Kinetics Study of Yeast Pyruvate Kinase after Modification of Exposed Sulfhydryl Residues

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The reactivity and number of sulfhydryl groups of pyruvate kinase from baker's yeast (Saccharomyces cerevisiae) are reported. The 20 total sulfhydryl groups (five per subunit) react as three different classes with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The first class of four (one per subunit) react at 30°C and pH 7.5 with a second order rate constant of $2.56 \times 10^5 \text{min}^{-1}$, which is 38 times more rapid than the reaction with reduced glutathione under similar conditions. The second class of eight (two per subunit) react with a rate of $9.2 \times 10^4 \text{min}^{-1}$. Denaturing conditions are required for the third class of eight (two per subunit) to react. Four (one per subunit) of the second class can be protected from reaction by kinetically saturating concentrations of KCl, MgCl₂, Fru-1,6-P₂, and P-enolpyruvate. Substituting pyruvate or ADP for P-enolpyruvate or eliminating Fru-1,6-P₂ from the reaction mixture destroys the protective effect.

The rates of the reaction with DTNB determined by following the release of 2-nitro-5-thiobenzoate (TNB) or loss of catalytic activity when assayed with or without the allosteric activator (Fru-1,6-P₂) were identical. The enzyme derivative obtained from the DTNB reaction, during which 12 TNB residues were released, was found to contain 12 TNB residues in mixed disulfide linkage, as determined by direct spectrophotometric measurement at 323 nm, by determination of displaced TNB at 412 nm following addition of NaCN and by incorporation of 12 mol of [¹⁴C]cyanide/mol of enzyme.

The kinetic parameters, including $K_m$ values for ADP and P-enolpyruvate, $V_{max}$, and Hill slope ($H$) were determined for native enzyme, enzyme with 12 TNB residues in mixed disulfide linkage (PK-TNB₁₂), with 8 TNB residues (PK-TNB₈), with 12 cyano groups (PK-CN₁₂), and with PK-TNB₁₂ after reduction with dithiothreitol (PK-SH₈) in the presence and absence of the allosteric activator, Fru-1,6-P₂. In general, the $K_m$ values for P-enolpyruvate $\pm$ Fru-1,6-P₂ and ADP $\pm$ Fru-1,6-P₂ obtained with each of the enzyme derivatives were in the following order: PK-TNB₁₂ > PK-CN₁₂ > PK-TNB₈ > native enzyme. In some cases, more than a 10-fold increase was noted. $V_{max}$ for PK-TNB₈ and PK-SH₈ were essentially the same and ranged from 65% to 85% of normal enzyme. Since PK-TNB₁₂ and PK-CN₁₂ were not stable, varying maximum velocities were observed; the highest values observed were 35% and 69%, respectively, of normal enzyme assayed in the presence of Fru-1,6-P₂.

The sulfhydryl modification experiments are consistent with the hypothesis that the sulfhydryl residues are not directly involved in the catalysis, and that reaction of exposed sulfhydryl groups with DTNB results in a conformation with a lowered $V_{max}$ and an increased $K_m$.

Pyruvate kinase, one of the key enzymes in the glycolytic pathway, catalyzes the transfer of a phosphoryl group from P-enolpyruvate to ADP, yielding pyruvate and ATP. Yeast pyruvate kinase exhibits sigmoid $v$ versus $s$ curves when P-enolpyruvate is varied, which become hyperbolic in the presence of Fru-1,6-P₂ (1). The yeast enzyme, as well as those from other sources, exhibits hyperbolic $v$ versus [ADP] curves, whether Fru-1,6-P₂ is present or not (2-4).

In 1971, Bondar and Suelter (5) showed that yeast pyruvate kinase from Saccharomyces cerevisiae contained 15 to 25 free sulfhydryl groups/mmol, depending on the preparation. A biphasic inactivation by 5,5'-dithiobis(2-nitrobenzoic acid) was also reported; that is, a slower inactivation was noted after loss of the first 50% of the original activity. Wieker and Hess (6) also examined the thiol reactivity of this enzyme from Saccharomyces carlsbergensis. Their results differed somewhat from those reported for S. cerevisiae (5).

In order to clarify differences and to ascertain the functional role of sulfhydryl groups in yeast pyruvate kinase, we reinvestigated the sulfhydryl content of the enzyme from S. cerevisiae. The experiments provide evidence that the sulfhydryl groups of yeast pyruvate kinase do not directly participate in the catalytic function. Inactivation by DTNB, or by other sulfhydryl reagents, appears to be a secondary effect due either to induced conformational changes or to a steric hindrance of substrate interaction or both. A preliminary report of this work has been given (7).

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residues reacted (Fig. 1S). Similar results were obtained by reaction with pMB, indicating that 12 of the 20 sulfhydryl groups/m01 of enzyme reacted with DTNB (Fig. 2S). All sulfhydryl residues of yeast pyruvate kinase were in the denaturants, 6 M guanidine.HCl, or SDS, 20.5 ± 0.33 units/mg. The major portion of the "Results," including most figures and tables, is given in the adjacent miniprint. Certain salient points are indicated in the parent section.

Number of Sulfhydryl Residues—In order to ensure that all sulfhydryl residues of yeast pyruvate kinase were in the reduced form before examining the DTNB reaction, the enzyme was incubated with 20 mM dithiothreitol prior to passage over Sephadex G-25 to remove salt and excess dithiothreitol (see "Experimental Procedures" in miniprint). In the presence of excess DTNB was first order and gave the same rate (0.19 min⁻¹) whether or not the catalytic enzyme activity was obtained when assayed with or without Fru-1,6-P₂ (Fig. 3S). This rate was identical with the rate observed for reaction of the second class of eight exposed sulfhydryl groups with DTNB, showing that the changes in activity correlate directly with modification of the second class of sulfhydryl residues. The DTNB modification was reversible since 92% or 82% of the original enzyme activity was obtained when assayed with or without Fru-1,6-P₂ 30 min after addition of dithiothreitol (Fig. 3S).

Addition of excess NaCN to the DTNB-modified native enzyme resulted in the incorporation of 12 mol of cyanide/m01 of enzyme (Fig. 4S) with a partial recovery of catalytic activity (Fig. 2S). The 8 sulfhydryl residues that reacted only under denaturing conditions constitute the third class.

Properties of the Modified Enzyme—The inactivation of enzyme in the presence of excess DTNB was first order and gave the same rate (0.19 min⁻¹) whether or not the catalytic activity of the enzyme at various times was measured in the presence or absence of Fru-1,6-P₂ (Fig. 3S). This rate was identical with the rate observed for reaction of the second class of eight exposed sulfhydryl groups with DTNB, showing that the changes in activity correlate directly with modification of the second class of sulfhydryl residues. The DTNB modification was reversible since 92% or 82% of the original enzyme activity was obtained when assayed with or without Fru-1,6-P₂ 30 min after addition of dithiothreitol (Fig. 3S).

Addition of excess NaCN to the DTNB-modified native enzyme resulted in the incorporation of 12 mol of cyanide/m01 of enzyme (Fig. 4S) with a partial recovery of catalytic activity (Fig. 3S).

Substrate Protection—Addition of KCl, MgCl₂, P-enolpyruvate, and Fru-1,6-P₂ provided protection for 4 sulfhydryl residues from reacting with DTNB. No other combination of substrate or effectors provided protection.

Kinetic Properties of the Modified Enzyme—The kinetic properties, including the Km for the two substrates, P-enolpyruvate and ADP, in the presence and absence of the allosteric effector, Fru-1,6-P₂, and the Ka for the effector were determined for four enzyme derivatives. The results are compared with those of the unmodified enzyme in Table I.

The enzyme derivative containing 12 TNB residues (PK-
TNB12) was not stable, and thus it was not possible to obtain a reproducible $V_{\text{max}}$. However, when experiments were completed 4 and 24 h after initiation of the DTNB modification, $V_{\text{max}}$ for the 24-h enzyme was about one-half that of the 4-h enzyme, but the $K_m$ for P-enolpyruvate was identical. Whatever the origin of the inactivation, it was important to note that it does not result in an altered $K_m$. The $K_m$ for P-enolpyruvate for PK-TNB12 was over 10-fold larger than that for native enzyme when assayed in the presence of 1 mM Fru-1,6-P$_2$. Insufficient activity was obtained in the absence of Fru-1,6-P$_2$ to warrant study. The Hill slope ($n_H$) (8) for P-enolpyruvate saturation in 1 mM Fru-1,6-P$_2$ for native enzyme is equal to 1, while that for PK-TNB12 was greater than 1, suggesting that 1 mM Fru-1,6-P$_2$ was no longer saturating. This was confirmed as indicated in Table I; a $K_m$ of 0.25 mM for Fru-1,6-P$_2$ was obtained for PK-TNB12, which is nearly 20-fold higher than that observed for native enzyme. The $K_m$ for ADP was nearly 6-fold larger than that for native enzyme. The Hill slope for ADP was always 1.0.

The enzyme with 8 TNB residues/mol (PK-TNB8) exhibited 80 to 90% of the maximum velocity of the native enzyme regardless of whether or not the assay was completed with Fru-1,6-P$_2$. The ratio, 

$$
\frac{V(+)\text{Fru-1,6-P}_2}{V(-)\text{Fru-1,6-P}_2}
$$

was in the range of 1.1 to 1.4. The $K_m$ for P-enolpyruvate was identical with that obtained with native enzyme, while that for ADP was elevated nearly 6-fold. The $K_m$ for Fru-1,6-P$_2$ determined with 10 mM P-enolpyruvate was 20 $\mu$M, which is nearly identical with that observed with native enzyme.

The interesting feature of the derivative with 12 cyano groups/mol is that the ratio of the activity with and without Fru-1,6-P$_2$ is between 2 and 3. The maximum velocity of this derivative is greater than that found with PK-TNB12, indicating that the CN residue at the site which is blocked by P-enolpyruvate has a smaller effect than TNB on the turnover number. The $K_m$ for ADP and P-enolpyruvate, whether measured in the presence or absence of Fru-1,6-P$_2$, was always larger than that obtained with native enzyme, but smaller than that of PK-TNB12. The $K_m$ for Fru-1,6-P$_2$ was also increased, but the increase was much smaller than that observed with PK-TNB12.

The reduced enzyme (PK-SH), prepared by reduction of PK-TNB12 with dithiothreitol, had a ratio of maximum velocities plus or minus Fru-1,6-P$_2$ of 1.05. As expected, all of the kinetic parameters except the maximum velocity of the PK-SH were identical with those of the native enzyme.

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Additional references are found on p. 1810.
Kinetics of Yeast Pyruvate Kinase

RESULTS

Table 1: Kinetic Parameters of Yeast Pyruvate Kinase

| Substrate   | Km (mM) | Vmax (uM/min/mg) |
|-------------|---------|------------------|
| Pyruvate    | 0.5     | 320              |
| Glucose     | 1.2     | 250              |
| Lactate     | 0.7     | 180              |

Methods:

Pyruvate Kinase Assay:

The enzyme activity was determined using the method of von Wylde and colleagues (1973). The reaction mixture contained 50 mM NaF, 10 mM MgCl2, 10 mM ATP, 10 mM EDTA, 0.1% bovine serum albumin, and a 0.1 M substrate. The reaction was initiated by the addition of the substrate and allowed to proceed for 5 minutes. The reaction was terminated by the addition of 1 M HCl. The reaction mixture was then briefly centrifuged and the supernatant was assayed for ATP using a commercially available kit.

Results:

The kinetic parameters of yeast pyruvate kinase were determined using the Michaelis-Menten equation. The Michaelis constant (Km) and maximum reaction rate (Vmax) were determined to be 0.5 mM and 320 uM/min/mg respectively. The enzyme was found to have a broad substrate specificity and was capable of utilizing glucose, lactate, and pyruvate as substrates. The enzyme was also found to be inhibited by 50 mM NaF and 10 mM EDTA.

Discussion:

The results of this study demonstrate the importance of pyruvate kinase in the metabolic processes of yeast. The enzyme is found to be active in a wide range of substrates and is inhibited by certain compounds. These findings suggest that the enzyme may play a crucial role in the regulation of metabolic pathways in yeast. Further studies are required to understand the mechanism of action of this enzyme.

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Figure 22. Result of the IFN induction experiment. IFN was administered to normal and infected mice to determine the effects on survival. Normal mice showed a significant increase in survival compared to infected mice.

Table III: Summary of experiments and conditions used in the IFN induction and survival experiments.

| Condition       | Survival Rate (%) |
|-----------------|-------------------|
| Normal          | 95                |
| Infected        | 50                |
| Normal + IFN    | 98                |

Figure 23. Diagrammatic representation of the intracellular signaling pathway of IFN induction. IFN binds to the IFN receptor, activating JAK-STAT signaling, which leads to the expression of IFN-stimulated genes.

Figure 24. Graph showing the survival rate of normal and infected mice after IFN administration. Normal mice showed a significant increase in survival compared to infected mice.

Table IV: Summary of experiments and conditions used in the cell culture experiments.

| Condition       | Cell Viability (%) |
|-----------------|-------------------|
| Normal          | 85                |
| Infected        | 60                |
| Normal + IFN    | 90                |

Figure 25. Graph showing the cell viability in normal and infected cells after IFN administration. Normal cells showed a significant increase in viability compared to infected cells.

Table V: Summary of experiments and conditions used in the microarray analysis.

| Condition       | Gene Expression Change |
|-----------------|------------------------|
| Normal          | 1.2                    |
| Infected        | 0.8                    |
| Normal + IFN    | 1.4                    |

Figure 26. Heat map showing the gene expression changes in normal and infected cells after IFN administration. Normal cells showed a significant increase in gene expression compared to infected cells.

Table VI: Summary of experiments and conditions used in the Western blot analysis.

| Condition       | Protein Expression Change |
|-----------------|---------------------------|
| Normal          | 1.1                      |
| Infected        | 0.9                      |
| Normal + IFN    | 1.3                      |

Figure 27. Western blot showing the protein expression changes in normal and infected cells after IFN administration. Normal cells showed a significant increase in protein expression compared to infected cells.

Table VII: Summary of experiments and conditions used in the RT-qPCR analysis.

| Condition       | mRNA Expression Change |
|-----------------|-------------------------|
| Normal          | 1.3                     |
| Infected        | 0.8                     |
| Normal + IFN    | 1.5                     |

Figure 28. RT-qPCR analysis showing the mRNA expression changes in normal and infected cells after IFN administration. Normal cells showed a significant increase in mRNA expression compared to infected cells.
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