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Regulation of the Nicotinamide Adenine Dinucleotide-specific Isocitrate Dehydrogenase from a Higher Plant

THE EFFECT OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE AND MIXTURES OF CITRATE AND ISOCITRATE*

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SUMMARY

The NAD^+-specific isocitrate dehydrogenase from pea stems is shown to respond to the mole fraction of NADH when total NAD (NAD^+ plus NADH) is kept constant. Fifty per cent inhibition occurs when the mole fraction of NADH is 0.1. In this region the enzyme is extremely sensitive to NADH concentration and it is suggested that this acts in a similar manner to energy charge in other systems. In an attempt to simulate conditions *in vivo*, the response of the enzyme to constant ratios of citrate to isocitrate was studied and it was found that even under these conditions the enzyme showed sigmoid kinetics.

There are two types of control of metabolic pathways. Firstly, there is a specific control by feedback regulation of a regulatory enzyme in the pathway by a particular compound or group of compounds produced by the pathway. Secondly, there is an energy control which depends on the general metabolic state of the cell. A description of the relationship between these two forms of control has been given by Atkinson (1). In the study of the regulation of enzymes *in vitro*, the usual approach is to vary one component of the assay system while maintaining the others constant. Atkinson (2) has suggested that, while such an approach is valid for enzymes involved in biosynthetic pathways, it may not be valid for enzymes concerned with energy metabolism. For these enzymes Atkinson (2) has put forward the adenylate control hypothesis in which he postulates that, while an enzyme may respond to an individual adenine nucleotide, a closer approach to physiological conditions may be approximated when AMP, ADP, and ATP are all varied simultaneously while the total adenine nucleotide concentration remains constant. To describe any particular mixture of nucleotides, Atkinson (2) has introduced the concept of energy charge and has shown that several enzymes respond more effectively to energy charge than to the individual components of the adenylate system.

The NAD^+-specific isocitrate dehydrogenase (*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating) EC 1.1.1.41) from yeast has been shown to respond to energy charge (3) but the higher plant enzyme is virtually unaffected (4). This has led some workers (4, 5) to suggest that the NAD^+:NADH ratio may be an important regulatory factor. The NAD^+:NADH system is similar to the adenylate system in that the fraction reduced varies *in vivo* but the total NAD (NAD^+ plus NADH) remains constant, at least over short periods of time. The response of the plant NAD^+-isocitrate dehydrogenase was, therefore, studied under conditions in which the NAD pool was kept constant while the mole fraction of NADH was varied.

NAD^+-isocitrate dehydrogenase is activated by citrate (4, 6, 7), which has been termed precursor activation (7). The function of precursor activation is obscure because aconitase will maintain citrate and isocitrate in equilibrium *in vivo* so that the concentrations of both will rise and fall together. Cox and Davies (8) have suggested that since this equilibrium favors citrate (9), the enzyme will always be fully activated and that the NAD^+-isocitrate dehydrogenase in plants possibly has no regulatory function. They have also concluded that citrate activation is of no regulatory significance.

It is more relevant to consider citrate and isocitrate *in vivo* as a single "citrates" pool in which the citrate to isocitrate ratio remains fairly constant because of the aconitase reaction. This ratio will be between 10:1 and 70:1 (citrate to isocitrate), depending on the magnesium concentration (9). Since the aconitase reaction will keep the isocitrate and citrate in equilibrium, the total citrates pool can be considered as the "substrate" for NAD^+-isocitrate dehydrogenase. The response of the enzyme to various ratios of citrate to isocitrate was, therefore, studied and the results indicate that even at high ratios of citrate to isocitrate, the enzyme still shows regulatory properties.

MATERIALS AND METHODS

A mitochondrial acetone powder was obtained from etiolated pea (*Pisum sativum* L.) epicotyls as described in the preceding paper (10). This preparation, at a concentration of 40 mg per ml, was suspended in 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid-NaOH buffer (pH 7.4) in 6 M methanol and

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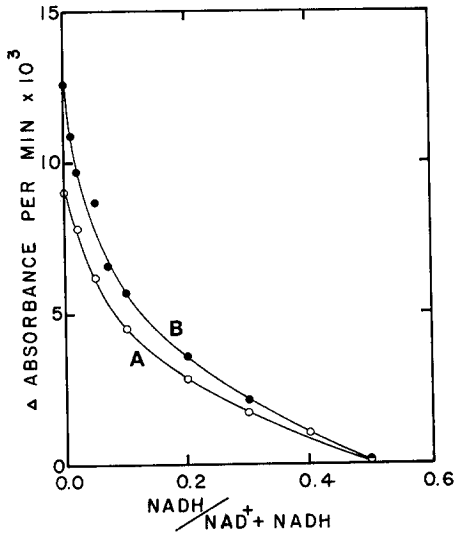

FIG. 1. The effect of the mole fraction of NADH at constant total NAD (NAD^+ plus NADH) on the activity of NAD^+-specific isocitrate dehydrogenase. The assay contained 1 mM magnesium sulfate, 0.2 mM isocitrate, 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid buffer (pH 7.4), and total NAD at 0.4 mM (○) or 1.0 mM (●). See the text for explanation of *Curves A* and *B*.

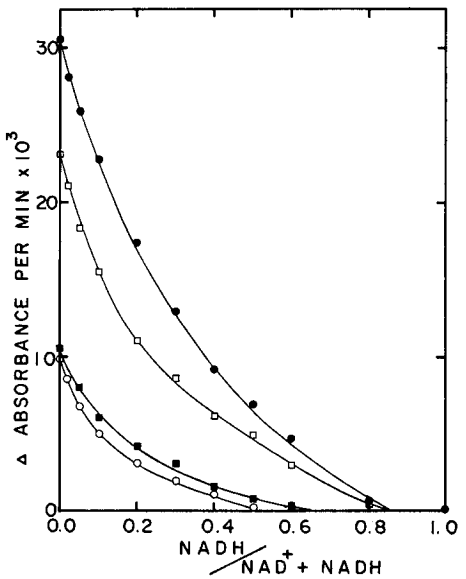

FIG. 2. The effect of the mole fraction of NADH at constant total NAD (NAD^+ plus NADH) on the activity of NAD^+-specific isocitrate dehydrogenase, in the presence of chloride ions and citrate. The assays contained 50 mM *N*-tris(hydroxymethyl)-methyl-2-ethane sulfonic acid buffer (pH 7.4), 1 mM magnesium sulfate, 0.4 mM total NAD, and 0.2 mM isocitrate (○), 0.2 mM isocitrate plus 4 mM citrate (●), 0.6 mM isocitrate (□), and 0.6 mM isocitrate plus 50 mM sodium chloride (■).

centrifuged at 3000 × *g* for 15 min at 0°, then at 100,000 × *g* for 30 min at the same temperature. The supernatant was stable at −20° for several weeks.

The enzyme was assayed by the method of Coultate and Dennis (11) using a preparation diluted with 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid-NaOH buffer (pH 7.4) in 6 M methanol, to give a ΔA_{340} per min of 0.04 per 0.1 ml of enzyme under the standard assay conditions of those workers.

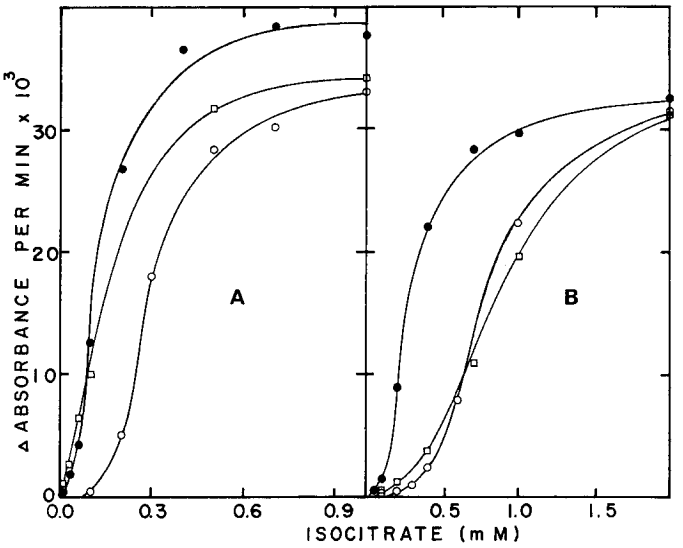

FIG. 3. *A*, the effect of sulfate and chloride ions on the activity of NAD^+-specific isocitrate dehydrogenase. The assay contained 1 mM NAD^+, 1 mM manganese sulfate, and 50 mM sodium chloride (○), 50 mM sodium sulfate (□), or no added anion (●). *B*, the effect of sulfate and chloride ions on the activity of NAD^+-specific isocitrate dehydrogenase. The conditions are as in *A* except that 1 mM magnesium sulfate is the metal cofactor.

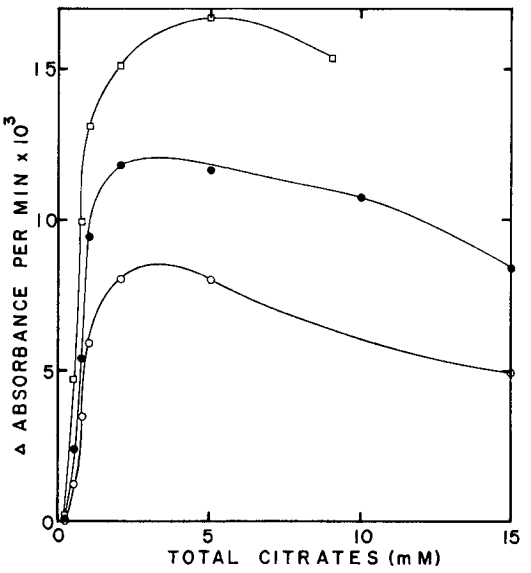

FIG. 4. The effect of various citrate to isocitrate ratios on the activity of NAD^+-specific isocitrate dehydrogenase. The assay contained 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid buffer (pH 7.4), 1 mM magnesium sulfate, 1 mM NAD^+. The citrate to isocitrate ratios were 60:1 (○), 28:1 (●), and 12:1 (□).

The concentrations of the various components of the assay system are indicated in the text and figures. Isocitrate concentrations refer to the *threo*-D_s,L_s-isocitrate isomers (12), unless otherwise stated. Biochemicals were purchased from the Sigma Chemical Company.

RESULTS

The response of the enzyme to increasing mole fraction of NADH at a total NAD concentration of 0.4 mM is shown in *Curve A* of Fig. 1. In the absence of NADH, 0.4 mM NAD^+

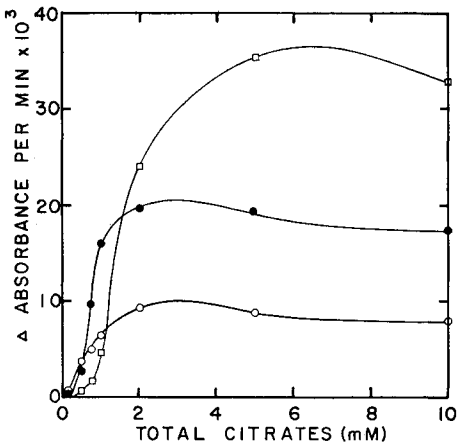

FIG. 5. The effect of different magnesium concentrations on the activity of NAD^+-specific isocitrate dehydrogenase in the presence of a constant citrate to isocitrate ratio of 36. Assays contained 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid buffer (pH 7.4), 1 mM NAD^+, and 0.25 mM (○), 1.0 mM (●), or 5.0 mM (□) magnesium sulfate.

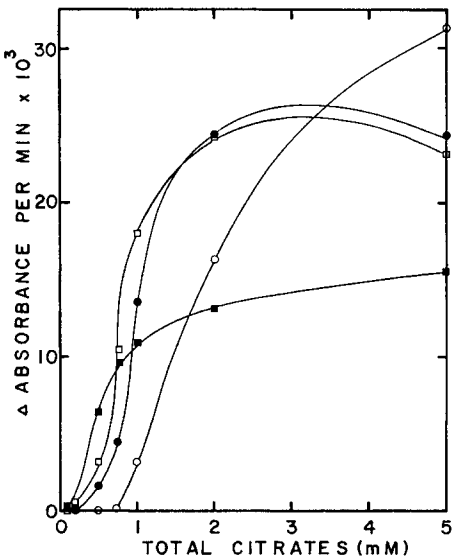

FIG. 6. The effect of various citrate to isocitrate ratios on the activity of NAD^+-specific isocitrate dehydrogenase. The amount of magnesium added for each ratio was that amount required to give this ratio in the aconitase equilibrium as indicated by the data of Blair (9). The assays contained 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid buffer (pH 7.4), 1 mM NAD^+, and citrate to isocitrate ratios of 55 (Mg^{2+}, 5 mM) (○), 36 (Mg^{2+}, 2 mM) (●), 22 (Mg^{2+}, 1 mM) (□), and 14 (Mg^{2+}, 0.25 mM) (■).

saturates the enzyme to 70%, and the isocitrate concentration (0.2 mM) is just below that required for half saturation. There is a very pronounced inhibition as the mole fraction of NADH is increased from 0 and 50% inhibition occurs at 0.1. Increasing the total NAD to 1.0 mM (a saturating concentration in the absence of NADH) raises the curve but does not alter its shape (*Curve B*, Fig. 1). Increasing the isocitrate concentration to 0.6 mM (85% saturating) or adding the activator, citrate, elevates the curve but again does not change its shape (Fig. 2). Chloride ions are known to inhibit this enzyme (5, 11), apparently behaving as negative effectors and possibly mimicking some form of regulation *in vivo* (11). Chloride ions (50 mM) at an isocitrate concentration of 0.6 mM depressed the curve but did not affect its shape.

Cox and Davies (5), using the pea enzyme, indicated that anion inhibition is restricted to monovalent ions, whereas the turnip enzyme (11) is also inhibited by sulfate ions. This discrepancy was resolved when it was found that sulfate inhibition is observed when magnesium is the metal activator (Fig. 3*B*), but not when manganese is the activator (Fig. 3*A*). Chloride is inhibitory in both cases. No explanation is available for this result, but certain features of the regulation of glutamine synthetase (13) and ADP glucose synthetase (14) involve a differential response to these two metals. Atkinson (1) has pointed out that this type of regulation may be far more widespread than is realized.

In an attempt to simulate conditions *in vivo*, assays were performed in the presence of fixed ratios of citrate to isocitrate.[1] The effect of the total citrates concentration (citrate plus isocitrate) on the saturation kinetics of the enzyme was then studied. When the citrate to isocitrate ratio is kept constant at values of 12, 28, and 60 (which covers the range of ratios observed with aconitase (9)), a sigmoid relationship between velocity and citrates concentration is found (Fig. 4). It is seen that the enzyme is not fully activated at all times, contrary to the suggestion of Cox and Davies (8). The $S_{0.5}$ (15) value of 0.75 mM citrates is not markedly altered by the different citrate to isocitrate ratios. However, there was a marked effect on the maximum velocity. All these curves were obtained at 1 mM magnesium, but it must be remembered that the citrate to isocitrate ratio in the mitochondrion is dependent on the magnesium concentration (9). The effect of changing the magnesium concentration at a constant citrate to isocitrate ratio was therefore studied (Fig. 5) and it was found that both the $S_{0.5}$ and the maximum velocity are dependent on the magnesium concentration.

While Figs. 4 and 5 illustrate the range of response that the enzyme can exhibit, neither represents a valid situation *in vivo* since the citrate to isocitrate ratio is determined by the aconitase reaction and will depend on the magnesium concentration. To study this relationship citrates saturation curves were prepared at various citrate to isocitrate ratios in the presence of a magnesium concentration which should give this ratio. *Curve B*, Fig. 2 of Reference 9 was used as a guide in choosing the required magnesium concentration for each citrate to isocitrate ratio. The results are shown in Fig. 6 and demonstrate that the kinetics is greatly affected by the citrate to isocitrate ratios. The shape of the curves changes as the ratio is lowered and there is also a profound effect on the maximum velocity.

DISCUSSION

A number of enzymes have been shown to be regulated by NADH (16–19). In some cases, the sensitivity of the enzyme is enhanced by cooperative binding involving some type of allosteric effect. However, as Atkinson (2) has suggested, the relative affinities of a substrate and product for the same site may be subject to selective pressures and hence competitive inhibition may form part of a control system. The effect of NADH on NAD^+-isocitrate dehydrogenase is an example of this type of

[1] This refers to the ratio of citrate to *threo*-D_s-isocitrate. Also present was *threo*-L_s-isocitrate at an equal concentration to the natural isomer.

control. Although the enzyme is irreversible under normal assay conditions (5), its affinity for NADH greatly exceeds that for NAD^+, indicating control by these compounds (11). The results in this paper indicate that the enzyme is very sensitive to the mole fraction of NAD in the reduced form. This is due both to a lowering of the substrate (NAD^+) concentration and an increase in the inhibitor (NADH) concentration as the mole fraction of NADH is increased. The enzyme will maintain the mole fraction of NADH at 0.1 to 0.2, a value similar to that found *in vivo* (20, 21). This type of approach has also been used to study NADH inhibition of pyruvate dehydrogenase (16).

The precursor activation of the enzyme by citrate (7) has never been assigned a clearly defined function. When the citrate to isocitrate ratio is kept constant and the enzyme activity measured as a function of the total concentration of these two acids, a sigmoid relationship is found with $S_{0.5}$ independent of the ratio (Fig. 4). The variation in maximum velocity is almost certainly due to inhibition by the higher concentrations of citrate in which the rate is limited by the concentration of magnesium isocitrate due to the chelation of magnesium by the high concentration of citrate. A similar situation can be seen in Fig. 5 in which differences in maximum velocity are again probably due to differences in the concentration of magnesium isocitrate. At low magnesium concentrations there is a higher apparent affinity of the enzyme for "substrate." This is probably due to higher levels of free citrates which activate the enzyme (10). Higher magnesium concentrations lower the free citrates to a point where they become limiting.

The attempt to simulate the aconitase equilibrium showed that the enzyme displays extremely complex kinetics (Fig. 6). It was anticipated that the maximum velocity would be the same at the different citrate to isocitrate ratios and this is clearly not the case. This may be due in part to the use of data from Blair (9), which may not have been entirely appropriate to the assay conditions used in this study (10). However, the data illustrate that under simulated *in vivo* conditions the enzyme shows complex regulatory properties.

It is clear that the concentration of magnesium ions is of great importance in determining the activity of the enzyme, and, since the mitochondrion is capable of metabolic ion uptake, this may be a means of regulating the tricarboxylic acid cycle. The suggestion of Cox and Davies (8) that this enzyme has no regulatory function is clearly not valid since it has been shown in this paper and in the companion paper (10) that the activity is controlled by NADH, citrate, and magnesium in a complex manner.

REFERENCES

1. Atkinson, D. E., *Ann. Rev. Microbiol.*, **23**, 47 (1969).
2. Atkinson, D. E., *Biochemistry*, **7**, 4030 (1968).
3. Atkinson, D. E., in *Metabolic roles of citrate*, Academic Press, New York, 1968, p. 23.
4. Dennis, D. T., and Coultate, T. P., *Life Sci.*, **6**, 2353 (1967).
5. Cox, G. F., and Davies, D. D., *Biochem. J.*, **105**, 729 (1967).
6. Hathaway, J. A., and Atkinson, D. E., *J. Biol. Chem.*, **238**, 2875 (1963).
7. Sanwal, B. D., Zink, M. W., and Stachow, C. S., *Biochem. Biophys. Res. Commun.*, **12**, 510 (1963).
8. Cox, G. F., and Davies, D. D., *Biochem. J.*, **113**, 813 (1969).
9. Blair, J. McD., *Eur. J. Biochem.*, **8**, 287 (1969).
10. Duggleby, R. G., and Dennis, D. T., *J. Biol. Chem.*, **245**, 3745 (1970).
11. Coultate, T. P., and Dennis, D. T., *Eur. J. Biochem.*, **7**, 153 (1969).
12. Vickery, H. B., *J. Biol. Chem.*, **237**, 1739 (1962).
13. Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 1703 (1967).
14. Gentner, N., and Preiss, J., *J. Biol. Chem.*, **243**, 5882 (1968.)
15. Atkinson, D. E., *Ann. Rev. Biochem.*, **35**, 85 (1966).
16. Bremer, J., *Eur. J. Biochem.*, **8**, 535 (1969).
17. MacElroy, R. D., Johnson, E. J., and Johnson, M. K., *Arch. Biochem. Biophys.*, **131**, 272 (1969).
18. Sanwal, B. D., *J. Biol. Chem.*, **244**, 1831 (1969).
19. Wright, J. A., and Sanwal, B. D., *J. Biol. Chem.*, **244**, 1838 (1969).
20. Yamamoto, Y., *Plant Physiol.*, **38**, 45 (1963).
21. Klingenberg, M., Slenczka, W., and Ritt, E., *Biochem. Z.*, **332**, 47 (1960).