Inhibition of Bovine Kidney α-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†

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ABSTRACT: Micromolar Ca^{2+} markedly reduces NADH inhibition of bovine kidney α-ketoglutarate dehydrogenase complex [Lawlis, V. B., & Roche, T. E. (1980) *Mol. Cell. Biochem. 32*, 147–152]. Product inhibition patterns from initial velocity studies conducted at $<10^{-9}$ M or at 1.5×10^{-5} M Ca^{2+} with NAD^+, CoA, or α-ketoglutarate as the variable substrate showed that NADH was a noncompetitive inhibitor with respect to each of these substrates, except at high NAD^+ concentrations, where reciprocal plots were nonlinear and the inhibition pattern for NADH vs. NAD^+ changed from a noncompetitive to a competitive pattern. From slope and intercept replots, 2-fold to 12-fold higher inhibition constants were estimated for inhibition by NADH vs. the various substrates in the presence of 1.5×10^{-5} M Ca^{2+} than for inhibition at $<10^{-9}$ M Ca^{2+}. These inhibition patterns and the lack of an effect of Ca^{2+} on the inhibition of the dihydrolipoyl dehydrogenase component suggested that Ca^{2+}-modulated NADH inhibition occurs at an allosteric site with competitive binding at the site by high levels of NAD^+. Decarboxylation of α-keto[1-^{14}C]glutarate by the resolved α-ketoglutarate dehydrogenase component was investigated in the presence of 5.0 mM glyoxylate which served as an efficient acceptor. NADH (0.2 mM) or 1.0 mM ATP inhibited the partial reaction whereas 15 μM Ca^{2+}, 1.0 mM ADP, or 10 mM NAD^+ stimulated the partial reaction and reduced NADH inhibition of this reaction. Thus these effectors alter the activity of the α-ketoglutarate dehydrogenase complex by binding at allosteric sites on the α-ketoglutarate dehydrogenase component. Inhibition by NADH over a wide range of NADH/NAD^+ ratios was measured under conditions in which the level of α-ketoglutarate was adjusted to give matching control activities at $<10^{-9}$ M Ca^{2+} or 1.5×10^{-5} M Ca^{2+} in either the presence or the absence of 1.6 mM ADP. These studies established that both Ca^{2+} and ADP decreased NADH inhibition under conditions compensating for the effects of Ca^{2+} and ADP on $S_{0.5}$ for α-ketoglutarate. ADP was particularly effective in reducing NADH inhibition; further studies are required to determine whether this occurs through binding of NADH and ADP at the same, overlapping, or interacting sites.

The α-ketoglutarate dehydrogenase complex is inhibited by the products NADH and succinyl-CoA (Garland, 1964; Smith et al., 1974). In addition, the activity of the α-ketoglutarate dehydrogenase complex from heart and kidney is enhanced both by micromolar Ca^{2+} and, independently, by ADP, through decreases in $S_{0.5}$ for α-ketoglutarate, and is inhibited by ATP due to an increase in this parameter (McCormack & Denton, 1979; Lawlis & Roche, 1981). In studies on the activity of bovine kidney α-ketoglutarate dehydrogenase complex, we noted that chelation of Ca^{2+} by EGTA[1] not only lowered the initial velocity of the complex by increasing the $S_{0.5}$ for α-ketoglutarate but also caused a sharp decrease in the rate of reaction with time. This suggested that one or more products became a more potent inhibitor at subnanomolar levels of Ca^{2+}. We subsequently presented evidence that this results, at least in part, from enhanced NADH inhibition (Lawlis & Roche, 1980). In the presence of NADH, Ca^{2+} decreased NADH inhibition of the kidney α-ketoglutarate dehydrogenase complex and produced greater than an 18-fold increase in the activity of the complex over the range of $<10^{-9}$–10^{-5} M Ca^{2+} whereas, in the absence of NADH, there was only a 2.2-fold increase in the activity of the complex due to a decrease in $S_{0.5}$ for α-ketoglutarate (Lawlis & Roche, 1980).

In the present communication, we have evaluated the effects of Ca^{2+} on the product inhibition patterns for NADH vs. the different substrates of the overall reaction catalyzed by the α-ketoglutarate dehydrogenase complex. These inhibition patterns together with the lack of an effect of Ca^{2+} on NADH inhibition of the dihydrolipoyl dehydrogenase suggested the possibility that there may be an allosteric site for inhibition by NADH. Evidence is presented that NADH directly inhibits the partial reaction catalyzed by the resolved α-ketoglutarate dehydrogenase component and that, in addition to Ca^{2+}, ADP or NAD^+ decreases NADH inhibition of the partial reaction.

Experimental Procedures

Materials. Chemicals were from the sources cited in the preceding paper (Lawlis & Roche, 1981), and the bovine kidney α-ketoglutarate dehydrogenase complex was prepared and stored as previously described (Roche & Cate, 1977). A preparation with an initial specific activity of 12.5 μmol min^{-1} mg^{-1} was used, and it lost less than 12% of its activity during storage in the course of these experiments. For each experiment, the enzyme was diluted to 1.0 mg mL^{-1} (at 4 °C) in a buffer consisting of 40 mM Mops–K^+ (pH 7.5) and 1.0 mM DTT.

We were unable to find conditions with different chaotropic

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; $S_{0.5}$, substrate concentration giving half-maximal velocity.

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salts to resolve the complex without inactivation of the α-ketoglutarate dehydrogenase component. However, a highly active component was obtained by using papain to specifically cleave the dihydrolipoyl transsuccinylase core. We utilized the conditions that Kresze et al. (1980) developed for resolution of the bovine kidney pyruvate dehydrogenase complex. As in the studies of Linn (1974), the cleaved transsuccinylase core did not disassemble, and we separated the free α-ketoglutarate dehydrogenase component by pelleting the core through a two-step (5% and 10%) sucrose pad. The resolved components were maintained in the presence of leupeptin (Sigma) to inhibit papain.

Assay of the α-Ketoglutarate Dehydrogenase Complex. Enzyme activity was determined as described in the preceding paper (Lawlis & Roche, 1981) except that all studies were conducted at pH 7.4. At this pH and a Mg^{2+} concentration of 2 mM, assays in the presence of 1.0 mM EGTA or 5.0 mM EGTA–Ca^{2+} were calculated to give free Ca^{2+} concentrations of $<10^{-9}$ M and 1.5×10^{-5}M, respectively. Free Ca^{2+} concentrations were calculated by using the association constants and procedures of Portzehl et al. (1964). Assays were initiated by addition of 5.0 μg of purified α-ketoglutarate dehydrogenase complex, unless otherwise indicated. Other conditions were as indicated in figure legends.

Assay of the Dihydrolipoyl Dehydrogenase Component. Dihydrolipoyl dehydrogenase activity was measured by monitoring production of NADH at 340 nm (30 °C). The assay medium contained 20 mM potassium phosphate (pH 7.4), 0.20 mM dihydrolipoamide, and 50 mM MOPS–K^+ with a final pyridine nucleotide concentration of 0.40 mM. The assays in the presence or virtual absence of Ca^{2+} contained 5.0 mM EGTA–Ca^{2+} or 1.0 mM EGTA, respectively. All of the reaction components, except the enzyme and dihydrolipoamide, were equilibrated at 30 °C, and the final pH was 7.4. Dihydrolipoamide was added just prior to initiation of the reaction with 5.0 μg of the kidney α-ketoglutarate dehydrogenase complex.

Assay of the α-Ketoglutarate Dehydrogenase Component. The activity of the α-ketoglutarate dehydrogenase component was measured by monitoring the release of $^{14}CO_2$ from α-keto[1-^{14}C]glutarate. In a total volume of 0.5 mL, the assay medium included 100 mM MOPS–K^+ (pH 7.4), 5.0 mM sodium glyoxylate, 4.0 mM $MgCl_2$, and 0.2 mM thiamin pyrophosphate. The Ca^{2+} concentration was controlled as described above. The enzyme was equilibrated in reaction medium at 30 °C for 1 min, and the reaction was initiated by the addition of 0.1 μmol of α-keto[1-^{14}C]glutarate (1.0 μCi/μmol) and terminated after 2.5 min by injection of 0.3 mL of 0.1 M H_2SO_4 through the rubber stopper of the serum bottle in which the assay was conducted. $^{14}CO_2$ was captured in a gelatin capsule containing phenethylamine, which was removed 1 h later for counting.

Results

Effects of Ca^{2+} on Product Inhibition of the α-Ketoglutarate Dehydrogenase Complex and Dihydrolipoyl Dehydrogenase Component. Ca^{2+} reduces NADH inhibition of the bovine kidney α-ketoglutarate dehydrogenase complex over a wide range of NADH/NAD^+ ratios with a half-maximal effect at about 1.0 μM free Ca^{2+} (Lawlis & Roche, 1980). We have measured the extent of inhibition of succinyl-CoA for a wide range of succinyl-CoA/CoA ratios and found that it was not significantly changed when free Ca^{2+} was present at 1.5×10^{-5} or $<10^{-9}$ M (data not shown).

The dihydrolipoyl dehydrogenase component, which catalyzes the partial reaction generating NADH, is interchangeable between the pyruvate and α-ketoglutarate dehydrogenase complex (Sakurai et al., 1970). However, NADH inhibition of the kidney pyruvate dehydrogenase complex, as noted previously, is not altered by the presence or absence of Ca^{2+} (Lawlis & Roche, 1980). Interestingly, in the presence of Ca^{2+}, the relative inhibition by NADH over a wide range of NADH/NAD^+ ratios was the same for the α-ketoglutarate dehydrogenase complex and for the pyruvate dehydrogenase complex (data not shown). This indicates that the enhanced NADH inhibition associated with Ca^{2+} reduction to subnanomolar concentration results from release of Ca^{2+} from a specific site on the bovine kidney α-ketoglutarate dehydrogenase complex which leads to an altered component or component interaction that is unique to the α-ketoglutarate dehydrogenase complex.

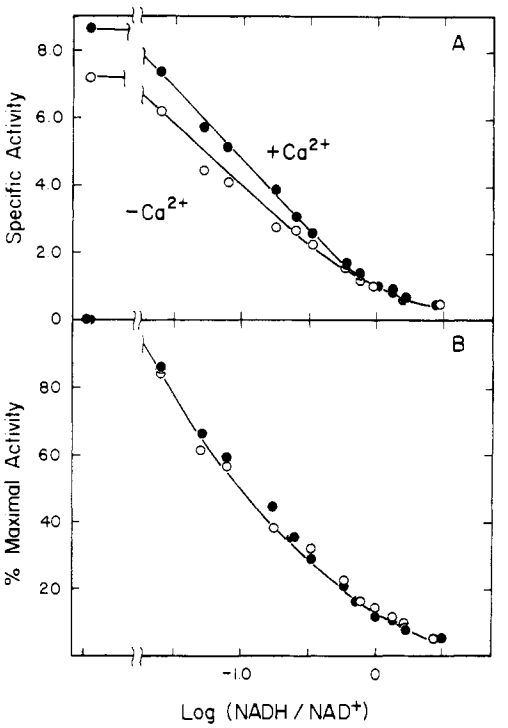

FIGURE 1: Effect of Ca^{2+} and the NADH/NAD^+ ratio on dihydrolipoyl dehydrogenase activity in the intact α-ketoglutarate dehydrogenase complex. Panel A presents changes in specific activity whereas panel B presents changes in the percent maximal activity for data shown in panel A. The points on the left side of the figure show control values in the absence of NADH. The dihydrolipoamide concentration was 0.20 mM for experiments in the presence (1.5×10^{-5} M (●) or absence ($<10^{-9}$ M) (○) of Ca^{2+}. (In the presence of a higher dihydrolipoamide concentration, 1.0 mM, specific activities of 24.4 or 18.6 μmol min^{-1} mg^{-1} protein was observed for studies in the presence or absence of Ca^{2+}.) Other conditions were as described under Experimental Procedures.

Figure 1 shows the effect of Ca^{2+} on NADH inhibition of the activity of the dihydrolipoyl dehydrogenase component (still associated with the α-ketoglutarate dehydrogenase complex) in a model reaction that utilizes free dihydrolipoamide as a substrate for reduction of NAD^+. Changes in specific activities are shown in Figure 1A whereas changes in the relative inhibition by NADH are shown in Figure 1B. Specific activities were slightly reduced at $<10^{-9}$ M Ca^{2+}, but there was no enhancement of the relative level of inhibition by NADH. Thus, the overall reaction catalyzed by the complex or a partial reaction catalyzed by a different component would appear to be required to detect Ca^{2+} modulation of NADH inhibition.

To evaluate the mechanism whereby Ca^{2+} decreases NADH inhibition, we investigated the inhibitory effects of NADH under conditions in which NAD^+, CoA, and α-ketoglutarate

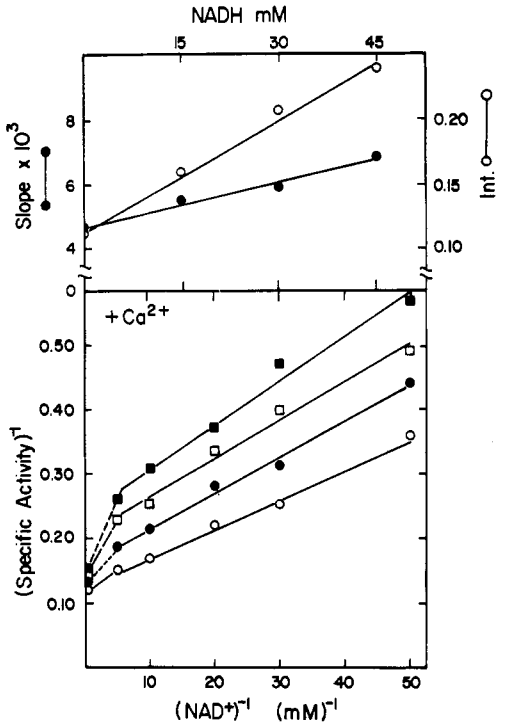

FIGURE 2: NADH inhibition with NAD^+ as the varied substrate in the presence of Ca^{2+}. The lower portion is a double-reciprocal plot in which each point is an average of duplicate assays. The upper panel shows replots of slopes and extrapolated y-intecept values from the linear portion of the double-reciprocal plot vs. NADH concentrations. α-Ketoglutarate and CoA concentrations were 1.0 and 0.12 mM, respectively; NAD^+ was present at the concentrations indicated. The concentrations of added NADH were 0 (○), 15.0 μM (●), 30.0 μM (□), and 45.0 μM (■). Other conditions were as described under Experimental Procedures.

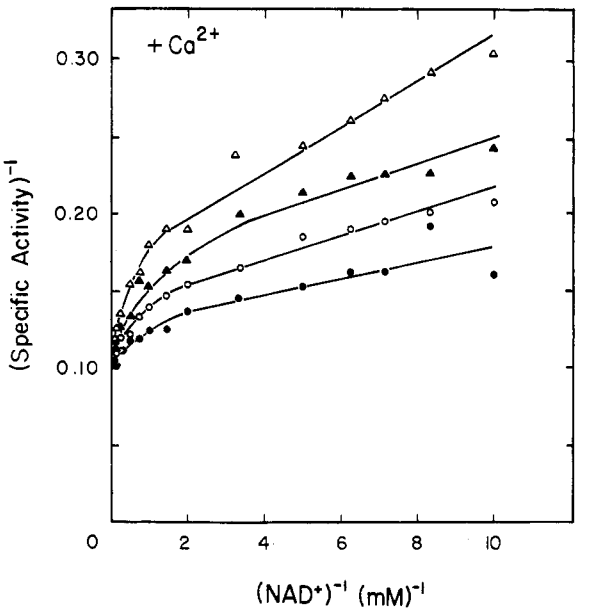

FIGURE 3: NADH inhibition with NAD^+ varied from 0.1 to 10.0 mM in the presence of Ca^{2+}. Except for the higher range of NAD^+ concentrations, conditions were as described in Figure 2 and Experimental Procedures. The concentrations of added NADH were 0 (●), 15.0 μM (○), 30.0 μM (▲), and 45.0 μM (△).

were the varied substrates in the presence of 1.5×10^{-5}M Ca^{2+} or $<10^{-9}$ M Ca^{2+}. The latter is subsequently referred to as "in the absence of Ca^{2+}". These studies were conducted at pH 7.4 [rather than pH 7.6 which was used in the companion paper (Lawlis & Roche, 1981)] because the $S_{0.5}$ for α-ketoglutarate is significantly lower in the absence of Ca^{2+} at pH 7.4 and control activities in the absence of Ca^{2+} can be matched at reasonable α-ketoglutarate concentrations to those in the presence of Ca^{2+}.

NADH Inhibition at Variable Levels of NAD^+. Previous initial velocity studies on the overall reaction catalyzed by the α-ketoglutarate dehydrogenase complex from pig heart indicated that NADH was either a noncompetitive inhibitor (Smith et al., 1974) or a competitive inhibitior (Hamand et al., 1975) vs. NAD^+. Micromolar Ca^{2+} would have been present in the assays used in these studies. In Figure 2 and 3, the inhibition pattern, for initial velocity studies conducted in the presence of Ca^{2+}, was noncompetitive with respect to NAD^+ for concentrations of NAD^+ less than 0.4 mM. Double-reciprocal plots were nonlinear due to a transition to enhanced reaction velocities at higher NAD^+ concentrations. Furthermore, the inhibition pattern for NADH changed from noncompetitive to a pattern closely approximating competitive at very high NAD^+ concentrations (Figure 3).

In the absence of Ca^{2+}, NADH was a more potent inhibitor, and, thus, lower concentrations of NADH were used, but a similar inhibition pattern with a change from noncompetitive to competitive inhibition was observed (data not shown). K_{ii} and K_{is} values were obtained from replots of slopes and extrapolated intercepts for the noncompetitive region of double-reciprocal plots, and these values are shown in Table I. Ca^{2+} caused a larger decrease in K_{ii} (from 101 to 8 μM) than for K_{is} (from 47 to 26 μM). Since Ca^{2+} also reduces the $S_{0.5}$ for α-ketoglutarate, it should be noted that NADH inhibition was investigated with 1.0 mM α-ketoglutarate in the presence of Ca^{2+} and with 10.0 mM α-ketoglutarate in the absence of Ca^{2+}. At pH 7.4, these concentrations of α-ketoglutarate yielded nearly identical maximum velocities at very high NAD^+ concentrations in the presence or absence of Ca^{2+}.

Table I: Product Inhibition Constants for NADH vs. the Different Substrates of the α-Ketoglutarate Dehydrogenase Complex in the Presence or Absence of Ca^{2+}

varied substrate	+Ca^{2+} K_{ii} (μM)	+Ca^{2+} K_{is} (μM)	−Ca^{2+} K_{ii} (μM)	−Ca^{2+} K_{is} (μM)
NAD^+	47	101	26	8
CoA	112	42	33	20
α-ketoglutarate	53	33	10	10

The transition to higher rates along with a change from noncompetitive to competitive inhibition at relatively high NAD^+ concentrations has not been previously reported in studies on NADH inhibition of the α-ketoglutarate dehydrogenase complex. On the basis of the results described below, it seems most likely that this transition results from binding of NADH at an allosteric site and removal of the inhibitory effect by competition of high concentrations of NAD^+ at this site.

NADH Inhibition with CoA and α-Ketoglutarate as the Varied Substrates. Since, as noted above, Ca^{2+} attenuation of NADH inhibition appears to require the overall reaction catalyzed by the α-ketoglutarate dehydrogenase complex (i.e., it is not observed with the model reaction catalyzed by the dihydrolipoyl dehydrogenase component), studies were conducted to determine the effect of the presence or absence of Ca^{2+} on the pattern for NADH inhibition with respect to CoA or α-ketoglutarate. In evaluating these inhibition patterns, it should be noted that initial velocity patterns for each substrate varied relative to the other substrates give parallel lines consistent with a multisite (at least three) Ping-Pong mechanism for the overall reaction catalyzed by the complex.[2]

The pattern for NADH inhibition vs. CoA or α-ketoglutarate was noncompetitive in the presence or absence of Ca^{2+}. The K_{ii} and K_{is} values were lower in the absence of Ca^{2+} and are shown in Table I. Similar patterns for NADH inhibition vs. α-ketoglutarate were observed by Smith et al. (1974) but not by Hamada et al. (1975). A three-site Ping-Pong mechanism predicts uncompetitive rather than noncompetitive inhibition (Cleland, 1973), and an observation of noncompetitive inhibition suggests either site–site interactions between components or a binding site for NADH on a component separate from the dihydrolipoyl dehydrogenase.

Preparation of Resolved α-Ketoglutarate Dehydrogenase Component and Effect of Glyoxylate on the Partial Reaction Catalyzed by the α-Ketoglutarate Dehydrogenase Component. Resolution of the α-ketoglutarate dehydrogenase complex by treatment with papain gave results similar to those of Linn (1974) achieved with a protease purified from extracts of kidney mitochondria. The isolated α-ketoglutarate dehydrogenase component was free of transsuccinylase and could be separated from the dihydrolipoyl dehydrogenase component by fractionation with ammonium sulfate. The latter fractionation lowered the specific activity of the α-ketoglutarate dehydrogenase component. The studies described below were done with a higher specific activity α-ketoglutarate dehydrogenase component which was contaminated by small amounts of the dihydrolipoyl dehydrogenase. However, qualitatively similar results were observed with lower specific activity enzyme that was essentially free of activity of the dihydrolipoyl dehydrogenase component.

Prior to papain treatment, the α-ketoglutarate dehydrogenase component gave two bands (M_r ~95 000 and ~88 000) in $NaDodSO_4$ gel electrophoresis. Papain treatment converted all of the α-ketoglutarate dehydrogenase to the lower molecular form (M_r ~88 000).

Effects of Ca^{2+} and ADP on the activity of the α-ketoglutarate dehydrogenase component were small when ferric cyanide reduction was assayed, (Massey, 1960), and effects of NADH could not be tested in the presence of dihydrolipoyl dehydrogenase because of electron transferase activity of the flavoprotein. Decarboxylation of α-keto[1-^{14}C]glutarate, in the presence of 0.2 mM thiamin pyrophosphate, was very slow, and it was very difficult to detect effects of ligands on the activity of the α-ketoglutarate dehydrogenase component. We found that addition of 5.0 mM glyoxylate caused a 21-fold increase in the rate of decarboxylation of 0.2 mM α-keto[1-^{14}C]glutarate, and effects of ligands could then be observed as described below. The increase in rate presumably results from an aldol condensation reaction in which the active aldehyde reacts with glyoxylate.

Effects of NADH, NAD$^+$, ADP, and ATP on the Activity of the Resolved α-Ketoglutarate Dehydrogenase Component in the Presence or Absence of Ca^{2+}. In Table II, effects on the activity of the α-ketoglutarate dehydrogenase component were measured by using 0.2 mM α-keto[1-^{14}C]glutarate in the presence or absence of Ca^{2+}. The activity of this component

Table II: Effects of NADH, NAD$^+$, ADP, and ATP on the Activity of the Resolved α-Ketoglutarate Dehydrogenase Component in the Presence or Absence of Ca^{2+}

additions[a]	α-ketoglutarate dehydrogenase activity or activity percent[b]			
	+Ca^{2+}	% of Ca^{2+} control	−Ca^{2+}	% of Ca^{2+} control
none	0.51	100	0.26	100
NADH	0.26	51	0.04	15
NAD$^+$ (0.2 mM)	0.59	116	0.24	92
NAD$^+$ (10.0 mM)	0.73	143	0.36	138
ADP	0.83	163	0.52	200
ATP	0.44	86	0.16	62
NADH, NAD (0.2 mM)	0.27	53	0.15	58
NADH, NAD (10.0 mM)	0.45	88	0.23	88
NADH, ADP	0.83	163	0.30	115
NADH, ATP	0.36	71	0.03	12

[a] NADH, ADP, and ATP were present at 0.2, 1.0, and 1.0 mM; NAD$^+$ was present at the indicated concentrations. [b] Activities are expressed as μmol min^{-1} mg^{-1}, and all values are an average of duplicate or triplicate assays; 4.0 μg of protein was present in each assays, and glyoxylate at a concentration of 5.0 mM was present in all assays. Other conditions were as described under Experimental Procedures.

was increased about 2-fold by Ca^{2+} (row 1), indicating that Ca^{2+} lowers the $S_{0.5}$ for α-ketoglutarate by binding to the α-ketoglutarate dehydrogenase component. As with other effectors, there is not quantitative agreement with the extent of change in the overall reaction catalyzed by the complex which, for instance, is increased more than 10-fold by Ca^{2+} at 0.2 mM α-ketoglutarate (pH 7.4). This difference, between the overall reaction and the partial reaction, is not surprising since the active aldehyde intermediate probably tends to accumulate in the partial reaction, which could change the concentration dependence for α-ketoglutarate.

NADH (0.2 mM) decreased the activity of the resolved component, and the extent of inhibition was much higher in the absence of Ca^{2+} (row 2). NAD$^+$ at 0.2 mM had little effect on activity (row 3), but 10.0 mM NAD$^+$ increased about 40% the activity of the resolved component (row 4). this suggests that the transition to competitive inhibition of NADH vs. NAD$^+$ (Figure 3) was due to NAD$^+$ preventing NADH inhibition at a site on the α-ketoglutarate dehydrogenase component. Consistent with this, in the absence of Ca^{2+}, 10.0 mM NAD$^+$ appreciably reduced NADH inhibition (row 2 vs. 8).

ADP (1.0 mM) increased the activity of the α-ketoglutarate dehydrogenase component by 63% or 100% in the presence or absence of Ca^{2+}, respectively (row 5). ATP (1.0 mM) decreased the activity of the component reaction 14% or 38% in the presence or absence of Ca^{2+} (row 6). The larger changes in the rates of the partial reaction in the absence of Ca^{2+} are consistent with the effects of ADP and ATP on the $S_{0.5}$ for α-ketoglutarate observed with the overall reaction catalyzed by the complex (Lawlis & Roche, 1981). In the presence of ADP, the activity of the resolved component was not inhibited by 0.2 mM NADH in the presence of Ca^{2+} but was reduced 54% by NADH in the absence of Ca^{2+} (row 5 vs. 9). Addition of 0.2 mM NAD$^+$ (in the presence or absence of NADH) to assays containing 1 mM ADP had no effect on activity (data not shown). The combination of 0.2 mM NADH and 1.0 mM ATP did not significantly reduce the activity of the resolved α-ketoglutarate dehydrogenase component below that observed

[2] A thorough study of initial velocity and product inhibition patterns for the α-ketoglutarate dehydrogenase complex from bovine heart is described in the M.S. Thesis of C. M. McGarity completed in L. J. Reed's laboratory at the University of Texas at Austin (1974). These studies demonstrated parallel patterns in double-reciprocal plots when substrates (α-ketoglutarate, CoA, or NAD$^+$) were varied at different fixed levels vs. each of the other substrates. Product inhibition patterns, which were studied only in the presence of Ca^{2+}, agree with those described in this article, although the changes in patterns at high NAD$^+$ concentrations were not noted. Hamada et al. (1975) also observed parallel initial velocity patterns in studies on the pig heart complex.

Table III: Selection of α-Ketoglutarate Concentrations To Give Nearly Matching Activities at 1.5×10^{-5} M Ca^{2+} ($+Ca^{2+}$) or $<1.0 \times 10^{-9}$ M Ca^{2+} ($-Ca^{2+}$) in the Presence or Absence of ADP and Change in Activity at Decreased NAD^+ Concentrations[a]

α-ketoglutarate concn (mM)		NAD^+ concn (mM)	other addition	sp act. (μmol min^{-1} mg^{-1})	
$+Ca^{2+}$	$-Ca^{2+}$			$+Ca^{2+}$	$-Ca^{2+}$
0.375	10.00	0.40		4.58	5.14
0.375	10.00	0.34		4.40	4.57
0.375	10.00	0.050		3.30	3.22
0.056	0.50	0.40	ADP	4.47	4.68
0.056	0.50	0.34	ADP	4.15	4.10
0.056	0.50	0.050	ADP	2.60	2.66

[a] Assays were conducted with 0.12 mM CoA and assays initiated by addition of 7.5 μg of α-ketoglutarate dehydrogenase complex. Other additions are noted in the table or are described under Experimental Procedures.

with NADH alone, and, in fact, ATP appeared to cause an increase (40%) in the presence of Ca^{2+} (row 2 vs. 10). This suggests that, in the presence of Ca^{2+}, ATP may decrease NADH binding to the α-ketoglutarate dehydrogenase component.

The results constitute strong evidence that Ca^{2+}, ADP, ATP, NADH, and NAD^+ bind to the α-ketoglutarate dehydrogenase component.

Relationship between Changes in the $S_{0.5}$ for α-Ketoglutarate and Degree of NADH Inhibition and Effects of ADP. On the basis of the above studies and previous observations (Lawlis & Roche, 1980), it would appear that Ca^{2+} and ADP are effective in increasing the activity of the α-ketoglutarate dehydrogenase complex not only by lowering the $S_{0.5}$ for α-ketoglutarate but also by decreasing NADH inhibition. We have further evaluated the effectiveness of Ca^{2+} and ADP in reducing NADH inhibition under conditions in which α-ketoglutarate levels were adjusted to compensate for the effects of Ca^{2+} and/or ADP on the $S_{0.5}$ for α-ketoglutarate in the absence of NADH. In assays containing 0.4 mM NAD^+, α-ketoglutarate concentrations were determined which gave nearly identical rates in the presence or absence of either Ca^{2+} or ADP (Table III). In the absence of NADH, these levels of α-ketoglutarate gave nearly identical activities for a wide range of NAD^+ concentrations. Since V_m is not changed in the presence or absence of Ca^{2+} or ADP (Lawlis & Roche, 1981), the approach of matching activities with different levels of α-ketoglutarate, in the presence or absence of Ca^{2+}, should eliminate changes in $S_{0.5}$ due to Ca^{2+} or ADP. As shown in Figure 4, under these conditions, much less inhibition by NADH was still observed in the presence of Ca^{2+} than in the absence of Ca^{2+} over a wide range of NADH/NAD^+ ratios. At a total pyridine nucleotide level of 0.40 mM, the NAD^+ concentrations of 0.34 and 0.050 mM in Table III were used in NADH/NAD^+ ratios of 0.176 and 7.0 in Figure 4. For these ratios, the activity of the α-ketoglutarate dehydrogenase complex was 2.8- and 2.6-fold higher, respectively, in the presence of Ca^{2+} than in the absence of Ca^{2+}. Thus the effects of Ca^{2+} on NADH inhibition are large under conditions that compensate for effects of Ca^{2+} on the $S_{0.5}$ for α-ketoglutarate.

As shown in Figure 4, in the absence of Ca^{2+}, 1.6 mM ADP reduced NADH inhibition to that observed in the presence of Ca^{2+}. In the presence of Ca^{2+}, ADP caused a further reduction in NADH inhibition over most of the range of NADH/NAD^+ ratios. Thus, in addition to Ca^{2+}, ADP appreciably decreases NADH inhibition under conditions compensating for the effect of ADP on the $S_{0.5}$ for α-ketoglutarate. This suggests that ADP either decreases the binding of NADH to the α-ketoglutarate dehydrogenase component (possibly in a competitive manner) or reduces the inhibition by bound NADH.

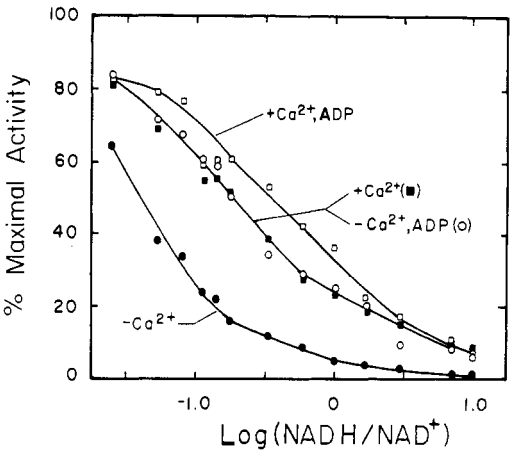

FIGURE 4: Effect of Ca^{2+} and ADP on the activity of the α-ketoglutarate dehydrogenase complex for a wide range of NADH/NAD^+ ratios with the concentration of α-ketoglutarate adjusted to give matching control activities (minus NADH) for the different conditions. Control (100%) activities in the absence of NADH were matched at the α-ketoglutarate concentrations shown in Table III. Total pyridine nucleotide was 0.40 mM, and 0.12 mM CoA was present in all assays. Where indicated, ADP was added at a concentration of 1.60 mM. Assays were initiated with 7.5 μg of complex. The conditions were $-Ca^{2+}$ (●), $+Ca^{2+}$ (■), $-Ca^{2+}$ plus ADP (○), and $+Ca^{2+}$ plus ADP (■). Other conditions were as described under Experimental Procedures.

Discussion

We have demonstrated that NADH inhibition is decreased in the presence of micromolar Ca^{2+} and that this is associated with an increase in K_{ii} and K_{is} values for NADH inhibition vs. NAD^+, CoA, and α-ketoglutarate. The gross inhibition pattern for NADH vs. all three substrates is noncompetitive (except at high NAD^+ concentrations) and is not altered by Ca^{2+} although there are differential increases in K_{ii} and K_{is} values for the different substrates. Although subject to other interpretations, these data and the observation that Ca^{2+} did not affect NADH inhibition of the dihydrolipoyl dehydrogenase suggest that Ca^{2+}-modulated NADH inhibition results from the binding of NADH at an allosteric site and that inhibition is reversed in a competitive manner by high concentrations of NAD^+.

Further studies established that Ca^{2+}, ADP, or NAD^+ activates the partial reaction catalyzed by the resolved α-ketoglutarate dehydrogenase component and NADH or ATP inhibits this reaction. The effects of Ca^{2+}, ADP, and ATP on the partial reaction were consistent with the effects of these ligands on the $S_{0.5}$ (or K_m) for α-ketoglutarate observed in studies on the intact complex from heart and kidney (McCormack & Denton, 1979; Lawlis & Roche, 1981). Consistent with studies on the overall reaction, high levels (10 mM) of NAD^+ both activate the partial reaction and reduce NADH inhibition. High levels of NAD^+ reduce the $S_{0.5}$ for α-ketoglutarate in the absence of Ca^{2+} (Lawlis & Roche, 1981), suggesting that NAD^+ and NADH may have opposing effects on the $S_{0.5}$ for α-ketoglutarate similar to ADP and ATP.

Detection of these effects on the partial reaction catalyzed by the α-ketoglutarate dehydrogenase component was facilitated by inclusion of 5.0 mM glyoxylate in the assay since glyoxylate increased the specific activity of the resolved α-ketoglutarate dehydrogenase component in the partial reaction from 0.8% to 17% of the specific activity of the complex in the overall reaction under similar conditions (i.e., 0.2 mM

α-ketoglutarate, at pH 7.4, in the presence of Ca^{2+}).

From the studies on the overall reaction, in Table III and Figure 4, it is clear that Ca^{2+} or ADP reduces NADH inhibition under conditions in which the effects of Ca^{2+} or ADP (in the absence of NADH) on the $S_{0.5}$ for α-ketoglutarate have been compensated for by changes in the α-ketoglutarate concentration. It is possible that ADP reduces NADH inhibition by binding at the same, overlapping, or interacting sites. Further studies are required to distinguish these possibilities. Interestingly, addition of ATP in assays containing NADH does not significantly reduce the rate of the partial reaction (or overall reaction, data not shown) below that in the presence of NADH and may even reduce NADH inhibition in the presence of Ca^{2+} (Table II). The data in Figure 4 suggest that NADH inhibition, even in the absence of Ca^{2+}, may not be very effective in the presence of a high level of ADP. However, we have observed with a phosphate potential (ATP/ADP × P_i) of 0.53 (adenylate pool = 10.0 mM) that an NADH/NAD^+ ratio of 0.5 (pyridine nucleotide pool = 0.4 mM) causes a 2-fold reduction in the activity of the α-ketoglutarate dehydrogenase complex in the absence of Ca^{2+} (Lawlis & Roche, 1980). It seems unlikely that the NADH inhibition observed was a result of inhibition of the dihydrolipoyl dehydrogenase since at 10-fold higher rates in the presence of Ca^{2+}, for which an equivalent degree of NADH inhibition of the activity of dihydrolipoyl dehydrogenase would be expected, a much smaller inhibition by NADH was observed (Lawlis & Roche, 1980).

The largest effects on the activity of the purified α-ketoglutarate dehydrogenase complex are elicited by Ca^{2+}. The mechanism of Ca^{2+} transport in isolated mitochondria has been shown to involve uptake through an electrophoretic transport of Ca^{2+} and release of Ca^{2+} by an electroneutral exchange of Ca^{2+} with Na^+ or H^+ [see Carafoli (1979) and Fiskum & Lehninger (1980) for reviews]. The range of concentrations for free intramitochondrial Ca^{2+} has not been established. Recently, Denton and co-workers (McCormack & Denton, 1980; Denton et al., 1980) have presented evidence that Ca^{2+} activated the α-ketoglutarate dehydrogenase complex (as well as the pyruvate dehydrogenase complex and NAD^+-dependent isocitrate dehydrogenase) in uncoupled heart and adipose tissue mitochondria with a half-maximal effect at about 1 μM Ca^{2+}. Based on a similar activation of these enzymes in coupled mitochondria, these authors suggest that free intramitochondrial levels of Ca^{2+} are only 2–3 times higher than free Ca^{2+} levels in the cytoplasm. This contrasts with the conclusions of Williamson & Murphy (1980) who have presented evidence that fairly high levels of free Ca^{2+} (30–60 μM) are maintained by an effective membrane potential of about 80 mV. A large number of physiological factors are reported to influence Ca^{2+} movement in mitochondria. Certainly, the effect of an independent uptake and efflux mechanism for Ca^{2+} is that the steady-state Ca^{2+} distribution can be regulated by altering the kinetic control or thermodynamic gradients of either pathway, and, therefore, the variation in the levels of free intramitochondrial Ca^{2+} may be fairly large.

Among the factors affecting Ca^{2+} efflux, some studies (Lehninger et al., 1978, Fiskum & Lehninger, 1980; Lotscher et al., 1980) presented evidence that a decrease in the NADH/NAD^+ ratio leads to release of Ca^{2+} from mitochondria; however, other studies (Nicholls & Brand, 1980; Beatrice et al., 1980) suggested that release is a secondary effect associated with collapse of the membrane potential. Because Ca^{2+} decreases NADH inhibition of the α-ketoglutarate dehydrogenase complex, it will be important in evaluating this regulatory effect to determine whether the level of free intramitochondrial Ca^{2+} is in some way coupled to the NADH/NAD^+ ratio.

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