

Catalytic properties of three L-Lactate dehydrogenases from saffron corms (*Crocus sativus L.*)

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Summary

Three L-lactate dehydrogenase isoenzymes were detected in saffron corms, using potassium ferricyanide as the electron acceptor. Their pH optima were 5.5, 7.5 and 9.5, respectively. All three dehydrogenases were substrate-inhibited by ferricyanide, but at different concentrations; maximum enzymatic activity was observed for 250, 100 and 600 μ M ferricyanide, at pH 5.5, 7.5 and 9.5, respectively. Catalytic efficiency, calculated per mg corm extract protein, was 1.9, 1.0 and 0.4 min^{-1} , respectively at pH 5.5, 7.5 and 9.5. Pseudo first order rate constant was also different under the three pH conditions. Malate was an inhibitor for the isoenzyme active at pH 9.5, but had no effect on the others.

Introduction

Flavocytochrome b_2 (L-(+)-lactate ferricytochrome c oxidoreductase, EC 1.1.2.3) contains both FMN and protoheme capable of taking part in a catalytic reaction [1] that transfers electrons from lactate to cytochrome c [2]. The electron transfer steps have been summarized by Capeillere-Blandin [3]. The first step is a two-electron transfer from L-Lactate to flavin mononucleotide within the flavodehydrogenase and the second a reversible intramolecular one-electron transfer between lactate-reduced flavin and heme b_2 taking place within an active site assembling these two related groups. Earlier studies characterized two molecular forms of cytochrome b_2 [4]. The molecular structure of the enzyme which is a tetramer of molecular weight 230,000 has been characterized [5]. Each subunit comprises two domains, one binding a heme and the other an FMN prosthetic group. The heme domain (residues 1 to 99) is folded in a fashion similar to the homologous soluble fragment of cytochrome b_5 ; the FMN domain (residues 100 to 486) is mostly sequestered from solvent. A cysteine cluster is critical for flavin binding [6]. Mutants determining the relationship between structure and function have also been investigated [7,8].

The purpose of this research was to investigate the kinetic properties of ferricytochrome b_2 in saffron (*Crocus sativus L.*) since few investigations have been reported so far on the enzymology of this plant.

Materials and Methods

Dormant saffron (*Crocus sativus L.*) corms were used throughout these studies. They showed neither roots nor shoots. Extracts were prepared from corms weighing each between 3 and 6 g, by homogenization in phosphate buffer 0.1 M, pH 7.0. After centrifugation at 3,000 g for 10 min, then at 35,000 g for 30 min, a clear, transparent supernatant termed "crude extract" was obtained and used for our studies. Protein concentrations were determined by the Lowry method.

L-Lactate dehydrogenase activity was determined by following the reduction of potassium ferricyanide at 420 nm with extinction coefficient $1040 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Assays were carried out at room temperature ($\sim 22\text{-}25^\circ\text{C}$), in 0.1 M phosphate buffer, pH 8.4, in the presence of 1 mM EDTA, 50 mM L-lithium lactate and 0.25 mM potassium ferricyanide, using Aminco DW2 and Milton Roy spectrophotometers. Results were average of 3 different experiments.

The pH activity curve was determined using a citrate-phosphate-borate buffer system (range 3-12) at a concentration of 0.1 M.

Results and Discussion

Figure 1 shows the reduction of ferricyanide by saffron corms extract in the presence of 50 mM L-lithium lactate at different pHs, ranging from 4.0 to 10.0, expressed as units of enzyme per mg extract protein. One unit was defined as the amount of enzyme reducing $1 \mu\text{mol}$ ferricyanide per min. Three peaks were found, respectively at pH 5.5, 7.5 and 9.5.

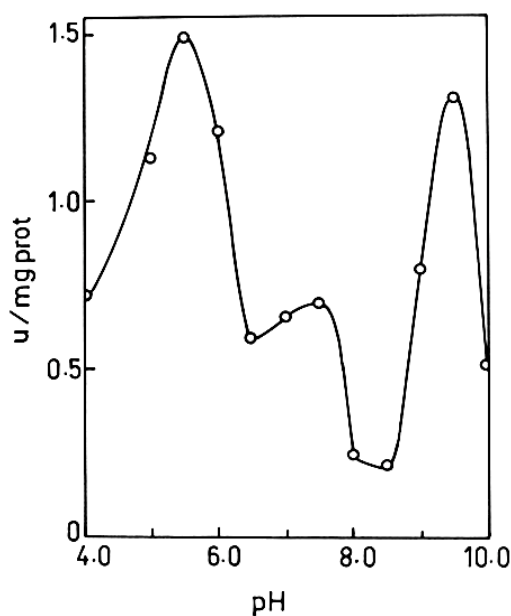


Figure 1 – pH dependency of L-lactate dehydrogenase activity in dormant saffron corm, using ferricyanide as electron acceptor.

As suggested in reference [9] the presence of various pH optima indicates the presence of distinct enzymes. Figure 2 shows the rate of reduction of ferricyanide by saffron corm extract as a function of substrate concentration (15 - 750 μM), at different pHs. At pH 5.5, the maximum rate was $130 \mu\text{M} \cdot \text{min}^{-1}$ for a substrate concentration of $225 \mu\text{M}$; thereafter, substrate inhibition was observed, and for $700 \mu\text{M}$ ferricyanide, the activity (expressed as rate of reduction) was reduced to $10 \mu\text{M} \cdot \text{min}^{-1}$ (Fig. 2A). At pH 7.5, maximum activity ($150 \mu\text{M} \cdot \text{min}^{-1}$) was observed for a substrate concentration of $70 \mu\text{M}$; thereafter, substrate inhibition reduced the activity to $20 \mu\text{M} \cdot \text{min}^{-1}$ for $350 \mu\text{M}$ ferricyanide (Fig. 2B). At pH 9.5, maximum activity ($100 \mu\text{M} \cdot \text{min}^{-1}$) was observed for $570 \mu\text{M}$ substrate; thereafter, substrate inhibition was observed and the activity was reduced to $60 \mu\text{M} \cdot \text{min}^{-1}$ for a substrate concentration of $750 \mu\text{M}$ (Fig. 2C). Table 1 shows the K_M , V_{MAX} , catalytic efficiency (calculated per mg extract protein) and the pseudo first order rate constant at the respective pHs. The K_M , catalytic efficiency and pseudo first order rate constant were different for all three pHs examined while the V_{MAX} was almost the same for pH 5.5 and 7.5, but diminished for pH 9.5. Malate inhibited the enzymatic activity observed at pH 9.5 (Fig. 3) but not that observed at pH 5.5 or 7.5. The type of inhibition at pH 9.5 was noncompetitive for

lower substrate concentrations (up to 100 μM) and competitive for higher substrate concentrations (100 to 200 μM).

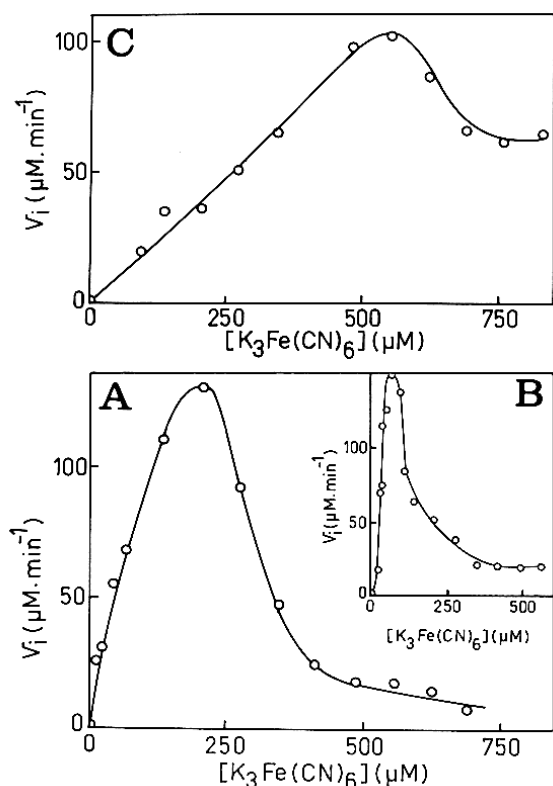


Figure 2 – Substrate inhibition (ferricyanide) of lactate dehydrogenase at different pHs. A) pH 5.5; B) pH 7.5; C) pH 9.5.

Table 1 : Kinetic Parameters at optima pHs

pH	K_M (μM)	V_{MAX} ($\mu\text{M}\cdot\text{min}^{-1}$)	V_{MAX}/K_M (min^{-1}) ^(*)	k (min^{-1})
5.5	70	130	1.9	1.3
7.5	138	140	1.0	2.2
9.5	270	100	0.4	0.2

(*) Calculated per mg extract protein

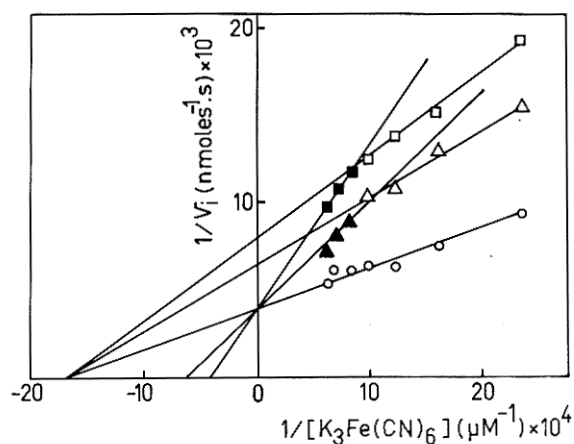


Figure 3 – Double reciprocal plot of the effect of malate on the reduction of ferricyanide. Control (open circles); 0.25 mM malate (triangles; open: noncompetitive inhibition; closed: competitive inhibition); 25 mM malate (squares; open: noncompetitive inhibition; closed: competitive inhibition).

Lactate dehydrogenase has been shown to be in single [10], or three [11] isoforms in various organisms. Our data suggested the possibility of at least three types of isoenzymes of L-lactate dehydrogenase in saffron (*Crocus sativus L.*).

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