MEASUREMENT OF INTERCONVERSION RATES OF BOUND SUBSTRATES OF PHOSPHORYL TRANSFER ENZYMES BY ^{31}P NUCLEAR MAGNETIC RESONANCE

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Kinases are phosphoryl transfer enzymes that catalyze the reaction ATP + X $\rightleftharpoons$ ADP + XP, where X is the second substrate, using a divalent metal ion (usually Mg^{2+}) as an obligatory component. On the basis of known dissociation constants of the substrates and products from their binary (or ternary) enzyme-bound complexes, sufficiently high concentrations of enzyme can be chosen in excess of those of the substrates or products so that either E-X-ATP or E-XP-ADP account for the predominant fraction of the enzyme-bound complexes. Such a mixture can be generated by setting up an equilibrium mixture with a small amount of Mg^{2+} and later removing Mg^{2+} from the reaction by adding a complexing agent like EDTA. A ^{31}P-NMR spectrum of such a solution consists of resonances that may be readily assigned to the six phosphate groups in the above complexes with line widths and chemical shifts governed by the interaction of the substrates and the enzyme.

Addition of sufficient Mg^{2+} to the sample sets up an equilibrium mixture of the reaction E-X-MgATP $\rightleftharpoons$ E-XP-MgADP. This interconversion of the reactants and products on the enzyme involves the following exchanges of the phosphate groups: α – P (ATP) $\rightleftharpoons$ α – P (ADP), β – P (ATP) $\rightleftharpoons$ β – P (ADP), and γ – P (ATP) $\rightleftharpoons$ P (XP), and the ^{31}P resonances in the spectrum display line shape changes. If τ_A and τ_B are the lifetimes at two sites for which ω_A and ω_B are, respectively, the resonance frequencies for τ_A^{-1}, $\tau_B^{-1} \gg |\omega_A - \omega_B|$ (fast exchange), a single resonance of frequency and line width given, by the weighted averages of frequencies and line widths in the absence of the exchange, is obtained. If τ_A^{-1}, $\tau_B^{-1} \ll |\omega_A - \omega_B|$ (slow exchange) the resonances are not shifted, but they become broadened due to the exchange by $\Delta\omega_A (= \tau_A^{-1}/\pi)$ and $\Delta\omega_B (= \tau_B^{-1}/\pi)$. In intermediate exchange condition both shifts and line width changes result. The α – P resonances of ATP and ADP are usually separated by <0.5 ppm whereas the corresponding β – P resonances are separated by >12.0 ppm. For ^{31}P-NMR operating frequencies of ~40 MHz, exchange rates (τ_A^{-1}, τ_B^{-1}) of over 100 s^{-1} generally bring the α – P resonances of ATP and ADP into fast exchange, but the β – P resonances easily satisfy slow exchange condition for rates up to ~1,000 s^{-1}. Therefore, from the difference in the line widths of the β – P resonances of ATP in the presence and absence of exchange, the lifetime of the E-X-MgATP complex, τ_A (reciprocal of the rate of ADP formation), may be determined. The β – P resonance of ATP is particularly suitable for line width measurements, since it is isolated at the high-field side of the ^{31}P-NMR spectrum. The lifetime

of E-XP-MgADP complex, τ_B, may then be deduced on the basis of the equilibrium constant, K'_{eq} = [E-XP-MgADP]/[E-X-MgATP] = τ_B/τ_A. The concentrations of the complexes at equilibrium are determined directly from the spectrum.

The rates determined above are the rates of interconversion of reactants and products on the surface of the enzyme. The NMR experiment on the bound substrates isolates and monitors exclusively the interconversion step in the reaction. The rate is thus obtained in a straightforward manner compared to the relatively cumbersome isotope exchange methods. A comparison of the measured rates with the corresponding overall rates of the reaction determines whether the interconversion step is the rate-limiting step. It is also possible to determine, by this method, whether compounds that alter the overall rate of the reaction (e.g. inhibitors or modifiers) do so by modifying the rate of the interconversion step or some other step in the overall kinetic scheme.

Measurements of interconversion rates were made for five different kinases: arginine kinase, adenylate kinase, creatine kinase, pyruvate kinase, and 3-phosphoglycerate kinase. The rates of ATP and ADP formation were 154 and 200 ± 15 s^{-1}, respectively for arginine kinase (at pH 7.25 and 12°C) and for adenylate kinase (at pH 7.0 and 4°C) 420 ± 40 s^{-1} and 690 ± 50^{-1}, respectively, and are the fastest among the enzymes studied. The overall rates of the reactions in either direction were an order of magnitude smaller. A similar result is obtained for creatine kinase. The interconversion process is, therefore, not rate-limiting for these reactions.

Pyruvate kinase and 3-phosphoglycerate kinase are enzymes responsible for ATP formation in glycolysis with equilibrium constants at catalytic enzyme concentrations of the order of 10^3 in favor of ATP and negligible overall rates for ADP formation. The ^{31}P-NMR experiments on bound substrates reveal similar interconversion rates for forward and reverse directions, indicating that (*a*) the equilibrium constant on the enzyme is ~1, and (*b*) while the interconversion step may be a rate-determining step for ATP formation, it is certainly not so in the reverse direction. Thus, the predominance of equilibrium in favor of ATP formation at low enzyme concentrations seems to be due to the very slow rates of dissociation of the products in the reverse direction especially of phosphoenol-pyruvate from pyruvate kinase and 1,3-diphosphoglycerate from 3-phosphoglycerate kinase.

Whenever the sample conditions may be easily chosen, such that the enzyme-bound complexes are predominantly in the two forms E-X-MgATP or E-XP-MgADP, the interconversion rates for these reactions may be measured simply and straightforwardly. The ability to obtain this condition is the only serious limitation for this method. The scope and accuracy of the method may be significantly improved by using the temperature to alter the magnitudes of the rates and employing higher NMR operating frequencies that provide both larger chemical shifts and better sensitivity.