E. COLI PYRUVATE OXIDASE: A HYSTERETIC ENZYME

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Pyruvate oxidase is a flavoprotein dehydrogenase isolated from E. coli which catalyzes the oxidative decarboxylation of pyruvate to acetate plus CO_2 (1,2). Each subunit of the isolated enzyme contains a molecule of non-covalently bound FAD, and Mg^{2+}-thiamin pyrophosphate is a required co-factor for catalytic activity (2). Recent studies have shown that the natural electron acceptor for the flavoprotein is probably ubiquinone-8 in the E. coli cytoplasmic membrane (3). In vitro kinetics studies, however, have primiarly used a water-soluble electron acceptor such as ferricyanide. The enzyme is isolated as a water-soluble species, but is considered to be a peripheral membrane protein.

In the absence of pyruvate, the enzyme appears to exist in a conformation with little membrane affinity. Upon addition of pyruvate, a conformational change occurs which results in a dramatic alteration in the solution properties of the protein, and the enzyme binds to phospholipids or other amphiphiles with substantially greater affinity (4). Once bound to lipids, the V_{max} of the enzyme measured in vitro using water-soluble electron acceptors is increased (5). In vivo, the enzyme must be bound to the membrane in order to reduce the long chain quinone substrate (3). Controlled proteolysis can also be used to activate the enzyme in vitro apparently by cleavage at a single site at or near the lipid-binding domain (6,7). Hence, the enzyme can be locked into an "activated" form by either "lipid activation" or "protease activation". In this paper, recent kinetics studies on this enzyme are summarized. The motivation of the work was to determine the kinetic mecha-

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nism and, in particular, the step(s) primarily accelerated upon lipid or protease activation. The strategy was to carry out single turnover experiments to observe the rates of reduction and re-oxidation of the bound flavin by, respectively, pyruvate and ferricyanide, and to compare the activated and unactivated forms of the enzyme.

The traditional kinetic scheme for pyruvate oxidase incorporates the Breslow mechanism expected for thiamin pyrophosphate-dependent enzymes, in a ping pong scheme (see 8). In the absence of any activation, the turnover number is about 7 sec^{-1}, while the activated form of the enzyme turns over at about 200-350 sec^{-1} depending on the specific conditions (2,5). Earlier work (M. O'Leary and R. B. Gennis, unpublished) clearly showed that the actual carbon-carbon bond breaking step is not rate determining either for the unactivated or activated forms of the enzyme.

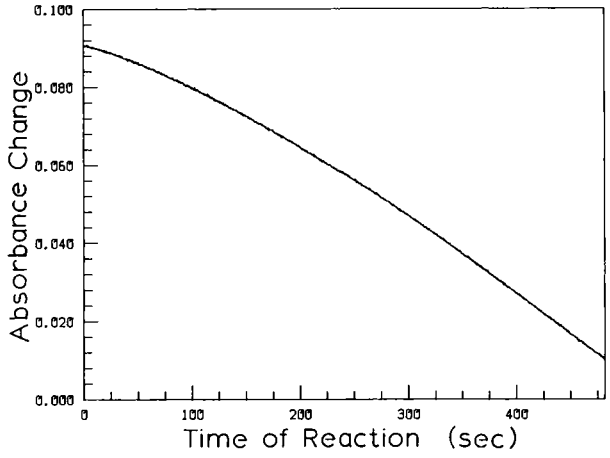

Figure 1: Progress curve for ferricyanide reduction by pyruvate oxidase at 25°C. Conditions are 6.25 mM pyruvate, 4 mM sodium ferricyanide, 0.15 mM thiamin pyrophosphate, 10 mM $MgCl_2$ 1% glucose, 0.1 M sodium phosphate at pH 6.0. Sufficient sodium sulfate was added to bring the ionic strength of 0.5. Protein concentration was 20 µg/ml.

Initial velocity studies of the ferricyanide reductase activity of the enzyme in the absence of an activator indicated that the enzyme is hysteric. The progress curves done both with manual mixing and by use of a

stop flow apparatus showed reproducible evidence of an increase in enzyme specific activity during the course of the reaction. This was most evident at low pyruvate concentration (Figure 1).

The rates of flavin reduction (by pyruvate) and oxidation (by ferricyanide) were measured in stop flow experiments in the absence of an activitor. Ordinarily, for a non-hysteretic enzyme, the results of these single turnover experiments should be compatible with data obtained in "steady state" initial velocity experiments. Previous initial velocity studies done in the absence of activator were largely performed at high pyruvate concentrations and the non-Michaelis-Menton behavior was not readily apparent (2,9). Using values of K_m and k_{cat} from a previous study (9), clear gross disparities were noticed. For example the rate of reoxidation of the flavoprotein by 2 mM ferricyanide is predicted to be 7 sec^{-1} from previous initial velocity data (9), but is measured to be 0.012 sec^{-1}. Hence, in the single turnover experiments the enzyme is reoxidized almost 600 times slower than expected. A comparison of the rate of flavin reduction rates at 3.1 mM pyruvate yields values of 0.25 sec^{-1} (predicted) and 0.05 sec^{-1} (measured). When similar experiments are performed with protease activated enzyme there is no accelerating phase in the progress curve and the predicted and measured rates of flavin reduction are in good agreement.

One kinetic scheme which can account for the hysteretic behavior of the unactivated enzyme is that the enzyme exists in equilibrium between two states, with low and high turnover. Each state is presumed to operate <u>via</u> a ping pong mechanism. The KINSIM kinetic programs (10) were used to simulate the kinetic behavior of the simplified two state ping pong system, and features of the unactivated pyruvate oxidase kinetics could be qualitatively duplicated. In single turnover experiments, the enzyme is largely in the unactivated state. However, in initial velocity measurements, as the system turns over, the amount of enzyme in the activated state progressively increases, resulting in the observed acceleration.

It is clear that lipid binding pulls the system into an activated state, as does protease activation. The stop flow results indicate that the rates of both flavin reduction and flavin reoxidation are accelerated upon activation. This is consistent with the general observation that the flavin active site "opens up" and appears to be more accessible when the enzyme is in the activated state (7). Further structural studies will be required to obtain detailed information about the exact nature of these alterations in the active site of this enzyme.

References

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