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Iron in relation to aconitate hydratase activity in *Glycine max.* Merr.

Aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) has been purified from animal tissue and found to be activated and stabilized by the addition of Fe^{2+} and cysteine[1].

BACON, DEKOCK AND PALMER[2] found that aconitate hydratase activity was restored to the normal level when iron-deficient mustard plants or leaves were supplied with chelated ferric iron. The enzyme activity of extracts, however, whether from iron-deficient or normal plants could not be increased by treatment with iron and cysteine.

PALMER[3] reported that Fe^{2+} and cysteine were not effective in activation of aconitate hydratase that had been purified from mustard.

Reports in the literature indicate that iron and cysteine are effective activating agents for aconitate hydratase prepared from animal tissue but have no effect on the enzyme from plant sources. The following experiments were initiated to study aconitate hydratase from plant tissue.

Glycine max, Merr. was grown in complete nutrient solution under controlled environmental conditions.

Leaf material was ground in an extraction medium (0.2 M Tris–HCl buffer containing 2 mM citric acid, 2 mM sodium citrate (pH 8.5) and 5 mM cysteine, added shortly before extraction), filtered through cheese cloth and centrifuged at 13 000 $\times$ *g* for 5 min. The extract was further purified using $(NH_4)_2SO_4$. The fraction collected between 60 and 90% saturation, with respect to $(NH_4)_2SO_4$, was taken up in 5 mM Tris–HCl buffer (pH 7.0) containing 1 mM sodium citrate.

The complete reaction mixture for aconitate hydratase determination contained the following constituents:

5.0 mM DL-isocitric acid (trisodium salt), 0.1 M Tris–HCl buffer (pH 8.0) and 0.1 ml enzyme extract.

The mixture containing a final volume of 3 ml was equilibrated for 5 min at 30° before initiation of the reaction by introduction of substrate.

The activity was measured at 240 mμ for 10 min and calculated as indicated by RACKER[4]. A unit of enzyme activity is defined as the amount of enzyme that formed 1 μmole of *cis*-aconitate in 1 min under these conditions. Specific activity is defined as the number of units/mg of protein. Protein was determined as outlined by LOWRY *et al.*[5].

Fig. 1 shows that a concentration of $5 \cdot 10^{-3}$ M isocitrate was necessary to saturate the enzyme. The K_m for isocitrate estimated from the saturation curve is $0.58 \cdot 10^{-3}$ M at 25° and pH 8.0.

Maximum activation of purified aconitase occurred with the addition of 12 mM Fe^{2+} (Fig. 2, A). The crude extract required 10 mM Fe^{2+} for optimum activity (Fig. 2, B). Maximum activation of the crude and purified extracts of aconitate hydratase by Fe^{2+} was 84 and 71%, respectively. The specific activity of aconitate hydratase increased with increasing concentrations of Fe^{2+} until maximum activation occurred, then decreased at higher levels of Fe^{2+}.

The effect of various concentrations of Fe^{2+} on the stability of aconitate hy-

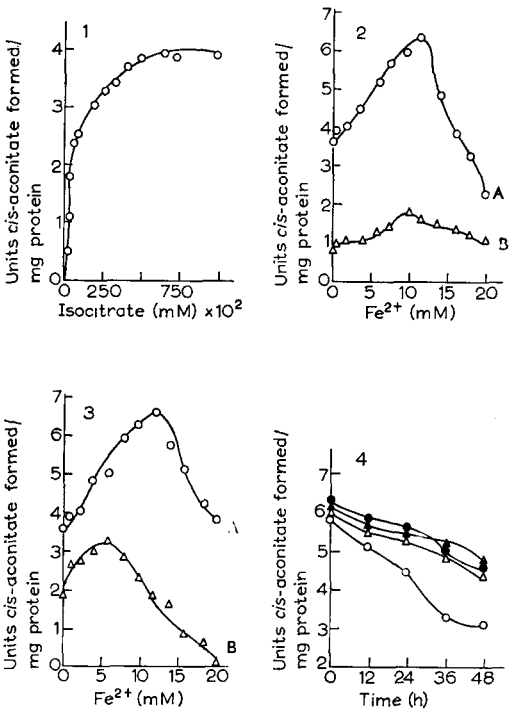

Fig. 1. Reaction rate related to concentration of substrate. Standard assay procedure was used (0.81 mg protein per ml) with variation in concentration of isocitrate as indicated.

Fig. 2. Effect of iron on the activation of aconitate hydratase. Purified and crude extract were incubated under standard conditions with isocitrate as substrate. ○—○, purified (0.43 mg protein/ml); △—△, crude (1.92 mg protein/ml).

Fig. 3. Effect of Fe^{2+} on aconitate hydratase stability. Extracts (0.51 mg protein per ml) were preincubated with various concentrations of Fe^{2+}. Specific activities of the enzyme extracts were measured before and after aging in an ice bath in a refrigerator. Standard assay procedure was used with isocitrate as substrate. ○—○, fresh extract; △—△, aged for 48 h.

Fig. 4. Activation and stability of aconitate hydratase by Fe^{2+}. ○—○, control (protein 0.56 mg/ml) without addition of any metal; △—△, 0.5 mM Fe^{2+} preincubated with enzyme extract (protein 0.56 mg/ml); ●—●, 0.5 mM Fe^{2+} added to the extraction medium of the enzyme extract (protein 0.56 mg/ml); ▲—▲, 0.5 mM Fe^{2+} added to the extraction medium, and the enzyme (protein 0.56 mg/ml) preincubated with 0.5 mM Fe^{2+}.

dratase (Fig. 3) was studied. Curve A illustrates specific activity of the fresh preparation and Curve B the aged preparation. The Fe^{2+} concentration that gave optimum activation of enzyme activity was different than that required for maximum stability. Stabilization occurred between 0.5 and 6 mM, compared to 10 and 12 mM for maximum activation of the fresh preparation. The stability of the enzyme extracts diminished rapidly at Fe^{2+} concentrations above 12 mM.

Neither crude nor purified aconitate hydratase was activated by preincubation with Mg^{2+} ($MgSO_4 \cdot 7\ H_2O$) at the same concentrations as those used with Fe^{2+}. Inhibition occurred with Zn^{2+} ($ZnSO_4 \cdot 7\ H_2O$) above 2 mM and with Cu^{2+} ($CuCl_2 \cdot 2\ H_2O$) at concentrations exceeding 0.5 mM.

The effects of Fe^{2+} on enzyme activity and stability when added to the extraction

medium or preincubated with the purified extract are shown in Fig. 4. When 0.5 mM Fe^{2+} was added to the extraction medium, about 73% of the specific activity was retained over a 48-h period. This compares to a retention of about 50% when no Fe^{2+} was added. Stability was enhanced in a likemanner whether Fe^{2+} was added to the extraction medium, preincubated with the extract or both. This low amount of Fe^{2+} was not sufficient to activate the enzyme; 10–12 mM being required to give maximum activation.

Citrate was added to the extraction medium and was, therefore, in this way included in the reaction mixture. PIERPOINT[6] found that when citrate was added to the reaction mixture, the activity of aconitate hydratase increased 5 to 10%. The presence of the substrate citrate partially stabilized enzyme activity. In the conversion of isocitrate to *cis*-aconitate and citrate, the increased citrate would favor the accumulation of *cis*-aconitate.

A reducing agent was required to keep iron in the ferrous state and perhaps to stabilize the enzyme protein. Addition of cysteine directly to the reaction mixture with or without the substrate isocitrate results in a decrease in absorbance readings at 240 mμ. Adding cysteine to the extraction media minimized to a negligible amount the influence of cysteine on the assay measurements.

The addition of iron and ascorbate[7] did not reactivate aconitate hydratase prepared from the kidneys of iron-deficient rats, but repeated experiments demonstrated that iron markedly increased the enzyme activity from kidney of normal rats. BEUTLER[7] interpreted this finding as an actual depletion of the protein moiety of the enzyme under iron-deficiency conditions. Apparently the lower enzyme activity in iron-deficient tissue is caused by a lack of the apoenzyme; consequently, activation by iron would not be expected.

DICKMAN AND CLOUTIER[8] using polarographic, spectrophotometric and titrimetric methods concluded an Fe^{2+}–substrate complex and an Fe^{2+}–protein complex were formed under conditions of optimum aconitate hydratase activity.

Iron is concluded to be an integral component of the enzyme system for stability and activity from animal or plant sources.

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