Transient Kinetics of Nicotinamide–Adenine Dinucleotide Phosphate-Linked Isocitrate Dehydrogenase from Bovine Heart Mitochondria

By KEITH DALZIEL, NEIL McFERRAN*, BRIAN MATTHEWS
and C. HUGH REYNOLDS†

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

(Received 26 October 1977)

Pre-steady-state studies of the isocitrate dehydrogenase reaction show that the rate constant for the hydride-transfer step is above 900s^{-1} , and that both subunits of the enzyme are simultaneously active. After the fast formation of NADPH in amounts equivalent to the enzyme subunit concentration, the rate of NADPH formation is equal to the steady-state rate if the enzyme has been preincubated with isocitrate and Mg^{2+} . If the enzyme has been preincubated with NADP^+ and Mg^{2+} , in 0.05M-triethanolamine chloride buffer, pH 7.0, with the addition of 0.1M-NaCl, the amount of NADPH formed in the fast phase is only 60% of the enzyme subunit concentration, and the turnover rate is at first lower than the steady-state rate. In 0.05M-triethanolamine chloride buffer, pH 7.0, if the enzyme is preincubated with NADP^+ or NADPH, the turnover rate increases 3-fold to reach the steady-state rate after about 5s. Preincubation of the enzyme with isocitrate and Mg^{2+} abolishes this lag phase, the steady-state rate being reached at once. It is suggested that the enzyme exists in at least two conformational forms with different activities, and that the lag phase represents the transition ($k = 0.4\text{s}^{-1}$) from a form with low activity to the fully active enzyme, induced by the binding of isocitrate and Mg^{2+} .

Isocitrate dehydrogenase [*threo*-D₈-isocitrate– NADP^+ oxidoreductase (decarboxylating), EC 1.1.1.42] purified from bovine heart mitochondria has two subunits and binds 2mol of NADPH/mol of enzyme (MacFarlane *et al.*, 1977). Steady-state kinetics showed that all the substrates must combine before any product can be formed, and maximum-rate relations were inconsistent with a simple ordered mechanism, suggesting a random sequential mechanism for reaction in both directions (Londesborough & Dalziel, 1970). These conclusions were also reached by Uhr *et al.* (1974) from steady-state kinetic studies of the pig heart enzyme, claimed by Colman (1972*b*) to be monomeric with mol. wt. 58000, and in addition more direct evidence in favour of a random mechanism was obtained by Northrop & Cleland (1974) from dead-end and multiple-inhibition studies. The random mechanism appears not to be of the rapid-equilibrium type; isotope-exchange rates suggested that catalysis in the central complexes is faster than product release (Uhr *et al.*, 1974).

There is kinetic evidence that the Mg^{2+} or Mn^{2+} complex of isocitrate is the main substrate of the enzyme from both these tissues (Londesborough &

Dalziel, 1970; Colman, 1972*a*), and that the product of the enzyme-catalysed decarboxylation is CO_2 , not HCO_3^- (Dalziel & Londesborough, 1968). Although the enzyme also catalyses the decarboxylation of oxaloacetate (Siebert *et al.*, 1957), it is not known whether the oxidative decarboxylation of isocitrate occurs by simultaneous hydride transfer and decarboxylation in a concerted reaction in the complex enzyme– NADP^+ – Mg^{2+} –isocitrate, or by successive steps with the formation of enzyme–NADPH– Mg^{2+} –oxalosuccinate as a molecular intermediate.

The aim of the work described here was to study the time course of NADPH formation in the transient phase by stopped-flow studies of the oxidative-decarboxylation reaction, in order to obtain information about the rate of hydride transfer, the number of intermediates and the rate-limiting step in catalysis, and to test whether or not the two subunits of the enzyme are simultaneously and independently active in catalysis.

Experimental

Isocitrate dehydrogenase from bovine heart mitochondria was purified and stored as described by MacFarlane *et al.* (1977). For each experiment a sample was dialysed overnight at 3°C against 0.1M-potassium phosphate buffer, pH 7.0, containing 0.1mM-EDTA. For experiments in 0.05M-

* Present address: Department of Biochemistry, The Queen's University, Belfast BT9 7BL, N. Ireland, U.K.

† Present address: Biochemical Research Department, The Wellcome Foundation Ltd., Temple Hill, Dartford, Kent DA1 5AH, U.K.

triethanolamine chloride buffer, pH 7.0, containing 0.1 mM-EDTA, with or without the addition of 0.1 M-NaCl, the enzyme was dialysed for a further 3 h against this buffer. The enzyme solution was then centrifuged (48 000g, 15 min) and the protein concentration and specific activity were determined as described by MacFarlane *et al.* (1977). The specific activities varied from 32 to 36 μ mol of NADPH formed/min per mg of protein. The enzyme concentrations stated are those of active enzyme calculated from the specific activity (39 units/min per mg) of the pure fully active enzyme, the mol.wt. (90 000) (MacFarlane *et al.*, 1977) and the protein concentration.

NADP⁺ and NADPH were from Boehringer Corp. (London) Ltd. (Lewes, Sussex BN7 1LG, U.K.) and DL-isocitrate (trisodium salt) was from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey KT2 7BH, U.K.). Concentrations of coenzymes and *threo*-D₅-isocitrate were estimated by enzymic assay (Londesborough & Dalziel, 1968). Triethanolamine hydrochloride was from Boehringer Corp. Other chemicals were of AR quality, purchased from Fisons Scientific Apparatus (Loughborough, Leics., U.K.).

Stopped-flow apparatus

The apparatus is a modification of that described by Gibson & Milnes (1964), thermostatically controlled by water circulation and with an optical path-length of 2.1 cm. The modifications include separate mounting of the signal photomultiplier (E.M.I. 6256) to minimize mechanical shock effects, and the addition of an incident-beam-splitter, reference photomultiplier and simple voltage-divider circuit to compensate for changes in source intensity and arc-wander. A logarithmic circuit for direct measurement of absorbance with the oscilloscope (Tektronix 849), a square-wave calibration source, a variable back-off potentiometer and a range of RF filters (time constant 0.1–10 ms) are also included. For the experiments described here, the light-source was a 250 W tungsten/iodide lamp operated from a stabilized power supply (Gresham Lion Electronics), with a monochromator (Bausch and Lomb, 33-86-50), heat filter and light filter (Farrand 7-54). At 340 nm, the band pass was 3 nm.

The dead-time of the apparatus was determined by recording progress curves for the reduction of 2,6-dichlorophenol-indophenol (approx. 0.03 mM) with excess of ascorbate (approx. 20 mM) in 0.1 M-sodium phosphate buffer, pH 6.0, and extrapolation of the first-order plots (k approx. 30 s⁻¹) to the initial absorbance of the reaction mixture, estimated by replacing ascorbate with buffer. Absorbance measurements were made at 522 nm, the isosbestic point for the acid and alkaline forms of the dye (pK 5.9). Although the reaction is usually carried out at pH 5.0, unlike Armstrong (1964), we found the absorbance of

the dye solution to be unstable at this pH. With a hydraulic drive (Gibson & Milnes, 1964) and by using 0.2 ml reactant solutions from each syringe, the dead-time was 2.5 ± 0.5 ms, and these conditions were used in the experiments described here. With a compressed-air activator (Durrum Instrument Corp.) operated at 500 kPa, the dead-time decreased from 10 to 3 ms, and the ram velocity increased in successive measurements as the ram advanced with progressive emptying of the drive syringes.

The mixing efficiency was estimated from progress curves with a large excess of ascorbate (approx. 0.4 M). The absorbance reached an apparently constant value of 1–2 % of the initial value in 20 ms, and thereafter decreased slowly, showing that the mixing efficiency was 98 % or more.

Kinetic measurements

All measurements were made at 340 nm and 25°C. The absorbances of the two reactant solutions were measured separately in the stopped-flow apparatus immediately before the kinetic experiment by mixing each in turn with buffer solution. Immediately after the kinetic experiment, in which several progress curves with different oscilloscope sweep-rates were obtained, the activity of a sample of the enzyme solution remaining in the syringe of the apparatus was measured; it was never less than 91 %, and usually 95 % or more, of the initial value.

In most of the experiments, the progress curves were recorded directly on the oscilloscope screen as absorbance changes, but for some experiments with large enzyme concentrations, the time course of the light transmission was recorded. This avoided exposure of the oscilloscope to large voltages generated by the large absorbance changes that accompanied reaction to equilibrium, which caused drift of the baseline. Photographs of the progress curves were analysed with a Nikon enlarger.

Control measurements of the steady-state rate were made in each experiment by conventional initial-rate measurements with a Zeiss spectrophotometer, with reaction mixtures of the same composition as those used in the stopped-flow experiment and low concentrations of enzyme.

Results

The early time course of NADPH formation in the oxidative-decarboxylation reaction was first studied with sufficiently large enzyme concentrations to detect any burst of enzyme-bound NADPH in the transient phase, and comparisons were made of the effects of preincubating the enzyme with isocitrate and Mg²⁺ and with NADP⁺ plus Mg²⁺. The oscilloscope records in Fig. 1 are examples from an experiment in which a solution containing 6.65 μ M-enzyme, isocitrate and MgCl₂ in 0.1 M-potassium phosphate/

Fig. 1. Oscilloscope records of changes of A_{340} accompanying the oxidative decarboxylation of isocitrate. Syringe 1 contained $6.65\mu\text{M}$ -enzyme, 0.9mM -threo- D_5 -isocitrate and 5mM - MgCl_2 , and syringe 2, 1mM - NADP^+ and 5mM - MgCl_2 , in 0.1M -potassium phosphate/ 0.1mM -EDTA buffer, $\text{pH}7.0$. The broken horizontal lines are square-wave signals, the lower one being ground, representing $0.170A$ unit in (a) and $0.200A$ unit in (b), equivalent to the back-off potentials. The initial absorbance of the reactants was 0.135 , and the time constant of the apparatus 0.1ms . The upper trace in (a) is a repeat sweep initiated 11ms after the end of the first sweep (from Expt. 1 in Table 1).

0.1mM -EDTA buffer, $\text{pH}7.0$, was mixed in the stopped-flow apparatus with one containing NADP^+ and MgCl_2 . The absorbance at the earliest time of observation is substantially greater than that of the initial reactants, showing that NADPH is formed rapidly in the dead-time of the apparatus and thereafter at a lower rate, constant for at least 0.2s .

The progress curves from three experiments, in Fig. 2, were obtained from such photographs by measuring the absorbance at several times and subtracting the initial absorbance of the two reactant solutions. Curve (1) is from Fig. 1(a) and curve (2) from an experiment under the same conditions except that twice the enzyme concentration was used. The ordinate intercepts, representing absorbance changes in the dead-time, are evidently proportional to the enzyme concentration, and are equivalent to $6.1\mu\text{M}$ -

Fig. 2. Effects of enzyme concentration and preincubation of the enzyme with NADP^+ on the progress curve in 0.1M -potassium phosphate buffer, $\text{pH}7.0$.

The data were obtained from photographs of oscilloscope records, and the initial absorbance of the reactants has been subtracted. Curve (1) was obtained from Fig. 1(a) and curve (2) from a similar experiment (Expt. 2 in Table 1) with twice the enzyme concentration. Curve (3) is from an experiment in which syringe 1 contained $7.3\mu\text{M}$ -enzyme, 1mM - NADP^+ and 5mM - MgCl_2 , and syringe 2, 0.9mM -threo- D_5 -isocitrate and 5mM - MgCl_2 (Expt. 4 in Table 1). The time constant was 0.1ms .

NADPH in curve (1) with $3.3\mu\text{M}$ -enzyme, and $14.1\mu\text{M}$ - NADPH in curve (2) with $6.7\mu\text{M}$ -enzyme, calculated from ϵ for free NADPH (6.22×10^3 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$) and the path-length of 2.1cm . The slopes of the subsequent progress curves are $1.89A$ units/s and $3.6A$ units/s respectively. Curve (3) in Fig. 2 is from an experiment in which enzyme was preincubated with NADP^+ and MgCl_2 ; the composition of the reaction mixture was unchanged, except that the enzyme concentration after mixing was $3.7\mu\text{M}$. The fast burst of NADPH formation is not quite complete in the dead-time in this experiment.

The results of these and other experiments with high concentrations of enzyme are summarized in Table 1. The amplitude of the rapid burst of absorbance is expressed as the concentration of NADPH formed divided by the enzyme concentration, the average and range of values from several progress curves being given for each experiment. The last column shows the average value for the ratio of the subsequent, constant rate of NADPH formation, in the time range $2\text{--}400\text{ms}$, to the steady-state rate. The latter was calculated for the enzyme concentration used in the stopped-flow experiment from conventional initial-rate measurements with the same concentrations of reactant and lower concentrations of enzyme. The results of Expts. 1–3 in 0.1M -potassium phosphate buffer and Expts. 5 and 6 in 0.05M -

Table 1. Kinetic data for the oxidative decarboxylation of isocitrate from stopped-flow experiments with high concentrations of enzyme at pH 7.0 and 25°C

In all the experiments, the NADP⁺ concentration after mixing was 0.5 mM and the MgCl₂ concentration 5 mM, except that the latter was replaced by 5 mM-MnCl₂ in Expt. 6. The enzyme and *threo*-D₂-isocitrate concentrations were as shown. The enzyme solution initially contained NADP⁺ and MgCl₂ for Expts. 4, 7 and 8, and isocitrate and MgCl₂ for the other experiments. The burst stoichiometry is the ratio of the concentration of NADPH formed in the dead-time of the apparatus to the enzyme concentration. The last column shows the ratio of the constant rate of NADPH formation from 2 to 400 ms to the final steady-state rate, the latter being calculated from conventional initial-rate measurements with small enzyme concentrations. The number of progress curves from which the average values and ranges were obtained in each experiment are shown in parentheses.

Expt.	Buffer	Isocitrate (mM)	Enzyme (μM)	Burst stoichiometry (mol of NADPH/mol of enzyme)	Initial rate steady-state rate
1	0.1 M-Phosphate	0.45	3.32	2.1 ± 0.3 (6)	1.15 ± 0.05 (5)
2	0.1 M-Phosphate	0.45	6.65	2.0 ± 0.2 (5)	1.23 ± 0.12 (4)
3	0.1 M-Phosphate	0.45	4.25	2.3 ± 0.2 (5)	0.97 ± 0.03 (6)
4	0.1 M-Phosphate	0.45	3.65	1.8 ± 0.02 (4)	0.92 ± 0.03 (5)
5	0.05 M-Triethanolamine + 0.1 M-NaCl	0.45	2.9	1.9 ± 0.14 (3)	1.19 ± 0.14 (3)
6	0.05 M-Triethanolamine + 0.1 M-NaCl	1.35	4.45	2.0 ± 0.08 (4)	1.28 ± 0.07 (6)
7	0.05 M-Triethanolamine + 0.1 M-NaCl	1.35	5.0	1.2 ± 0.2 (6)	0.50 ± 0.03 (8)
8	0.05 M-Triethanolamine + 0.1 M-NaCl	1.35	5.5	1.2 ± 0.06 (2)	0.52 ± 0.04 (6)

Fig. 3. Progress curves for the isocitrate dehydrogenase reaction in 0.05 M-triethanolamine chloride/0.1 M-NaCl/0.1 mM-EDTA, pH 7.0, after preincubation of enzyme, NADP⁺ and MgCl₂.

Syringe 1 contained 10.0 μM-enzyme, 1 mM-NADP⁺ and 5 mM-MgCl₂, and syringe 2, 2.7 mM-*threo*-D₂-isocitrate and 5 mM-MgCl₂. Duplicate progress curves obtained with a sweep rate of 50 ms/cm and time constant 1.0 ms are shown. The initial absorbance of the reactants has been subtracted (Expt. 7 in Table 1).

triethanolamine chloride/0.1 M-NaCl/0.1 mM-EDTA buffer, pH 7.0, for which the enzyme was preincubated with isocitrate and MgCl₂, show that 2 mol of NADPH/mol of enzyme is formed within the dead-time of the apparatus, and that the subsequent rate of NADPH formation, constant for at least 0.4 s, is in satisfactory agreement with the steady-state rate measured with small enzyme concentrations. In

Expt. 4 in phosphate buffer, with enzyme, NADP⁺ and MgCl₂ preincubated, similar results were obtained except that the rapid burst extends for about 4 ms after the dead-time. However, in triethanolamine chloride buffer with enzyme, NADP⁺ and MgCl₂ preincubated (Expts. 7 and 8) the amplitude of the dead-time burst was only 60% of the enzyme subunit concentration, and the subsequent, apparently constant, rate was only half the steady-state rate. These findings are illustrated by progress curves from Expt. 7 in Fig. 3.

This latter discrepancy was further investigated in stopped-flow experiments with lower concentrations of enzyme, so that the progress of the reaction could be followed accurately for longer times. The results showed that the turnover rate increased slowly from 2 ms after mixing, reaching the steady-state rate after about 4 s. This is illustrated by a progress curve in Fig. 4 from an experiment with 215 nM-enzyme, again in 0.05 M-triethanolamine chloride/0.1 M-NaCl/0.1 mM-EDTA, pH 7.0, and after preincubation of the enzyme with NADP⁺ and MgCl₂. By contrast, when the enzyme (final concn. 0.15 μM) was preincubated with isocitrate and MgCl₂, the rate remained constant and equal to the steady-state rate from 2 ms to 4 s after mixing (Fig. 4).

The progress curves in Fig. 5 from experiments in 0.05 M-triethanolamine chloride/0.1 mM-EDTA buffer, pH 7.0, again show a slow approach to the steady-state rate when the enzyme is preincubated with NADP⁺ (curve 1), but not when it is preincubated with isocitrate and MgCl₂ (curve 2). There is also a lag when the enzyme is preincubated with NADPH (curve 3) and, to a lesser degree, when

reaction is initiated by mixing a solution of enzyme in buffer alone with one containing NADP^+ , isocitrate and MgCl_2 (curve 4).
The results of these and other experiments in

Fig. 4. Effects of preincubation of the enzyme with NADP^+ and MgCl_2 and with isocitrate and MgCl_2 on the time course of NADPH formation in 0.05M-triethanolamine chloride/0.1M-NaCl/0.1mM-EDTA, pH7.0
For (a), syringe 1 contained 0.43 μM -enzyme, 0.5mM- NADP^+ and 5mM- MgCl_2 , and syringe 2 contained 0.9mM-threo- D_3 -isocitrate and 5mM- MgCl_2 . For (b) syringe 1 contained 0.30 μM -enzyme, 0.9mM-threo- D_3 -isocitrate and 5mM- MgCl_2 , and syringe 2, 0.5mM- NADP^+ and 5mM- MgCl_2 . The time constant of the apparatus was 1.0ms (Expts. 12 and 11, Table 2).

Fig. 5. Effects of preincubation of the enzyme with NADP^+ , NADPH and isocitrate on the progress curve in 0.05M-triethanolamine chloride/0.1mM-EDTA, pH7.0
The enzyme was preincubated with NADP^+ (●), with isocitrate and MgCl_2 (○), with NADPH (■) or with buffer alone (□). The progress curves are from Expts. 13, 10, 14 and 9 respectively in Table 2, where the concentrations are stated.

Table 2. Effects of preincubating isocitrate dehydrogenase with ligands on the rate of approach to the steady-state rate of the oxidative-decarboxylation reaction

The solutions in the two syringes of the stopped-flow apparatus were made up in 0.05M-triethanolamine chloride/0.1mM-EDTA, pH7.0, with the addition of 0.1M-NaCl in Expts. 11 and 12 only. The isocitrate concentrations stated are those of the threo- D_3 -isomer. Initial rates were estimated from progress curves recorded with an oscilloscope sweep rate of 0.1 s/cm, and final steady-state rates were obtained with a sweep rate of 2s/cm. The number of progress curves used in each estimation is given in parentheses. The first-order rate constant (k) for the transition from the initial to the final rate was estimated as illustrated in Fig. 6, and described in the text.

Expt.	Syringe 1	Syringe 2	Initial rate final rate	k (s^{-1})
9	0.26 μM -Enzyme	0.5mM- NADP^+ , 10mM- MgCl_2 and 0.9mM-isocitrate	0.94 (9)	—
10	0.4 μM -Enzyme, 10mM- MgCl_2 and 0.9mM-isocitrate	0.5mM- NADP^+	1.0 (6)	—
11	0.3 μM -Enzyme, 5mM- MgCl_2 and 0.9mM-isocitrate	0.5mM- NADP^+ and 5mM- MgCl_2	1.0 (9)	—
12	0.43 μM -Enzyme, 5mM- MgCl_2 and 0.5mM- NADP^+	0.9mM-Isocitrate and 5mM- MgCl_2	0.58 (9)	~ 0.4
13	0.4 μM -Enzyme and 0.5mM- NADP^+	0.9mM-Isocitrate and 10mM- MgCl_2	0.35 (9)	0.46
14	0.26 μM -Enzyme and 21 μM -NADPH	0.9mM-Isocitrate, 10mM- MgCl_2 and 0.5mM- NADP^+	0.33 (9)	0.31
15	0.4 μM -Enzyme, 19 μM -NADPH and 0.5mM- NADP^+	0.9mM-Isocitrate and 10mM- MgCl_2	0.29 (8)	0.41
16	0.4 μM -Enzyme, 5mM- MgCl_2 , 0.5mM- NADP^+ and 2mM-2-oxoglutarate	0.9mM-Isocitrate and 5mM- MgCl_2	0.29 (5)	0.40
17	0.26 μM -Enzyme, 5mM- MgCl_2 , 0.9mM-isocitrate and 42 μM -NADPH	0.5mM- NADP^+ and 5mM- MgCl_2	1.0 (7)	—

triethanolamine chloride buffer with enzyme concentrations in the range 0.13–0.22 μM are summarized in Table 2. In each experiment, several progress curves were recorded with an oscilloscope sweep rate of 0.1 s/cm, to estimate the initial rate, and several with a sweep-rate of 2 s/cm, to estimate the final steady-state rate, and the ratio of the initial to the final rate is given. In 0.05 M-triethanolamine chloride/0.1 mM-EDTA, when the enzyme is preincubated with NADP^+ , with NADPH, or with NADP^+ , MgCl_2 and 2-oxoglutarate, the initial rate after mixing with isocitrate and MgCl_2 in the stopped-flow apparatus is about one-third of the steady-state rate reached after about 4 s. When the enzyme is preincubated with isocitrate and MgCl_2 , or with these ligands and NADPH, the steady-state rate is reached at once. When the ionic strength is increased by the addition of 0.1 M-NaCl to the buffer, the initial rate after preincubation of the enzyme with NADP^+ and MgCl_2 is 60% of the steady-state rate.

A quantitative description of the relatively slow increase in turnover rate induced by preincubation of the enzyme with NADP^+ or NADPH was sought on the basis of a model in which the enzyme is assumed to be present initially in a form E_1 , with specific activity a , which isomerizes to a form E_2 , with specific activity b . If $[E]_T$ is the total enzyme concentration, and k the rate constant for the isomerization, the concentrations of these two forms at any time t will be $[E]_T e^{-kt}$ and $[E]_T(1 - e^{-kt})$ respectively, and the rate of formation of NADPH will be:

$$\frac{d[\text{NADPH}]}{dt} = [E_1]a + [E_2]b = [E]_T \{b - (b-a)e^{-kt}\}$$

By integration:

$$[\text{NADPH}]_t = [E]_T \left\{ bt - \left(\frac{b-a}{k} \right) + \left(\frac{b-a}{k} \right) e^{-kt} \right\}$$

and this equation should describe the progress curve throughout its time course. When t is large, the exponential term will be negligible and the progress curve linear, described by:

$$[\text{NADPH}]_t = [E]_T \left\{ bt - \frac{(b-a)}{k} \right\}$$

Therefore, if the linear portion of the progress curve is extrapolated to a smaller value of t , and $A_{\text{calc.}}$ is the absorbance thus obtained at any early time t , then:

$$A_{\text{obs.}} - A_{\text{calc.}} = \Delta A = \left(\frac{b-a}{k} \right) e^{-kt}$$

where $A_{\text{obs.}}$ is the measured absorbance at time t , Hence:

$$\log \Delta A = \log \left(\frac{b-a}{k} \right) - \frac{kt}{2.3}$$

Fig. 6. First-order plot for the supposed transition of the enzyme, after preincubation with coenzyme, to the fully active form during the lag phase

Values of $\log(\Delta A)$ from Expt. 13 (○), Expt. 15 (□) and Expt. 16 (●), described in Table 2, are plotted to test conformity to the simple model described in the text.

and a plot of $\log \Delta A$ versus t should be linear, with slope $-k/2.3$. The experimental data in Fig. 6 conform well with this requirement, and values for k obtained from several experiments are shown in Table 2.

Discussion

Isocitrate dehydrogenase from bovine heart mitochondria has two binding sites for NADPH and for NADP^+ in the dimeric molecule (MacFarlane *et al.*, 1977; Reynolds *et al.*, 1978a). It also binds isocitrate in the presence of Mg^{2+} , and in the absence of coenzyme (Reynolds *et al.*, 1978a), as does the enzyme from pig heart (Colman & Chu, 1970), and this is consistent with the evidence for a random mechanism from steady-state kinetics (Londesborough & Dalziel, 1970; Uhr *et al.*, 1974; Northrop & Cleland, 1974). From the dissociation constants for these complexes of the bovine heart enzyme in the three buffer/salt systems used in the present work (Reynolds *et al.*, 1978a), it can be shown that the concentrations of coenzyme and substrate were sufficient in all the experiments to saturate the enzyme with either ligand, to the extent of 95% or more, so far as equilibrium binding is concerned. The concentrations are also much larger than the K_m values (Londesborough & Dalziel, 1970).

We have shown that when the enzyme is preincubated with isocitrate and MgCl_2 in 0.1 M-potassium phosphate buffer, or in 0.05 M-triethanolamine chloride/0.1 M-NaCl buffer, pH 7.0, and then mixed with NADP^+ in the stopped-flow apparatus, the formation of NADPH is biphasic. Steady-state

turnover at a rate of 36kat/mol is preceded by a fast burst of NADPH formation of amplitude proportional to the enzyme concentration and equivalent to the enzyme subunit concentration (Table 1). This transient phase is completed (>90%) within the dead-time of 2.5ms. The rate constant for hydride transfer must therefore be at least 900s^{-1} , and one of the later steps, which might include decarboxylation and conformational changes as well as product release, must be rate-limiting in the overall reaction. For the enzyme from pig heart, Uhr *et al.* (1974) suggested that catalysis in the central complexes is faster than product release, on the basis of isotope-exchange rates.

The close agreement between the concentration of NADPH formed in the transient phase and the enzyme subunit concentration in these experiments establishes that both subunits are simultaneously active in catalysis, and also that the proportion of the enzyme remaining as enzyme-NADP⁺ or enzyme-NADP⁺-Mg²⁺-isocitrate complexes after the transient phase is small. This suggests that the reversible hydride transfer is prevented from equilibrating by rapid dissociation of one of the products, perhaps CO₂. These conclusions are based on the assumption that the molar absorbance of the NADPH formed in the transient phase is the same as that of free NADPH. We have shown that in its complex with enzyme and Mg²⁺ the absorbance of NADPH at 340nm is unchanged. The burst of NADPH may include other complexes, but it is most unlikely that their molar absorbance at 340nm would be substantially greater than that of free NADPH, as would be needed to invalidate the conclusions drawn.

When the enzyme has been preincubated with NADP⁺ and MgCl₂ in 0.1 M-phosphate buffer, there is again a 'full burst' of NADPH formation, followed immediately by steady-state turnover at 36kat/mol, but the transient phase is not quite completed in the dead-time of the apparatus. In this experiment (Expt. 4, Table 1), the combination of isocitrate with the enzyme-NADP⁺ complex might have been rate-limiting for hydride transfer; in a similar experiment in triethanolamine buffer with a 3-fold higher isocitrate concentration, the burst, although smaller in amplitude, was completed in the dead-time.

The time course of NADPH formation in 0.05M-triethanolamine chloride/0.1M-NaCl buffer, pH7.0, after preincubation of the enzyme with NADP⁺ and Mg²⁺, is more complex. First, the amplitude of the 'burst' of NADPH formation was equivalent to only 60% of the enzyme subunit concentration. Secondly, the rate after this fast phase, although apparently constant for 0.4s, was only 18kat/mol, half the steady-state rate. It was shown in similar experiments under the same conditions, but with smaller enzyme concentrations, that the turnover rate slowly increased and reached the steady-state rate after about

4s. A qualitatively similar time course was obtained in 0.05M-triethanolamine chloride buffer, pH7.0, without the addition of 0.1M-NaCl, when the enzyme was preincubated with NADP⁺ and Mg²⁺, with NADP⁺ alone, or with NADPH. However, in this medium of lower ionic strength the initial rate in the stopped-flow apparatus was only one-third of the final steady-state rate, and the lag phase was therefore more marked. There was also a less marked but reproducible lag phase when the enzyme in buffer alone was mixed with NADP⁺, isocitrate and MgCl₂ in the stopped-flow apparatus. As in the experiments at higher ionic strength, the steady-state rate was reached at once if the enzyme has been preincubated with isocitrate and Mg²⁺, or with NADPH, isocitrate and Mg²⁺. In the latter case, the enzyme would be present as the abortive complex enzyme-NADPH-Mg²⁺-isocitrate before it was mixed with NADP⁺ (Reynolds *et al.*, 1978a).

The simplest explanation of these results, we suggest, is that the enzyme can exist in two conformations with different catalytic activities. A conformation with low activity is favoured by low ionic strength and induced particularly by ligation with NADP⁺ or NADPH; its activity is at most 30% of that of fully active enzyme, and it may represent inactive enzyme in equilibrium with active enzyme. The enzyme conformation with full activity is formed by high ionic strength and by phosphate, and is completely induced by ligation of isocitrate and Mg²⁺, even when NADP⁺ or NADPH are already bound to the enzyme, forming respectively the reactive quaternary complex and the abortive complex. The time course of NADPH formation in the oxidative-decarboxylation reaction after preincubation of the enzyme with NADP⁺, with NADPH, with NADP⁺ and NADPH, or with NADP⁺, 2-oxoglutarate and Mg²⁺ has been shown to be quantitatively consistent with a hypothesis of this kind, and to give the same value, within the admittedly large experimental uncertainty, for the apparent first-order rate constant for the transition of about 0.4s^{-1} . It seems likely that this relatively slow conformational change occurs in the reactive complex enzyme-NADP⁺-Mg²⁺-isocitrate in all these experiments. It would be premature to attribute any regulatory significance to these findings. Indeed, since in the presence of activating metal ions isocitrate is very firmly bound by the enzyme (Reynolds *et al.*, 1978a), it is conceivable that the enzyme exists in the tissue largely as its complex with this substrate, and therefore in the fully active conformation.

Enhancement or quenching of the protein fluorescence caused by equilibrium binding of substrates and coenzymes, by changes of ionic strength and by phosphate have also indicated conformational changes (Reynolds *et al.*, 1978a). There is no simple correlation between these changes of protein fluor-

escence and the effects on enzymic activity described here. For example, NADP⁺ binding causes an enhancement of enzyme protein fluorescence, and NADPH and isocitrate each quench the fluorescence. There have also been many previous reports of time lags from steady-state kinetic studies of NADP-linked isocitrate dehydrogenase from various sources, including bovine heart (Londesborough, 1969), pig heart (Sanner & Ingebrechtsen, 1976; Ingebrechtsen & Sanner, 1976; Ehrlich & Colman, 1976) and pig liver (Carlier & Pantaloni, 1976). Again no correlation with the effects that we describe is apparent; the time lags reported by these earlier workers were generally more prolonged, or were observed under different conditions, notably in phosphate buffer and with very small concentrations of metal activator, and were abolished by NADPH in some cases.

Although we have established that the rate-limiting step in the oxidative decarboxylation occurs after the redox reaction, and probably after decarboxylation (if indeed this is a separate step), it has yet to be identified. Preliminary kinetic studies of the dissociation of NADPH from its binary complex with the enzyme, by displacement with NADP⁺ and fluorescence stopped-flow measurements, indicate that it is slower than the maximum specific rate of oxidative decarboxylation. In the presence of Mg²⁺, the affinity of the enzyme for NADPH is decreased (Reynolds *et al.*, 1978a) and it is therefore possible that the specific rate of dissociation of NADPH from the complex enzyme-Mg²⁺-NADPH, which has not yet been studied directly, may be the rate-limiting step in oxidative decarboxylation. If this is so, then from the maximum turnover number of 18 s⁻¹ (per active centre) for the latter reaction and the dissociation constant for NADPH of 0.01 μ M for this complex, the second-order rate constant for the combination of NADPH with the enzyme in the presence of Mg²⁺ must be about 2 nM⁻¹·s⁻¹, which is close to the diffusion-controlled limit (Alberty & Hammes, 1958). A more likely rate-limiting intermediate under maximum rate conditions is the abortive enzyme-Mg²⁺-NADPH-isocitrate complex, in which NADPH is much less firmly bound than it is to the enzyme alone (Reynolds *et al.*, 1978a).

Many other dehydrogenases form analogous abortive complexes, in which, however, the reduced coenzyme is more firmly bound than it is to the enzymes alone, and this results in substrate inhibition (cf. Dalziel, 1975). The isocitrate dehydrogenase reaction does not exhibit substrate inhibition with concentrations of isocitrate up to that used in the present work. 'Malic' enzyme, which catalyses an oxidative-decarboxylation reaction analogous to the isocitrate dehydrogenase reaction, does exhibit such substrate inhibition, and the results of recent tran-

sient kinetic studies with 'malic' enzyme in this laboratory (Reynolds *et al.*, 1978b) should be mentioned. The hydride-transfer step is again fast ($k > 900$ s⁻¹), but in contrast with isocitrate dehydrogenase the burst of NADPH formation in the transient phase is equivalent to only half the active-centre concentration, and is followed by turnover at a rate that is at first substantially higher than the steady-state rate, reached after about 0.5 s. Evidence has been presented that this slow decrease in the turnover rate is related to the slow onset of substrate inhibition by the formation of an abortive enzyme-Mn²⁺-NADPH-malate complex.

We are indebted to the Science Research Council for financial support. K. D. is a member of the Oxford Enzyme Group. We thank Dr. C. G. Morgan for designing the voltage-divider and ancillary circuits.

References

- Alberty, R. A. & Hammes, G. G. (1958) *J. Phys. Chem.* **62**, 154-159
- Armstrong, J. McD. (1964) *Biochim. Biophys. Acta* **86**, 194-197
- Carlier, M.-F. & Pantaloni, D. (1976) *Biochemistry* **15**, 1761-1766
- Colman, R. F. (1972a) *J. Biol. Chem.* **247**, 215-223
- Colman, R. F. (1972b) *J. Biol. Chem.* **247**, 6727-6733
- Colman, R. F. & Chu, R. (1970) *J. Biol. Chem.* **245**, 601-607
- Dalziel, K. (1975) *Enzymes 3rd Ed.* **11A**, 1-60
- Dalziel, K. & Londresborough, J. C. (1968) *Biochem. J.* **110**, 223-230
- Ehrlich, R. S. & Colman, R. F. (1976) *Biochemistry* **15**, 4034-4041
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161-171
- Ingebrechtsen, O. C. & Sanner, T. (1976) *Arch. Biochem. Biophys.* **176**, 442-448
- Londesborough, J. C. (1969) D.Phil. Thesis, University of Oxford
- Londesborough, J. C. & Dalziel, K. (1968) *Biochem. J.* **110**, 217-222
- Londesborough, J. C. & Dalziel, K. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 315-323, Springer-Verlag, Heidelberg
- MacFarlane, N., Matthews, B. & Dalziel, K. (1977) *Eur. J. Biochem.* **74**, 553-559
- Northrop, D. B. & Cleland, W. W. (1974) *J. Biol. Chem.* **249**, 2928-2931
- Reynolds, C. H., Kuchel, P. W. & Dalziel, K. (1978a) *Biochem. J.* **171**, 733-742
- Reynolds, C. H., Hsu, R. Y., Matthews, B., Pry, T. A. & Dalziel, K. (1978b) *Arch. Biochem. Biophys.* in the press
- Sanner, T. & Ingebrechtsen, O. C. (1976) *Arch. Biochem. Biophys.* **172**, 59-63
- Siebert, G., Dubuc, J., Warner, R. C. & Plaut, G. W. E. (1957) *J. Biol. Chem.* **226**, 965-975
- Uhr, M. L., Thompson, V. W. & Cleland, W. W. (1974) *J. Biol. Chem.* **249**, 2920-2927