Yeast Diphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase. Regulation of Activity and Unidirectional Catalysis†

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ABSTRACT: Both DPNH and α-ketoglutarate inhibit the oxidation of isocitrate catalyzed by yeast DPN-specific isocitrate dehydrogenase (EC 1.1.1.41); AMP decreases the kinetic orders (slopes of Hill plots) with regard to both inhibitors. The kinetic orders for α-ketoglutarate and DPNH as substrates for the reverse reaction at pH 6.5 are both 1. Citrate decreases the concentration of either substrate required for half-maximal velocity by a factor of 2 or 3. The velocity of the forward reaction increases as the DPN^+ mole fraction of the pyridine nucleotide pool (DPN^+ + DPNH) increases. The rate is about 50% of maximal when the mole fraction of DPN^+ is 0.8. The concentration of isocitrate required for half-maximal velocity increases by a factor of fifteen between adenylate energy charge values of 0 and 1. The steep slope of the curve at high adenylate energy charge is the response expected for a regulatory enzyme in an ATP-regenerating sequence. Because of positive cooperativity of both substrates (DPN^+ and isocitrate), as well as inhibition by DPNH and α-ketoglutarate, the catalytic activity of the enzyme decreases rapidly with increase in the reaction parameter Q [Q = (α-ketoglutarate)(DPNH)(CO_2)/(isocitrate)(DPN^+)], and becomes essentially zero before equilibrium is reached. The reverse reaction, reductive carboxylation of α-ketoglutarate, is not catalyzed at a significant rate at pH 7.6. Thus, the enzyme is effectively a unidirectional catalyst, being almost inactive under conditions where the oppositely directed reaction is thermodynamically possible. At pH 6.5, where the reverse reaction is feebly catalyzed, the rate first increases and then falls as the value of Q is reduced from infinity to its equilibrium value. This response appears to result mainly from the positive modifier action of isocitrate.

The kinetic and binding properties of yeast DPN-specific isocitrate dehydrogenase (*threo*-D_s(+)-isocitrate + DPN^+ → α-ketoglutarate + DPNH + CO_2, EC 1.1.1.41) have been partly characterized (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965; Barnes *et al.*, 1971; Kuehn *et al.*, 1971). The binding of isocitrate is highly cooperative. Citrate appears to bind cooperatively at isocitrate sites, and thus may be either an inhibitor or activator, depending upon the citrate and isocitrate concentrations. AMP is a positive modifier. The rate of the reaction catalyzed by partially purified enzyme responds to the adenylate energy charge as expected for an enzyme involved in ATP regeneration (Atkinson, 1968a). Although the purified enzyme exhibits a lower degree of cooperativity among ligand sites than partially purified enzyme, both preparations are equally activated by AMP (Barnes *et al.*, 1971).

Kornberg and Pricer (1951) reported that the enzyme did not catalyze the reverse reaction (reductive carboxylation of α-ketoglutarate) at pH 7.6. Yeast TPN-specific isocitrate dehydrogenase readily catalyzed the reaction in both directions. Hathaway and Atkinson (1963) demonstrated that DPN isocitrate dehydrogenase catalyzed the reverse reaction at a measurable rate at pH 6.5 when enzyme concentrations about 50 times greater than those employed for the forward reaction were used. The addition of isocitrate or citrate at low concentration eliminated a short time lag in the initiation of the reverse reaction. AMP was required for the reverse reaction if citrate was omitted.

Regulation of enzymic activity by the DPN^+ mole fraction and the response of the $(isocitrate)_{0.5}$ to the adenylate energy charge are reported here. Some aspects of the dependence of reaction velocity on the concentrations of substrates and products as the reaction approaches equilibrium from both directions are presented.

Materials and Methods

Materials. Adenine and pyridine nucleotides were obtained from P-L Biochemicals. DL-Isocitric acid (allo free), Hepes,[1] dithiothreitol, and α-ketoglutarate were from Calbiochem. Pig heart TPN isocitrate dehydrogenase was from Sigma. All other chemicals used were either reagent or analytical grade. Yeast DPN isocitrate dehydrogenase was isolated according to Barnes *et al.* (1971). The preparation used has a specific activity of 26.5 (based on the purified enzyme as the protein standard).

Enzyme Assays. Isocitrate dehydrogenase activity was determined by following the formation or oxidation of DPNH at

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $(S)_{0.5}$, the concentration of substrate required for half-maximal activity (occasionally S is replaced by the name of the specific substrate); $(M)_{0.5}$, the concentration of modifier which causes half-maximal effect (occasionally M is replaced by the name of the specific modifier).

340 nm with a Gilford Model 2000 spectrophotometer and accessory system. An assay used to study the kinetics of the forward reaction (oxidative decarboxylation of isocitrate) contained 100 mM Hepes–KOH (pH 7.6), 3 mM dithiothreitol, enzyme preparation containing 0.5–1 μg of protein, and variable concentrations of substrates and modifiers as indicated. An assay used to study the kinetics of the reverse reaction (reductive carboxylation of α-ketoglutarate) contained 100 mM sodium cacodylate–H_2SO_4 (pH 6.5), 3 mM dithiothreitol, 4 mM $MgSO_4$, 0.4 mM AMP, 5 μM isocitrate, enzyme preparation containing 30–60 μg of protein, and variable concentrations of substrates and citrate as indicated.

Assays used to demonstrate the unidirectional preference of the enzyme for the forward reaction contained 100 mM Hepes–KOH (pH 7.6), 4 mM $MgSO_4$, and 3 mM dithiothreitol. Concentrations of DPN^+ and DPNH were varied in a complementary manner with their total concentration held constant at 0.2 mM. Isocitrate and α-ketoglutarate were varied in a similar manner, with their total concentration fixed at 2 mM. Because the concentration of $NaHCO_3$ required for half-saturation is large, each assay contained 20 mM $NaHCO_3$ in addition to a variable concentration equal to that of α-ketoglutarate. All components were incubated together at 25° for 2 min, and reaction was initiated by addition of enzyme. The experiment was performed in the same manner using pig heart TPN isocitrate dehydrogenase instead of yeast DPN isocitrate dehydrogenase, except that TPN^+ and TPNH replaced DPN^+ and DPNH.

Stock 30 mM adenylate energy charge solutions were prepared by mixing appropriate amounts of AMP, ADP, and ATP to establish the desired energy charge (Atkinson, 1968b), assuming the equilibrium constant of the adenylate kinase reaction to be 0.8 (Markland and Wadkins, 1966). The adenylate pool in the assay mixtures was 3 mM.

Substrate, ADP, and ATP concentrations were determined enzymatically and the AMP concentration was calculated from its absorbance at 259 nm.

Results

Inhibition of the Forward Reaction by α-Ketoglutarate and DPNH. High concentrations of α-ketoglutarate completely inhibited the oxidation of isocitrate when isocitrate was present at a concentration approximately equal to its $(S)_{0.5}$ value (Figure 1). In the absence of AMP, the $(M)_{0.5}$ for α-ketoglutarate was 4.9 mM, which is about 15 times the $(isocitrate)_{0.5}$ value (Barnes *et al.*, 1971). When AMP was added, maximal inhibition by α-ketoglutarate was 90%, and the $(M)_{0.5}$ for α-ketoglutarate was increased slightly to 6.3 mM (Figure 1). In the presence of AMP, the value of $(M)_{0.5}$ for α-ketoglutarate is about 300 times the $(isocitrate)_{0.5}$ value (Barnes *et al.*, 1971). The Hill slope for α-ketoglutarate as an inhibitor was 1.6 in the absence of AMP, and 1.0 in the presence of the modifier.

In the absence of AMP and at a DPN^+ concentration of 0.8 mM, the addition of 0.2 mM DPNH caused 50% inhibition of the forward reaction (Figure 2). Maximal inhibition varied between 70 and 90% under these conditions. The kinetic order with respect to DPNH (slope of the Hill plot) was 1.6. The order decreased to 1.0 when AMP was added, the isocitrate concentration increased, and the DPN^+ concentration decreased.

A concentration of α-ketoglutarate comparable to its $(M)_{0.5}$ value increased the $(isocitrate)_{0.5}$ by approximately 50% when the reaction was observed in the absence of AMP,

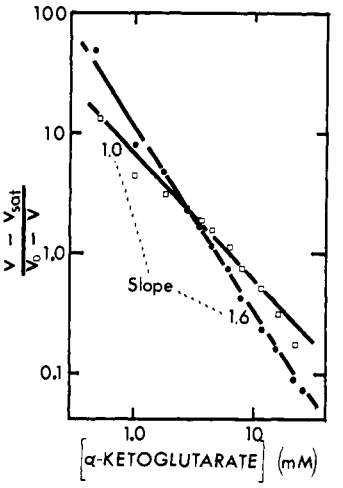

FIGURE 1: Inhibition by α-ketoglutarate of the oxidation of isocitrate catalyzed by yeast DPN isocitrate dehydrogenase. Reaction mixtures contained 100 mM Hepes–KOH (pH 7.6), 4 mM $MgSO_4$, 3 mM dithiothreitol, 0.4 mM DPN^+, 0.35 mM isocitrate, α-ketoglutarate at the concentrations indicated, AMP as indicated, and enzyme. The reaction velocity in the presence of saturating concentrations of α-ketoglutarate (V_{sat}) was 0 in the absence of AMP and 10% of V_0 in the presence of 1 mM AMP.

and doubled the $(isocitrate)_{0.5}$ when AMP was present. The inhibitor decreased the kinetic order for isocitrate from 3.5 to 3.1 in the absence of AMP and from 2.6 to 2.2 in the presence of AMP. Concentrations of DPNH that caused 50% inhibition had negligible effect on the kinetic order for DPN^+. In the absence of AMP and at an isocitrate concentration about equal to its $(S)_{0.5}$ value, 0.2 mM DPNH increased the $(DPN^+)_{0.5}$ by a factor of about 2.5. At saturating concentrations of AMP and isocitrate, 0.1 mM DPNH increased the $(DPN^+)_{0.5}$ by a factor of about four. Kinetic parameters for the forward reaction are tabulated in Table I.

Control by the DPN^+ Mole Fraction and the Adenylate Energy Charge. The rate of the reaction catalyzed by yeast DPN isocitrate dehydrogenase increases as the mole fraction of DPN^+ in the pyridine nucleotide (DPN^+ + DPNH) pool is increased. Response to this mole fraction was measured at pyridine nucleotide pool concentrations of 0.4 and 0.8 mM, and is shown in relation to the rate as a function of DPN^+

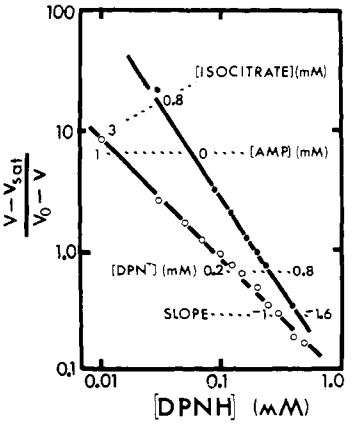

FIGURE 2: Inhibition by DPNH of the oxidation of isocitrate catalyzed by yeast DPN isocitrate dehydrogenase. Reaction mixtures were as in Figure 1, except for concentrations specified in the figure.

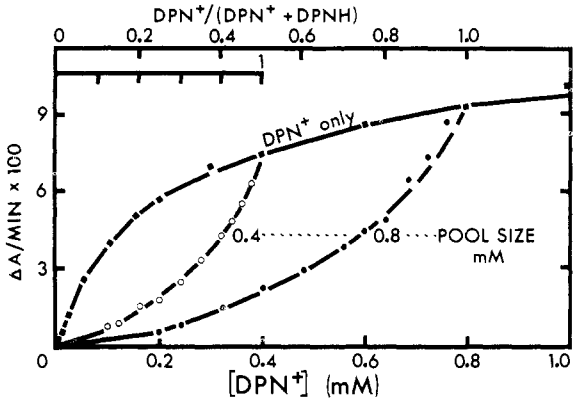

FIGURE 3: Response of yeast DPN isocitrate dehydrogenase to the DPN^+ mole fraction of the pyridine nucleotide pool. Reaction mixtures contained 100 mM Hepes–KOH (pH 7.6), 4 mM $MgSO_4$, 3 mM dithiothreitol, 1 mM AMP, 3 mM isocitrate, enzyme, and DPN^+ at the concentrations indicated on the lower horizontal scale. Curves labeled 0.4 and 0.8 present results obtained when DPNH was added to bring the total pyridine nucleotide pool (DPN^+ + DPNH) to the concentration indicated (millimolar). The DPN^+ mole fraction for each pool is indicated on the upper horizontal scale.

concentration alone in Figure 3. The increase in rate was sharpest between DPN^+ mole fraction values of 0.8 and 1.0. The DPN^+ concentration in both pool sizes was less than saturating, and to compare the responses the rates were replotted as a percentage of the maximal rate occurring at a DPN^+ mole fraction of 1.0 for each pool size (Figure 4). The relative rates were almost identical, which indicates that over this range of concentrations the enzyme responds to the ratio of the pyridine nucleotide levels rather than to their absolute concentrations.

The rate of the reaction catalyzed by yeast DPN isocitrate dehydrogenase responds to variation in the adenylate energy charge in the way expected for a regulatory enzyme in an ATP-regenerating sequence (Atkinson, 1968a). The effect is on the affinity of the enzyme for substrate as illustrated by the sharp response of the $(isocitrate)_{0.5}$ value to variation in energy charge (Figure 5). $(Isocitrate)_{0.5}$ increased by a factor of 15 between energy charge values of 0 and 1. Most of this change occurred in the range from 0.75 to 1.0. The vertical scale is inverted in Figure 5, in conformity with the convention that a decrease in velocity or affinity should be indicated by a negative slope.

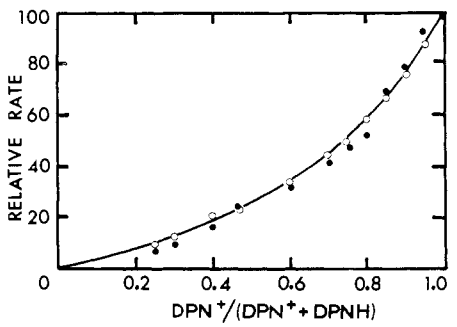

FIGURE 4: Response to the DPN^+ mole fraction. Results from Figure 3 are replotted on a single mole fraction scale, and normalized to a relative rate of 100 at a mole fraction of 1. Open circles, pyridine nucleotide pool 0.4 mM; filled circles, 0.8 mM pool; line, response curve calculated on the basis of simple competition between DPN^+ and DPNH at a catalytic site having effective Michaelis dissociation constants of 0.35 mM for DPN^+ and 0.1 mM for DPNH, assuming a 1 mM pool.

TABLE I: Kinetic Parameters for the Oxidative Decarboxylation of Isocitrate by Yeast DPN Isocitrate Dehydrogenase.

Varied Component	Other Assay Components[a] (mM)	Hill Slope	$(S)_{0.5}$ (μM)	$(M)_{0.5}$ (μM)
Isocitrate[b]		3.5	320	
Isocitrate[b]	AMP (1.0)	2.6	18	
DPN^+ [b]	Isocitrate (0.4)	1.2	620	
DPN^+ [b]	Isocitrate (3.0) AMP (1.0)	1.0	210	
Mn^{2+} [b]	Isocitrate (1.5)	1.0	5	
Mn^{2+} [b]	Isocitrate (4.0) AMP (1.0)	1.0	0.95	
AMP [b]	Isocitrate (0.05)	1.7		90
AMP [b]	Isocitrate (0.2)	1.5		6
α-Ketoglutarate	Isocitrate (0.35)	−1.6		4900
α-Ketoglutarate	Isocitrate (0.02) AMP (1.0)	−1.0		6300
DPNH	Isocitrate (0.8) DPN^+ (0.8)	−1.6		200
DPNH	Isocitrate (3.0) DPN^+ (0.2) AMP (1.0)	−1.0		100
Isocitrate	α-Ketoglutarate (5.4)	3.1	540	
Isocitrate	α-Ketoglutarate (5.4) AMP (1.0)	2.2	35	
DPN^+	Isocitrate (0.4) DPNH (0.2)	0.96	1500	
DPN^+	Isocitrate (3.0) DPNH (0.1) AMP (1.0)	0.95	780	

[a] Assay solutions contained 100 mM Hepes–KOH (pH 7.6), 4 mM $MgSO_4$ (except when Mn^{2+} was the varied component), 3 mM dithiothreitol, 0.4 mM DPN^+ (except when DPN^+ was the varied component), enzyme, and the indicated components. [b] From Barnes *et al.* (1971).

Interaction between the adenylate energy charge and the DPN^+ mole fraction is illustrated in Figure 6. In these experiments, isocitrate was present at a concentration of 0.3 mM, which is saturating at an energy charge of zero. As the DPN^+ mole fraction of the 1 mM pyridine nucleotide pool was decreased from 1 to 0.8 and 0.6, the rate was inhibited by about 50 and 70%, respectively. These levels of inhibition agree with the results given in Figure 4. As the energy charge was increased, the rate decreased because the $(isocitrate)_{0.5}$ increased and isocitrate was no longer saturating. A decrease in the DPN^+ mole fraction decreased the steepness of the response to energy charge because of the pronounced inhibition at low values of charge.

Kinetics of the Reductive Carboxylation of α-Ketoglutarate. The reverse reaction was first-order with respect to α-ketoglutarate in the absence or presence of citrate (Figure 7). A 90-sec time lag observed in cacodylate buffer was reduced to less than 10 sec when 5 μM isocitrate was added. AMP was

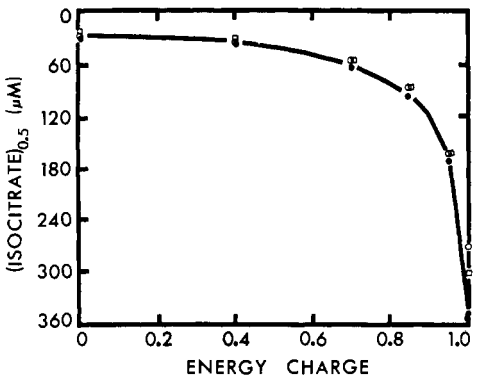

FIGURE 5: (Isocitrate)$_{0.5}$ of yeast DPN isocitrate dehydrogenase as a function of the adenylate energy charge. Reaction mixtures contained 100 mM Hepes–KOH (pH 7.6), 5 mM $MgSO_4$, 3 mM dithiothreitol, 0.3 mM isocitrate, and enzyme. The adenine nucleotide pool (AMP + ADP + ATP) was constant at 3 mM. Sizes of pyridine nucleotide pools and mole fractions of DPN^+ were (●) 0.4 mM and 1; (□) 1.0 mM and 1; (○) 1.0 mM and 0.8.

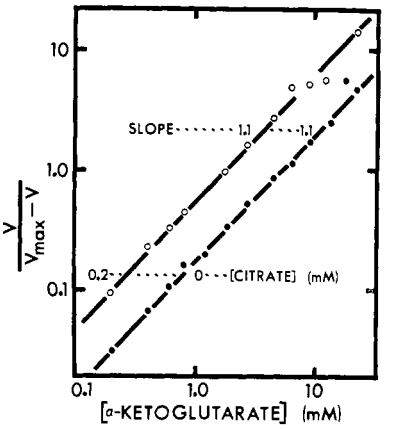

FIGURE 7: Dependence of the reductive carboxylation of α-ketoglutarate, catalyzed by yeast DPN isocitrate dehydrogenase, on the concentration of α-ketoglutarate; effect of citrate. Reaction mixtures contained 100 mM sodium cacodylate–H_2SO_4 (pH 6.5), 4 mM $MgSO_4$, 3 mM dithiothreitol, 20 mM $NaHCO_3$, 0.1 mM DPNH, 0.4 mM AMP, 5 μM isocitrate, α-ketoglutarate and citrate at the concentrations indicated, and enzyme.

included in the assay solution to obtain readily measurable rates. The (α-ketoglutarate)$_{0.5}$ value was 5.2 mM in the absence of citrate, and decreased to 1.8 mM in the presence of 0.2 mM citrate. These values were obtained in the presence of 5 μM isocitrate. When isocitrate was omitted and the linear rates were measured after the time lag, the value of (α-ketoglutarate)$_{0.5}$ was 1.7 mM in the presence of 0.2 mM citrate. The kinetic order with respect to α-ketoglutarate remained unchanged when isocitrate was omitted (data not shown).

The order with respect to DPNH was 1.3 in the presence of 5 μM isocitrate (Table II). This value was not changed when citrate was added at 50 μM, but (DPNH)$_{0.5}$ decreased from 54 to 27 μM.

Dalziel and Londesborough (1968) showed that dissolved CO_2 rather than bicarbonate ion is the substrate in the reductive carboxylation of α-ketoglutarate catalyzed by the TPN isocitrate dehydrogenase from ox heart. In an experiment with yeast DPN isocitrate dehydrogenase, half-maximal velocity was observed at a HCO_3^- concentration of 21 mM in the presence of 4.5 mM α-ketoglutarate and 0.2 mM DPNH at pH 6.5. The kinetic order for HCO_3^- was 1.0 under these conditions (data not shown). The 21 mM concentration of HCO^{3-} corresponds to a concentration of CO_2 of 15 mM, based on an apparent first ionization constant of 4.31×10^{-7} M for carbonic acid, assuming that the H_2CO_3 concentration is negligible with respect to the CO_2 concentration (Londesborough and Dalziel, 1968).

Unidirectional Catalysis. Initial concentrations of the reaction components were used to calculate the reaction parameter Q. For the yeast enzyme: Q = [DPNH][α-ketoglutarate][CO_2]/[DPN^+][isocitrate]. TPN^+ and TPNH replaced DPN^+ and DPNH in the expression for the pig heart enzyme. Net initial reaction rates were observed at various initial values of Q. The concentration of CO_2 was calculated from

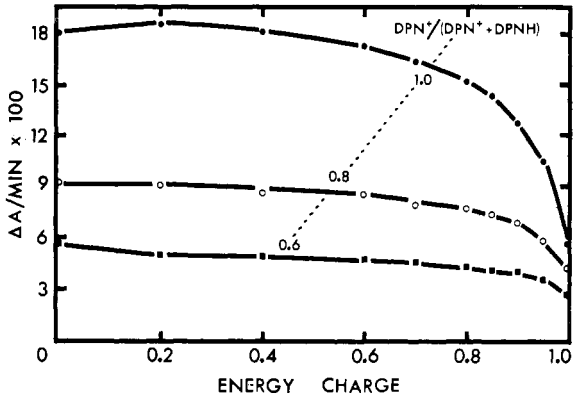

FIGURE 6: Velocity of the oxidation of isocitrate catalyzed by yeast DPN isocitrate dehydrogenase as a function of the adenylate energy charge; effect of mole fraction of DPN^+. Reaction mixtures contained 100 mM Hepes–KOH (pH 7.6), 5 mM $MgSO_4$, 3 mM dithiothreitol, 0.3 mM isocitrate, and enzyme. The adenine nucleotide pool (AMP + ADP + ATP) was constant at 3 mM and the pyridine nucleotide pool (DPN^+ + DPNH) at 1 mM. The compositions of these pools varied as indicated by the values of the adenylate energy charge and the DPN^+ mole fraction.

TABLE II: Kinetic Parameters for the Reductive Carboxylation of α-Ketoglutarate by Yeast DPN Isocitrate Dehydrogenase.

Varied Substrate	Other Assay Components[a] (mM)	Hill Slope	$(S)_{0.5}$ (mM)
α-Ketoglutarate	DPNH (0.1) Isocitrate (0.005)	1.1	5.2
α-Ketoglutarate	DPNH (0.1) Isocitrate (0.005) Citrate (0.2)	1.1	1.8
α-Ketoglutarate	DPNH (0.1) Citrate (0.2)	1.1	1.7
DPNH	α-Ketoglutarate (4.5) Isocitrate (0.005)	1.3	0.054
DPNH	α-Ketoglutarate (4.5) Isocitrate (0.005) Citrate (0.05)	1.3	0.027
HCO_3^-	α-Ketoglutarate (4.5) DPNH (0.2)	1.0	21

[a] Assay solutions contained 100 mM sodium cacodylate–H_2SO_4 (pH 6.5), 4 mM $MgSO_4$, 3 mM dithiothreitol, 20 mM $NaHCO_3$ enzyme, and the indicated components.

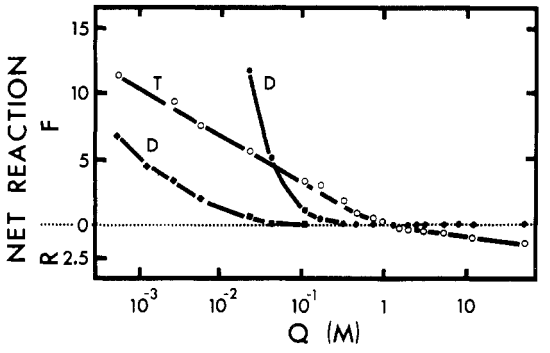

FIGURE 8: Dependence on the parameter Q (see text) of the reaction catalyzed by isocitrate dehydrogenases. Reaction mixtures contained 100 mM Hepes–KOH (pH 7.6), 4 mM $MgSO_4$, 3 mM dithiothreitol, 0.2 mM pyridine nucleotide pool (DPN^+ + DPNH or TPN^+ + TPNH), 2 mM substrate–product pool (isocitrate + α-ketoglutarate), $NaHCO_3$ at 20 mM plus the α-ketoglutarate concentration, and enzyme. T: pig heart TPN isocitrate dehydrogenase; D: yeast DPN isocitrate dehydrogenase; ●, enzyme concentration 20 times that designated by ■. Direction of net reaction indicated by: F, forward (oxidation of isocitrate); R, reverse (reductive carboxylation of α-ketoglutarate).

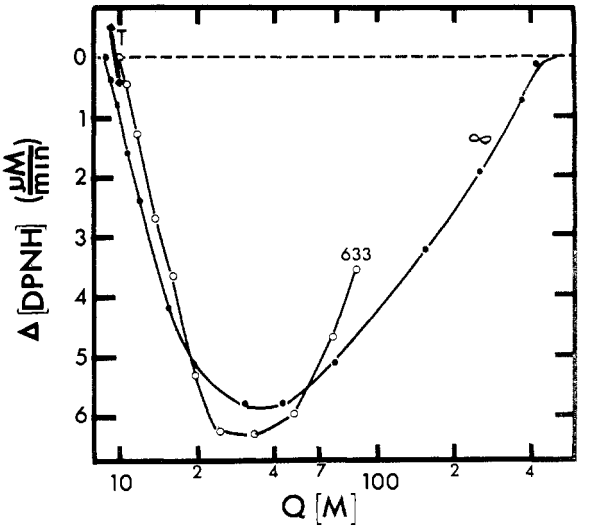

FIGURE 9: Velocity of the reductive carboxylation of α-ketoglutarate catalyzed by yeast DPN isocitrate dehydrogenase as a function of the reaction parameter Q. Initial values of Q are indicated on the curves. For an initial Q value of infinity, the reaction mixture contained 100 mM sodium cacodylate–H_2SO_4 (pH 6.5), 4 mM $MgSO_4$, 2 mM α-ketoglutarate, 6 mM dithiothreitol, 0.2 mM DPNH, 22 mM $NaHCO_3$, and 1 mM AMP. To obtain an initial Q value of 633, the DPNH/DPN^+ and (α-ketoglutarate, $NaHCO_3$)/isocitrate pools were varied stoichiometrically by an appropriate amount. T, response of pig heart TPN isocitrate dehydrogenase.

the amount of HCO_3^- added. Results are shown in Figure 8. Pig heart TPN isocitrate dehydrogenase was an effective catalyst for both the forward and reverse reactions. The apparent equilibrium constant was 1.33 M compared to a value of 0.86 M reported for the reaction catalyzed by ox heart TPN isocitrate dehydrogenase (Londesborough and Dalziel, 1968). The value obtained here is only approximate because of uncertainties as to exact concentrations; in particular, careful precautions were not taken with regard to gain or loss of CO_2. The purpose of these experiments was not confirmation of the equilibrium constant, but demonstration of the differential behaviors of the enzymes at different ratios of reactants and products.

The yeast DPN enzyme was used at two levels, one corresponding to 3.5 times the activity of the pig heart TPN enzyme under standard assay conditions (Q = 0), and the other to 70 times. Even at the higher concentration, the net reaction rate of the reaction catalyzed by the yeast enzyme became negligible before the equilibrium value was reached, and the reverse reaction was not calalyzed at a measurable rate at pH 7.6.

Reverse Reaction at pH 6.5. It was earlier observed that the reductive carboxylation of α-ketoglutarate proceeds at a slow but measurable rate at pH 6.5, and that an initial lag was reduced or abolished by the addition of isocitrate at a low concentration (Hathaway and Atkinson, 1963). From these observations it appeared that the dependence on concentrations of reactants and products of the reverse reaction is of a highly unusual nature: at high values of Q the rate increases as the reaction proceeds toward equilibrium. Experiments analogous to those reported in Figure 8 confirmed this behavior. Because of the large number of points needed to establish the shape of the curves and the degree of scatter obtained, however, that approach did not seem feasible for more quantitative work. Accordingly an alternate procedure was adopted. From a plot of absorbance at 340 nm as a function of time, the increase in DPNH concentration at any point may be calculated from the value of absorbance. This allows calculation of the changes in concentrations of all other reactants and products, and hence of the value of Q. The slope of the line at the point in question is proportional to the velocity of the reaction. Thus a full curve of velocity as a function of Q may be obtained from each reaction mixture allowed to proceed from a known value of Q (usually infinitely large) to equilibrium. All estimates of rate were made before calculation of Q values to eliminate the possibility of unconscious bias in the evaluation of slope. Curves of the type described are shown in Figure 9. Initial conditions are at high values of Q (at the right-hand edge of the figure). As the reaction proceeds the value of Q decreases, while the velocity of the net reaction first increases and then decreases to zero at equilibrium. To test the possibility that a time-dependent change in enzyme conformation, rather than the decrease in Q, might account for the increase in rate, some reactions were initiated at Q values other than infinity. One such experiment is shown in Figure 9. Here the initial rate was much faster, and the time required to reach any given level of Q was much less than in the control experiment. From the close correspondence of the curves, it is evident that the reaction rate depends primarily on the momentary value of Q and not on the time during which the enzyme has been in contact with the reaction components. In other experiments, the addition of 5 or 12.5 μM D$_s$-isocitrate, which accelerates the reaction without affecting the initial value of Q (since no DPN^+ was added), gave similar results. Although this acceleration caused the early stages of the reaction to proceed more rapidly in actual time, the curves of velocity as a function of Q were similar to that for the control mixture over the measurable range.

Addition of DPN^+ affected the course of the reaction quite differently from addition of isocitrate. Rather than accelerating the initiation of reaction and then having little further effect, DPN^+ at low concentration inhibited at all values of Q (Figure 10).

Citrate, as noted above, binds cooperatively with isocitrate and may enhance the forward reaction at low concentrations of isocitrate, and also decreases or abolishes the lag period

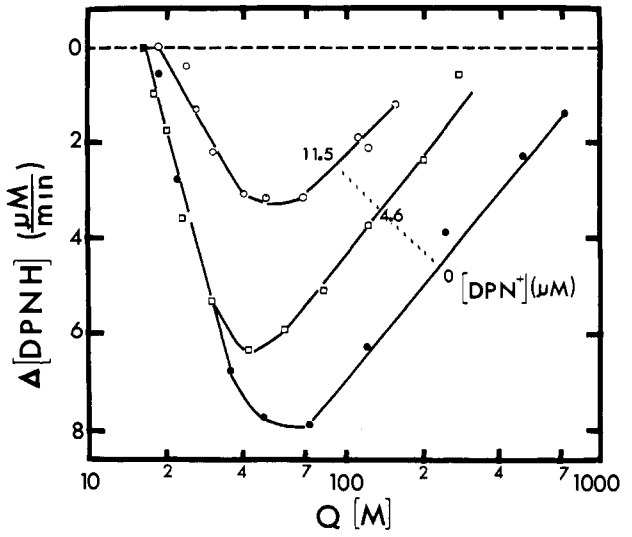

FIGURE 10: Effect of DPN^+ on the response of yeast DPN isocitrate dehydrogenase (reverse reaction) to the value of the reaction parameter Q. Reaction mixtures were the same as the infinite-Q mixture of Figure 9, except that DPN^+ was added at the concentrations indicated.

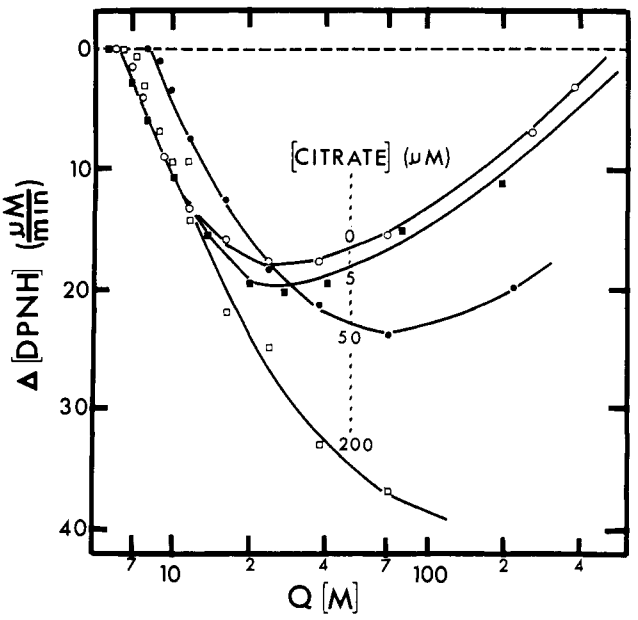

FIGURE 11: Effect of citrate on the response of yeast DPN isocitrate dehydrogenase (reverse reaction) to the value of the reaction parameter Q. Reaction mixtures were the same as the infinite-Q mixture of Figure 9, except that citrate was added at the concentrations indicated.

typically observed with the reverse reaction. In keeping with this latter observation, citrate at relatively low concentrations converts the curve of velocity as a function of Q to a more normal shape (Figure 11). When citrate was added at concentrations higher than those shown in the figure, curves were generally similar to that corresponding to 200 μM until a level of 2–3 mM was reached, after which further increase in citrate concentration was inhibitory, probably because of competition with α-ketoglutarate at catalytic sites.

Discussion

Control by the DPN^+ Mole Fraction and the Adenylate Energy Charge. The nonintegral order for α-ketoglutarate in the absence of AMP and the decrease in order upon addition of AMP are analogous to the results on cooperativity for isocitrate obtained with the purified enzyme (Barnes *et al.*, 1971). The increase in $(M)_{0.5}$ for α-ketoglutarate and a small decrease in maximal inhibition on addition of AMP are probably consequences of the large decrease in $(S)_{0.5}$ for isocitrate under these conditions.

Although DPNH did not inhibit totally, it must bind at the catalytic site since it is a product of the forward reaction. Observation of cooperativity of DPNH inhibition was unexpected since DPN^+ displayed no cooperativity (Barnes *et al.*, 1971) with the purified enzyme used in these experiments. It is not clear why DPNH should elicit a cooperative interaction between the catalytic sites when DPN^+ does not. This interaction was abolished on the addition of AMP. High concentrations of isocitrate and AMP increased the affinity for both DPN^+ and DPNH.

The relative affinities of the enzyme for DPN^+ and DPNH appear to be the basis for the response of isocitrate dehydrogenase to the DPN^+ mole fraction. As was pointed out previously (Atkinson, 1968b, 1970), response curves of the type reported here with respect to the mole fraction of the pyridine nucleotide pool that is oxidized (or reduced), as well as responses to adenylate energy charge of the type observed for kinases in biosynthetic pathways, may result from simple competition at the catalytic site if the affinity for the product nucleotide (DPNH or ADP) is greater than for the conjugate reactant (DPN^+ or ATP). Comparison of the experimental points of Figure 4 with the curve calculated for the response of isocitrate dehydrogenase to the DPN^+ mole fraction, using values for $(DPN^+)_{0.5}$ and $(DPNH)_{0.5}$ approximating those estimated experimentally, lends support to this suggestion. Thus a definitive advantage is seen for the otherwise anomalously high relative affinity for product.

The increase in $(\text{isocitrate})_{0.5}$ with increasing adenylate energy charge is the basis for the decrease in the velocity of reaction with increasing energy charge reported previously (Atkinson, 1968a). The general nature and steepness of the response in the region of 0.85–0.95 energy charge is that expected for an enzyme involved in the regeneration of ATP (Atkinson, 1968b).

Both the adenylate energy charge and the DPN^+ mole fraction appear to be factors enabling isocitrate dehydrogenase to participate in stabilization of metabolite concentrations in the cell (Atkinson, 1969). These control parameters will also influence partitioning of metabolites between fatty acid biosynthesis (energy storage) and total oxidation by way of the citric acid cycle (energy regeneration) (Hathaway and Atkinson, 1963). If the energy charge increases in the region from 0.8 to 1, the rate of isocitrate oxidation will decrease and the citrate level will increase (because of the equilibrium of the aconitase reaction). Citrate activation of acetyl-CoA carboxylase (Rasmussen and Klein, 1967) will increase the rate of storage of acetyl-CoA as fat. The consequent decrease in ATP regeneration will tend to restore the adenylate pool to its original charge value. Conversely, any decrease in the energy charge will be opposed by an increase in the rate of isocitrate oxidation. The resulting decrease in citrate concentration will lower the activity of acetyl-CoA carboxylase, which will enhance the flow of acetyl-CoA into the citric acid cycle. Similarly, if the DPN^+ mole fraction decreases in the region from 0.9 to 0.7, the resulting decrease in the rate of DPNH production will tend to restore the DPN^+ mole frac-

tion to its original value. Any increase in the DPN^+ mole fraction would increase the rate of DPNH production, which would again oppose the change. Of course, an increase in DPNH will usually be coupled *in vivo* with an increase in ATP. This increase in energy charge will be opposed by a decrease in rate of isocitrate oxidation, which will also oppose the increase in DPNH. Thus the effects of adenylate energy charge and DPN^+ mole fraction on isocitrate dehydrogenase work in the same direction in stabilizing metabolic conditions. These interactions will obviously be affected by the transport properties of the mitochondrial membranes.

Pea DPN isocitrate dehydrogenase is reported not to be affected by the adenine nucleotides (Cox and Davies, 1967), but does respond to the DPN^+ mole fraction (Duggleby and Dennis, 1970) in the same manner as demonstrated for the yeast enzyme. Pyruvate dehydrogenase from *Escherichia coli*, another enzyme involved in the production of DPNH and the regeneration of ATP, is controlled by the DPN^+ mole fraction as well as the adenylate energy charge (Shen and Atkinson, 1970), and the shape of the DPN^+ mole fraction response is very similar to that reported here.

Kinetics of the Reductive Carboxylation of α-Ketoglutarate. Because of negligible rates in the absence of AMP, even with high enzyme concentrations it was necessary to add this nucleotide in all studies of the reverse reaction. This fact may account for the lack of cooperativity observed for α-ketoglutarate and DPNH, since addition of AMP decreased interactions of these compounds when tested as inhibitors of the forward reaction. Low concentrations of citrate activate the reverse reaction by decreasing the $(S)_{0.5}$ for the substrates. The increased affinity is presumably a consequence of citrate binding to regulatory isocitrate sites. High concentrations of citrate inhibit (Hathaway and Atkinson, 1963) probably by competing with α-ketoglutarate for the catalytic sites. Elimination of the time lag for initiation of the reverse reaction by isocitrate is probably the result of its binding to regulatory isocitrate sites.

The number of moles of isocitrate bound per mole of enzyme is the same at pH 6.5 and 7.6 (Kuehn *et al.*, 1971). Although 5.3 mM α-ketoglutarate did not affect isocitrate binding at pH 7.6, it abolished binding by 0.3 mM isocitrate at pH 6.5. These results suggest that the reverse reaction becomes observable at the lower pH because of increased binding of α-ketoglutarate.

Unidirectional Catalysis. The results illustrated in Figure 8 show that yeast DPN isocitrate dehydrogenase behaves kinetically as a one-way catalyst at pH 7.6. Catalysis of the reaction in both directions by the pig heart TPN isocitrate dehydrogenase demonstrates the position of equilibrium under the experimental conditions. Since the equilibrium constant is the same for the reactions catalyzed by both isocitrate dehydrogenases (because the values of E^0 for the DPN^+/DPNH and TPN^+/TPNH couples are nearly identical), the apparent inability of DPN isocitrate dehydrogenase to catalyze the reverse reaction must be related to the kinetic properties of the enzyme.

Physiologically the yeast enzyme will encounter values of Q much less than 1 because of the DPN^+ mole fraction and substrate concentrations *in vivo*. Consequently, it is doubtful that unidirectionality has any metabolic significance for the yeast DPN isocitrate dehydrogenase. Probably this property, as well as the unusual kinetic behavior seen when the reverse reaction was followed at pH 6.5, is a fortuitous consequence of properties evolved for catalysis and control of the forward reaction. Nevertheless, these results demonstrate that an enzyme may be effective in catalyzing a reaction in one direction, but be almost totally inactive under conditions where the reverse reaction is thermodynamically favored.

LéJohn (1967, 1968; LéJohn and Stevenson, 1970) reported that the activities of several glutamate dehydrogenases, which readily catalyze the reaction in both directions, are unidirectionally stimulated or inhibited by various metabolites. The observations were consistent with the metabolic functions of the sequences in which the enzymes participated. In these cases, unidirectional response is probably a control mechanism as suggested by LéJohn.

Ready reversibility of the reaction catalyzed by the TPN isocitrate dehydrogenase from pigeon liver (Grisolia and Vennesland, 1947), pig heart (Ochoa, 1948), yeast (Kornberg and Pricer, 1951), and *Azotobacter vinelandii* (Chung and Franzen, 1969) has been demonstrated. Reversibility has not been observed for the DPN isocitrate dehydrogenase from yeast at pH 7.6 (Kornberg and Pricer, 1951; this paper), beef heart (Plaut and Sung, 1954), and pea (Cox and Davies, 1967). Evidence from product inhibition studies indicated that the DPN enzyme from *Blastocladiella emersonii* catalyzes the reverse reaction very weakly (LéJohn *et al.*, 1969). Isocitrate dehydrogenase from *Acetobacter peroxydans* is the only DPN enzyme yet shown to catalyze the reaction readily in both directions (Hathaway and Atkinson, 1963). This enzyme is not affected by the adenine nucleotides, a finding probably related to the apparent nonparticipation of the citrate cycle in the energy metabolism of *Acetobacter*.

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Preparation of Highly Purified Ribonucleic Acid Polymerase; Separation from Polynucleotide Phosphorylase and Polyphosphate Kinase†

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ABSTRACT: RNA polymerase was prepared from *Escherichia coli*, suitable for studies on the initiation of RNA synthesis. The enzyme appeared >95% pure on sodium dodecyl sulfate polyacrylamide gels. The enzyme preparations were shown to be free of phosphatase and ribonuclease activities. Contamination by deoxyribonuclease was measurable in a highly sensitive assay but was insignificant. Methods are described for the removal or control of trace amounts of polynucleotide phosphorylase. The incorporation of the γ-phosphate from ATP into acid-precipitable form by RNA polymerase preparations in the absence of template did not represent the formation of a phosphoenzyme intermediate, but resulted from the synthesis of polyphosphate, probably by trace amounts of contaminating polyphosphate kinase. Methods are described and assessed for the control of this enzyme. The activities of these contaminants are not usually assayed in studies of this kind but could cause serious misinterpretation of initiation studies if not controlled.

Several methods have been described for the preparation of RNA polymerase from *Escherichia coli* (Chamberlin and Berg, 1962; Furth *et al.*, 1962; Stevens and Henry, 1964; Babinet, 1967; Burgess, 1969); it is now possible to obtain highly purified enzyme in lots of several milligrams. This paper describes the analysis of preparations of such enzyme from *E. coli* D-10 which were intended for use in studies of initiation of transcription. They appeared to be >95% pure on sodium dodecyl sulfate–polyacrylamide gels and were analyzed for a number of potential contaminating activities. No phosphatase activity was detected while the amount of deoxyribonuclease as measured in a sensitive assay was shown to be insignificant.

The preparations used in this study were found to incorporate both adenine and γ-phosphate from ATP into acid-insoluble form in the absence of added template. There were two reasons for studying these reactions more closely. Firstly it was possible that the enzyme was being phosphorylated and adenylated. It has been suggested from kinetic data and from the effects of various inhibitors that RNA polymerase may undergo a number of transitions during initiation (Fuchs *et al.*, 1967; Zillig *et al.*, 1970). There are several well-known cases in which phosphorylated or adenylated enzymes are intermediates in a reaction sequence (Krebs and Fischer, 1962; Little *et al.*, 1967; Shapiro *et al.*, 1967a,b). Goff and Weber (1970) have shown that RNA polymerase does possess sites at which the covalent addition of AMP can occur *in vivo* after T4 infection. The second possible explanation for incorporation of adenine and γ-phosphate was that the enzyme preparations were contaminated by polynucleotide phosphorylase and polyphosphate kinase. It was shown that this was the case.

Methods are described for the control or removal of these activities which can lead to misinterpretation of studies of initiation. In particular any experiments which presume to measure the initiation of RNA synthesis by following the incorporation of the γ-phosphate of ATP into acid-insoluble form (for example, Bautz and Bautz, 1970) must consider the possible artifacts caused by small amounts of contaminating polyphosphate kinase.

Materials and Methods

Cells. Frozen cells of *E. coli* D-10 (one-fourth log phase) were obtained from Grain Processing Corporation, Muscatine, Iowa, and of *E. coli* B ATCC 11303 (midlog phase) from General Biochemicals, Chagrin Falls, Ohio.

RNA Polymerase Assay. The enzyme was assayed in 0.25 ml of 0.05 M Tris (pH 7.9), 0.008 M $MgCl_2$, 0.0001 M Cleland's reagent, and nucleoside triphosphates (0.2 mM each; radioactive ATP, 1 Ci/mole) in the presence of 10–100 μg of DNA, for 10 min at 37°. Acid-precipitable counts were collected on nitrocellulose (25 mm B-6, Schleicher and Schuell) or glass fiber (Whatman GFC) filters. The filters were washed with 5 × 10 ml volumes of 10% trichloroacetic acid, and counted. The GFC filters gave a lower background (about 50% of the nitrocellulose) which could be further reduced by making the trichloroacetic acid solution 0.1 M in sodium pyrophosphate and 0.05 mM in ATP. In some assays 0.8 mM K_2HPO_4 and 0.05 mM ADP were added to inhibit polynucleotide phosphorylase and polyphosphate kinase, respectively. High salt assays contained 0.15 M KCl in addition. One unit of enzyme activity is that which incorporates 1 nmole of ATP/10 min at 37° into acid-insoluble form in the low salt

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