

Stopped Flow and Steady State Kinetic Studies of the Effects of Metabolites on the Soluble Form of NADP⁺:Isocitrate Dehydrogenase*

Harold M. Farrell, Jr.‡, Jude T. Deeney, Erich K. Hild, and Thomas F. Kumosinski

The cytosolic form of NADP⁺:isocitrate dehydrogenase, a primary source of the NADPH required for *de novo* fatty acid synthesis in lactating bovine mammary gland, was studied to determine possible mechanisms of regulation by metabolites. Stopped flow kinetics showed a distinct lag time, followed by attainment of an apparently linear final velocity. Direct nonlinear regression analyses of the reaction progress curves allowed for the calculation of the rate constant (*k*) for the transition of the enzyme from an inactive to an active form; this transition is best catalyzed by its metal-substrate complex. Preincubation with metal-substrate or metal-citrate nearly abolished the lag by increasing *k* 10-fold. In steady state experiments, analyses of velocity *versus* metal-citrate complex as a binding isotherm, following the assumptions of Wyman's theory of thermodynamic linkage, showed that binding of metal-citrate complex could both activate and inhibit the enzyme. This analysis suggested: (a) activation by binding to sites with an average dissociation constant of 0.25 mM; (b) inhibition by binding to sites with an average dissociation constant of 3.83 mM; and (c) modulation (reactivation) by binding to sites with an average dissociation constant of 1.54 mM. Concentration ranges observed for these transitions are compatible with physiological conditions, suggesting that complexes of metal-citrate and metal-isocitrate serve to modulate the activity of NADP⁺:isocitrate dehydrogenase.

NADP⁺-dependent isocitrate dehydrogenase (*threo*-D₅-isocitrate:NADP⁺ oxidoreductase (decarboxylating) EC 1.1.1.42) can serve as a source of NADPH for synthesis of metabolic end products in a variety of tissues (1, 2). For example, in lactating ruminant mammary gland, this enzyme may be a primary source of the NADPH required for fatty acid synthesis (3). A survey of the distribution of Krebs cycle enzymes in mammary tissue showed that NADP⁺:isocitrate dehydrogenase (IDH)¹ is predominately cytosolic (>90%) in nature, and that little or no NAD⁺:IDH activity is present (4). Since the latter enzyme is known to be allosterically regulated by metabolites and is thought to control Krebs cycle activity (5, 6), its absence in mammary gland prompted a study of the possible effects of metabolites on the kinetics of NADP⁺:IDH. Although the kinetics of NADP⁺:IDH from heart tissue have

been investigated in detail (1) Plaut and coworkers (7) have demonstrated that the heart enzyme is of mitochondrial origin, while the purified mammary enzyme is clearly a cytosolic or soluble form. These two forms of the enzyme are now known to be immunologically and electrophoretically distinct (7) and so investigation of the kinetic properties of the mammary enzyme seemed warranted. In this study the purified cytosolic enzyme (8), which gives essentially linear steady state kinetics, was investigated using stopped flow techniques. Analysis of the reaction progress curves showed a distinct lag time followed by a linear progress curve; this nonlinear behavior indicates that NADP⁺:IDH may be classified as a substrate-activated, hysteretic enzyme as delineated by Frieden (9). Effects of substrate, metal-substrate complex, metal ion, citrate, and metal-citrate complex on enzyme activity and activation were studied to gain further insight into possible mechanisms which may regulate this enzyme.

EXPERIMENTAL PROCEDURES

Materials

All coenzymes, substrates, and biochemicals used in this study were purchased from either Sigma or Calbiochem. [1,5-¹⁴C]Citric acid, 110 mCi/mmol, was from Amersham Crop. Blue Sepharose CL-6B and Sephacryl S-200 were products of Pharmacia LKB Biotechnology Inc., and DE32 was obtained from Whatman. All other chemicals were reagent grade.

Whole mammary glands from cows of known good health and productivity were obtained through the cooperation of Drs. J. E. Keys and R. H. Miller of the United States Department of Agriculture, Beltsville, MD. The whole mammary glands were obtained at the time of slaughter, trimmed to remove adipose tissue, and sectioned into pieces approximately 10 × 15 × 5 cm. The tissue was frozen on dry ice and stored at -80 °C until used. NADP⁺:IDH was isolated as described by Farrell (8).

Enzyme Assays

Steady State—NADP⁺:isocitrate dehydrogenase activity was measured at 25 °C by monitoring the increase in A_{340 nm}. The standard reaction mixture consisted of 100 mM Tris-HCl at pH 7.4, 1.10 mM MnSO₄, 1.50 mM DL-isocitrate, and 100 μM NADP⁺ in a total volume of 2.70 ml; 10–50 μl of enzyme were added to start the reaction. Throughout this study only Mn²⁺ was used, therefore metal from here on refers to Mn²⁺. Concentrations of metal-citrate, metal-isocitrate, and free metal ion were computed as previously described (10). By fixing the free Mn²⁺ concentration, as well as the desired metal-substrate and metal-modifier concentrations, the required total concentrations can easily be calculated. These values ranged as follows: Mn²⁺, 0.3–6.3 mM; isocitrate, 0.08–6 mM; citrate, 3.5–24.2 mM. Reactions were carried out in 1-cm cells and monitored on a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) with a scale setting of 0–0.150 absorbance units. One enzyme unit catalyzes the formation of 1.0 μmol of NADPH per min at 25 °C. Specific activity is defined as enzyme units/mg protein.

Kinetic data were obtained by averaging triplicate assays for each point. The results were analyzed by iterative techniques as recommended by Cleland (11). Surface denaturation and adsorption was minimized when cuvettes were prewashed three to four times with the

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‡ To whom correspondence should be addressed: United States Department of Agriculture, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118.

¹ The abbreviation used is: IDH, isocitrate dehydrogenase.

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10% glycerol buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 100 μM dithiothreitol, and 10% glycerol) used to purify the enzyme (8); the cuvettes were allowed to drain dry and then were used for the assays.

Stopped Flow—Reactions were also carried out and monitored at 340 nm on a Durrum stopped flow spectrophotometer (Dionex, Sunnyvale, CA) equipped with a storage oscilloscope (scale settings of 1–5 V per division, where 1 V = 0.1 absorbance units). The time course of a reaction was generally observed from 0 to 500 ms; linearity for the assay, once achieved, continued for over 10 s. Polaroid pictures of the oscilloscope traces were digitized with a pad (Houston Instrument, Austin, TX) and the data were entered directly into a Mod-comp computer. In all of the stopped flow experiments *threo*-D₅-isocitrate, the stereo-active form of the substrate, was employed. Syringes were prewashed with 10% glycerol buffer. Dead time of 5 ms was determined as described by Dalziel *et al.* (12).

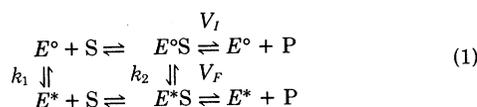
Data Analysis—Nonlinear regression analyses of progress curves, and variance of other parameters with concentration were carried out using the program Abcus which is based on the Gauss-Newton iterative method and which was developed by Dr. William Damert of this Center. Choices between fits of models and statistical methods of analysis of the nonlinear fit are as described by Meites (13).

Binding Studies—The binding of metal-¹⁴C]citrate to IDH was studied at 25 °C using Ultrafree PF-filter units (Millipore/Continental Water Systems, Bedford, MA) containing polysulfone membranes with a nominal cut-off of 30,000 molecular weight for proteins. The initial total concentrations of metal-citrate were determined as described above.

Enzyme (0.040 mM) and metal-citrate (stock concentration 81.3 mM citrate with a specific activity of 53.3 cpm/nmol) were allowed to equilibrate at 25 °C in 0.1 M Tris-HCl, pH 7.4, for 10 min. The samples (300 μl) were filtered to near dryness under N₂ at 25 p.s.i.; 100 μl of Tris-HCl was then filtered to purge the Luer tip of the cartridge. Bound metal-citrate was washed through the filter under N₂ by three successive 300-μl washes with 0.1 M Tris-HCl containing 0.1 M NaCl. The pooled washings were counted in a scintillation counter (LS-8100, Beckman Instruments). In preliminary experiments at three levels of metal-citrate (0.25, 1.0, and 6.0 mM) it was determined that greater than 97% of the added cpm could be accounted for in the combined first filtrate and three washings. Blank experiments with no protein present were conducted at each concentration of metal-citrate. These blank values (which represent ~1% of total cpm or 5–8% of cpm bound to protein) were subtracted at each concentration.

RESULTS

Stopped Flow Kinetics—In the determination of K_m for DL-isocitrate for soluble NADP⁺:IDH (8), particularly at low metal ion concentrations, early nonlinear portions of the initial velocity curves were observed when the reaction was initiated with substrate. Examination of the early phases of the reaction progress curves in the stopped flow spectrophotometer showed a nonlinear response with a characteristic lag time prior to the achievement of a linear steady state (Fig. 1A). This behavior was nearly abolished when the enzyme was preincubated with metal ion and substrate prior to mixing in the stopped flow apparatus (Fig. 1B). Preincubation with NADP⁺, metal ion alone, or substrate alone did not appear to abolish the lag time, and results nearly identical to Fig. 1A were obtained. These preliminary results were consistent with an apparent metal-isocitrate-induced elimination of the lag time. Previous results on the soluble (8) and the mitochondrial forms (1) of NADP⁺:IDH have shown that the metal-isocitrate complex is the true substrate for these enzymes. According to the terminology developed by Frieden (9) the general mechanism for hysteretic (nonlinear) kinetics of an enzyme is as follows,



and

$$V_{\text{obs}} = V_F + (V_I - V_F) e^{-kt}, \quad (2)$$

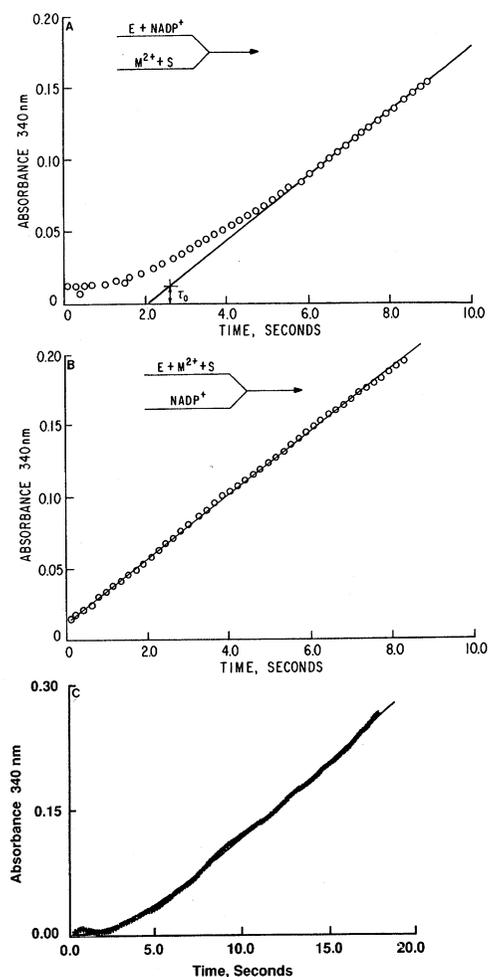


FIG. 1. Time course of the reaction of NADP⁺:IDH. The enzyme was incubated under two conditions in the syringes of the stopped flow spectrophotometer. The insets show the contents of the syringes prior to mixing. A, enzyme incubated with NADP⁺ only; B, enzyme incubated with M²⁺ (Mn²⁺) and S (*threo*-D₅-isocitrate). Both syringes contain buffer, and the concentrations after mixing are the standard conditions of the assay. Each circle represents five data points. The final linear portion can be thought of as V_F ; the intercept of this line at $A_{340 \text{ nm}} = 0$ is the lag time, τ , its reciprocal is k . C, actual fit of data, not preincubated with metal-substrate and fitted with Equation 4.

where V_{obs} is the observed velocity, V_I is velocity of enzyme in state E° , V_F is the velocity of enzyme in state E^* , k_1 is the rate constant for conversion of enzyme from E° to E^* , k_2 is the rate constant for conversion of $E^{\circ}S$ to E^*S , and t is time.

The enzyme can be considered to occur in two states E° and E^* and substrate binding may facilitate the transition between these states. Equation 1 states the case in which cooperativity occurs, that is, both V_F and V_I contribute to the observed velocity, but $V_I < V_F$. Stopped flow experiments on NADP⁺:IDH were conducted without preincubation and at metal-substrate concentrations ranging from one-tenth to ten times K_m (the Michaelis-Menten constant). Each curve was analyzed by nonlinear regression techniques and attempts to fit the data with the integrated form of Equation 2 were unsuccessful. Fits obtained were only at 10% confidence level. V_F and k were then estimated graphically as shown in Fig. 1A; k was taken to be the reciprocal of τ , the extrapolated lag time. This allowed for an estimate of V_I from Equation 2. The values of V_I as a function of metal-substrate were found to be small or near 0. Therefore, it was concluded that in the absence of preincubation with metal-substrate, $E^{\circ} \gg E^*$.

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Since V_f is taken to be 0, then the reaction observed in the stopped flow experiments proceeds through the conversion of $E^\circ S$ to E^*S , k_2 . Equation 2 thus becomes

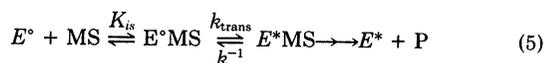
$$V_{obs} = V_F - V_F e^{-kt} \quad (3)$$

and the integrated form for appearance of product (P) is as follows.

$$P_{obs} = V_F t - V_F \left(\frac{1 - e^{-kt}}{k} \right) \quad (4)$$

When the stopped flow data were fitted with Equation 4, excellent root mean squares were obtained and the confidence levels for the fits were all in excess of 95%. Fig. 1C shows an example of the actual fit of data to Equation 4. This analysis also uses all data points obtained as contrasted to graphical estimates which require an arbitrary choice for the line drawn and which ignore the actual data obtained at early times. In preliminary experiments, it was observed that V_F was often lower in the absence of preincubation with metal substrate. Dalziel *et al.* (12) had also observed this effect for the mitochondrial enzyme. Steady state experiments were conducted to determine the rate of reaction as a function of time following dilution. At 25 °C, activity remained constant for enzyme diluted into metal-substrate for up to 15 min; enzyme activity declined by 10–20% during this same period of time for enzyme diluted into buffer, or buffer + NADP⁺. More substantial declines occurred for dilutions in water or in metal ion-containing solutions. Keeping the solutions cold and dark prevented some of the losses. Thus for stopped flow experiments, adding the cold enzyme to diluent just prior to filling preinsulated syringes maintains the highest activity.

The general scheme given above (Equation 1) can be simplified as follows,



where E° is the inactive enzyme, E^* is the active enzyme, K_{is} is the dissociation constant for $E^\circ MS$, k_{trans} is the rate constant for the conversion of $E^\circ MS$ to E^*MS , and k^{-1} is the reverse rate constant for E^*MS to $E^\circ MS$.

The terms in Equation 5 can be solved for in the following form.

$$k_{obs} = \frac{k_{trans}[MS]}{K_{is} + [MS]} \quad (6)$$

Here k of Equation 4 is taken to be k_{obs} and the parameters k_{trans} and K_{is} determined by the variance of k_{obs} with [metal-substrate] as shown in Fig. 2. This assumption is valid if k^{-1} is negligible, as it should be in the early times of stopped flow data collection. A second assumption is that the binding of NADP⁺ does not affect the transition; this is seen to be the case and is discussed below. The data given in Fig. 2 were fitted with Equation 6 by nonlinear regression yielding root mean squares within 95% confidence limits. The *inset* of Fig. 2 shows the double reciprocal plot computed from nonlinear regression analysis of k_{obs} as a function of metal-substrate concentration; k_{trans} was found to be 0.189 s⁻¹ and K_{is} was found to be 25 μM. Stopped flow experiments of NADP⁺:IDH activity as a function of metal-substrate concentration were repeated on two other preparations from different animals. The free metal ion concentration was held constant at 80 μM; average values for K_{is} and k_{trans} are given in Table I. The experiment was repeated at higher free metal ion concentration (300 μM) and this did not appreciably alter K_{is} , but k_{trans} was somewhat increased. At lower free metal concentration (30 μM) K_{is} appears to be increased.

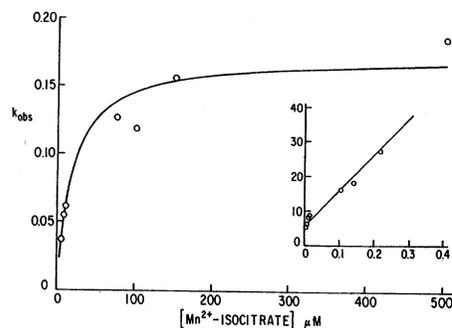


FIG. 2. Variation of k_{obs} (s⁻¹) obtained by analysis of the reaction progress curves (Equation 4) with [Mn²⁺-isocitrate]. The *inset* shows a double reciprocal plot generated from nonlinear regression analysis of the primary data, here $1/k$ in s is plotted against the concentrations in μM⁻¹ of metal-isocitrate.

TABLE I

Kinetic parameters calculated from the variation of k_{obs} with concentration of metal-substrate

From plots of k_{obs} against [Mn²⁺ - D₂-isocitrate] as in Equation 6; the two syringes were filled with $E + NADP^+$, and Mn²⁺ + isocitrate, respectively, concentrations of metal-substrate used are as in Fig. 2 while free Mn²⁺ was fixed as noted in column 1.

Free Mn ²⁺ μM	K_{is}	k_{trans} s ⁻¹
300 ^a	21	0.280
80 ^b	25 ± 8	0.189 ± 0.32
30 ^a	87	0.244

^a Average of two runs.

^b Average of three runs with three preparations ± σ.

TABLE II

Effects of various preincubation conditions on k_{obs} and τ, the lag time

Average of three determinations using fits to Equation 4. Here the lag time, τ, is defined as $1/k_{obs}$.

Enzyme cosolutes ^a	k_{obs} s ⁻¹	τ s	V_F μmoles/min·mg
Enzyme alone	0.169 ± 0.015	5.92	30.4
Enzyme + NADP ⁺	0.180 ± 0.013	5.55	29.5
Enzyme + MS	1.76 ± 0.06	0.56	38.3
Enzyme + M ²⁺ _T	0.262 ± 0.014	3.82	32.2
Enzyme + S _T	0.156 ± 0.017	6.38	37.4

^a Standard reaction concentrations except the concentration of metal-substrate (MS) complex after mixing was fixed at 150 μM, and the free Mn²⁺ concentration was fixed at 80 μM. The total metal concentration (M²⁺_T) was 0.245 mM and the total substrate concentration (S_T) was 1.78 mM.

Having established the applicability of Equation 4, the effects of various preincubation conditions could now be plotted on a more quantitative basis by solving for k_{obs} . The results are given in Table II. Here it is seen that preincubation with metal-substrate at 150 μM, a concentration six times that of K_{is} produces a 10-fold increase in k_{obs} resulting in a lag time of only 0.56 s. Thus, even with preincubation some lag time is observed; neither NADP⁺ alone nor substrate alone causes as large a decrease in lag time. Metal ion alone decreases the lag but not nearly to the same extent as metal substrate.

Inhibition by Citrate—In the case of human heart NADP⁺:IDH, a mitochondrial form of the enzyme, citrate has been reported to be a simple competitive inhibitor of DL-isocitrate (14). This was found to be true here for the soluble NADP⁺:IDH as well in steady state experiments; data for citrate inhibition at 6, 9, and 12 mM fit the classical case of competitive inhibition. Replots of the slopes of the double-

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TABLE III

Parameters obtained for linked function analysis of the variation of V_{max} with metal-citrate

Average values and error of coefficients for two complete and two partial runs each point in triplicate; total error of each individual fit averaged 1%. Data of Fig. 3A were fitted with Equation 9.

State ^a	Exponents ^b	k	$K_D \pm S.E.$ ^c	V_i ^d
	<i>integer</i>	M^{-1}	<i>mM</i>	$\mu\text{mole}/\text{min}\cdot\text{mg}$
V_0				50.7 ± 0.4
V_1	2	3790	0.246 ± 0.113	54.6 ± 2.1
V_2	2	260	3.83 ± 0.32	-3.0 ± 6.3
V_3	4	650	1.54 ± 0.04	39.1 ± 15.1

^a State of the enzyme $\pm I$ as defined in Equation 7.

^b Integer exponents n , m , and q of Equation 9.

^c Calculated dissociation constant ($1/k$).

^d V_i represents the predicted theoretical V contributed by each state and produced by the binding of each class of metal-citrate as described in Equation 7; V_0 represents calculated V_{max} in the absence of metal-citrate.

TABLE IV

Effects of metal-citrate on the activation of NADP⁺:IDH

From plots of k_{obs} against [metal-isocitrate] as in Equation 6, concentrations of metal-isocitrate were as in Fig. 2 and free Mn^{2+} was fixed at 80 μM (Table I). NADP⁺ and enzyme were preincubated; [metal-citrate] held constant at the values given.

Concentration of metal-citrate	k_{trans}	τ	K_{is}
<i>mM</i>	s^{-1}	<i>s</i>	μM
0	0.189 ± 0.032	5.29	25 ± 8
1	0.704 ± 0.101	1.42	9 ± 5
5	0.669 ± 0.080	1.49	22 ± 4

TABLE V

Effects of various preincubation conditions on k_{obs} and τ , the lag time

Experimental procedure as in Table I; abbreviations as in Table II except C = citrate.

Enzyme cosolute	k_{obs}	τ
	s^{-1}	<i>s</i>
Enzyme alone	0.169	5.92
Enzyme + MS (150 μM)	1.76	0.56
Enzyme + MC (1 mM)	1.38	0.72
Enzyme + MC (5 mM)	2.46	0.41
Enzyme + C _{total} (1 mM)	0.368	2.72
Enzyme + C _{total} (5 mM)	0.318	3.15

and allows assessment of the relative contributions of V_i values to V_{obs} .

Effects of Metal-Citrate on Activation—The stopped flow kinetics of NADP⁺:IDH were next studied to determine the effects of metal, citrate, and metal-citrate complex on K_{is} and k_{trans} (Equation 6). In the first series of experiments, the concentration of metal-isocitrate was varied as in Fig. 2, while metal-citrate was held constant; the enzyme was not preincubated with metal, citrate, or isocitrate. From the plots of k_{obs} against [metal-isocitrate] values for k_{trans} and K_{is} were obtained (Table IV). The data show that metal-citrate has two effects. At 1 mM metal-citrate, k_{trans} increases showing an accelerated conversion of the enzyme from the inactive to the active form (reduced lag time) and also causes an apparent tighter binding of substrate to the inactive enzyme. At 5 mM metal-citrate, k_{trans} is again increased but K_{is} is only slightly increased over its value in the absence of inhibitor, the effect here is predominately inhibition. When the NADP⁺:IDH is preincubated with metal-citrate alone at 1 mM, the lag time is drastically decreased (Table V). Metal-citrate at 5 mM is as effective as metal-substrate at 150 μM in decreasing the lag time. Citrate alone can partially depress τ , but not nearly as

effectively as the metal-citrate complex.

Binding of Metal-Citrate—Studies on the binding of metal-¹⁴C]citrate to IDH were carried out using porous membrane filters as described under "Experimental Procedures." Results of the binding studies are given in Fig. 4, and nonlinear regression analysis was used to fit the data with Equation 9. Here \bar{v}_i (moles of ligand bound/mol of monomer) replaces V_i . The results of this analysis are consistent with the binding of up to 24 mol of metal-citrate per mol of IDH monomer. For the first transition observed, \bar{v}_1 was found to be 4.3 with $K_D = 0.257 \pm 0.049$ mM; the dissociation constant is similar to that found for metal-citrate activation (V_1 , Table III). The second transition of Fig. 4 represents a class of sites with $\bar{v} = 10$ and $K_D = 1.63 \pm 0.05$; this K_D value is close to the value found for V_3 in Table III. The third transition represents binding to an additional 10 sites with K_D equal to 3.02 ± 0.04 mM. These values probably are composites of a number of binding sites and actually represent an average class sites (15, 16). The overall analysis of Fig. 4 has an error of 3%.

DISCUSSION

The apparent lack of NADP⁺:IDH in bovine mammary gland (4) has led to the speculation that the activity of the soluble form of NADP⁺:IDH might be influenced by metabolites and thus in some way serve a regulatory function in this tissue and perhaps elsewhere. Work carried out primarily on heart (mitochondrial) NADP⁺:IDH (12, 17) had shown nonlinear kinetics under certain conditions, indicating a lag-burst hysteretic mechanism as delineated by Frieden (9). When the mammary NADP⁺:IDH was injected into the stopped flow cell without prior incubation with metal-substrate, nonlinear progress curves were observed (Fig. 1A). Such curves were first noted by Carlier and Pantaloni (18) for the cytosolic beef liver enzyme, but they made no calculations regarding the magnitude of the lag time, nor did they study its dependence upon substrate concentration. In the current study, unique fits of these progress curves were obtained directly by nonlinear regression analysis with Equation 4. This analysis allows for calculation of the observed rate constant k for the transition from inactive to active form under a variety of conditions (e.g. Table II). The values of k obtained here by direct fitting

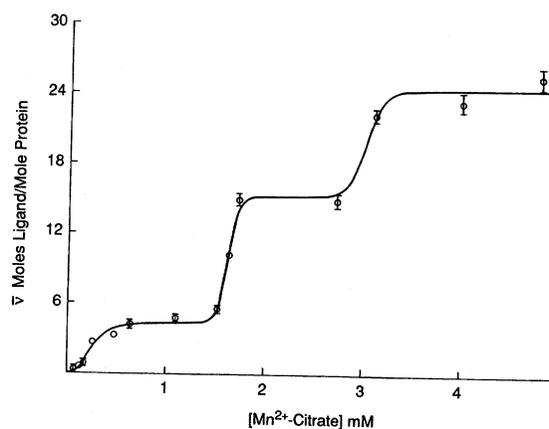


FIG. 4. Binding of Mn^{2+} -¹⁴C]citrate to NADP⁺:IDH; the number of moles of metal-citrate bound per mol of IDH are plotted against the concentration of free metal-citrate. The monomeric concentration of IDH was 85.3 μM . The concentration of free Mn^{2+} was fixed at 80 μM , and the concentration of metal-citrate varied. The experiment was conducted three times with two preparations of enzyme. Data points represent three to four replicates and error bars show standard deviations. The composite data were then fitted with Equation 9; the line represents the best fit with n , m , and q set at 3, 34, and 34, respectively.

are significantly lower than those given by Dalziel *et al.* (12) for heart (mitochondrial) enzyme; their values of 0.4/s were obtained graphically. The direct fitting of the reaction progress curve by Gauss-Newton nonlinear regression analysis as carried out here allows for careful analysis of the data even when, to the eye, the lag is abolished and k_{obs} is rather large (Table II). This was not done in prior studies (12, 17, 18).

The value of k_{trans} (in analogy with V_{max}) can be thought of as the transition rate for conversion of $E^{\circ}\text{MS}$ to $E^*\text{MS}$ at saturation with MS and without preincubation (Equation 6). The transition time, τ_{min} , the inverse of k_{trans} , calculated for NADP⁺:IDH is 5 s and is relatively long when one considers that the rotational correlation time of a molecule such as IDH is of the order of 10^{-9} s and diffusion controlled binding (the maximal upper rate) occurs at rates of the order of 10^{11} /s. Thus the rate-limiting step here is probably not binding, but perhaps a slow conformational transition of the protein itself. Such transitions can range from a few milliseconds, for an aromatic side chain rotation, to up to minutes for large chain displacements (19). This transition is clearly catalyzed by metal-isocitrate (Table II).

The data presented thus far do not rule out monomer-dimer association as being rate limiting; this has been proposed for the heart (mitochondrial) enzyme (17). Carlier and Pantaloni (18) also suggest, on the basis of their qualitative observations, that ligand induced association is the rate-limiting step for activation of the beef liver enzyme. In contrast, Bailey and Colman (20) did not observe concentration-dependent changes in enzyme activity for heart NADP⁺:IDH. In a recent study, Serry and Farrell (21) estimated K_D for dimer dissociation of mammary IDH to be 2 nM in the presence or absence of metal-substrate and metal-citrate. Under the conditions of the stopped-flow experiments (60 nM) the enzyme thus exists as a dimer. The latter authors also noted that decreases in activity occur for the enzyme diluted and held in the absence of protectants.

The total concentration of citrate in homogenized mammary gland is ~ 3 mM (22), but the calculated K_I (250 μM), although in agreement with literature for this metabolite, is much smaller, thus metal-citrate should be the actual inhibitor. Analysis of V versus I (Fig. 3A), using the assumptions of thermodynamic linkage, shows that metal-citrate binding can indeed stimulate activity (k_1) with a K_D of 0.25 mM; the maximum stimulation would be to 54.6 $\mu\text{mol}/\text{min}/\text{mg}$ or an 8% increase (Table III). In contrast, binding to inhibitory sites with $K_D = 3.83$ mM dramatically decreases activity with a predicted total repression of activity. At concentrations of metal-citrate between these values, binding to sites with K_D equal to 1.54 mM, produces an apparent alleviation of inhibition ($V_3 = 39.1$). This latter effect can be considered a reactivation. The curve of Fig. 3A is thus the composite of these three interactions. Contributions from the various terms of Equation 9 are plotted individually in Fig. 3B. The linked-function analysis (Equation 9) thus appears to provide a more quantitative basis for discussion of these phenomena than the Dixon plot. Since the best data (lowest [I]) are used, the statistical fits are better, although qualitatively the Dixon plot suggests a similar result. As noted above the average total citrate content of lactating bovine mammary gland is ~ 3 mM and the average free $\text{Mg}^{2+} + \text{Mn}^{2+}$ content is ~ 1 mM (22); these effects could thus occur in the physiological range.

In protein-ligand studies n , m , and q derived from Equation 9 have been correlated with the number of bound ligands giving rise to a selected change in properties (16). Here it may mean that 2 mol of metal-citrate bind per dimer for activation to occur, then additional binding of 2 mol of metal-citrate

complex leads to strong inhibition (k_2) but binding of an additional 4 mol to modulation (k_3). Interpreted another way, these exponents may be analogous to Hill coefficients (15) and could represent a measure of the degree of cooperativity within a class of sites. Direct binding studies of metal-citrate showed three classes of binding sites with a total of 24 mol bound/mol of monomer. The first two classes have comparable K_D values to those found from linked-function analysis (0.257 and 1.63 as compared with 0.246 and 1.54 from Table III). The third class has a K_D of 3.08 which is lower than 3.58 from Table III. In each case \bar{v} was found to be $>n$, m , or q . While at first this could appear to be contradictory, it must be remembered that these values represent either relatively large numbers of individual sites with composite K_D values or cooperativity within the classes of sites, *e.g.* the Hill coefficient is 3.0 for binding of 4 mol of O_2 to hemoglobin (15). Furthermore, studies involving thermodynamic linkage contain the qualification that only binding sites which cause a change in the measured parameter, in this case V_{max} , are disclosed by linkage; other binding not associated with changes in activity can occur (16). Thus linkage can be a more powerful means of uncovering functional relationships than binding studies alone; changes in enzyme activity are thus used to follow only that binding which influences activity.

Metal-citrate can also cause the same transition as metal-substrate from the inactive to the active form of NADP⁺:IDH; k_{trans} , as calculated from Equation 6, is the rate of conversion at saturation of metal substrate without preincubation, thus it can be seen (Table IV) that metal-citrate at 1 mM produces nearly a 4-fold increase in the transition rate. Considering the reciprocal of this constant (τ_{min} as discussed above), 1 mM metal-citrate reduces the lag time to 1.42 s which is nearly equivalent to preincubation with substrate (0.56 s, Table V). Preincubation with metal-citrate at 5 mM is as effective (0.41 s) as metal-substrate in reducing the observed τ (Table V).

The analysis presented here is in line with the random mechanism for NADP⁺:IDH proposed by Northrop and Cleland (23) based on steady state kinetics. The initial binding of metal-substrate or metal-citrate activates the enzyme whether or not NADP⁺ is present during the incubation.

The importance of enzyme-substrate interactions in the regulation of enzymes has long been recognized (9, 11) as have the principles of allosteric regulation (5-7). The more recent focus on regulation through metal ion-activated cascades has emphasized the importance of regulatory binding sites with association constants of approximately 10^9 (24). The data reported above point to the potential significance of weaker binding sites as regulators of enzyme activity since metabolite concentrations in mammary tissue occur in the same range as the derived constants. The effects of metal-citrate and metal-isocitrate on enzyme activation are noteworthy but only if the pools of Krebs cycle metabolites in mammary tissue oscillate in the same fashion (1-5 s) as the glycolytic pools have been shown to do in other tissues (25); under these circumstances, the 1-6-s lag times observed in this study could be relevant. From the linked function analysis, metal-citrate could also stimulate activity, while higher metal-citrate concentrations produce either inhibition or modulation. Soluble NADP⁺:IDH could thus be positively and negatively controlled by the metabolite of the first step in the Krebs cycle, citrate synthesis, which occurs within the mitochondria (2, 4). Since it was recently shown by Gabriel and Plaut (6) that metal-citrate may control NAD⁺:IDH as well, it could thus be speculated that metal-citrate plays a more important role than previously expected in Krebs cycle metabolism.

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