Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 233–236, 1983. © Ankho International. Printed in the U.S.A.

Kinetics of Malate Dehydrogenase and Control of Rates of Ethanol Metabolism in Rats

KATHRYN E. CROW, TERENCE J. BRAGGINS, RICHARD D. BATT AND MICHAEL J. HARDMAN

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand

CROW, K. E., T. J. BRAGGINS, R. D. BATT AND M. J. HARDMAN. *Kinetics of malate dehydrogenase and control of rates of ethanol metabolism in rats.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 233–236, 1983.—The theory that the rate of ethanol oxidation is governed by rates of NADH reoxidation is based in part on the observation that the ratio of free cytosolic [NADH]/[NAD^+] increases during ethanol metabolism. However, it has recently been suggested that the amount of alcohol dehydrogenase governs rates of ethanol metabolism, which then leaves the change in cytosolic redox state unexplained. In this paper the kinetic parameters for rat liver malate dehydrogenase, determined at 37°C and pH 7.4, are used to provide an explanation for the change in cytosolic redox state that is compatible with rate control by alcohol dehydrogenase.

Ethanol metabolism Cytosolic redox state Rat liver Malate dehydrogenase Kinetic parameters Simulations

VERY often, it is assumed that kinetic parameters determined *in vitro* are of little use or relevance in assessing rates of reactions *in vivo*. However, we feel that a more useful approach is to assume that kinetic properties determined *in vitro* are applicable *in vivo* unless there is good evidence to suggest otherwise. This approach allowed formulation of the theory of rate control of ethanol metabolism by alcohol dehydrogenase [4].

Previously, it was widely accepted that the rate of ethanol oxidation *in vivo* was governed by the rate at which NADH could be reoxidized [9,15]. This view appears to have initially arisen from the fact that the free [NAD^+]/[NADH] ratio in the cytosol of liver cells changes during ethanol oxidation. To quote a typical example from a recent review [11] "A change toward a more reduced NADH:NAD ratio in the cytosol following ethanol indicates that the production of NADH is faster than the rate at which NADH is reoxidized into NAD. This suggests that NADH reoxidation, and thus NAD availability to ADH, is an important rate-limiting step in the metabolism of ethanol."

Implicit in this statement are a number of assumptions; we consider that some of these are incorrect and have hampered understanding of the control of ethanol metabolism. One such assumption is that NADH reoxidation has its effect through changing the NAD^+ concentration. This is incorrect. Because of the large concentration of free NAD^+ relative to that of free NADH, the cell does not 'run out of NAD^+' when the redox state changes during ethanol metabolism. As shown in Table 1, the change in redox state results from an increase in free [NADH] is so small that there is no significant decrease in free [NAD^+].

A second assumption is that changes in NAD^+ or NADH concentration giving rise to the change in NAD^+/NADH ratio are sufficient to significantly affect alcohol dehydrogenase. This is only partially correct, as has been shown previously by kinetic analysis of rat liver alcohol dehydrogenase [4]. A third assumption is that NADH is continually being produced faster than it can be reoxidized. If NADH were being produced faster than it could be reoxidized, a steady-state could not be reached and the concentration of NADH in the cytosol would increase continually during ethanol oxidation. This would mean that a continual increase in [lactate]/[pyruvate] would be observed during ethanol oxidation, but we have shown previously [1] that this is not the case.

Incorrect assumptions such as those above have been made because the theory of rate limitation by NADH reoxidation was based on the observation of a change in ratio of two metabolites, without reference to the actual concentration of the metabolites or to the kinetic properties of the enzymes interacting with the metabolites.

If we accept an alternative theory, that the rate of ethanol metabolism is actually governed by the amount of alcohol dehydrogenase [4], there is one question left unanswered: why does a change in cytosolic redox state occur during ethanol metabolism? The fact that free [NADH] increases during ethanol oxidation implies that the reoxidation of cytosolic NADH does influence the cytosolic redox state in some way. If there were an absolute limitation on the rate of NADH reoxidation, then NADH reoxidation would have to be the rate limiting step for ethanol oxidation since NADH would accumulate until alcohol dehydrogenase was inhibited

TABLE 1

FREE NAD^+ AND NADH CONCENTRATIONS

	− ethanol	+ ethanol
[NAD^+]	500 μM	498.5 μM
[NADH]	0.5 μM	2.0 μM
[NAD^+]/[NADH]	1000	249
[lactate]/[pyruvate]	10	40

back to the rate of NADH reoxidation. In this case, increasing the amount of alcohol dehydrogenase would produce an increase in [lactate]/[pyruvate] (or free cytosolic [NADH]) but no change in the rate of ethanol oxidation. An increased rate of ethanol oxidation would only be obtained by increasing the NADH reoxidation rate, which would lead to a decrease in [lactate]/[pyruvate]. However, data in the literature show that increasing ethanol oxidation rates can be accompanied by increasing [lactate]/[pyruvate] [1, 10, 14]. These results suggest that the rate of ethanol metabolism controls the free cytosolic [NADH], rather than the free [NADH] controlling the rate of ethanol metabolism. It appeared to us that the only way in which these results could be satisfactorily explained was if the rate of reoxidation of cytosolic NADH was dependent on the concentration of NADH.

One of the major routes of NADH reoxidation during ethanol metabolism is the malate-aspartate shuttle. The first enzyme reaction in the shuttle sequence is the oxidation of NADH to NAD^+ by malate dehydrogenase. A rate-limiting role in NADH reoxidation for this enzyme appeared very unlikely since the maximal velocity was reported to be about 400 μmoles per minute per g wet weight liver [12] while the rate of NADH production by alcohol dehydrogenase is about 3–4 μmoles per minute per g [4]. However, published K_m values for cytosolic malate dehydrogenases were much higher than the probable free cytosolic NADH and oxaloacetate concentrations [2,8]. When this factor was considered, it appeared that the combined limitation of low [oxaloacetate] and low [NADH] might reduce the capability of the enzyme for oxidizing NADH to as low as 3–4 μmoles per minute per g. All the kinetic parameters we needed to confirm this were not available, so we decided to purify rat liver malate dehydrogenase and determine the necessary values at 37°C and pH 7.4

METHOD

Cytoplasmic malate dehydrogenase was purified from livers of 300 g male Sprague-Dawley rats (manuscript submitted for publication). Assays for kinetic characterization were carried out at 37°C, in 0.1 M phosphate buffer, pH 7.4, using an Aminco DW 2a UV/Visible Spectrophotometer. Data were fitted to appropriate equations for an ordered bi bi mechanism [3,13] using a non-linear least squares curve fitting program [7] with initial estimates of parameters obtained from reciprocal plots and replots [16]. The maximum velocities of rat liver cytosolic malate dehydrogenase were determined in 0.1 M phosphate buffer, pH 7.4, using 1.2 mM oxaloacetate and 0.2 mM NADH for oxaloacetate reduction and 60 mM malate and 2.0 mM NAD^+ for malate oxidation. The net rate of NADH oxidation by malate dehydrogenase under conditions *in vivo* was calculated using the rate equation for an ordered bi bi system [5]. Simulations were carried out on a CROMEMCO CS2 computer using 16K extended BASIC. For each enzyme, an appropriate steady state rate equation was used to calculate flux. For alcohol and aldehyde dehydrogenase, the equation for an ordered bi bi system [5] was used and for aldehyde dehydrogenase the simple Michaelis-Menten equation. Differential equations representing the rate of change of metabolite concentrations were integrated by the Euler method [19].

TABLE 2

KINETIC CONSTANTS FOR RAT LIVER CYSTOSOLIC MALATE DEHYDROGENASE

Constant	Values (μM)*
K_a	26 ± 3
K_{ia}	4.9 ± 0.6
K_b	88 ± 4
K_{ib}	63 ± 5
K_p	1100 ± 100
K_{ip}	7100 ± 400
K_q	114 ± 7
K_{iq}	940 ± 100

*Results expressed as means±SEM for at least 5 separate determinations.

RESULTS AND DISCUSSION

The kinetic parameters determined and used for subsequent calculations are shown in Table 2. We also determined the maximum velocity of rat liver cytosolic malate dehydrogenase, and found values of 533±19 μmoles per minute per g wet weight liver for oxaloacetate reduction and 48±2 μmoles per minute per g for malate oxidation.

By inserting these maximal velocities, the kinetic parameters shown in Table 2 and physiological concentrations of NAD^+, NADH, malate and oxaloacetate into the rate equation for an ordered bi bi system [5] we were able to calculate the rate of NADH oxidation by malate dehydrogenase under physiological conditions. At likely physiological concentrations in the absence of ethanol, NADH 0.5 μM, see Table 1; NAD^+ 0.5 mM [4]; malate 0.5 mM [18] and oxaloacetate 10 μM [17,18], the calculated rate of NADH oxidation by malate dehydrogenase was −1.86 μmoles per minute per g wet weight of liver. In other words, the enzyme was operating in the direction of NAD^+ reduction. However, the calculated rate of NADH oxidation in the presence of ethanol, where the NADH concentration was assumed to be 2.0 μM (Table 1), was 4.0 μmoles per minute per g. These calculations show than under physiological conditions malate dehydrogenase can be switched from NADH oxidation to NAD^+ reduction by a relatively small change in oxaloacetate or NADH concentrations.

We therefore proposed the following explanation for the change in redox state during ethanol metabolism. When ethanol first reaches the liver NADH is produced at a faster rate than it is being reoxidized, so that its concentration increases. The increased concentration of NADH produces an increase in rate and/or change in direction of operation of MDH. The rate of NADH oxidation by MDH rises with the

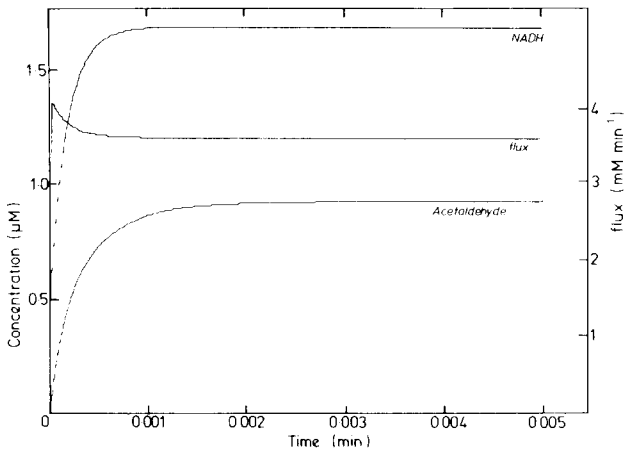

FIG. 1. Results of simulation of flux rate and changes in NADH and acetaldehyde concentrations. Conditions were as described in the text. The simulation was initiated with the addition of 10 mM ethanol, and allowed to run until all parameters reached a steady state.

TABLE 3

SIMULATION OF THE EFFECT OF ALCOHOL DEHYDROGENASE ACTIVITY ON STEADY STATE CONCENTRATIONS AND FLUXES

V_{max} of ADH* (mM min^{-1})	[NADH] (μM)	Flux (mM min^{-1})	$\%V_{max}$†
6.3	1.7	3.60	57
12.6	2.3	6.35	50

*V_{max} for ethanol oxidation.
†Flux expressed as a percentage of the V_{max} in column 1.

TABLE 4

EFFECT OF MALATE DEHYDROGENASE ACTIVITY ON STEADY STATE CONCENTRATIONS AND FLUXES

V_{max} of MDH* (mM min^{-1})	NADH (μM)	Flux (mM min^{-1})
666	1.7	3.60
1332	1.3	3.73

*V_{max} for oxaloacetate reduction.

increasing [NADH] until it equals the rate of production of NADH and a new steady state is achieved.

We then used our kinetic data for malate dehydrogenase, combined with published kinetic parameters for rat liver alcohol dehydrogenase [4] and estimated K_m (acetaldehyde) and V_{max} values for aldehyde dehydrogenase in computer simulation of changes in metabolite concentrations and flux rates during ethanol metabolism. Our simulations were started with initial metabolite concentrations representing those in the absence of ethanol. Ethanol (10 mM) was introduced, and the system allowed to adjust to produce an overall steady state by varying the concentrations of NAD^+, NADH and acetaldehyde. Oxaloacetate (10 μM) and malate (0.5 mM) were held constant. Initial values for [NADH] and [acetaldehyde] were 0.5 μM and zero, respectively.

Unless specified otherwise, the maximum velocity for alcohol dehydrogenase was 6.3 $mM \cdot min^{-1}$ (5.05 μmoles $\cdot min^{-1} \cdot g^{-1}$) [4], for aldehyde dehydrogenase V_{max} was 7.5 $mM \cdot min^{-1}$ and K_m (acetaldehyde) was 1 μM. Figure 1 illustrates the results of one simulation. When ethanol was introduced, the free cytosolic [NADH] increased by a factor of 3 fold—equivalent to a change from 10 to about 30 in [lactate]/[pyruvate]. The acetaldehyde concentration increased to about 0.9 μM and remained constant. The system flux, or ethanol oxidation rate, settled to a value of 3.6 $mM \cdot min^{-1}$ (about 3.0 μmoles $min^{-1} \cdot g^{-1}$), which was 60% of the allocated V_{max}. This agrees with the previously published suggestion that *in vivo* the rate of ethanol oxidation is 50–80% of the V_{max} of alcohol dehydrogenase [6]. The fact that alcohol dehydrogenase is not operating at V_{max} does not imply, as has been suggested recently [11], that changes in alcohol dehydrogenase activity will not result in equivalent changes in the rate of ethanol metabolism *in vivo*. This is illustrated well by the use of simulations. Table 3 shows that doubling the V_{max} of alcohol dehydrogenase led to an almost 2-fold (1.8) increase in the rate of ethanol oxidation. The [NADH] increased by 1.4 fold. In contrast, doubling the maximal velocity of malate dehydrogenase had little effect on flux through the pathway (Table 4). This is an example of a case where the amount of enzyme is not rate limiting. It also illustrates that the value we obtain for the maximum velocity of MDH is not critical in determining the outcome of simulations.

CONCLUSIONS

Our results show that the initial observation that prompted development of the theory of rate control by NADH reoxidation, the change in cytosolic redox state, can be explained by the kinetic properties of malate dehydrogenase. Our simulations support the theory that the amount of alcohol dehydrogenase is an important rate limiting factor for ethanol oxidation in rats.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of New Zealand. The Cromemco computer was purchased with the aid of a grant from the Research Committee of the New Zealand University Grants Committee.

REFERENCES

1. Braggins, T. J. and K. E. Crow. The effects of high ethanol doses on rates of ethanol oxidation in rats. *Eur J Biochem* **119:** 633–640, 1981.

2. Busquets, M., J. Baro, A. Cortes and J. Bozal. Separation and properties of the two forms of chicken liver cytoplasmic malate dehydrogenase. *Int J Biochem* **10:** 823–835, 1979.

3. Cleland, W. W. Steady state kinetics. In: *The Enzymes*, vol. 2, edited by P. D. Boyer. New York and London: Academic Press, 1970, p. 8.
4. Cornell, N. W., K. E. Crow, M. G. Leadbetter and R. L. Veech. Rate-determining factors for ethanol oxidation *in vivo* and in isolated hepatocytes. In: *Alcohol and Nutrition*, edited by T. K. Li, S. Schenker and L. Lumeng. Washington, DC: US Government Printing Office, 1977, pp. 315–330.
5. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*. London and Boston: Butterworths, 1979, p. 105.
6. Crow, K. E., N. W. Cornell and R. L. Veech. The rate of ethanol metabolism in isolated rat hepatocytes. *Alcoholism: Clin Exp Res* **1:** 43–47, 1977.
7. Daniel, C. and F. S. Wood. *Fitting Equations to Data*. New York: Wiley Interscience, 1971.
8. Englard, S. Extramitochondrial L-malate dehydrogenase of beef heart. In: *Methods in Enzymology*, vol. 13, edited by J. M. Lowenstein. New York and London: Academic Press, 1969, pp. 123–128.
9. Hawkins, R. and H. Kalant. The metabolism of ethanol and its metabolic effects. *Pharmacol Rev* **24:** 67–157, 1972.
10. Higgins, J. J. Control of ethanol oxidation and its interaction with other metabolic systems. In: *Biochemistry and Pharmacology of Ethanol*, vol. 1, edited by E. Majchrowicz and E. P. Noble. New York and London: Plenum Press, 1979, pp. 249–351.
11. Khanna, J. M. and Y. Israel. Ethanol metabolism. In: *Liver and Biliary Tract Physiology 1*, International Review Physiology, vol. 21, edited by N. B. Javitt. Baltimore: University Park Press, 1980, pp. 275–315.
12. Krebs, H. A. and R. L. Veech. Regulation of the redox state of the pyridine nucleotides in rat liver. In: *Pyridine Nucleotide Dependent Dehydrogenases*, edited by H. Sund. Berlin, Heidelberg, New York: Springer-Verlag, 1970, pp. 413–434.
13. Plapp, B. V. Enhancement of the activity of horse liver alcohol dehydrogenase by modification of amino groups at the active sites. *J Biol Chem* **245:** 1727–1735, 1970.
14. Pösö, A. R. and O. A. Forsander. Influence of ethanol oxidation rate on the lactate/pyruvate ratio and phosphorylation state of the liver in fed rats. *Acta Chem Scand (B)* **30:** 801–806, 1976.
15. Scholz, R. Mechanism of the fructose effect on ethanol oxidation. In: *Alcohol and Aldehyde Metabolising Systems*, vol. 3, edited by R. G. Thurman, J. R. Williamson, H. R. Drott and B. Chance. New York: Academic Press, 1977, pp. 305–312.
16. Segel, I. H. *Enzyme Kinetics*. New York: John Wiley and Sons, 1975, pp. 560–588.
17. Siess, E. A., D. G. Brocks and O. H. Wieland. Subcellular distribution of key metabolites in isolated liver cells from fasted rats. *FEBS Lett* **69:** 265–271, 1976.
18. Tischler, M. E., D. Friedrichs, K. Coll and J. R. Williamson. Pyridine nucleotide distributions and enzyme mass action ratios in hepatocytes from fed and starved rats. *Arch Biochem Biophys* **184:** 222–236, 1977.
19. Wiberg, K. B. Use of computers. In: *Techniques of Chemistry*, vol. 6, part 1, *Investigation of Rates and Mechanisms of Reactions*, edited by E. S. Lewis. New York: John Wiley, 1974, pp. 764–772.