Kapoor, M. Pyruvate kinase of N. crassa: conformational changes induced by Tris buffer.

Pyruvate kinase (PK) was isolated from N. crassa (St. Lawrence strain 79a; FGSC#533). The inoculum was grown on Vogel's minimal medium with 2% sucrose for 30 hrs. at 28°C, in an Environmental (New Brunswick) rotatory shaker. The mycelium was harvested on four layers of cheesecloth, lyophilirod and stored at -65°C. The enzyme was isolated from lyophilised mycelium powder in 0.05 M phosphate buffer containing 5 x $10^{-4}$ EDTA and $10^{-4}$ M ß-mercaptoethanol, pH7.5, and purified to near homogeneity by the application of several fractionation steps including heat treatment of the crude extract, ammonium sulphate precipitation (0.40 to 0.65 saturation), ion exchange chromatography on columns of DEAE sephadex, a second ammonium sulphate fractionation and finally gel filtration on a Sephadex G-200 column, followed by ammonium sulphate precipitation of the active fractions.

The enzyme preparation thus obtained was found to be approximately 95% purified. Upon electrophoresis on polyacrylamide gels, one major bond containing about 95% of the total protein (the pyruvate kinase bond) and two minor ones were detected. The two minor components are probably contaminants although the presence of subunits produced as an artifact of electrophoresis has not been ruled out. Sedimentation in the Analytical Ultracentrifuge (Spinco model E) revealed the presence of a single symmetrical peak; no minor peaks could be discerned on sedimentation of enzyme solutions containing 2 and 4 mg protein per ml. Details of the purification procedure will be published elsewhere. The final enzyme preparation was stored in 0.2 M phosphate buffer, pH 7.5, containing $10^{-4}$ M EDTA and 2 x $10^{-3}$ M dithiothreitol. It was stable for at least two months at -20°C. Before use the enzyme was routinely diluted to a concentration of 10µg/ml in the some buffer, 0.1 ml being used in individual assays.

Enzyme activity was determined by measuring the pyruvate produced from phospho-enol-pyruvate (PEP) ond ADP in the presence of magnesium chloride by coupling the reaction with lactate dehydrogenase (LDH) and following the disappearance of reduced NAD at 340 mµ in a Beckmon-DU spectrophotometer. The quantity of LDH in individual assay systems was maintained at a 100-fold excess over that of PK. The pH curve for pyruvate kinase reaction, using phosphate buffer in the assay mixture, showed a broad pH optimum between 6.8 and 8.0 with very little variation in activity within this range. A progress curve of the reaction ($OD_{340m\mu}$ versus time) at all pH valuer tested (6.5 to 9.5) in phosphate buffer was linear. With Tris-HCl buffer, on the other hand, the reaction showed a pronounced initial log, lasting for approximately 2.0 min and then a linear reaction rate war attained. The reaction velocity during the linear phase was observed to be no more than 60% of that obtained in phosphate buffer.

Thus there are two distinct aspects of the effect of Tris buffer: (a) the introduction of a lag period and (b) a diminished reaction velocity *during* the *linear phase*. *These two* effects of *Tris* buffer *were observed* in *reactions* performed *at pH values ranging* from 7.5 - 9.5, the log becoming more pronounced at higher pH. It would appear that the first effect is connected with a conformational change induced by iris leading to a lag in the reaction and that the attainment of linearity is a consequence of a subsequent

reversal of this conformational change by the substrates added to the reaction mixture. The reduction in the reaction velocity after the log period could be a direct consequence of the conformotionol change.

To determine whether the inhibitory effect was confined to the Trir buffer alone or not, a number of other buffers, including HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), MES (2-(N-morpholino)ethanesulfonic acid), TES (N-tris (hydroxymethyl)-methyl-2-amino ethanesulfonic acid), Tricine (N-tris (hydroxymethyl)methylglycine), and Bicine (N,N-bis (2-hydroxyethyl)glycine) were tested in the assay system at the some concentrotionr as Tris ond phosphate buffers. If the reaction war conducted at pH 7.5, only in TES buffer was a short log observed; none of the other buffers produced any log. However, in TES the reaction velocity during the linear phase was not significantly different from that in phosphate buffer. At pH 7.9, TES, MES, Tricine and Bicine, but not HEPES, elicited a brief lag lasting from 0.5 to 0.8 minutes; the reaction rate in the linear phase was again compamble to that in phorphote buffers.

The effect of substrates, PEP and ADP, on the reversal of the conformotionol alteration induced by Tris buffer was studied by adding varying amounts of these substrates to the assay system. In all cases, substrate concentrotionr employed were higher than those required for maximum velocity in phosphate buffer, which was 0.33 mM for PEP and ADP. It was demonstrated that by addition of 1 mM PEP to the reaction mixture containing 33.3 mM Trir buffer, the initial log period could be reduced to a considerable extent. ADP was not so effective as PEP. Similarly, the reaction velocity during the linear phase could also be partially restored and a value approaching that realized in phosphate buffer could be attained. Incubation of the enzyme for varying time intervals of up to 30 minutes with Tris buffer also resulting in conformational changes that could be reversed by PEP. However, prolonged incubation led to on irreversible change in the enzyme, resulting in a loss of as much as 90% of its activity.

Since the Trir-induced conformational alteration of pyruvate kinase is reversed by the binding of substrates to the enzyme, it would appear reasonable to assume that either this effect is brought about by binding of Trir close to the substrate site, or, the binding of Trir is independent of the active site but the binding of substrates, particularly PEP, results in a further conformational change, thus negating the effect of the first alteration. Details of the conformotionol changes induced by Tris and other buffers and their reversal ore being currently investigated. - - - Deportment of Biology, The University of Calgary, Calgary, Alberta, Canada.