APPLICATION OF EQUILIBRIUM EXCHANGE KINETICS TO STUDIES OF KINETIC PROPERTIES AT HIGH ENZYME CONCENTRATIONS: EXPERIMENTS WITH YEAST HEXOKINASE P-II*

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Summary

The usefulness of isotope exchange at equilibrium measurements to define both the kinetic parameters and the kinetic reaction mechanism of enzymes at high levels of enzyme is demonstrated by studies of yeast hexokinase P-II. Additional support for this new application of equilibrium exchange kinetics, based upon previously published studies of a variety of enzymes, is also presented, and the advantages and limitations of this approach are discussed.

The utility of isotope exchange at equilibrium studies of enzymic reactions, as originally proposed by Boyer (1) and extended by Boyer and Silverstein (2), has been amply demonstrated. For example, studies of the equilibrium kinetics of enzyme catalyzed reactions can provide evidence supporting results of initial rate studies on the order of substrate binding and product release, as illustrated by studies of yeast hexokinase (3), lactate dehydrogenase (4), creatine kinase (5), and adenylate kinase (6, 7). In addition, this approach for probing enzyme mechanism has been

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extended to involve measurement of rates of loss, or exchange, of all possible atoms or functional groups of substrates, determination of various possible primary and secondary isotope effects, and definition of feedback modifier effects on the catalytic power of regulatory enzymes. Studies of fumarase (8), enolase (9), the Escherichia coli glutamine synthetase (10), malate dehydrogenase (11), and alcohol dehydrogenase (12) demonstrate the versatility of isotopic probes in examining the above aspects of enzyme catalysis. It appears, however, that this technique has been overlooked as a valuable and convenient procedure for determining the kinetic reaction mechanism and kinetic parameters of those enzymes known to exist at high concentrations within the cell. This report attempts to draw attention to the feasibility, advantages, and limitations of this new application.

RESULTS AND DISCUSSION

To investigate the utility of equilibrium isotope exchange studies as probes of enzyme kinetic properties at high concentrations of enzyme, we have carried out measurements of the yeast hexokinase reaction. In 1964, we reported that equilibrium exchange studies were in accord with our previous initial rate studies of this phosphotransferase (3, 13). Yeast hexokinase appeared to be an appropriate enzyme for studies at high concentration because our previous studies could serve as an experimental guide and the results could be directly compared with rapid reaction studies of this enzyme done at 60 μg hexokinase per ml (14). Moreover, the use of the P-II isozyme, in place of commercial hexokinase preparations, could serve to confirm our previous studies (3), especially in the light of the numerous seemingly conflicting studies on the reaction mechanism of this enzyme (see reference 15 for an extended discussion of this problem). The exchange measurements were carried out at pH 6.5 to decrease the preponderence of ADP and glucose-6-P at equilibrium. The findings presented in Fig. 1 illustrate that the apparent maximal rate

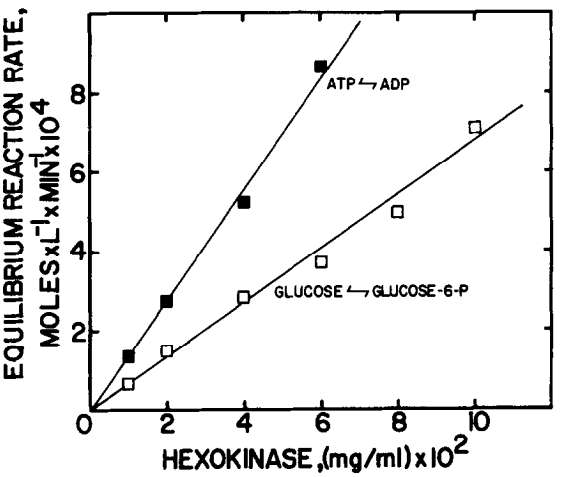

Fig. 1. Plot of the equilibrium exchange rate of the glucose-glucose-6-P and ATP-ADP exchanges versus the concentration of yeast hexokinase. Reaction mixtures (final volume, 0.125 ml) contained 58 mM imidazole-nitrate buffer (pH 6.5), 13 mM magnesium nitrate, 50 mM glucose-6-P, 2.5 mM glucose, 38.0 mM ADP, 2.0 mM ATP, and a variable concentration of the P-II isozyme of yeast hexokinase. Reaction samples were pre-incubated for 75 min at 28° before addition of approximately 200,000 cmp of either 14C-glucose-6-P (specific radioactivity, 20 mC/mmole). Reactions were stopped 1.0 min after the addition of the isotopic compound by addition of 0.5 ml of either 4 mM silver nitrate-0.1 M glucose or 4 mM silver nitrate-0.02 M ATP, and the samples were immediately placed in an ice-water bath. Substrate and product radioactivities were separated and counted as described elsewhere (15), and the rate of isotopic exchange was calculated by use of Eqn 1 as described elsewhere (1). The concentrations of hexokinase substrates and products were assayed by enzymatic procedures previously described (3, 13, 15).

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of the ATP⇌ADP and glucose ⇌glucose-6-P equilibrium exchanges are proportional to the hexokinase concentration over the indicated range. In Fig. 2, the exchanges were measured by holding one substrate-product pair constant while the other substrate-product pair was varied from below to approximately 10-12 times their respective Michaelis constants, but in a constant ratio. These measurements are in agreement with our previous studies of this phosphotransferase (3, 13, 15), and suggest that accurate measurements of the order of substrate binding and product release can be conveniently achieved at high enzyme concentrations. The data suggest that the dissociation constants for the hexokinase substrates and products remain essentially unchanged at these enzyme levels, and that

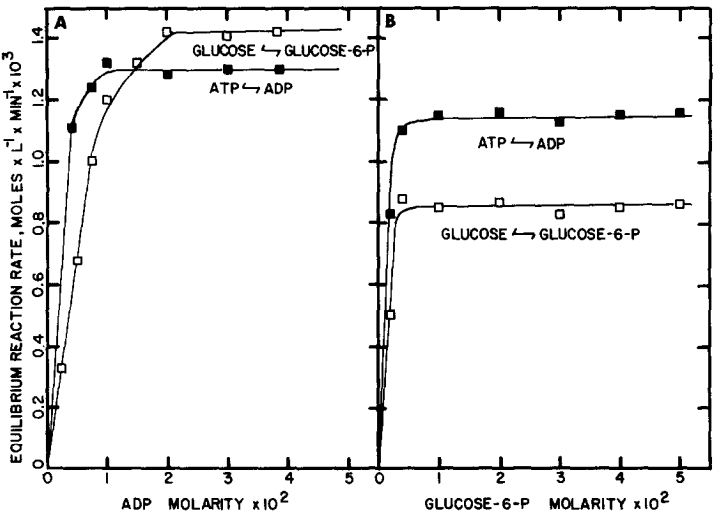

Fig. 2. A. The effect of ADP and ATP concentrations of the equilibrium exchange rates of the yeast hexokinase reaction. The reaction mixtures contained 58 mM imidazole-nitrate buffer (pH 6.5), 13 mM magnesium nitrate, 50 mM glucose-6-P, 2.5 mM glucose, and a variable concentration of ADP and ATP, maintained at a constant ADP/ATP ratio of 19. The hexokinase P-II concentration was 47 µg/ml for measurements of the ADP-ATP exchange and approximately 100 µg/ml for the glucose-glucose-6-P exchange, and the incubation time was 0.5 min. B. The effect of glucose-6-P and glucose concentrations of the equilibrium exchange rates of the yeast hexokinase reaction. The reaction mixtures contained 58 mM imidazole-nitrate buffer (pH 6.5), 13 mM magnesium nitrate, 38.0 ADP, 2.0 mM ATP, and a variable concentration of glucose-6-P and glucose, maintained at a constant glucose-6-P/glucose ratio of 20. The hexokinase P-II concentration was approximately 100 µg/ml, and the incubation time was 1.0 min. Reactions were initiated and terminated as described in the legend to Fig. 1.

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the maximal exchange rates are linearly dependent upon the hexokinase concentration. This latter observation is in harmony with rapid reaction measurements of the maximal velocity (V_m) at hexokinase concentrations of approximately 60 µg/ml (14) where the rate of the reaction was monitored by the production of hydrogen ion.

Although the concentration of hexokinase in yeast is nearly 10 times higher than the enzyme levels used in these experiments (14), it seems clear that equilibrium exchange rates could be measured using an inexpensive flow device to permit reaction times of around 1 second. A flow device of this sort has been described by Barman and Gutfreund (16)

and modified by Bridger et al. (17) to measure reactions occurring within several hundredths of a second. To further illustrate the potential usefulness of such measurements, a listing of a variety of enzymes for which there exists adequate published data is presented in Table I. The enzyme concentrations tabulated are estimates of the levels of enzyme one could study provided that the reaction time was 1 second.

TABLE I

Estimated Enzyme Concentrations At Which Isotope Exchange Measurements Could Be Made Provided That The Reaction Periods Were 1.0 Second

Enzyme and Reference Number	Exchange Reaction[a]	Estimated Enzyme Concentration (mg/ml)
Hexokinase (3)	Glucose-6-P-Glucose	22
	ADP-ATP	9.8
Maltodextrin Phosphorylase (19)	P_i-Glucose-1-P	3.7
	Glucose-1-P-Dextrin	4.4
Creatine Kinase (5)	Creatine-Creatine-P	5.9
	ADP-ATP	14
Galactokinase (20)	Galactose-Galactose-1-P	40
	ADP-ATP	19
Malate Dehydrogenase (11)	Oxaloacetate-Malate	1.6
	DPNH-DPN^+	0.3
Alcohol Dehydrogenase (12)	Ethanol-Acetaldehyde[b]	1.8
	DPNH-DPN^+,[b]	4.0

a. For clarity, only the exchange reactions for the varied substrate-product pair are presented. Since the non-varied substrate-product pair can be at any experimentally convenient concentration, it does not generally limit the method.

b. Estimates for the alcohol dehydrogenase exchange rates were made from the values given in Fig. 1 of reference 12 in the absence of imidazole.

These estimates were obtained by the use of Eqn. 1 which relates the

$$R = \frac{-(A)(P)\ln(1-F)}{[(A)+(P)]\ t} \qquad (1)$$

rate of isotope exchange, R, between substrate A and product P to the time of reaction, t, the concentrations of A and P, and the fractional attainment of isotopic equilibrium, F. The estimates presented in Table I were made for the substrate-product pair concentration that yields one-half of the apparent maximal exchange rate. We have assumed that F was 0.5, and that the exchange rates are proportional to the enzyme concentration. These estimates also assume that the fraction of substrate or product associated with the enzyme is negligible to the total substrate or product concentrations. The estimates suggest that it may be possible to study a great variety of enzymes by this approach.

Aside from the obvious requirements of rapid quenching of the reaction, separation of the substrate and product radioactivities, and a knowledge of the apparent equilibrium constant, the method outlined here requires that the enzymic reaction under study be reversible. The reversibility is not only a function of the apparent equilibrium constant, but, perhaps most importantly, dependent upon the kinetic parameters as indicated by the Haldane relation (18). On the other hand, the major advantage of this new application of equilibrium kinetics lies in its versatility, and on the basis of this brief survey, it offers a promising approach to the understanding of enzymatic catalysis at high enzyme concentrations.

References

1. Boyer, P. D., Arch. Biochem. Biophys., 82, 387 (1959).
2. Boyer, P. D., and Silverstein, E., Acta Chem. Scand., 17, Suppl. 1, S195 (1963).
3. Fromm, H. J., Silverstein, E., and Boyer, P. D., J. Biol. Chem., 239, 3645 (1964).

4. Silverstein, E., and Boyer, P. D., J. Biol. Chem., 239, 3901 (1964).
5. Morrison, J. F., and Cleland, W. W., J. Biol. Chem., 239, 673 (1966).
6. Rhoads, D. G., and Lowenstein, J. M., J. Biol. Chem., 243, 3963 (1968).
7. Su, S., and Russell, P. J., Jr., J. Biol. Chem., 243, 3826 (1968).
8. Hansen, J. N., Dinovo, E. C., and Boyer, P. D., J. Biol. Chem., 244, 6270 (1969).
9. Dinovo, E. C., and Boyer, P. D., J. Biol. Chem., 246, 4586 (1971).
10. Wedler, F. C., and Boyer, P. D., J. Biol. Chem., 247, 984, 993 (1972).
11. Silverstein, E., and G. Sulebele, Biochemistry, 8, 2543 (1969).
12. Silverstein, E., Biochim. Biophys. Acta, 198, 148 (1970).
13. Fromm, H. J., and Zewe, V., J. Biol. Chem., 237, 3027 (1962).
14. Barwell, C. J., and Hess, B., Hoppe-Seyler's Z. Physiol. Chem., 351, 1531 (1970).
15. Rudolph, F. B., and Fromm, H. J., J. Biol. Chem., 246, 6611 (1971).
16. Barman, T. E., and Gutfreund, H., in Rapid Mixing and Sampling Techniques in Biochemistry, Chance, B., Gibson, Q. H., Eisenhardt, R. H., and Lonberg-Holm, K. K., Eds., 1964, Academic Press: New York, p 339.
17. Bridger, W. A., Millen, W. A., and Boyer, P. D., Biochemistry, 7, 3608 (1968).
18. Haldane, J. B. S., in Enzymes, 1930, Longmans Press: London, p 80.
19. Chao, J., Johnson, G. F., and Graves, D. J., Biochemistry, 8, 1459 (1969).
20. Gulbinsky, J. S., and Cleland, W. W., Biochemistry, 7, 566 (1968).