activities and had low levels of contaminating PDH and NADH-oxidase activities. The effect of KG, NAD, CoA, TPP and NAD/NADH on the cauliflower KGDH activity, were also examined.
MATERIALS AND METHODS

Cauliflower (*Brassica oleracea* L.) was purchased from a local store. Chemical reagents were obtained from either the Sigma Chemical Co. or Fisher Scientific Co.

Mitochondrial preparation

The mitochondria were prepared by a variation of the Craig and Wedding (1980) procedure, using a high speed mechanical tissue disruptor. Routinely, one kg of the top one cm of cauliflower (*Brassica oleracea*) florettes was homogenized in two liters of 0.05 M MOPS pH 7.4, 1 mM KCl, 5 mM EDTA, 10 mM MgCl₂, and 0.6 M sucrose. The homogenization was accomplished using a Tekmar Ultraturrax homogenizer (Model SD45) for 40 s, at a power setting of 80. The homogenate was filtered through one layer of Miracloth (CalBiochem.) and the filtrate centrifuged at 8000 x g for 15 min. The pellet was resuspended in 100 ml of 0.05M MOPS pH 7.0, 0.1 mM EDTA, 10 mM MgCl₂ and 0.4 M sucrose, then centrifuged at 300xg for 10 min. The supernatant fluid was then centrifuged at 12000xg for 10 min and the ensuing pellet used as a crude mitochondrial preparation.

Enzyme preparation

The mitochondrial pellet from one kg of cauliflower florettes was resuspended in 100 ml of 0.05 M MOPS pH 7.0, 0.1 mM EDTA, 1 mM
EGTA and 10 mM MgCl₂ and containing: 0.1 mg/ml trypsin inhibitor, 34 uM leupeptin, 1 mM DTT, and 55 uM amphotericin B. The mitochondrial solution was shaken at 120 rpm for 20 min at room temperature in a Lab-Line orbital shaker, then centrifuged at 20200xg for 10 min. The pellet was resuspended in 50 ml of the above buffer in the absence of amphotericin B and the solution sonicated for 10 s at 3 mA and at 0 °C, in batches of 3ml each. The sonicated mitochondria were then centrifuged at 20200xg for 30 min and the supernatant diluted to 90 ml with the above buffer (less amphotericin B) and centrifuged at 150000xg for 90 min. The pellet was resuspended in 10 ml of 20 mM HEPES pH 7.5, 1 mM EGTA, 0.4 M sucrose and 0.1 mM DTT and incubated overnight at 8 °C. The enzyme solution was then cleared twice by centrifugation at 20000xg for 60 min, and the final supernatant fluid used as the source of enzyme activity.

Modulation by NAD, CoA, TPP, and the NAD/NADH

The response of KGDH to modulation by NAD, CoA, or TPP was measured by incubating the enzyme in an assay mixture of 0.5 ml, and in the presence of 250 uM ketoglutarate (KG), for 30 s. The reaction was initiated by the addition of the studied cofactor. The effect of NAD/NADH on the KGDH activity was evaluated by incubating the enzyme in the presence of 0.1 mM, 0.5 mM, or 1mM NAD and with increasing concentrations of NADH (0-0.1 mM), at pH 7.4. The reaction was initiated by the addition of 250 uM ketoglutarate.
Assay procedures

The enzyme activity was measured spectrophotometrically in a Beckman Model 3600 recording spectrophotometer by following NAD reduction at 340 nm. The standard assay mixture consisted of: 1.5 mM NAD, 0.3 mM CoA, 0.3 mM TPP, 3 mM DTT and 1 mM EGTA in 50 mM Tris-Ac (pH 7.0 or 7.4), and KG (5-1500uM). A unit of enzyme activity was defined as the amount of enzyme that converted 1 nmole of NAD/min.

The velocity curves of the enzyme were constructed as described by Segel (1975) and the kinetic values were calculated according to Cleland (1979). For the measurement of optimal pH activity, the pH of the assay mixture was adjusted to the specific value between pH 6.8 and 7.8, in the presence of 250 uM KG, and the reaction initiated by the addition of enzyme to a concentration of 100 ug/ml. NADH oxidase was assayed in 50 mM MOPS pH 7.4 and 1 mM NADH. PDH was assayed according to Paynter et al., 1985. Protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.
RESULTS AND DISCUSSION

The method used to prepare the enzyme yielded a preparation of about 25 ug protein per gram of cauliflower with a KGDH specific activity of 4-7 units/mg protein which was equal or greater than that obtained by Wedding and Black (1971). The amount of PDH obtained in the preparation (20% of the KGDH activity) was comparable to that obtained by Poulsen and Wedding (1970). More than 50% of the PDH activity was lost upon storage at 8 °C for 24 h.

Addition of amphotericin B to the mitochondria prior to sonication resulted in an increased yield of KGDH activity and a decreased NADH oxidase activity. In the presence of amphotericin B, the endogenous NADH oxidase obtained in some preparations represented no more than 10% of the total KGDH activity. The occasional NADH oxidase contamination could not be separated from the KGDH by either elution on a cellulose-phosphate column or by centrifugation in a 30-60% sucrose density gradient. Treatment by hydroxylapatite resulted in the deactivation of both enzymes. The oxidase was more resistant to successive freeze-thaw and heating cycles (2 min at 90 °C) than KGDH. In addition, the oxidase was resistant to KCN, rotenone, antimycin A, amytal, and salicyl hydroxamate (SHAM). Use of these inhibitors caused a complete inhibition of the KGDH. In contrast to the PDH from molluscs (Paynter et al., 1985), the cauliflower PDH was not inhibited by high concentrations of EDTA (5 mM).
Kinetics

Using a standard assay system with 50 mM Tris-Ac between pH 6.6-7.8 and in the presence of 250 μM KG and 1 mM EGTA, KGDH showed optimal activity at pH 7.4 (Fig. 1). Poulsen and Wedding (1970) and Wedding and Black (1971) found an optimal KGDH activity at pH 6.9 and 7.0, respectively. Addition of EGTA to 2 mM did not inhibit the enzyme activity.

The KGDH activity showed rectangular hyperbolic kinetic responses at pH 7.0 and pH 7.4 when the velocities were plotted against KG concentrations (Fig. 2). Analysis of the Hill plots showing no cooperative effects (n_app of 1.0) at pH 7.0 and 7.4 was in agreement with the previous results of Wedding and Black (1970).

The V_max at saturating [KG] at pH 7.4 was about 3.4-fold higher than that observed at pH 7.0. The K_m for KG at pH 7.4 (564 μM), was higher than that found at pH 7.0 (116 μM). The latter K_m value was in agreement with that of 120 μM observed by Poulsen and Wedding at pH 6.9 (1970) but differed from the values of 260-270 μM reported by Wedding and Black (1971) and Craig and Wedding (1980).

At pH 7.0, the K_m values for NAD, CoA, and TPP were about 441 μM, 16 μM, and 106 μM, respectively, whereas at pH 7.4, these values were 981 μM, 12 μM, and 88 μM, respectively (Table 1). The K_m values for NAD, CoA and TPP were 3-fold higher than those of KGDHs from other sources (Karam et al. 1987) but 3.3 and 7.5-fold lower than
Fig. 1. Optimal pH for the activity of KGDH. Aliquots of 0.5 ml assay mixtures containing 250 uM ketoglutarate were adjusted to different pH values (6.8-7.8) and prerun for one min, at 23 °C. Then, aliquots of 50 ug enzyme were added to each mixture and the NAD reduction followed at 340 nm.
Fig. 2. Kinetics of KGDH with increasing concentrations of substrate. Aliquots of 100 ug enzyme were assayed for 10 min at either pH 7.0 (A) or 7.4 (B). The assays were carried in the presence of 50 mM Tris-Ac, 1 mM EGTA, and with increasing concentrations of ketoglutarate, and at 23 °C.
the $K_m$ values for NAD and TPP and 3-fold higher than the $K_m$ for CoA reported previously by Craig and Wedding (1980).

The differences between the $K_m$ values for CoA and KG at pH 7.0 reported here (Table 1) and those observed by Poulsen and Wedding (1970) at the same pH may be related to differences in the preparation of the enzyme or the source of the plant (see also Day et al., 1984; Douce, 1985). The $K_m$ value at pH 7.4 probably reflects a more realistic picture of the in vivo physiological situation because plant mitochondrial pH is probably between 7.3 and 7.5 (Martin et al., 1982; Roberts et al., 1982).

**NADH oxidase activity**

The insensitivity of the NADH oxidase activity to rotenone, EGTA and Ca$^{2+}$, indicates that this enzyme is the internal rotenone-resistant, low affinity NADH oxidase (Moller and Palmer, 1982) that is linked to the respiratory chain via the ubiquinone pool (Palmer, 1976; Marx and Brinkmann, 1979). This would mean that the enzyme would be insignificant in competing for the binding of mitochondrial NADH except under state 4 conditions or any condition that would restrict electron flow and lead to an increase in mitochondrial NADH levels. In addition, this NADH oxidase has a $K_m$ for NADH of about 84 uM (Moller and Palmer, 1982; Moller and Lin, 1986). Therefore, the much lower amounts of NADH produced during the assay of the KGDH reaction means that the oxidase would play an insignificant role in
Table 1. Effect of NAD, CoA, and TPP on the kinetics of KGDH at pH 7.0 and 7.4. Assays for NAD, CoA and TPP were run in the presence of 250 μM ketoglutarate and at 23 °C, for 10 min. The reaction was initiated by the addition of NAD, CoA or TPP. NAD reduction was monitored spectrophotometrically at 340 nm.

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>pH</th>
<th>Km (μM)</th>
<th>Vmax (nmol.min⁻¹.ml⁻¹)</th>
<th>n_app</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoglutarate</td>
<td>7.0</td>
<td>115.53 ± 20.78</td>
<td>28.7 ± 1.91</td>
<td>0.94</td>
</tr>
<tr>
<td>Ketoglutarate</td>
<td>7.4</td>
<td>564.16 ± 63.57</td>
<td>97.04 ± 5.20</td>
<td>1.13</td>
</tr>
<tr>
<td>NAD</td>
<td>7.0</td>
<td>440.97 ± 105.23</td>
<td>60.94 ± 5.59</td>
<td>1.17</td>
</tr>
<tr>
<td>NAD</td>
<td>7.4</td>
<td>980.98 ± 120.88</td>
<td>36.47 ± 1.88</td>
<td>1.10</td>
</tr>
<tr>
<td>CoA</td>
<td>7.0</td>
<td>15.74 ± 4.56</td>
<td>23.96 ± 1.86</td>
<td>1.50</td>
</tr>
<tr>
<td>CoA</td>
<td>7.4</td>
<td>12.23 ± 2.05</td>
<td>30.53 ± 1.35</td>
<td>1.14</td>
</tr>
<tr>
<td>TPP</td>
<td>7.0</td>
<td>105.77 ± 36.67</td>
<td>20.15 ± 2.43</td>
<td>1.75</td>
</tr>
<tr>
<td>TPP</td>
<td>7.4</td>
<td>87.47 ± 17.13</td>
<td>34.53 ± 2.27</td>
<td>0.96</td>
</tr>
</tbody>
</table>
Fig. 3. Response of KGDH to the NAD/NADH ratio. Aliquots of 100 ug enzyme were incubated at pH 7.4, in assay mixtures containing 0.1 mM (●), 0.5 mM (△), or 1 mM (▲) NAD, with increasing concentrations of NADH (0-0.1 mM). The reaction was started by the addition of 250 μM ketoglutarate, at 23°C.
the utilization of the NADH produced and would not compromise the measurement of the KGDH activity.

**Effect of NAD/NADH on the enzyme activity**

The effect of the NAD/NADH at pH 7.4 and 0.1 mM NAD with increasing concentrations of NADH (Fig. 3) indicated a decline in the enzyme activity at a NAD/NADH of about 5. This indicated a lower tolerance of the cauliflower KGDH to higher reducing equivalents than the PDH and KGDH activity from animal sources (Paynter et al., 1985; Karam et al., 1987).

Since the inner mitochondrial NADH oxidases present in the matrix may share a common NAD pool with the NAD-linked enzymes of the Krebs' cycle (Douce, 1985), the intolerance of the KGDH to high concentrations of NADH relative to NAD would mean that the activity could be reduced considerably during anaerobiosis or inhibition of the oxidase capacity of the mitochondria.

With the exception of the inhibition by NADH and the low $K_m$ for CoA and TPP, the KGDH from cauliflower appears to be fairly similar to the activity in animal mitochondria. The use of the Ultraturrax, amphotericin B and the differential centrifugation of the sonicated mitochondria provides a rapid method for the preparation of this important plant mitochondrial enzyme.
LITERATURE CITED


Marx, R. and K. Brinkmann (1979) Effect of temperature on the pathway
of NADH-oxidation in broad-bean mitochondria. Planta 144, 359-365


presence of a rotenone-resistant NADH dehydrogenase on the inner
surface of the inner membrane of plant mitochondria. Physiol.
Plant., 54, 267-274

in plant mitochondria. Arch. Biochem. Biophys., 229, 253-258

Biochem. J., 216, 443-450

Palmer, J. M. (1976) The organization and regulation of electron
133-157

Pyruvate dehydrogenase complex from ribbed mussel gill
mitochondria. J. Exp. Zool., 236, 251-257

of the ketoglutarate dehydrogenase complex of cauliflower
mitochondria. J. Biol. Chem., 245, 5709-5717

Regulation of cytoplasmic and vacuolar pH in maize root tips under
different experimental conditions. Plant Physiol., 69, 1344-1347

Segel, I.H., (1975) Enzyme kinetics: Behavior and Analysis of Rapid
Equilibrium and Steady-State Enzyme Systems. A. Wiley Interscience
Publ., John Wiley & Sons, New York

Tobin, A., B. Djerdjour, E. journet, M. Neuburger, and R. Douce
(1980) Effect of NAD+ on malate oxidation in intact plant

Wedding, R. T. and M. K. Black (1971) Nucleotide activation of
cauliflower ketoglutarate dehydrogenase. J. Biol. Chem., 246,
1638-1643
PART III. ACTIVATION OF CAULIFLOWER KETOGLUTARATE DEHYDROGENASE BY CALCIUM IONS
ACTIVATION OF CAULIFLOWER KETOGLUTARATE
DEHYDROGENASE BY CALCIUM IONS

George A. Karam, M.Sc.
Stephen H. Bishop, Ph.D.

From the Department of Zoology, Iowa State University, Ames, IA 50011
ABSTRACT

The intramitochondrial enzyme system ketoglutarate dehydrogenase (KGDH) (E.C. 1.2.4.2) was partially purified from cauliflower (Brassica oleracea L.) florettes in the presence of EGTA. KGDH was found to be highly activated by Ca\(^{2+}\) with an optimal activation at pH 7.0. Addition of Ca\(^{2+}\) at pH 7.0 and pH 7.4 caused an increase in $V_{\text{max}}$ of 14-fold and 4.3-fold, respectively. Addition of Ca\(^{2+}\) decreased the $K_m$ for ketoglutarate from 115.5 uM to 37.3 uM at pH 7.0, and from 564 uM to 126 uM at pH 7.4. The $n_{\text{app}}$ was equal to 1.0, indicating the absence of any cooperative binding. Titration of the protein with increasing concentrations of Ca\(^{2+}\) and in the presence of 1 mM EGTA, showed a $K_s$ value of 1 uM at pH 7.0 and 0.28 uM at pH 7.4. Measurement of the Ca\(^{2+}\) in the mitochondrial lysate indicated a total amount of 300 nmol/mg protein and about 20-50 nmol of free Ca\(^{2+}\)/mg protein. Addition of Ca\(^{2+}\) at pH 7.0 or 7.4, increased the $V_{\text{max}}$ in the presence of NAD, CoA, and thiamine pyrophosphate (TPP) but, decreased the $K_m$ only in the presence of NAD and TPP and increased it in the presence of CoA. No calcium activating effect could be shown on the pyruvate dehydrogenase, NAD-isocitrate dehydrogenase, or the internal NADH-oxidase both in the presence or absence of EGTA. We suggest that the exit of the mitochondrial calcium to the cytoplasm will result in a decrease in
the activity of KGDH. The subsequent entry of Ca$$^{++}$$ into the mitochondrion will activate KGDH.
INTRODUCTION

The physiological role of calcium as an intracellular messenger is important in the regulation of animal mitochondrial matrix enzymes and is well established (Hansford, 1985). In animals, several mitochondrial enzymes of intermediary metabolism are known to be regulated by changes in mitochondrial calcium levels (Campbell, 1983; Denton and McCormack, 1980; Akerman and Nicholls, 1983). In the Krebs' cycle, three key enzymes catalyze reactions that are known to be activated by Ca\(^{2+}\). These are: ketoglutarate dehydrogenase (KGDH), NAD-Isocitrate dehydrogenase (NAD-ICDH), and pyruvate dehydrogenase (PDH), (Denton et al., 1980; McCormack and Denton, 1981, Hansford, 1985; Carafoli, 1987).

In plants on the other hand, calcium plays an important role in the regulation of various structural, electrical, and physiological processes (Smith, 1978; Weisenseel and Ruppert, 1977; Gillet and Lefebvre, 1978; Beilby, 1984; Williamson, 1984; Macklon, 1975; Robinson, 1977; Salisbury and Floyd, 1978; Williamson and Ashley, 1982). The role of calcium as a messenger and a regulator of mitochondrial matrix enzymes is still debatable. In the last few years, some evidence has been presented that supports its role as a messenger (Anderson and Cormier, 1978; Dieter, 1984; Marme, 1986a, 1986b; McEuen et al. 1981; Sabnis and McEuen, 1986). But, with the exception of a few measurements of intracellular (Ashley and
Williamson, 1981; Williamson and Ashley, 1982; Sabnis and McEuen, 1986) and mitochondrial plant Ca\(^{2+}\) (Moore and Akerman, 1982; Akerman and Moore, 1983), little is known about the role of plant mitochondria as a calcium buffering organelle and the effect of changing calcium levels on the activity of plant mitochondrial enzymes.

Recently, a role for calcium in sensing changes in cell volume has been suggested (Kauss, 1987). This implies that in osmotically stressed plants, calcium might play an important role in relaying cellular changes to the mitochondrial matrix and controlling both the uptake and the utilization of cofactors needed for the activation of matrix enzymes. It is therefore of considerable importance to study the interaction of the various cofactors with key mitochondrial enzymes and the role of calcium in regulating the utilization of these compounds.

In higher plants, cells that are under osmotic stress accumulate high concentrations of proline and proline turnover occurs in the mitochondrion (Aspinall and Paleg, 1981). The Krebs' cycle intermediate, ketoglutarate (KG) serves as a precursor for proline biosynthesis and as an end product of proline catabolism. Control of carbon flow between proline and the Krebs' cycle requires regulation of KGDH, the enzyme controlling KG utilization by the Krebs' cycle. KGDH catalyses the oxidative decarboxylation of KG in the presence of NAD, TPP and CoA from succinyl-CoA, CO\(_2\), and NADH. Blockage of KGDH...
will result in a shunting of carbon toward proline biosynthesis whereas the activation of KGDH will promote proline catabolism.

In higher plants, with the exception of glutamate dehydrogenase activation by calcium in mitochondrial extracts of *Pisum* and green tobacco callus (Joy, 1973; Takahashi and Furuhashi, 1980; Furuhashi and Takahashi, 1982), no evidence on the calcium activation of any mitochondrial matrix enzyme has been presented (Denton and McCormack, 1980; Randall et al. 1981; McCormack and Denton, 1981, 1986). In this study, we report the activation of cauliflower KGDH by μM concentrations of Ca^{2+} and its interaction with the various cofactors necessary for its regulation.
MATERIALS AND METHODS

Cauliflower (Brassica oleracea L.) was purchased from a local store. Chemical reagents were obtained from either the Sigma Chemical Co. or Fisher Scientific Co.

Enzyme preparation

The mitochondria were prepared by a variation of the procedure described by Craig and Wedding (1980) as described in (Karam and Bishop, 1988). The KGDH activity was prepared by the previously described method (Karam and Bishop, 1988). All isolation steps were performed at 4 °C. All labware necessary for the experiments was pretreated with 1 mM EGTA before use. For the measurement of intramitochondrial calcium levels, only plasticware was used in order to avoid interaction with calcium ions from glass.

Measurement of intramitochondrial calcium

For the measurement of intramitochondrial Ca\(^{2+}\), the mitochondria were isolated in 0.05 M MOPS buffer pH 7.4, 0.6 M sucrose and, in the presence or absence of 1 mM EGTA. The final mitochondrial pellet was resuspended in 0.05 M MOPS buffer at pH 7.0 and frozen for 24 h at -20 °C. The mitochondrial fraction was then thawed, sonicated for 10 s at 7 mA then, cleared by centrifugation at 20000 x g for 15 min and the supernatant stored at 4 °C. For measuring the total calcium
content, an amount of 100 μL of mitochondrial lysate containing 0.1 mg protein, was resuspended in 1.9 ml of 0.1% Sterox and the calcium content determined with a Perkin-Elmer model 51 Ca, Coleman flame photometer, using 0.5 mM CaCl₂, and 1 mM CaCl₂ as low and high standards, respectively. Free calcium levels in mitochondrial extracts were measured with a calibrated Orion Research calcium electrode.

The level of free Ca²⁺ in the standards was controlled by Ca²⁺-EGTA buffers at pH 7.0 and 7.4 in 56 mM Tris-Ac and 1 mM EGTA. The dissociation constants for Ca²⁺-EGTA binding were calculated from the tables of Martell and Smith (1974), as discussed in (Blinks et al., 1982; Grynckiewicz et al., 1985; and Tsien and Rink, 1980).

Assay procedures

The KGDH activity was measured using a Beckman model 3600 spectrophotometer by following NAD reduction at 340 nm in an assay mixture consisting of 1.5 mM NAD, 0.3 mM CoA, 0.3 mM TPP, 3 mM DTT and 1 mM EGTA, in 50 mM Tris-Ac pH 7.0 or 7.4, and varying concentrations of KG (5-1500μM) at 23 °C. The velocity curves of the enzyme were constructed as described by Segel (1975) and the kinetic values were calculated according to Cleland (1979). For the measurement of the variation in activity with pH, the pH of the assay mixture was adjusted to the specific value (6.6-8.0) in the presence of 250 μM KG, 1 mM EGTA and 2 mM CaCl₂. The reaction was initiated
by the addition of enzyme to a concentration of 100 ug/ml, and the NAD reduction followed at 23 °C.

Routinely, PDH was assayed at pH 7.2, according to Paynter et al. (1985), and in the presence of 750 uM pyruvate and 1 mM EGTA.

The inner rotenone-insensitive NADH oxidase found in the preparation (10%), was assayed in the presence of 0.2 mM NADH for 3 min, then 1 mM EGTA and 2 mM CaCl₂ were added and the change in absorption recorded at 340 nm.

The NAD-ICDH activity was measured according to (Kargbo and Swift, 1983) in the presence of 1 mM EGTA and increasing concentrations of CaCl₂ (1-5 mM).

Protein concentration was measured by the method of Lowry et al. (1951), using BSA as a standard. A unit of activity was defined as the amount of enzyme that reduced one nmole of NAD per min.

**Ca²⁺ titration of KGDH activity**

Aliquots of 100 ug of protein were incubated for one min in an assay mixture of 0.5 ml containing 120 uM ketoglutarate (KG) at pH 7.0 or 250 uM KG at pH 7.4, and in the presence of 1 mM EGTA. The reaction mixture was incubated for two min, then increasing concentrations of CaCl₂ were added and the velocity recorded.
**Activation of KGDH by Ca^{2+}**

For assays of KGDH activation by Ca^{2+}, aliquots of 100 ug of enzyme were incubated for 30 s in the KGDH assay mixture at either pH 7.0 or 7.4 in the presence of 1 mM EGTA, then CaCl_{2} (1 mM) (19 uM free Ca^{2+} at pH 7.0 and 7.4 uM free Ca^{2+} at pH 7.4) was added and the change in the reaction rate recorded. The reaction incubations were for 5 min at 23 °C, with increasing concentrations of KG (1-750 uM at pH 7.0 and 5-1250 uM at pH 7.4).

**Ca^{2+} activation of KGDH in the presence of NAD, CoA, and TPP**

Assays of KGDH activation were performed at increasing concentrations of NAD (30-1200 uM at pH 7.0, 6-3000 uM at pH 7.4), CoA (0.3-450 uM at pH 7.0, 1.2-300 at pH 7.4), and TPP (0.06-42 uM at pH 7.0, 0.3-450 uM at pH 7.4). Aliquots of 100 ug enzyme were incubated for 30 s in an assay mixture containing 1 mM EGTA and 250 uM KG at either pH 7.0 or 7.4. The reactions were run for 5 min in the presence of the studied cofactor, then CaCl_{2} was added to 1 mM (19 uM free Ca^{2+} at pH 7.0, and 7.4 uM at pH 7.4) and the NAD reduction recorded.

**Response of PDH to added Ca^{2+}**

To measure the possible calcium reversal of PDH inhibition by ATP in cauliflower, 7 units of PDH were incubated in the presence of 100 mM ATP at pH 7.4 and for 10 min, then dialyzed twice against 250
ml of 50 mM MOPS buffer at 4 °C. The dialysed fraction was then assayed in the presence of 1 mM EGTA and 2.0 mM CaCl₂ (1 mM free Ca²⁺).
RESULTS

The total mitochondrial Ca\(^{2+}\) measured by the atomic absorption spectrometric procedure was 300-400 nmol/mg protein, in mitochondria isolated either in the presence or absence of 1 mM EGTA. Alternatively, the extract from mitochondria isolated in the presence of 1 mM EGTA showed free Ca\(^{2+}\) levels of 15-20 nmol/mg protein. The extract from those isolated in the absence of EGTA showed free Ca\(^{2+}\) levels of 40-50 nmol/mg protein.

Titration of the KGDH activity in the assay medium at increasing concentrations of Ca\(^{2+}\) and in the presence of 1 mM EGTA as a Ca\(^{2+}\) buffering system showed a K\(_s\) of 1 uM at pH 7.0, and 0.28 uM at pH 7.4, or 1 nmol free Ca\(^{2+}\)/mg protein and 0.28 nmol free Ca\(^{2+}\)/mg protein, respectively (Fig. 1). Saturating concentrations of free Ca\(^{2+}\) (19 uM at pH 7.0 and 7.4 uM at pH 7.4) obtained from Fig. 1 and corresponding to a total concentration of 1 mM CaCl\(_2\) were used in assays in which calcium activation of the enzyme was studied.

Assay of the enzyme in the presence of 1 mM EGTA and both in the presence or absence of 1 mM CaCl\(_2\) at pH 6.6-8.0, indicated an optimal enzyme activity at pH 7.4 but optimal activation by Ca\(^{2+}\) at pH 7.0 (data not shown). In the presence of Ca\(^{2+}\) and at pH 7.0, the K\(_m\) for KG was 3.4-fold lower than that observed at pH 7.4. The V\(_{\text{max}}\) of KGDH remained essentially unchanged (Table 1).
Kinetic studies of the enzyme activity at pH 7.0 or 7.4, in the presence or absence of Ca$^{2+}$, showed rectangular hyperbolic responses when the reaction velocities were plotted against KG concentrations. Addition of 1 mM Ca$^{2+}$ at pH 7.0 in the presence of 1 mM EGTA (19 uM free Ca$^{2+}$, caused an increase in $V_{\text{max}}$ for KG of 12-fold and a decrease in $K_m$ of 3.10-fold. (Fig. 2A). At pH 7.4, addition of 1 mM Ca$^{2+}$ and 1 mM EGTA (7.4 uM free Ca$^{2+}$) caused an increase in $V_{\text{max}}$ of 3.18-fold and a decrease in $K_m$ of 4.47-fold (Fig. 2B). Analysis of the Hill plots showed no cooperative effects ($n_{\text{app}}=1.0$) with respect to changing KG concentrations in the presence or absence of Ca$^{2+}$.

Assay of KGDH activity in the absence and presence of 1 mM Ca$^{2+}$ and 1 mM EGTA at pH 7.0 (19 uM free Ca$^{2+}$), and at varying NAD, CoA and TPP concentrations (Table 1), indicated a 6-fold increase in the $K_m$ for CoA and a decrease in the $K_m$ for NAD and TPP by 1.7-fold and 69-fold, respectively, with added calcium ions. Under these conditions, the $V_{\text{max}}$ was increased by 6 to 20-fold with varying NAD, CoA and TPP concentrations. Alternatively, under these substrate conditions, addition of Ca$^{2+}$ at pH 7.4 (7.4 uM free Ca$^{2+}$) increased the $K_m$ for CoA by 3.65-fold and lowered the $K_m$ for NAD and TPP by 3.7-fold and 19-fold, respectively and increased the $V_{\text{max}}$ by 5 to 6-fold with varying NAD, TPP and CoA concentrations (Table 1).
Fig. 1. Titration of the cauliflower KGDH by Ca\(^{2+}\) ions. Aliquots of 100\,\mu\text{g} Enzyme were preincubated for one min at 23°C, in the KGDH assay medium and in the presence of 1\,\text{mM} EGTA and 120\,\mu\text{M} KG at pH 7.0 (IA), or 1\,\text{mM} EGTA and 250\,\mu\text{M} KG at pH 7.4 (IB). Increasing concentrations of CaCl\(_2\) (0.1-1.7\,\text{mM}) were added and the NAD reduction recorded at 340 nm.
Fig. 2. Kinetic study of the cauliflower KGDH activity. Aliquots of 125 μg enzyme were preincubated for two min at 23 °C, in the presence of 1 mM EGTA and in the absence of KG. Increasing concentrations of KG were added in the presence (●) or absence (○) of 1 mM Ca^{2+}, and at pH 7.0 (19 μM free Ca^{2+}) (2A) or pH 7.4 (7.4 μM free Ca^{2+}) (2B) and the NAD reduction recorded at 340 nm.
Table 1. Effect of NAD, CoA, and TPP on the kinetics of KGDH at pH 7.0 and 7.4. Assays were run for 5 min in the presence of 1 mM EGTA, and at 23 °C, in the presence of increasing concentrations of NAD, CoA, and TPP. KG was used at a concentration of 120 μM at pH 7.0 or 250 μM at pH 7.4. CaCl₂ was then added (1 mM) and the NAD reduction recorded for another 5 min at 340 nm.

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>pH</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol.min⁻¹.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG</td>
<td>pH 7.0</td>
<td>115.53 ± 20.78</td>
<td>28.7 ± 1.91</td>
</tr>
<tr>
<td>KG</td>
<td>pH 7.4</td>
<td>564.16 ± 63.57</td>
<td>97.04 ± 5.20</td>
</tr>
<tr>
<td>KG + Ca²⁺</td>
<td>pH 7.0</td>
<td>37.31 ± 2.74</td>
<td>345.00 ± 5.65</td>
</tr>
<tr>
<td>KG + Ca²⁺</td>
<td>pH 7.4</td>
<td>126.12 ± 11.22</td>
<td>308.43 ± 8.11</td>
</tr>
<tr>
<td>NAD</td>
<td>pH 7.0</td>
<td>440.97 ± 105.23</td>
<td>60.94 ± 5.59</td>
</tr>
<tr>
<td>NAD</td>
<td>pH 7.4</td>
<td>980.98 ± 120.88</td>
<td>36.47 ± 1.88</td>
</tr>
<tr>
<td>NAD + Ca²⁺</td>
<td>pH 7.0</td>
<td>254.00 ± 45.18</td>
<td>383.54 ± 21.58</td>
</tr>
<tr>
<td>NAD + Ca²⁺</td>
<td>pH 7.4</td>
<td>265.84 ± 18.10</td>
<td>205.26 ± 3.82</td>
</tr>
<tr>
<td>CoA</td>
<td>pH 7.0</td>
<td>15.74 ± 4.56</td>
<td>23.96 ± 1.86</td>
</tr>
<tr>
<td>CoA</td>
<td>pH 7.4</td>
<td>12.23 ± 2.05</td>
<td>30.53 ± 1.35</td>
</tr>
<tr>
<td>CoA + Ca²⁺</td>
<td>pH 7.0</td>
<td>95.55 ± 9.48</td>
<td>470.70 ± 16.00</td>
</tr>
<tr>
<td>CoA + Ca²⁺</td>
<td>pH 7.4</td>
<td>44.58 ± 5.75</td>
<td>187.33 ± 4.84</td>
</tr>
<tr>
<td>TPP</td>
<td>pH 7.0</td>
<td>105.77 ± 36.67</td>
<td>20.15 ± 2.43</td>
</tr>
<tr>
<td>TPP</td>
<td>pH 7.4</td>
<td>87.47 ± 17.13</td>
<td>34.53 ± 2.27</td>
</tr>
<tr>
<td>TPP + Ca²⁺</td>
<td>pH 7.0</td>
<td>1.53 ± 0.21</td>
<td>404.92 ± 13.68</td>
</tr>
<tr>
<td>TPP + Ca²⁺</td>
<td>pH 7.4</td>
<td>4.58 ± 1.53</td>
<td>197.42 ± 15.50</td>
</tr>
</tbody>
</table>
PDH is a key enzyme of the Krebs' cycle that could be activated by Ca\textsuperscript{2+} (McCormack and Denton, 1986) therefore, affecting the flow of carbon towards the production of KG. In animal sources, phosphorylation of the enzyme by a kinase in the presence of ATP leads to the inhibition of its activity (Paynter et al. 1985). This inhibition could be reversed by the Ca\textsuperscript{2+} activation of a phosphatase that dephosphorylates the enzyme and makes it responsive to the effect of Ca\textsuperscript{2+}. When assayed at pH 7.2, the PDH activity in this mitochondrial extract showed no response to added calcium (1-5 mM) both in the presence or absence of 1-5 mM EGTA. Incubation of PDH with 100 mM ATP caused a complete inhibition of the PDH activity. Addition of 2 mM CaCl\textsubscript{2} to the inhibited PDH did not cause any activation either in the presence or absence of 1 mM EGTA indicating the absence of any phosphatase activation by Ca\textsuperscript{2+}.

Response of ICDH and NADH oxidase to added Ca\textsuperscript{2+}

The assayed NAD-ICDH showed no response to the addition of Ca\textsuperscript{2+}.

The NADH oxidase obtained in some extracts was found to be the low affinity rotenone-insensitive enzyme of the inner mitochondrial membrane (Karam and Bishop, 1988). This oxidase activity was not affected by the addition of 1 mM EGTA and showed no response to added CaCl\textsubscript{2} (2 mM).
DISCUSSION

Throughout the experiments, calcium levels in the medium were controlled at low concentrations by the use of the EGTA buffering system which circumvented the interference of calcium released from the cells during enzyme preparation. This caused a more pronounced calcium effect on the activity of KGDH.

The calcium concentrations observed in the cauliflower mitochondria are higher than values previously obtained in animal cells which show a total calcium concentration of 1-2 nmol/mg protein (Hansford, 1985; Carafoli, 1987). These high values from the cauliflower reflect the high rate of calcium uptake in plants (McCormack and Denton, 1986) and the wide range of total calcium content of 1-50 mg/g dry weight observed in many plants (Kirkby and Pilbeam, 1984). Except for the mitochondrial calcium values of 40-700 µM observed by Moore and Akerman (1982) and Akerman and Moore (1983) and the difficulties of measuring plant mitochondrial calcium content (Gilroy et al., 1986), no accurate and widely accepted values have been reported.

In this study, the mitochondria were lysed by freeze-thawing and sonication but were not solubilized which would tend to reduce the amount of protein obtained and raised the total relative calcium content. The values reported here for cauliflower mitochondria are somewhat higher than those observed in isolated corn mitochondria.
which had a total Ca\(^{2+}\) of 80 nmol/mg protein and a free Ca\(^{2+}\) of 0.5-3 nmol/mg protein in the soluble high speed mitochondrial extract (Karam and Bishop, unpublished). The total mitochondrial calcium obtained in mitochondria isolated in the absence of EGTA was not changed by the addition of EGTA to the isolation medium. This indicated the absence of any leakage of calcium ions across the mitochondrial membrane either independently or due to its binding by EGTA.

Titration of the KGDH activity showed a \(K_s\) for calcium of 0.28 and 1.0 \(\mu\)M at pH 7.4 and 7.0, respectively. This is in agreement with the widely accepted idea that mitochondrial matrix enzymes are activated at calcium concentrations of 0.1-10 \(\mu\)M with a half-maximal activation around 1 \(\mu\)M (Somlyo, 1984; McCormack and Denton, 1986).

The increase in \(V_{\text{max}}\) upon calcium addition and in the presence of NAD, CoA and TPP indicates that the enzyme was dependent on Ca\(^{2+}\) for maximal activation in the presence of these cofactors. Alternatively, the increase in \(K_m\) for CoA in the presence of Ca\(^{2+}\) indicates that the calcium binding site affects the CoA binding site and decreases the affinity of the enzyme to CoA.

These results show clearly the activation of cauliflower KGDH by micromolar, physiological concentrations of Ca\(^{2+}\). This activation is manifested mainly as an increase in the \(V_{\text{max}}\) of the KGDH activity and a decrease in the \(K_m\) for KG when assayed as a function of KG concentrations. In addition, preliminary studies with corn KGDH show
that although present at a much lower concentration than the cauliflower KGDH, it is also activated (V_{max}) by 15-20-fold with uM amounts of Ca^{2+} (Karam and Bishop, unpublished).

The fact that cauliflower PDH and NAD-ICDH are unresponsive to Ca^{2+} makes KGDH a key regulatory enzyme in the Krebs' cycle. These results are in contradiction with the postulation by McCormack and Denton (1981, 1986) that plant enzymes of the Krebs' cycle and specifically PDH, NAD-ICDH, and KGDH are not calcium regulated. In addition, it appears that at such a relatively high mitochondrial Ca^{2+} concentration, KGDH is most likely to be under continuous activation by Ca^{2+} and therefore highly dependent on Ca^{2+} for its key regulatory function.

It is evident that in salt-stressed plants, the concentration of intracellular calcium is increased reflective of any changes in cell volume (Kauss, 1987). This calcium has to be drawn from the external medium or the internal stores, specifically the endoplasmic reticulum and the mitochondria. This will most likely result in a decrease in the activity of KGDH and directs the carbon flow at the level of KG towards the production of glutamate and the subsequent accumulation of proline as an osmolyte. Under this situation, the activity of the Krebs' cycle is reduced to the minimum. Upon the relief of the stress, the subsequent uptake of Ca^{2+} by the mitochondria would lead to the activation of KGDH and glutamate dehydrogenase (GDH). At the intracellular pH (7.3-7.5) (Martin et al., 1982; Roberts et al.,
1982), the presence of mM amounts of Ca$^{2+}$ causes a 6-fold increase in the $V_{\text{max}}$ of tobacco GDH (Furuhashi and Takahashi, 1982). Therefore, the presence of Ca$^{2+}$ in the mitochondria will lead to a fast utilization of KG, thereby favoring a reduction in glutamate levels. It follows then, that activation of KGDH and GDH by Ca$^{2+}$ will favor the utilization of accumulated proline and, shifts the carbon flow from proline synthesis and toward proline catabolism. The effect of Ca$^{2+}$ on the proline-glutamate interconversion in the plant mitochondria remains to be investigated.
LITERATURE CITED


Ashley, C. C., and R. E. Williamson (1981) Cytoplasmic free Ca\textsuperscript{2+} and streaming velocity in characean algae; measurement with microinjected aequorin. J. Physiol. (London), 319, 103


Gillet, C., and J. Lefebvre (1978) Ionic diffusion through the Nitella cell wall in the presence of calcium. J. Exp. Bot., 29, 1155-1159


Macklon, A. E. S. (1975) Cortical cell fluxes and transport to the stele in excised root segments of *Allium cepa* L. II. Calcium. *Planta (Berlin)*, 122, 131-141


PART IV. REGULATION OF CAULIFLOWER KETOGLUTARATE DEHYDROGENASE BY ADENINE NUCLEOTIDES, CoA DERIVATIVES, AND NaCl
REGULATION OF CAULIFLOWER KETOGLUTARATE
DEHYDROGENASE BY ADENINE NUCLEOTIDES, CoA
DERIVATIVES, AND NaCl

George A. Karam, M.Sc.
Stephen H. Bishop, Ph.D.

From the Department of Zoology, Iowa State
University, Ames, IA 50011
ABSTRACT

The enzyme ketoglutarate dehydrogenase (KGDH) plays an important role in the regulation of proline accumulation in salt-stressed plants. In this study, KGDH was partially purified from the mitochondria of the cauliflower *Brassica oleracea* L. and was inhibited by ATP at both pH 7.0 and 7.4. Alternatively, AMP and ADP activated the enzyme by increasing the $V_{\text{max}}$ and lowering the $K_m$ at both pH 7.0 and 7.4. The activation of the enzyme was optimal at 0.5 mM AMP. This activation could be increased by the addition of either MgCl$_2$ or CaCl$_2$ to a final concentration of 1 mM. The addition of acetyl-, butyryl-, propionyl-, and succinyl-CoA inhibited the enzyme by 8-21%. Addition of AMP, CaCl$_2$, or MgCl$_2$ reversed the inhibition by the CoA derivatives. In the presence of guanine and inosine nucleotides and ATP analogs, the enzyme showed a response ranging from 33% inhibition to 150% activation. Addition of Mg$^{2+}$ and Ca$^{2+}$ in the presence of the above nucleotides reactivated the enzyme to a range of 200-700%. NaCl inhibited the enzyme by increasing the $K_m$ for KG at pH 7.0 and by both increasing the $K_m$ for KG and decreasing the $V_{\text{max}}$ at pH 7.4. The inhibition by NaCl was reversed by both Ca$^{2+}$ and AMP at all the studied pH values.
INTRODUCTION

Under conditions of osmotic stress, high amounts of proline were shown to accumulate in the tissues of higher plants (Buhl and Stewart, 1983). The accumulation, oxidation, and incorporation of proline into the Krebs' cycle are regulated by the nonequilibrium catalyzing multienzyme system of ketoglutarate dehydrogenase (KGDH). Some key intramitochondrial parameters play an important role in the regulation of plant KGDH and the carbon flux into the Krebs' cycle. The role of the various cofactors: CoA, NAD, and Thiamine pyrophosphate (TPP) and the role of NAD/NADH as an energy-linked modulator have been discussed earlier (Karam and Bishop, 1988a). In addition, Ca\(^{2+}\) has been found to play an important role in the regulation of plant KGDH (Karam and Bishop, 1988b). The complexity of mitochondria from higher plants and the role that that the metabolically important substrates play as major sources of energy are reflected on the operation of the Krebs' cycle and the recycling of carbon intermediates. Studies on KGDH from various insect (Hansford, 1972), animal (Lawlis and Roche, 1981a, 1981b; McCormack and Denton, 1979) and plant sources (Wedding and Black, 1971) showed that the enzyme is profoundly inhibited by ATP and activated by ADP and AMP. On the other hand, Lawlis and Roche (1981a) showed that the $K_m$ for KGDH from bovine kidney was increased when nucleotides were added in the presence of Ca\(^{2+}\). In addition, GTP was found to have an
inhibitory effect on the enzyme. The role of Mg-ATP as a major form under which ATP is present in the cell and its effect on the activation of KGDH remain to be clarified. The role played by CoA as an activator of NAD-linked malic enzymes and a cofactor for pyruvate dehydrogenase and KGDH is well established (Macrae, 1971; Neuberger et al., 1984; Day et al., 1984; Dry and Wiskich, 1987). The regulation of mammalian KGDH by the CoA/succinyl-CoA ratio is well established (Garland, 1964; Smith et al., 1974). It follows that the various CoA derivatives as well as the ATP analogs might play a major regulatory role of the Krebs' cycle in the presence of Ca^{2+}, Mg^{2+}, and the low energy nucleotides. In addition, the response of KGDH to increasing concentrations of NaCl in response to osmotic stress and in the presence of AMP and the divalent cations would give an insight into the regulation of the enzyme under environmental changes and in the presence of the possibly accumulated modulators of enzyme activity.

In this study, we report the response of cauliflower KGDH activity to various nucleotides, ATP analogs, CoA derivatives, and NaCl both in the presence or absence of Ca^{2+} and Mg^{2+}. We also discuss the role played by these modulators in the control of the Krebs' cycle via the regulation of KGDH and the subsequent effect of this phenomenon on the regulation of the mitochondrial energy metabolism.
MATERIALS AND METHODS

Mitochondrial and enzyme preparation

Cauliflower (Brassica oleracea L.) was purchased from a local store. Chemical reagents were obtained from the Sigma Chemical Co. and Fisher Scientific Co. The mitochondria were prepared by a variation of the procedure described by Craig and Wedding (1980) as described in (Karam and Bishop, 1988a). The enzyme was isolated as described in (Karam and Bishop, 1988a). Intracellular calcium measurements involved were carried as in (Karam and Bishop, 1988b). All isolation steps were carried at 4 °C.

Activation of KGDH by Ca²⁺ and Mg²⁺

Assays of KGDH activation by Ca²⁺ were performed as described by Karam and Bishop (1988b). Assays of enzyme activation by 1 mM Mg²⁺ were run under the same conditions and in the presence of 1 mM EGTA except that the total free [Mg²⁺] obtained was equal to 0.97 mM at pH 7.0, and 0.92 mM at pH 7.4.

The velocity curves were constructed as described by Segel (1975). Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. One unit of enzyme activity was defined as the amount of enzyme that would reduce one nmole of NAD per min.
Regulation of KGDH activity by adenine nucleotides and ATP analogs

The AMP concentration necessary for optimal activation of KGDH was determined by adding increasing concentrations of AMP (50-2000 uM) to aliquots of KGDH in the presence of 120 uM (pH 7.0) or 250 uM (pH 7.4) KG. These KG concentrations represent $K_m$ values of KGDH at the specified pH and in the absence of any enzyme modulator. The kinetics of KGDH activation by AMP, ADP and ATP were studied by assaying the enzyme in the presence of 0.5 mM AMP, ADP, or ATP and increasing concentrations of KG (10-400). The response of KGDH to the various nucleotides and ATP analogs was studied by assaying the enzyme in the presence of GMP, GDP, GTP, IMP, IDP, AMP-PCP, and AMP-PNP, in the presence of 250 uM KG and at pH 7.4. The response of KGDH to the activation by Ca$^{2+}$ or Mg$^{2+}$ after subjecting it to modulation by the nucleotides, was studied by adding CaCl$_2$ or MgCl$_2$ to the assay medium (in the presence of 1 mM EGTA) and to a final total concentration of 1 mM and recording the change in NAD reduction.

Regulation of KGDH activity by the CoA derivatives

To find the optimal CoA concentration necessary for the modulation of KGDH, aliquots of the enzyme were assayed at pH 7.0 in the presence of 250 uM KG, 1 mM EGTA, and increasing concentrations of acetyl-CoA (50-500 uM). Finally, CaCl$_2$ was added to a final total concentration of 1 mM and the activation of KGDH recorded.
To study the effect of CoA derivatives on KGDH activity, the enzyme was assayed at pH 7.4 and in the presence of 250 uM KG and 0.1 mM of each of the CoA derivatives: acetyl-CoA, butyryl-CoA, propionyl-CoA, and succinyl-CoA. Finally, AMP (0.5 mM), CaCl$_2$ (1 mM), or MgCl$_2$ (1 mM) were added to the mixture and the response of the enzyme recorded.

Effect of NaCl on the KGDH activity

Aliquots of the enzyme were assayed at pH 7.0 (120 uM KG) or 7.4 (250 uM KG) and in the presence of increasing concentrations of NaCl (5-120 mM). To test for the role of Ca$^{2+}$ in reactivating the NaCl-inhibited KGDH, the enzyme was assayed in the presence of NaCl for two min then CaCl$_2$ was added and the enzyme activation recorded. The effect of pH on the salt-inhibited KGDH was tested by incubating the enzyme for two min in the presence of 10 mM NaCl and at various pH values (7.0-7.6) then assaying it in the presence of 250 uM KG. The effect of AMP on the activity of the inhibited KGDH at those pH values was studied by adding AMP (0.5 mM) to the assayed aliquots and recording the NAD reduction.
RESULTS AND DISCUSSION

In contrast with the results obtained by Wedding and Black (1971), the optimal AMP concentration necessary for the activation of KGDH at both pH 7.0 and pH 7.4 was 0.5 mM (Fig. 1). Activation of the enzyme by AMP at both pH values showed a 3.3-fold decrease in $K_m$ at pH 7.0 in contrast with a 7.5-fold decrease at pH 7.4. On the other hand, the $V_{max}$ was increased by 9.8-fold and 2.8-fold at both pH 7.0 and 7.4, respectively. In the presence of 0.5 mM ADP, the $K_m$ was decreased by 1.7-fold at pH 7.0 and 6.2-fold at pH 7.4 with an increase in $V_{max}$ of 4.3-fold and 1.2-fold at pH 7.0 and 7.4, respectively (Table 1, Fig. 2). Addition of 0.5 mM ATP caused a complete inhibition of the enzyme at both pH values. The addition of 1 mM MgCl$_2$ in the presence of AMP, ADP, or ATP caused no change in the response of the enzyme to AMP or ADP at pH 7.4, but decreased the $K_m$ in the presence of AMP by 2.3-fold with no change in the $K_m$ obtained in the presence of ADP at pH 7.0 (Table 1). On the other hand, at pH 7.0, the $V_{max}$ was increased by an additional 2-fold and 4.6-fold in the presence of AMP and ADP, respectively. Addition of MgCl$_2$ in the presence of ATP reactivated the enzyme that was inhibited in the presence of ATP alone and resulted in a $K_m$ that is 1.3-fold and 19.5-fold lower than the one obtained at both pH 7.0 and pH 7.4, respectively. Addition of Mg$^{2+}$ in the presence of ATP and at pH 7.0 and pH 7.4, increased the $V_{max}$ by 9.6-fold and decreased it by
Fig. 1. Optimal AMP concentration for the activation of KGDH. Aliquots of 100 μg/ml KGDH were assayed in the presence of 120 μM KG at pH 7.0 (●) or 250 μM KG at pH 7.4 (○), for 2 min then, increasing concentrations of AMP (50-2000 μM) were added and the NAD reduction monitored at 340 nm.
Table 1. Effect of the various modulators on the kinetics of KGDH at varying KG concentrations. Assays were run in the presence of 1 mM EGTA, and at 23 °C, with increasing [KG] (5-1200 uM). CaCl₂ or MgCl₂ were added (1mM) and the NAD reduction monitored at 340 nm.

<table>
<thead>
<tr>
<th>Measured Substrate</th>
<th>pH</th>
<th>Kₘ (uM)</th>
<th>Vₘₐₓ (nmol.min⁻¹.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>pH 7.0</td>
<td>115.53 ± 20.78</td>
<td>28.7 ± 1.91</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>pH 7.0</td>
<td>37.31 ± 2.74</td>
<td>345.00 ± 5.65</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>pH 7.0</td>
<td>138.08 ± 21.90</td>
<td>332.36 ± 18.54</td>
</tr>
<tr>
<td>NONE</td>
<td>pH 7.4</td>
<td>564.16 ± 63.57</td>
<td>97.04 ± 5.20</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>pH 7.4</td>
<td>126.12 ± 11.22</td>
<td>308.43 ± 8.11</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>pH 7.4</td>
<td>512.87 ± 44.60</td>
<td>157.43 ± 6.30</td>
</tr>
<tr>
<td>AMP</td>
<td>pH 7.0</td>
<td>34.96 ± 3.23</td>
<td>280.01 ± 7.86</td>
</tr>
<tr>
<td>AMP + Mg²⁺</td>
<td>pH 7.0</td>
<td>15.25 ± 9.22</td>
<td>569.79 ± 99.81</td>
</tr>
<tr>
<td>AMP ± Mg²⁺</td>
<td>pH 7.4</td>
<td>74.97 ± 5.37</td>
<td>270.76 ± 8.24</td>
</tr>
<tr>
<td>ADP</td>
<td>pH 7.0</td>
<td>69.50 ± 6.75</td>
<td>123.74 ± 4.41</td>
</tr>
<tr>
<td>ADP + Mg²⁺</td>
<td>pH 7.0</td>
<td>68.87 ± 13.82</td>
<td>573.79 ± 35.15</td>
</tr>
<tr>
<td>ADP ± Mg²⁺</td>
<td>pH 7.4</td>
<td>91.96 ± 8.70</td>
<td>117.74 ± 4.76</td>
</tr>
<tr>
<td>ATP + Mg²⁺</td>
<td>pH 7.0</td>
<td>92.96 ± 19.64</td>
<td>275.59 ± 22.69</td>
</tr>
<tr>
<td>ATP + Mg²⁺</td>
<td>pH 7.4</td>
<td>28.88 ± 8.68</td>
<td>23.97 ± 3.27</td>
</tr>
<tr>
<td>NaCl</td>
<td>pH 7.0</td>
<td>336.88 ± 172.78</td>
<td>31.31 ± 8.77</td>
</tr>
<tr>
<td>NaCl + Ca²⁺</td>
<td>pH 7.0</td>
<td>33.02 ± 3.41</td>
<td>324.60 ± 7.30</td>
</tr>
<tr>
<td>NaCl</td>
<td>pH 7.4</td>
<td>191.82 ± 25.52</td>
<td>33.80 ± 2.11</td>
</tr>
<tr>
<td>NaCl + Ca²⁺</td>
<td>pH 7.4</td>
<td>258.55 ± 62.50</td>
<td>433.30 ± 54.14</td>
</tr>
<tr>
<td>NaCl + AMP</td>
<td>pH 7.4</td>
<td>70.14 ± 9.28</td>
<td>194.76 ± 7.83</td>
</tr>
</tbody>
</table>
Fig. 2. Kinetics of KGDH activation by AMP and ADP. Aliquots of 100 μg/ml KGDH were assayed for two min, in the presence of increasing concentrations of KG (10-400 μM) (○) at pH 7.0 (2 A) or at pH 7.4 (2 B). Independently, 0.5 mM AMP (△) or ADP (□) were added to each assay and the increase in NAD reduction recorded.
1.2-fold, respectively. The above results indicate that the effect of Mg\(^{2+}\) in the presence or absence of AMP, ADP or ATP is mainly on the \(V_{\text{max}}\) of the enzyme at pH 7.0 while in the absence of Mg\(^{2+}\), AMP and ADP activated the enzyme by increasing the \(V_{\text{max}}\) at pH 7.0 and lowering the \(K_m\) at pH 7.4.

The fact that AMP and ADP activate KGDH while ATP inhibits it indicates that KGDH is activated at a lower energy state. This is supported by the fact that cauliflower KGDH is active under a low NADH concentration and subsequently, a high NAD/NADH (Karam and Bishop, 1988a).

In contrast with the effect observed with MgCl\(_2\), addition of 1-2 mM CaCl\(_2\) both in the presence or absence of 1 mM EGTA, and to samples of KGDH that are inhibited by ATP fail to reverse the inhibition of the enzyme.

Assay of the enzyme activity at pH 7.4, in the presence of 250 uM KG and various nucleotides and ATP analogs (Fig. 3) reveal a response ranging from about 30% inhibition in the presence of GTP or AMP-PNP to 5-50% activation in the presence of the other modulators. Addition of 1 mM CaCl\(_2\) in the presence of 1 mM EGTA (free Ca\(^{2+}\) = 7.4 uM) cause an activation of all samples by 3.5 to 10.5-fold as compared to the activity recorded in the absence of Ca\(^{2+}\). Activation of the enzyme by Ca\(^{2+}\) and in the presence of GMP, GDP, and GTP show a 1.2 to 1.4-fold activation more than the samples with CaCl\(_2\) alone. Addition of 1 mM MgCl\(_2\) in the presence of EGTA (free Mg\(^{2+}\) = 90 uM).
Fig. 3. Response of KGDH to the various nucleotides and ATP analogs in the presence of CaCl$_2$, and MgCl$_2$. Aliquots of KGDH were assayed at pH 7.4 and 250 mM KG (■), in the presence of 0.5 mM GMP, GDP, GTP, IMP, IDP, AMP-PCP, and AMP-PNP. Then, 1 mM of either CaCl$_2$ (■) or MgCl$_2$ (■) were added to each sample and the NAD reduction recorded.
and the various nucleotides caused an increase in activity of 1.5 to 3.5-fold when compared to the samples assayed in the absence of Mg\(^{2+}\) (Fig. 3). These results show that Ca\(^{2+}\) is more effective than Mg\(^{2+}\) as an activator of KGDH.

Assay of KGDH at pH 7.0 in the presence of increasing concentrations of acetyl-CoA (50-500 \(uM\)) (Fig. 4), showed 86% inhibition of the enzyme at a CoA concentration of 0.1 mM. Addition of 1 mM CaCl\(_2\) in the presence of 1 mM EGTA (free Ca\(^{2+}\) = 19 \(uM\)) reversed the inhibition and activated the enzyme to about 97% of the activity observed in the presence of Ca\(^{2+}\) alone. The activation by Ca\(^{2+}\) was maintained to a CoA concentration of 0.2 mM. In the presence of 500 \(uM\) acetyl-CoA which resulted in the complete inhibition of the enzyme, the addition of CaCl\(_2\) reactivated the enzyme to about 57% on the activity observed in the presence of Ca\(^{2+}\) and the absence of CoA.

Assay of KGDH at pH 7.4 in the presence of 250 \(uM\) KG and 0.1 mM acetyl-CoA, butyryl-CoA, propionyl-CoA, and succinyl-CoA (Fig. 5) caused an inhibition of the enzyme activity by 8-21%. Addition of 0.5 mM AMP reversed the inhibition by the CoA derivatives completely and reactivated the enzyme by about 4.7-fold, a value equal to the activation of the enzyme by AMP in the absence of any CoA derivatives. Addition of 1 mM CaCl\(_2\) in the presence of EGTA reactivated the enzyme by 5 to 6-fold both in the presence or absence
Fig. 4. Calcium reversal of KGDH inhibition by acetyl-CoA. An amount of 85 µg/ml KGDH was assayed at pH 7.0, in the presence of 250 µM KG, 1 mM EGTA and increasing concentrations of acetyl-CoA (50-500 µM) (•). At the end, 1 mM CaCl₂ (○) was added to each assay and the NAD reduction recorded.
Fig. 5. Response of KGDH to the various CoA derivatives in the presence of AMP, CaCl₂, and MgCl₂. Aliquots of KGDH were assayed at pH 7.4 and 250 μM KG (■), in the presence of 0.1 mM acetyl-CoA, butyryl-CoA, propionyl-CoA, or succinyl-CoA. The assay mixtures were made 1 mM in CaCl₂ (■), 1 mM in MgCl₂ (■), or 0.5 mM in AMP (□) and the activation of KGDH recorded.
of CoA. In the presence of 1 mM MgCl₂ and 1 mM EGTA, the enzyme was activated by 5.6 to 6.6-fold, similar to the effect observed in the presence of Ca²⁺ except that the addition of MgCl₂ in the absence of CoA derivatives resulted in a modest 1.9-fold activation as compared to the activity observed in the absence of Mg²⁺.

The inhibition of KGDH by NaCl and its reactivation by CaCl₂ were studied at pH 7.0 in the presence of 120 μM KG and at 7.4 in the presence of 250 μM KG (Fig. 6). Assay of the enzyme in the presence of 5 mM NaCl resulted in about 25-50% inhibition at both pH 7.0 and pH 7.4. This inhibition was increased to 75% with increasing concentrations of NaCl (120 mM). The addition of 1 mM CaCl₂ in the presence of 1 mM EGTA and at pH 7.0 reversed the inhibition and activated the enzyme to about 85% of the activity observed in the presence of Ca²⁺ alone (Fig. 6A) and to about 90% of the activity observed at pH 7.4 under the same conditions (Fig. 6B). The reactivation of the enzyme by CaCl₂ decreased with increasing concentrations of NaCl and reached a level of 22-24% at 120 mM for both pH 7.0 and pH 7.4. Higher concentrations of NaCl (120-500 mM) did not result into further inhibition of the enzyme but caused an almost complete loss in the ability of Ca²⁺ to reverse the inhibition by NaCl (2% reactivation) (data not shown).

Inhibition of KGDH at various pH values (7.0-7.6) in the presence of 250 μM KG and 10 mM NaCl (Fig. 7) showed an increase in
Fig. 6. Calcium reversal of KGDH inhibition by NaCl. Aliquots of 200 ug/ml KGDH were assayed for 2 min at pH 7.0, and in the presence of 120 uM KG (6 A) or at pH 7.4 with 250 uM KG (6 B). The assays were run in the presence of increasing concentrations of NaCl (5-120 mM) (●). At the end of each assay, CaCl₂ was added to a total concentration of 1 mM and the increase in NAD reduction monitored (○).
Fig. 7. AMP reversal of KGDH inhibition by NaCl at different pH values. Aliquots of KGDH (100 ug/ml) were incubated for two min at various pH values (7.0-7.6), and in the presence of 10 mM NaCl then, KG was added to a concentration of 250 uM and the enzyme assayed for 3 min (○). At the end, 0.1 mM AMP (●) was added to each assay and the NAD reduction recorded at 340 nm.
the inhibition of the enzyme by NaCl with increasing pH. The enzyme inhibition showed a constant increase from 20% at pH 7.0 to 80% at pH 7.4. Addition of 0.1 mM AMP to the inhibited KGDH resulted in the reactivation of the enzyme to 90-105% of the original activity observed in the absence of NaCl with an optimal activation at pH 7.2-7.4.

Kinetic studies of KGDH in the presence of NaCl and at pH 7.0 and 7.4 (Table 1) showed a 3-fold increase in the $K_m$ for KG with no change in the $V_{max}$ at pH 7.0, and a 3-fold decrease in the $K_m$ for KG with a 3-fold decrease in $V_{max}$ at pH 7.4, when compared to the values observed in the absence of NaCl. Addition of calcium ions to the NaCl-treated KGDH, activated the enzyme by both lowering the $K_m$ and increasing the $V_{max}$ by 10-fold at pH 7.0. At pH 7.4, addition of calcium increased both the $K_m$ and the $V_{max}$ by 1.4-fold and 13-fold, respectively, as compared to values observed in the presence of NaCl alone.

Kinetic studies on the effect of 0.5 mM AMP on NaCl-treated KGDH at pH 7.4 and increasing concentration of KG (Table 1), showed an activation of the enzyme with a 2.7-fold decrease in the $K_m$ and a 5.9-fold increase in the $V_{max}$ as compared to salt-treated samples assayed in the absence of AMP.
LITERATURE CITED


Garland, P. B. (1964) Some kinetic properties of pig heart oxoglutarate dehydrogenase that provide a basis for metabolic control of the enzyme activity and also a stoichiometric assay for coenzyme A in tissue extracts. Biochem. J., 92, #2, 11C-12C


Lawlis, V. B. and T. E. Roche (1981a) Regulation of bovine kidney ketoglutarate dehydrogenase complex by calcium ion and adenine nucleotides. Effects on S0.5 for ketoglutarate. Biochem., 20, 2512-2518

Lawlis, V. B. and T. E. Roche (1981b) Inhibition of bovine kidney ketoglutarate dehydrogenase complex by reduced nicotinamide adenine dinucleotide in the presence or absence of calcium ion and effect of adenosine 5'-diphosphate on reduced nicotinamide adenine dinucleotide inhibition. Biochem., 20, 2519-2524
110


GENERAL DISCUSSION AND SPECULATIONS

In the mitochondria, KGDH participates in the control of the flow of carbon from proline, glutamate and isocitrate into the Krebs' cycle. It appears from the results discussed above that the enzyme from bivalve molluscs, that from plant sources (cauliflower) and that from mammalian heart, kidney, or liver (Lawlis and Roche, 1980, 1981a, 1981b; McCormack and Denton, 1981; Roche and Lawlis, 1982) are regulated in basically different ways. A major factor in the regulation of the Krebs' cycle in both animals and plants is the concentration of ADP which is the result of ATP turnover and leads to the control of mitochondrial oxidation. The fact that succinyl-CoA and other CoA derivatives are generally unable to leave the mitochondrion after production necessitates the need for their cycling and regeneration of CoA. These derivatives should not accumulate therefore, the inhibition or activation of KGDH by the nucleotides may not be a very effective regulatory mechanism unless this regulation is relayed to the enzyme through the regulation of NAD/NADH or some other modulators. It is of some interest then that the CoA derivatives inhibit the activity of the enzyme from both mussel gills and cauliflower to a much greater extent than that seen with the mammalian enzyme. The finding that KGDH from mussel gills operates at a lower NAD/NADH than that of the enzyme from cauliflower may indicate the greater tolerance for the hypoxic or reduced
environment of the mussel KGDH compared to the cauliflower or mammalian system. The inability of cauliflower KGDH to maintain its optimal activity at high NADH values may also be related to the fact that plant NADH oxidases which lower NADH levels, are more numerous and more resistant to inhibitors of electron flow than the enzymes from animal sources (Storey, 1980). The results discussed above showed that the NADH oxidase obtained in the KGDH preparation is the internal rotenone-insensitive, low-affinity oxidase. The cyanide-insensitive plant dehydrogenases can contribute reducing power to the external NADH oxidase at high ATP concentrations (Meeuse, 1975). Since the KGDH from cauliflower is cyanide-sensitive and is inhibited by 0.5 mM ATP, then the NADH oxidase obtained in some KGDH preparations would not interfere with the oxidation of ketoglutarate. The relative function of the two oxidase systems in plants is not well understood in that some plants can shift from one pathway to the other and deliver their NADH to a different oxidase in response to stress or injury (Day et al., 1980). Possible coupling of the KGDH reaction to these oxidase systems needs to be investigated.

The role of calcium as an important intracellular messenger and regulator of mitochondrial enzymes is well established in animal systems (Hansford, 1985). In plants on the other hand, the role of calcium and its levels in the cell and in the mitochondrion is important but still debatable (Duncan, 1976; Dieter, 1984). In plants, calcium ions play an important role in maintaining the
integrity of the cell membrane and its deficiency renders the membrane permeable and the enzymes susceptible to the effect of metabolites and inhibitory modulators (Simon, 1978). The isolation of calmodulin from plants (Anderson and Cormier, 1978; Van Eldik et al., 1980; Grand et al., 1980; Dieter, 1984) indicates the presence of very low intracellular concentrations which makes the determination of the exact amounts of free and bound calcium ions and the measurement of its uptake uncertain. Calcium ions also bind to cytoplasmic and mitochondrial membranes and appear to affect many factors related to plant growth such as temperature, salinity, light, growth period, age, etc. (Clark, 1976).

McCormack and Denton (1986) have suggested from data obtained with potato KGDH, PDH and ICDH that calcium ion is probably not a regulator of mitochondrial metabolism in plants. From the studies presented here it is evident that these ideas need to be revised. Calcium ion is a strong regulator of plant KGDH and probably all of plant mitochondrial metabolism.

From the point of view of osmotic stress in plant cells, the transport of salt causes loss of some carrier and solute-transporting proteins (Heppel, 1969; Nieman and Willis, 1971). The transport of salt also consumes ATP and releases ADP therefore stimulating respiration and activating glycolysis. This process promotes the production of sugars and other compatible solutes such as proline (Adams, 1970; Adams and Rowan, 1972). This new ionic environment
might last for a short period after salt is removed (Poole, 1976) probably due to the inhibition of membrane transporter proteins that release the osmolites from the cell.

Positive regulation of KGDH during salt stress is a real possibility. For instance in a plant cell, osmotic stress is sensed by the membrane. This triggers a series of events that open the calcium stores and tend to promote removal of calcium ions from the mitochondria (Kauss, 1987). If the plant accumulates proline during salt stress, the plant mitochondria would have to exclude calcium ions, thereby reducing KGDH activity and diverting carbon flow from the Krebs' cycle towards glutamate and proline. With relief from the salt stress, calcium ions would have to enter the mitochondrion and cause activation of KGDH to promote the removal or oxidation of the proline carbon skeleton by the Krebs' cycle.
LITERATURE CITED


Lawlis, V. B. and T. E. Roche (1981b) Inhibition of bovine kidney -ketoglutarate dehydrogenase complex by reduced nicotinamide adenine dinucleotide in the presence or absence of calcium ion and effect of adenosine 5'-diphosphate on reduced nicotinamide adenine dinucleotide inhibition. Biochemistry, 20, 2519-2524


