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CONCERNING THE EQUILIBRIUM EXCHANGE KINETICS AND MECHANISM OF THE SHEEP BRAIN GLUTAMINE SYNTHETASE REACTION*

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Summary: Isotope exchange measurements of sheep brain glutamine synthetase have yielded conflicting experimental findings and interpretation, leaving in doubt the question of whether the enzyme operates via ordered or random pathways of enzyme-substrate interactions. We now report new experimental evidence that demonstrates the earlier discrepant results may be attributed to the choice of reaction conditions used to achieve equilibration of the chemical reaction prior to addition of isotopic tracer. A random kinetic mechanism, perhaps not of the rapid-equilibrium type, is most compatible with the exchange data. We also discuss other potential time-dependent processes that may compromise the equilibration of reaction systems and affect the outcome of exchange experiments, and criteria for equilibration are suggested for the aid of other workers.

An understanding of the order of substrate addition to and product release from an enzyme can provide valuable information for the elucidation of mechanisms for enzymic catalysis and regulation (1-3). During the characterization of the ovine brain glutamine synthetase reaction kinetics, Allison et al. (4) applied a combination of initial rate and equilibrium isotope exchange measurements, but they overlooked an earlier report of Wedler (5) who first applied the exchange technique to this enzyme. Both studies involved the Wedler and Boyer (6) protocol which permits distinction of compulsorily ordered- and random-substrate addition mechanisms by searching for noncompetitive and competitive substrate effects, respectively, as all substrates and products are raised in their absolute concentrations while still maintaining both their relative concentrations and the mass action ratio for the reaction. In particular, the Wedler-Boyer protocol is most useful for distinguishing between random mechanisms with one or more dead-end complexes and the compulsorily ordered scheme, both of which may give depression in exchange upon raising a single substrate-product pair(2,3,6). Yet, it is

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disconcerting that the latter set of experiments (4) yielded results favoring the random sequence while the earlier results (5) were interpreted to favor a sequential ordered mechanism. The most straightforward retrospective evaluation is that Allison et al. (4) failed to achieve adequate saturation of the enzyme by not raising the reactant concentrations sufficiently high to observe the depressed exchange phenomenon found by Wedler (5). Yet, the reactant concentration range in the later study (4) exceeded that used in the earlier inquiry (5), indicating that substrate saturation of the enzyme cannot be the source of disagreement. To reconcile this rather fundamental discrepancy and to achieve a better perspective on the kinetic reaction pathway, we re-evaluated the experimental conditions applied in the two studies. In addition, new experimental data were obtained by determining the equilibrium exchange velocities from both sides of the equilibrium to ascertain whether inadequate attainment of reaction equilibration could explain the disagreement. These efforts provided further support for the random kinetic reaction scheme for the sheep brain enzyme.

Experimental Procedures

Sheep brain glutamine synthetase was isolated from brain acetone powder as described by Rowe et al. (7), yielding a final specific activity of 125-130 units/mg with the glutamyl-hydroxamate assay. All substrates, products, radiolabeled P_i and ATP, and assay chemicals were obtained commercially as indicated elsewhere (4). All exchange measurements were carried out by the so-called initial rate method, using three or four reaction times to establish the reaction rate. Methods for preparing the exchange reaction samples, initiating the reactions, quenching the reactions, processing samples by DE-81 ion-exchange paper chromatography, and scintillation counting are fully described in reference (4). Reported exchange rates are expressed as millimolarity/min.

Results

A little more than a decade after Boyer (1) proposed the equilibrium exchange kinetic technique, Wedler and Boyer (6) introduced a reliable shortcut method avoiding the need for numerous exchange experiments to distinguish ordered and random kinetic mechanisms. They recognized that the two cases differ in that the former involves noncompetitive interactions whereas the latter can only display competitive interactions amongst the substrates and products. By simultaneously raising the levels of all substrates and products for maintaining chemical equilibrium, competitive interactions cannot alter the relative distribution of enzyme-reactant complexes, but noncompetitive effects can. In their studies of the Escherichia coli glutamine synthetase, for example, Wedler and Boyer (6) adduced evidence for a random pathway of enzyme-substrate interactions. As noted in the Introduction, the discrepancy between the findings with the ovine brain enzyme (4,5) cannot be attributed to failure of Allison et al. (4) to achieve sufficiently high

reactant concentrations to reveal the noncompetitive interactions diagnostic for ordered reaction mechanisms.

Upon further scrutiny of the experimental conditions imposed in the two equilibrium exchange studies of the sheep brain enzyme, we recognized that the preincubation conditions of Wedler (5) might have been insufficient to allow attainment of thermodynamic equilibrium. Because the theory developed for equilibrium exchange studies absolutely depends upon attainment of thermodynamic equilibrium prior to radiotracer addition (especially in the one-point assays depending on the first-order exchange behavior used by Wedler (5)), we carefully considered this possibility. The enzyme level used by Allison et al. (4) exceeded that of the earlier study (5) by eight-fold, and the preincubation period was three-fold longer than that applied in the earlier study. It may be noted that Allison et al. (4) verified that equilibration had occurred in their experiments by measuring the rate of exchange from both sides of the glutamine synthetase reaction. Measurement of the same exchange from either side of an equilibrated system should yield identical exchange rates (2,3), and such a determination provides the test sine qua non for equilibration. Thus, we set out to test whether the lower enzyme level and shorter preicubation period, as applied by Wedler (5), would be sufficient to ensure equilibration. We used the mass action ratio of 444 to represent the apparent equilibrium constant at pH 6.5.

The results presented in Fig. 1 illustrate that there is a net inbalance in the exchange rates for the ATP$\rightleftharpoons$$P_i$ exchange when probed in the forward reaction direction with radiolabeled ATP and in the reverse reaction direction with radiolabeled orthophosphate. Indeed, the observed rates are about two-fold slower for the forward direction, suggesting that the system was still displaced from equilibrium under the conditions applied by Wedler (5). We were also surprised to note that the ATP*$\rightleftharpoons$$P_i$ exchange rose monotonically, while the $P_i^*$$\rightleftharpoons$ATP exchange reached an apparent maximum, as the absolute levels of all substrates and products were increased. It is noteworthy that Wedler's results were obtained from measurements starting with P_i^*, and he observed a slight depression in the exchange rate profile. Furthermore, one may note that Wedler (4) used a mass action ratio of 500, and use of such a value would only serve to displace the reaction even further from the equilibrium poise. Furthermore, although ovine glutamine synthetase has an intrinsic ATPase activity of relatively low activity compared to its synthetic capacity (8), this hydrolase cannot explain the observed inbalance because ATP*$\rightleftharpoons$$P_i$ exchange is slower than $P_i^*$$\rightleftharpoons$ATP exchange, not vice versa. Thus, these findings weigh against the validity of the earlier exchange data.

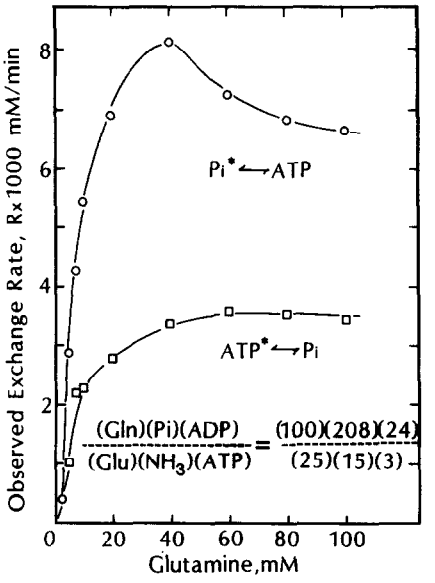

Figure 1. Comparison of the forward and reverse ATP⇌P_i exchange rates catalyzed by sheep brain glutamine synthetase. All experimental conditions, with the exception of an 8-fold lower enzyme level and a 3-fold shorter preincubation time, are identical to the conditions described elsewhere (4). The numbers in the brackets correspond to the millimolar levels of each of the reactants shown in the corresponding mass action ratio. (P_i^* and ATP* refer to the radiotracer used in the measurement of exchange rate.) The free magnesium concentration was held constant at 1 mM.

Discussion

The findings presented in this communication illustrate one of the problems that can potentially preclude an unambiguous interpretation of equilibrium exchange behavior. There are other factors that can introduce kinetic effects on a system presumed to be at equilibrium. One such case is enzyme instability; the fraction of active catalyst in each assay may be affected by varying protective effects of substrates, products, and even effectors against enzyme inactivation, and these protective effects may not be the same from sample to sample if the concentrations of these ligands are altered. Another source of error can occur in the case of substrate instability, and Silverstein and Sulebele (9), for example, deliberately chose to examine malate dehydrogenase exchange behavior at 1°C to avoid problems arising from thermal decomposition of oxaloacetate. Other errors are introduced whenever a presumed allosteric effector turns out to be an alternative substrate; the equilibrium mass action ratio will necessarily change from sample to sample whenever the "modifier" concentration is changed. Finally, contaminating enzymatic activities such as adenylate kinase and nucleotide hydrolases may lead to ambiguity by preventing true thermodynamic equilibration of the reaction system.

All of the aforementioned obstacles to accurate equilibrium exchange measurements can be surmounted through the judicious application of suitable control experiments. Simultaneous measurement of the same exchange from both sides of a reaction can help, but alternative substrate effects of the sort described in the preceding paragraph may be more difficult to ascertain. Nonetheless, regarding the particular problem of allowing sufficient time for equilibration of the reaction system, there does appear to be valuable information available from the observed exchange kinetics per se. From computer-assisted simulation (10) of enzyme systems, we have found that the time allowed for preincubation should be at least five times greater than the period necessary for achieving a F value (i.e., a fractional attainment of isotopic equilibrium value (2)) of 0.5. An intuitive observation is that if exchange of substrate and product is slow, then the approach toward thermodynamic equilibrium is also probably quite slow.

Finally, until additional mechanistic information to the contrary is available, the ovine glutamine synthetase should be regarded as displaying a random kinetic pathway. Such a random order of enzyme-substrate interactions does not prevent the enzyme from displaying some steric preference, and the pathway need not be of the rapid equilibrium random type. Instead, because Meister's work so overwhelmingly favors the intermediacy of a γ-glutamyl-phosphate to activate the carboxylate toward amide formation (8), the major pathway could be weighted to favor a sterically-preferred path for substrate adsorption and release. These are matters that will ultimately require the further development of enzyme kinetic theory.

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