The Effects of pH and Citrate on the Activity of NicotinamideA-denine Dinucleotide-Specific Isocitrate Dehydrogenase from Pea Mitochondria

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1. The effect of pH on the co-operative activation of the NAD-specific isocitrate dehydrogenase from pea mitochondria by isocitrate is shown. 2. The interlinked effects of pH on the affinity of the NAD-specific isocitrate dehydrogenase for isocitrate and the dependence of the pH optimum on the substrate concentration are presented. 3. A consideration of the conditions of pH and substrate concentration under which citrate activates the NAD-specific isocitrate dehydrogenase demonstrates similarities between the binding of isocitrate and citrate. 4. A comparison of the effects of citrate and pH on the gross structure of the enzyme is investigated by density-gradient centrifugation. 5. The kinetic interpretations of these results are briefly considered. 6. The metabolic significance of these studies is discussed.

The characterization of the NAD-specific isocitrate dehydrogenase [*threo*-D₂-isocitrate-NAD oxidoreductase (decarboxylating), EC 1.1.1.41] from yeast (Hathaway & Atkinson, 1963; Atkinson, Hathaway & Smith, 1965), *Neurospora crassa* (Sanwal, Zink & Stachow, 1963, 1964), locust flight muscle (Klingenberg, Goebell & Wenske, 1965) and mammalian heart (Chen & Plaut, 1963) has led to the suggestion that this enzyme may be a site of regulation of the tricarboxylic cycle. This postulate has been based on the observation of a stimulation of NAD-specific isocitrate dehydrogenase activity from yeast and *N. crassa* by AMP and a similar activation by ADP of the enzyme from locust and mammalian heart. Further, the NAD-specific isocitrate dehydrogenase from yeast, *N. crassa* and locust showed a sigmoid relationship between initial rate and isocitrate concentration at pH values above 7.0. This sigmoid relationship was eliminated for the enzyme from yeast and *N. crassa* by the presence in the assay system of saturating concentrations of citrate.

The NAD-specific isocitrate dehydrogenase from pea mitochondria has been purified by Cox & Davies (1967). Initial observations indicated that the characteristics of the enzyme with respect to isocitrate and citrate were similar to those reported for the enzyme from yeast and *N. crassa*. However, the absence of any activation effects by AMP or ADP at any concentration of isocitrate with the NAD-specific isocitrate dehydrogenase from pea mitochondria indicated a significant difference between the plant enzyme and those isolated from other sources. These observations have been confirmed by similar studies on the NAD-specific iso-

citrate dehydrogenase from swede (Dennis & Coulgate, 1967). The present studies are a continuation of those reported by Cox & Davies (1967), and were aimed at establishing the possible significance of the enzyme in the control of the tricarboxylic acid cycle in higher plants.

MATERIALS AND METHODS

The materials and methods employed in the isolation, stabilization, purification and assay of the NAD-specific isocitrate dehydrogenase were described by Cox & Davies (1967).

Enzyme purification. The shortened purification scheme described by Cox & Davies (1967) was usually used for the preparation of the enzyme for kinetic experiments and this is termed the 'partially purified' preparation. The only modification was the use of 50 mM-Hepes* buffer (Good *et al.* 1966) adjusted to pH 7.6 with NaOH in the solutions used for the isolation and purification of the enzyme. This was done because the NAD-specific isocitrate dehydrogenase from plants is inhibited by univalent anions (Cox & Davies, 1967), and Cennamo, Montecuccoli & Bonaretti (1965) have shown that the yeast enzyme is also inhibited by univalent anions; tris-HCl is therefore unsuitable for kinetic experiments with these two dehydrogenases. Further, Hepes has pK 7.55 at 20° (Good *et al.* 1966), which is clearly advantageous since the plant enzyme has a pH optimum of 7.6 under standard assay conditions.

Density-gradient centrifugation. The 40 ml. linear density gradients running from 3M- to 5M-glycerol were prepared by using an apparatus suggested by Samis (1966). The gradients were buffered throughout by 25 mM-Hepes-NaOH adjusted to the required pH. A 1 ml. sample of

* Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

crude mitochondrial isocitrate dehydrogenase isolated in a solution containing 25 mM-Hepes-NaOH buffer, pH 7.6, and 2.5 M-glycerol was placed on each gradient. Crude extracts of the enzyme were used in these experiments because the purified enzyme preparations were insufficiently stable in 2.5 M-glycerol. Liquid paraffin was added to fill the cellulose nitrate tubes and the gradients were centrifuged in the SW 25 head of a Beckman Spinco model L-2 ultracentrifuge at 24 000 rev./min. (63 000 $g_{av.}$) at -2° . The period of centrifugation is quoted for each experiment. After centrifugation the gradients were fractionated by lowering a fine glass capillary tube attached to peristaltic pump tubing into the gradient until it reached the bottom. The gradients were then removed by the use of a peristaltic pump and 16-drop fractions were collected by using a drop counter.

RESULTS

A study of the effects of pH on the binding by the plant NAD-specific isocitrate dehydrogenase of both its substrate, isocitrate, and its activator, citrate, has shown marked similarities in the characteristics of the reaction with the two ligands.

Effect of pH on the binding of isocitrate. The co-operative binding of isocitrate by the plant NAD-specific isocitrate dehydrogenase was investigated at the pH optimum of 7.6 by Cox & Davies (1967). By conducting similar experiments at pH 6.4, 7.0, 7.6, 8.0 and 8.6, not only have changes in the co-operative activation of the

Fig. 1. Effect of pH on the reaction of isocitrate with NAD-specific isocitrate dehydrogenase. Conditions of assay: 0.66 mM-NAD⁺, 1 mM-Mn²⁺, 50 mM-Hepes-NaOH buffer at: (a) pH 6.4; (b) pH 7.0; (c) pH 7.6; (d) pH 8.0; (e) pH 8.6.

Table 1. *Effect of pH on the n value from the Hill plot determined from the reaction between isocitrate and NAD-specific isocitrate dehydrogenase*

pH	n
6.4	1.0
7.0	1.6
7.6	2.6
8.0	2.8
8.6	2.0

Table 2. *Effect of pH on the affinity of NAD-specific isocitrate dehydrogenase for isocitrate as determined from the $s_{0.5}$ values*

For details see the text.

pH	$s_{0.5}$ (μM)
6.4	8
7.0	33
7.6	96
8.0	310
8.6	1700

enzyme been identified, but information on the change in affinity of the enzyme for isocitrate with change in pH and the possible manner in which pH affects the enzyme has been obtained.

A partially purified preparation of the enzyme was used in these experiments. The effects of isocitrate concentration on the initial reaction rates at each pH are presented in Fig. 1. Examination of these plots suggested that the co-operative activation of the enzyme by isocitrate diminished as the pH decreased. This was confirmed by plots of $\log[v/(V_m - v)]$ against $\log s$, where v is the rate at a given concentration of isocitrate, V_m is the maximum velocity at saturating concentration of isocitrate and s is the concentration of isocitrate. The n values (Hill, 1913), which describe the slope of this linear plot at half V_m , are presented in Table 1. These values represent the minimum order of the reaction with respect to isocitrate (Atkinson *et al.* 1965). The plots shown in Fig. 1 also indicated a pronounced change in the affinity of the enzyme for isocitrate with changes in pH. The results were therefore replotted as v against $\log s$, as suggested by Koshland, Nemethy & Filmer (1966), and the isocitrate concentration at half-maximal velocity ($s_{0.5}$) was determined. The values obtained from these plots are presented in Table 2. Klingenberg *et al.* (1965), observing a similarly significant change in the affinity of the locust NAD-specific isocitrate dehydrogenase for isocitrate, investigated the role of H^+ ions in the binding of isocitrate by comparing plots of $-\log s_{0.5}$ against pH and

Fig. 2. Relationship between $s_{0.5}$ and pH ($\circ$) and $pV_{\max}$ and pH ($\bullet$).

Fig. 3. Effect of isocitrate concentration on the pH optimum for NAD-specific isocitrate dehydrogenase. Assay conditions: 0.66 mM-NAD⁺, 1 mM-Mn²⁺, 50 mM-Hepes-NaOH buffer. $\square$, 0.05 mM-Isocitrate; $\triangle$, 0.15 mM-isocitrate; $\circ$, 0.5 mM-isocitrate.

$-\log V_m$ against pH (Dixon, 1953). Similar treatment of the $s_{0.5}$ and $V_{\max}$ values obtained with the plant enzyme at each pH are presented in Fig. 2. Although the plot of $-\log V_{\max}$ against pH shows only a small slope, the slope of the plot of $-\log s_{0.5}$ against pH is -1.2 , suggesting the binding of a single proton to the enzyme before the binding of each isocitrate molecule. This results (Fig. 3) in a movement of the pH optimum from 7.6

at 1mM-isocitrate to 7.5 at 0.5mM-isocitrate, 7.3 at 0.15mM-isocitrate and 6.9 at 0.05mM-isocitrate.

Effect of pH on the binding of citrate. These studies were performed at pH 6.4, 7.6 and 8.6 (a) with constant citrate concentration and variable isocitrate concentration and (b) with variable citrate concentration and constant isocitrate concentration.

(a) Cox & Davies (1967) have reported that the

Table 3. *Effect of citrate on the affinity of NAD-specific isocitrate dehydrogenase for isocitrate at two pH values*

pH	$s_{0.5}$ (μ M)	
	Citrate absent	1mM-Citrate present
7.6	87	83
8.6	1700	1300

co-operative activation of the plant NAD-specific isocitrate dehydrogenase at pH 7.6 by isocitrate is eliminated by the inclusion of 1mM-citrate in the assay system, a hyperbolic plot of initial rate against isocitrate concentration resulting. Construction of Hill plots from such results showed a decrease in the n value from 2.6 in the absence of citrate to 1.1 in its presence, thus confirming the change in the order of the reaction.

Similar experiments were performed at pH 6.4 and 8.6 with partially purified preparations of NAD-specific isocitrate dehydrogenase. No activation by 1mM-citrate was observed at pH 6.4. At pH 8.6, although substantial activation was observed at low isocitrate concentrations, 1mM-citrate was insufficient to eliminate totally the co-operative activation by isocitrate. It was also observed that at all pH values 1mM-citrate inhibited the reaction with isocitrate at high isocitrate concentrations. This is consistent with the hypothesis that citrate binds to the active site.

Fig. 4. Activation of NAD-specific isocitrate dehydrogenase by citrate at two isocitrate concentrations at: (a) pH 6.4; (b) pH 7.6; (c) pH 8.6. Assay conditions: 0.66mM-NAD⁺, 1mM-Mn²⁺, 50mM-Hepes-NaOH buffer. ●, 0.05mM-Isocitrate; ○, 0.2mM-isocitrate.

To determine the effect of citrate on the affinity of the enzyme for isocitrate at pH 7.6 and 8.6, plots of rate against $\log[\text{isocitrate}]$ were constructed, and the $s_{0.5}$ values in the presence and absence of 1 mM-citrate determined. These results (Table 3) show that although the $s_{0.5}$ value decreases in the presence of citrate the effect is not marked.

(b) The effect of varying the citrate concentration on a partially purified preparation of NAD-specific isocitrate dehydrogenase at each pH was studied at two constant isocitrate concentrations, 0.05 mM and 0.2 mM. The plots of initial rate against citrate concentration at each pH are presented in Fig. 4. Clearly the activation by citrate was more significant at higher pH values and low isocitrate concentrations. The most notable aspect of these results, however, was the appearance of the sigmoid curve at pH 8.6 at both high and low isocitrate concentrations.

To obtain some information about the affinity of the enzyme for citrate and the degree of co-operativity observed in the plots showing the greatest activation effects, some of the results were replotted as plots of $V - V_0$ against $\log[\text{citrate}]$, where V is the rate in the presence of citrate and V_0 is the rate in its absence (Nakazawa & Hayaishi, 1966).

From such plots the citrate concentration at half-maximum velocity ($c_{0.5}$) and the R_s value (Koshland *et al.* 1966) can be calculated. The values obtained from three such plots are presented in Table 4.

Although these values are only approximate they demonstrate that: (i) the affinity of the enzyme for citrate decreases with increasing pH; (ii) remembering that some isocitrate is present in each assay, the affinity of the enzyme for citrate appears to be similar to that for isocitrate at the same pH; (iii) the activation by citrate is highly co-operative, especially at higher pH values.

The manner in which the R_s value corresponds to the n value of the Hill plot is shown in Fig. 5.

Density-gradient centrifugation. The effects of citrate and of low pH on the plant NAD-specific isocitrate dehydrogenase show considerable simi-

larity, since they both eliminate the co-operative activation of the enzyme by isocitrate. In view of this a comparison of the effects of citrate and pH on the gross molecular structure of the enzyme was carried out by means of density-gradient-centrifugation studies.

In the first experiment, performed at pH 7.6, the gradients were prepared as described in the Materials and Methods section. A crude extract of NAD-specific isocitrate dehydrogenase was layered on to two gradients, one containing 100 mM-citrate throughout and the other without citrate. Yeast alcohol dehydrogenase was used as a marker enzyme by layering 1 ml. of enzyme solution in 2.5 M-glycerol on to the third gradient. After centrifugation for 22½ hr. the gradients were fractionated and assayed for enzymic activity (Fig. 6). This experiment indicates that the distance of sedimentation from the origin of the NAD-specific isocitrate dehydrogenase had been decreased in the presence of citrate. In a subsequent experiment, the citrate concentration was lowered to 1 mM and a second marker, catalase, was included in the third gradient. The period of centrifugation was again 22½ hr. The result of this experiment is presented in Fig. 7. This experiment suggests that as the concentration of citrate is decreased the tendency of the enzyme to disaggregate decreases and two peaks of activity are observed, one corresponding to the faster-sedimenting peak observed

Fig. 5. Relationship between the R_s value determined from the plot of rate against $\log[\text{ligand}]$ and the n value determined from the Hill plot.

Table 4. Effect of pH and isocitrate concentration on the affinity of the NAD-specific isocitrate dehydrogenase for citrate as shown by the $c_{0.5}$ value and the degree of co-operative activation by citrate as shown by the R_s value

pH	Concn. of		R_s
	isocitrate (mM)	$c_{0.5}$ (μM)	
7.6	0.05	140	10.7
8.6	0.05	2800	5.8
8.6	0.2	2100	4.1

Fig. 6. Sedimentation of NAD-specific isocitrate dehydrogenase in the presence and absence of 100 mM-citrate by using density-gradient centrifugation. ■, NAD-specific isocitrate dehydrogenase in the absence of citrate; △, NAD-specific isocitrate dehydrogenase in the presence of 100 mM-citrate; ○, yeast alcohol dehydrogenase.

Fig. 7. Sedimentation of NAD-specific isocitrate dehydrogenase in the presence and absence of 1 mM-citrate by using density-gradient centrifugation. ■, NAD-specific isocitrate dehydrogenase in the absence of citrate; △, NAD-specific isocitrate dehydrogenase in the presence of 1 mM-citrate; ○, yeast alcohol dehydrogenase; ●, bovine liver catalase.

in the absence of citrate and the second corresponding to a slower-sedimenting peak observed in the presence of high citrate concentrations.

To investigate the effect of pH on the sedimentation of NAD-specific isocitrate dehydrogenase, three gradients at pH 6.4, 7.6 and 8.6 were prepared and a crude extract of enzyme was layered on to each. Centrifugation was carried out for 24 hr. and the gradients were fractionated and assayed. Although a greater recovery of activity was obtained with increasing pH no significant change in the sedimentation of the enzyme could be detected from these results. The results obtained in these studies were used in the manner suggested by Martin & Ames (1961) to calculate the $S_{20,w}^{0.725}$ value and the molecular weights of the two species of NAD-specific isocitrate dehydrogenase. These values are presented in Table 5, with the marker enzymes used for the calculations as a comparison.

Martin & Ames (1961) have pointed out the disadvantages of the use of crude enzyme preparations for sedimentation studies and the accuracy of the results presented must be treated with appropriate caution. However, the changes in the $S_{20,w}^{0.725}$ value and molecular weight of the NAD-specific isocitrate dehydrogenase on the

Table 5. Determination of $S_{20,w}^{0.725}$ and molecular-weight values for NAD-specific isocitrate dehydrogenase in the presence and absence of citrate by comparison with marker enzymes (alcohol dehydrogenase and catalase) by the method of Martin & Ames (1961)

Enzyme	$S_{20,w}^{0.725}$ (s)	Mol. wt.
Alcohol dehydrogenase	7.4	150 000
Catalase	11.3	250 000
NAD-specific isocitrate dehydrogenase (no citrate)	18.0	535 000
NAD-specific isocitrate dehydrogenase (plus citrate)	11.0	260 000

inclusion of citrate in the gradient are considered sufficient to suggest the existence of at least two distinct molecular forms of the enzyme.

These results may be interpreted as indicating that the NAD-specific isocitrate dehydrogenase molecule is composed of two major subunits each of mol. wt. approx. 260 000, which disaggregate in the presence of the activator citrate. Further,

although the effect of both citrate and low pH is to eliminate the co-operative activation of the enzyme by isocitrate, the effect on the gross molecular structure of the enzyme is not the same.

DISCUSSION

The studies reported in this paper have dealt with the binding of isocitrate and citrate to NAD-specific isocitrate dehydrogenase at various pH values and have demonstrated pronounced similarities between the binding of the two acids.

The change from a sigmoid plot of rate against isocitrate concentration at high pH values to a hyperbolic plot at pH 6.4 and below has been observed with the enzyme from yeast (Hathaway & Atkinson, 1963), locust (Klingenberg *et al.* 1965) and *N. crassa* (Sanwal & Stachow, 1965). Further activation of the enzyme by citrate has been reported with the enzyme from yeast (Atkinson *et al.* 1965) and *N. crassa* (Sanwal *et al.* 1964).

Of the various mechanisms of action put forward for the NAD-specific isocitrate dehydrogenase from different sources, that proposed by Atkinson *et al.* (1965) for the yeast NAD-specific isocitrate dehydrogenase appears to be of the greatest relevance to the NAD-specific isocitrate dehydrogenase from pea.

The order of the reaction between the plant NAD-specific isocitrate dehydrogenase and isocitrate at high pH values appears to be at least 3.0. This may indicate the presence of four binding sites on the plant enzyme for isocitrate, but the degree of interaction between sites may be less than with the yeast NAD-specific isocitrate dehydrogenase, which shows an order of reaction of 4 with respect to isocitrate. Study of the calculations provided by Atkinson *et al.* (1965) indicates that the degree of interaction between binding sites is above 5 if the number of sites is four. Thus the binding of an isocitrate molecule should result in at least a fivefold decrease in the dissociation constant for isocitrate at the remaining binding sites on the enzyme. This corresponds to a decrease in the standard free energy of binding of 1.5 kcal./mole for each further molecule of isocitrate bound.

The possibility that there may be four isocitrate/citrate binding sites on the plant NAD-specific isocitrate dehydrogenase is not discounted by a consideration of the density-gradient-centrifugation studies. These suggest that the enzyme may be composed of two major subunits of mol.wt. approx. 260 000 into which the enzyme disaggregates in the presence of citrate. This may imply that the number of sites for binding isocitrate on the enzyme is an even number. Correlation of this information with the kinetic results, which show a minimum order of reaction with respect to isocitrate/citrate

of 3.0, suggests that the number of isocitrate/citrate binding sites may be four as for the yeast NAD-specific isocitrate dehydrogenase.

Most of the results can be interpreted satisfactorily in terms of the general model for the mechanism of action of allosteric proteins proposed by Monod, Wyman & Changeux (1965). Thus the effect of pH on the co-operative binding of citrate or isocitrate may be viewed as an effect on the R_0 - T_0 equilibrium. If such a mechanism were operative, a lowering of the pH would favour the conformation of the enzyme having the greater affinity for isocitrate or citrate. The terms of the model suggest the existence of separate sites for activator and substrate, however, and it is difficult to explain the inhibition by citrate at high isocitrate concentrations at all pH values except by the binding of citrate to the same sites as isocitrate.

The study of the activation of the plant NAD-specific isocitrate dehydrogenase by citrate has consistently suggested that citrate binds to the same sites on the enzyme as isocitrate. A similar conclusion has been put forward by Atkinson *et al.* (1965) for the yeast NAD-specific isocitrate dehydrogenase. A brief resumé of the evidence supporting this point of view is presented.

(a) Activation by citrate is greatest at low isocitrate concentrations. At high isocitrate concentrations citrate appears to be a competitive inhibitor.

(b) The co-operative activation by citrate, as well as by isocitrate, is increased at high pH values. The order of reaction with respect to citrate at high pH and low isocitrate concentrations was near to 3.0, suggesting that, as for isocitrate, there may be four binding sites with a degree of interaction between sites above 5.

(c) Citrate inhibited the enzyme at all pH values when present in high concentration, even when the activation effects were small, as at low pH values. This suggests that citrate can bind at catalytic sites.

(d) Citrate repressed the inhibition by anions in a similar manner to isocitrate.

(e) The affinity of the enzyme for isocitrate and citrate showed similar changes with changes in pH.

The proposition that the operation of the tricarboxylic acid cycle in certain plant tissues is limited by the activity of NAD-specific isocitrate dehydrogenase has been advanced by Laties (1967).

Although the results presented in this paper and by Cox & Davies (1967) show that the plant NAD-specific isocitrate dehydrogenase possesses characteristics typical of enzymes that have been suggested to have a regulatory significance in metabolism, including the NAD-specific isocitrate dehydrogenase isolated from other sources, it is

difficult with the plant enzyme to propose confidently any such regulatory role in plant metabolism. This is chiefly due to the apparent absence of any activating effects by AMP or ADP or inhibition by ATP. In any case, the importance of such effects in reflecting the energy state of the mitochondrion requires a tight coupling between NADH oxidation and oxidative phosphorylation. It has been suggested that the NAD^+/NADH concentration ratio in the plant mitochondrion may be important in the control of NAD-specific isocitrate dehydrogenase activity (Cox & Davies, 1967), but the importance of this ratio in reflecting the energy state of the plant mitochondrion again depends on the tight coupling of the oxidation of NADH to oxidative phosphorylation. Further, the permeability of the plant mitochondrial membrane to external NADH has been firmly established by Ikuma & Bonner (1967), and the possibility that internal NADH may be transported outside the plant mitochondrion further weakens the argument for the importance of the NAD^+/NADH concentration ratio.

The co-operative activation of the plant NAD-specific isocitrate dehydrogenase by isocitrate at high pH suggests a finer control of reaction rate with respect to isocitrate concentration than would be possible with an enzyme showing a hyperbolic relationship between initial rate and substrate concentration. Further, the activation by citrate may be viewed as an example of precursor activation. However, it has been known for many years (Krebs, 1953) that the aconitase equilibrium lies very much in favour of citrate, and unless one postulates the existence of compartmentation of isocitrate and citrate within the mitochondrion it is clear that the NAD-specific isocitrate dehydrogenase is likely to be permanently in its 'activated' state. Thus it appears that the activation by citrate, far from giving the enzyme a 'regulatory' role, in fact ensures that the enzyme exists in its apparently kinetically 'active' state. Only at pH values above 8.0, when the affinity of the NAD-specific isocitrate dehydrogenase for citrate and isocitrate would become much smaller and the responses of the enzyme to both activator and substrate would be greater than first-order, is it

likely that the enzyme could play a regulatory role.

It is suggested that the plant NAD-specific isocitrate dehydrogenase may be an example of a class of enzymes that, although they possess multiple sites and show co-operative binding of ligands, have no regulatory significance in metabolism. Concepts of 'normality' of enzyme action may require adjustment to include such enzymes. The regulatory significance of enzymes showing the characteristics of co-operative binding of ligands requires entirely separate consideration.

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