The Effect of pH on the Characteristics of the Binding of Nicotinamide-Adenine Dinucleotide by Nicotinamide-Adenine Dinucleotide-Specific Isocitrate Dehydrogenase from Pea Mitochondria

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1. The effect of pH on the $V_{\max}$ and concentration of NAD^+ at half-maximum velocity at a constant isocitrate concentration was examined, and the results were related to the requirements for binding of H^+ ions to the enzyme. 2. The effect of varying the NAD^+ concentration on the pH optimum with constant isocitrate concentration was studied. 3. A comparison has been made between the effect of isocitrate concentration on the characteristics of binding of NAD^+ and the effect of NAD^+ concentration on the characteristics of isocitrate binding at three different pH values. 4. The mechanistic and metabolic significance of these studies is considered.

The isolation, stabilization, purification and initial characterization of the NAD-specific isocitrate dehydrogenase [*threo*-D₂-isocitrate-NAD oxidoreductase (decarboxylating), EC 1.1.1.41] from pea mitochondria was first reported by Cox & Davies (1967). Co-operative activation of the enzyme by isocitrate at the pH optimum was observed, and the activation of the enzyme by low concentrations of citrate was shown to eliminate this co-operative binding. Dennis & Coultate (1967) have made similar observations with the enzyme from swede.

More extensive investigation of the binding characteristics of isocitrate and citrate by the pea enzyme (Cox & Davies, 1969) have shown that at low pH the co-operative binding of isocitrate disappears, and the activation of the enzyme by citrate is also eliminated. At high pH, especially beyond the pH optimum, co-operative activation by citrate at low isocitrate concentrations was observed. It was concluded from these studies that both citrate and isocitrate bind to the same sites. It was considered necessary that a single H^+ ion bind to the free enzyme before the binding of isocitrate or citrate.

Density-gradient centrifugation of the NAD-specific isocitrate dehydrogenase from peas showed that in the presence of citrate the enzyme tended to dissociate into two subunits. It was tentatively suggested that four isocitrate-citrate binding-sites exist on the intact enzyme molecule.

The determination of the characteristics of the plant NAD-specific isocitrate dehydrogenase is

important for the consideration of the enzyme as a possible regulatory point in the tricarboxylic acid cycle, as has been suggested for the enzyme from other sources. Coultate & Dennis (1969) have stressed the importance of the NAD^+/NADH concentration ratio in the regulation of the NAD-specific isocitrate dehydrogenase from swede, since the apparent affinity of the enzyme for NADH was greater than for NAD^+ . An almost equal apparent affinity of the pea enzyme for NAD^+ and NADH has been observed (Cox & Davies, 1967). Although the activation by citrate of the NAD-specific isocitrate dehydrogenase from *Neurospora crassa* has been suggested by Sanwal & Stachow (1965) as an example of precursor activation, and a similar role for citrate has been suggested by Coultate & Dennis (1969) for the swede enzyme, it has been pointed out by Cox & Davies (1969) that the position of the aconitase equilibrium will ensure that higher concentrations of citrate rather than isocitrate prevail. The enzyme will therefore tend to exist in its 'activated' state and the regulatory advantages of co-operative activation by isocitrate will therefore not be apparent.

The pH at which the NAD-specific isocitrate dehydrogenase acts within the mitochondrion must be important in determining whether the enzyme plays a significant part in the regulation of the tricarboxylic acid cycle, owing to the considerable variation in the binding characteristics of citrate and isocitrate with pH. In this paper the effect of pH on the characteristics of binding of NAD^+ is investigated

MATERIALS AND METHODS

The materials and methods employed in the isolation, stabilization, purification and assay of the NAD-specific isocitrate dehydrogenase from pea mitochondria have been described in detail by Cox & Davies (1967). Slight modifications to the purification of the enzyme were reported by Cox & Davies (1969). The 'partially purified' preparation of the enzyme described by Cox & Davies (1967, 1969) has been used throughout these studies.

50 mM-Hepes* buffer adjusted to the required pH with NaOH was used in these experiments.

RESULTS

Effect of pH on the binding of NAD⁺. The characteristics of the binding of NAD⁺ by NAD-specific isocitrate dehydrogenase has been determined previously only under standard assay conditions in which the isocitrate concentration was saturating and the pH was at the optimum value of 7.6 (Cox & Davies, 1967). Under these conditions a hyperbolic plot of rate against NAD⁺ concentration was observed.

Cox & Davies (1969) observed effects of pH on the degree of co-operativity and the affinity of the enzyme for isocitrate. To assess the importance of H⁺ ions in the binding of NAD⁺ and the effect of NAD⁺-binding on the binding of isocitrate a series of experiments were performed with variable NAD⁺ concentration and constant isocitrate concentration. The degree of co-operativity and the apparent affinity of the enzyme for NAD⁺ was established at pH values in the range 6.4–8.7. Two constant isocitrate concentrations were used, 500 μ M and 200 μ M.

A representative series of plots of rate versus NAD⁺ concentration when the isocitrate concentration was 200 μ M are shown in Fig. 1. The results obtained from these experiments were used to calculate the $V_{\max}$ and the NAD⁺ concentration at half $V_{\max}$ ($N_{0.5}$), by constructing reciprocal plots of $1/v$ versus $1/[S]$ or, in instances where the co-operativity of the rate versus $[NAD^+]$ plot demanded it, $1/v$ versus $1/[S]^2$. The values for $N_{0.5}$ were checked by constructing plots of v versus $\log[NAD^+]$, as suggested by Koshland, Nemethy & Filmer (1966). The order of the reaction was determined in each case by determining the slope, n , of the plot of $\log[v/(V_{\max} - v)]$ versus $\log[S]$ (Hill, 1913), where v is the velocity at a given substrate concentration, $V_{\max}$ the maximum velocity and $[S]$ the substrate concentration. The results obtained in these experiments are presented in Table 1.

The role of H⁺ in the binding of NAD⁺ was further investigated by constructing plots of $-\log N_{0.5}$ ($pN_{0.5}$) and $-\log V_{\max}$ ($pV_{\max}$) against pH for both

* Abbreviation: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

Fig. 1. Effect of pH on the reaction of NAD⁺ with NAD-specific isocitrate dehydrogenase. The plots are representative of a series obtained from pH 6.4 to 8.6. Conditions of assay: 200 μ M-isocitrate, 1 mM-Mn²⁺ and 50 mM-Hepes-NaOH buffer at: (a) pH 6.4; (b) pH 7.6; (c) pH 8.2.

series of results. Such a plot was first suggested by Dixon (1953), and has been employed by Klingenberg, Goebell & Wenske (1965) in an investigation of the change in the affinity of the locust NAD-specific isocitrate dehydrogenase for isocitrate. These plots are presented in Fig. 2. Clearly these plots are rather more complex than those observed when isocitrate was the variable ligand (Cox & Davies, 1969). At higher pH values the slope of the plot of $pN_{0.5}$ versus pH is 2.2 for the

Table 1. *Effect of pH on the apparent affinity of NAD-specific isocitrate dehydrogenase for NAD⁺ and on the order of the reaction, at two constant isocitrate concentrations*

For details see the text.

(a) 0.5mM-Isocitrate		
pH	$N_{0.5}$ (μM)	n
6.5	23	1.4
6.9	65	1.2
7.3	150	0.97
7.6	170	1.0
7.85	220	1.13
8.15	260	1.18
8.45	930	1.61
8.7	1400	2.18

(b) 0.2mM-Isocitrate		
pH	$N_{0.5}$ (μM)	n
6.4	49	1.04
6.8	100	1.09
7.2	140	1.08
7.6	170	1.04
7.9	300	1.25
8.2	3000	2.32
8.6	4800	4.84

Fig. 2. Relationship between (a) $pN_{0.5}$ and pH and (b) $pV_{\max}$ and pH, at two isocitrate concentrations. 200 μM -Isocitrate: $\circ$, $pN_{0.5}$; $\triangle$, $pV_{\max}$. 500 μM -Isocitrate: $\bullet$, $pN_{0.5}$; $\blacktriangle$, $pV_{\max}$.

200 μM -isocitrate plot and 1.8 for the 500 μM -isocitrate plot. The downward curvature of the plot may be interpreted as indicating the requirement for the binding of 2H⁺ ions to the free enzyme before the binding of NAD⁺. The apparent pK of the ionizable group on the protein responsible appears to be about 7.9.

The second curvature of the $pN_{0.5}$ plot occurs at lower pH values and the apparent pK of the ioniz-

Fig. 3. Effect of NAD⁺ concentration on the pH optimum for NAD-specific isocitrate dehydrogenase. Assay conditions: 500 μM -isocitrate, 1 mM-Mn²⁺ and 50 mM-Hepes-NaOH buffer. $\blacktriangle$, 66 μM -NAD⁺; $\blacksquare$, 200 μM -NAD⁺; $\bullet$, 660 μM -NAD⁺.

able group in this case appears to be about 7.0. Since the deflexion of the curve in this instance is upward rather than downward, the binding of an H⁺ ion to the enzyme-substrate complex is indicated (Dixon, 1953). This is further suggested by a similar change in slope of the $pV_{\max}$ plot, showing that the binding of an H⁺ ion to the enzyme-substrate complex also affects the velocity of the reaction.

As indicated in Table 1, the value of n from the Hill plot shows a marked increase above pH 7.8, which corresponds to the apparent pK of the ionizable group affecting binding to the free enzyme.

Effect of NAD⁺ concentration on the pH optimum. Three concentrations of NAD⁺ were used: 66, 200 and 660 μM . The concentration of isocitrate was 0.5mM throughout. As shown in Fig. 3, no significant effect on the pH optimum was observed. This is in marked contrast with the effect of isocitrate concentration on the pH optimum, where the optimum moved to lower pH values with decreasing isocitrate concentration (Cox & Davies, 1969). The marked asymmetry of the plots shown in Fig. 3 should also be noted.

Effect of isocitrate concentration on the binding of NAD⁺ at three pH values. The effect of isocitrate concentration on the affinity of NAD-specific isocitrate dehydrogenase for NAD⁺ and the order of the reaction was investigated at pH 6.4, 7.6 and 8.6. The two concentrations of isocitrate chosen at each pH were as follows: pH 6.4, 5 and 20 μM ; pH 7.6, 60 and 500 μM ; pH 8.6, 750 and 3000 μM . The concentrations were chosen to represent similar relationships to the saturating isocitrate concentrations at each pH value. The results are presented

Fig. 4. Effect of isocitrate concentration on the binding of NAD^+ at three pH values. (a) pH 6.4: $\bullet$, $5\text{ }\mu\text{M}$ -isocitrate; $\circ$, $20\text{ }\mu\text{M}$ -isocitrate. (b) pH 7.6: $\bullet$, $60\text{ }\mu\text{M}$ -isocitrate; $\circ$, $500\text{ }\mu\text{M}$ -isocitrate. (c) pH 8.6: $\bullet$, $750\text{ }\mu\text{M}$ -isocitrate; $\circ$, $3000\text{ }\mu\text{M}$ -isocitrate. Assay conditions: 1 mM-Mn^{2+} and 50 mM-Hepes-NaOH .

in Fig. 4. Table 2 shows the $N_{0.5}$ values calculated for each plot and the order of reaction calculated from the Hill plot. Clearly the effect of isocitrate on the affinity of the enzyme for NAD^+ is greatest at

Table 2. Effect of isocitrate concentration on the binding characteristics of NAD^+ by NAD -specific isocitrate dehydrogenase at three pH values

pH	Concn. of isocitrate (μM)	$N_{0.5}$ (μM)	n
6.4	5	160	1.0
	20	120	1.18
7.6	60	430	1.32
	500	210	1.1
8.6	750	1400	1.76
	3000	400	0.96

Fig. 5. Effect of NAD^+ concentration on the binding of isocitrate at pH 7.6. $\blacktriangle$, $130\text{ }\mu\text{M-NAD}^+$; $\triangle$, $330\text{ }\mu\text{M-NAD}^+$; $\bullet$, $660\text{ }\mu\text{M-NAD}^+$; $\circ$, $1300\text{ }\mu\text{M-NAD}^+$. Assay conditions: 1 mM-Mn^{2+} and 50 mM-Hepes-NaOH .

Table 3. Effect of NAD^+ concentration on the binding characteristics of isocitrate by NAD -specific isocitrate dehydrogenase

pH	Concn. of NAD^+ (μM)	$S_{0.5}$ (μM)	n
6.4	50	40	1.2
	660	53	1.15
7.6	130	110	2.7
	330	80	2.4
	660	83	2.4
	1300	69	2.2
8.6	500	1500	2.04
	2000	480	1.96

higher pH values. Further, at high pH and low isocitrate concentrations the order of the reaction becomes greater than unity.

Effect of NAD^+ concentration on the binding of isocitrate at three pH values. In an attempt to compare the activating effects of NAD^+ and isocitrate,

the effect of NAD^+ concentration on the apparent affinity of the enzyme for isocitrate and on the order of the reaction was investigated at pH 6.4, 7.6 and 8.6. The following concentrations of NAD^+ were chosen: pH 6.4, 50 and $666\mu\text{M}$; pH 7.6, 130, 330, 660 and $1300\mu\text{M}$; pH 8.6, 500 and $2000\mu\text{M}$. The results of the experiment at pH 7.6 are shown in Fig. 5. The $S_{0.5}$ values calculated for each of the plots, and the n values determined from Hill plots constructed from the same data, are presented in Table 3. Although some increase in the apparent affinity of the enzyme for isocitrate is seen with increasing NAD^+ concentrations at each pH, at higher pH, where isocitrate binding is co-operative, very little effect of NAD^+ on the order of the reaction is observed. This is in sharp contrast with the effect of isocitrate on the order of the reaction with NAD^+ .

DISCUSSION

The results presented enable some useful comparisons to be made between the characteristics of binding of NAD^+ and isocitrate by NAD-specific isocitrate dehydrogenase and the interacting effects of one ligand on the other. The most important points arising from such comparisons are summarized below.

(1) A sharp contrast is evident between the H^+ -binding requirements for NAD^+ and those for isocitrate previously published (Cox & Davies, 1969). Thus with isocitrate only a single H^+ ion appears to be required to be bound to the free enzyme before the binding of the isocitrate molecule. The pK of the ionizing group responsible is difficult to assess from the data available. With NAD^+ as the variable ligand, however, two regions of slope in the plot of $\text{pN}_{0.5}$ versus pH are observed. The first slope, which occurs at low pH, has a gradient approaching 1.0, and since it has an upward curvature at the position of change in gradient this may be considered to represent the binding of a single H^+ ion to the enzyme-substrate complex (Dixon, 1953). The apparent pK of the ionizing group involved is about 7.0. The ionizing group must be associated with the enzyme since the nearest pK for NAD^+ is 3.9 (Kaplan, Ciotti, Stolzenbach & Bacur, 1955). Since the plot of pV_{max} versus pH also shows a similar gradient and position of pK to the $\text{pN}_{0.5}$ plot, it is likely that the binding of the H^+ ion affects the rate of breakdown of the enzyme-substrate complex to the free enzyme and products.

The second slope of the $\text{pN}_{0.5}$ plot, which occurs at higher pH, represents the binding of H^+ ions to the free enzyme before the binding of NAD^+ . The apparent pK of the ionizing group(s) responsible is about 7.9. In this instance the pV_{max} plot shows no change in gradient.

This second slope is also seen in the plot of

$\text{pS}_{0.5}$ versus pH (Cox & Davies 1969), although the slope is only 1.2 in this instance. It should be noted, however, that the concentrations of isocitrate used in this experiment were not saturating at the higher pH values, and the second slope observed in the $\text{pN}_{0.5}$ plot is therefore likely to represent a mixed effect of pH on isocitrate and NAD^+ binding. This possibility is further indicated by the smaller gradient observed with $500\mu\text{M}$ -isocitrate than with $200\mu\text{M}$ -isocitrate. It seems probable that isocitrate and NAD^+ each require the binding of a single H^+ ion to the free enzyme before being bound.

(2) Comparison of the effect of isocitrate and NAD^+ concentration on the pH optimum shows considerable differences. Thus decrease in isocitrate concentration in the presence of high NAD^+ concentrations causes a shift in the pH optimum from 7.6 to 6.9. However, decrease in the NAD^+ concentration in the presence of high isocitrate concentrations causes no significant movement of the pH optimum from 7.6.

(3) Whereas high isocitrate concentrations completely eliminate the co-operative binding of NAD^+ at all pH values, saturating NAD^+ concentrations have only a small effect on the co-operative binding of isocitrate. However, in both cases the apparent affinity of the enzyme for either NAD^+ or isocitrate is increased by increasing the concentration of the other ligand. This result highlights a significant difference between the effects of NAD^+ and isocitrate on the NAD-specific dehydrogenase.

(4) Associated with the results discussed in (1) is the observation that co-operative activation by isocitrate begins above pH 7.0 and co-operative activation by NAD^+ begins above pH 7.9. It seems possible that these characteristics may be closely associated with the pK values of the ionizable groups determined from the plot of $\text{pN}_{0.5}$ versus pH. However, with the concentrations of isocitrate used in those experiments (200 and $500\mu\text{M}$) the enzyme would be close to saturation with isocitrate up to pH 7.8–8.0. It is likely that if the concentrations of isocitrate had been significantly lower the co-operative activation by NAD^+ would have been observed at pH values approaching 7.0. Since NAD^+ concentration has little effect on the co-operative activation by isocitrate this was observed above pH 7.0 even in the presence of high NAD^+ concentrations.

The characteristics of the NAD-specific isocitrate dehydrogenase do not fit satisfactorily the 'concerted' mechanism of allosteric action proposed by Monod, Wyman & Changeux (1965), nor the alternative mechanism proposed by Koshland, Nemethy & Filmer (1966). This may indicate that such mechanisms, in which each binding site for substrates is considered to be involved in catalysis, are not applicable to this enzyme.

The mechanism of action proposed by Atkinson, Hathaway & Smith (1965) for the yeast NAD-specific isocitrate dehydrogenase appears to have considerable relevance to the characteristics of the pea NAD-specific isocitrate dehydrogenase. As discussed previously (Cox & Davies, 1969) the characteristics of binding of citrate and isocitrate to the latter suggest that both ligands are bound to the same sites. If there are four sites the degree of interaction between sites must be smaller than that observed with yeast NAD-specific isocitrate dehydrogenase, resulting in an order of reaction lower than 4.0 (Cox & Davies, 1969). Two catalytic and two regulatory isocitrate-binding sites were proposed by Atkinson *et al.* (1965) for the yeast enzyme. The same authors also proposed that NAD^+ could only be bound at the two catalytic sites and that a preferential binding mechanism for NAD^+ before isocitrate operated. However, only isocitrate binding was considered to bring about conformational changes. They suggested that increases in NAD^+ concentration, by facilitating isocitrate binding, result in an increase in affinity of the enzyme for all ligands, so that the co-operative activation by NAD^+ is only an indirect effect achieved through the increased affinity of the enzyme for isocitrate.

A similar mechanism of action satisfactorily fits the characteristics of the pea NAD-specific isocitrate dehydrogenase. Such a model explains the observation that high concentrations of isocitrate eliminate the co-operative activation of the enzyme by NAD^+ , but that high NAD^+ concentrations do not eliminate the co-operative binding of isocitrate although the affinity of the enzyme for isocitrate is increased.

From the studies of the effect of pH on the binding of NAD^+ and isocitrate it is possible to speculate on the possible importance of the ionizing groups, the apparent pK values of which have been determined, in regulatory and active sites. Thus it seems likely that the ionizing group affecting the enzyme-

substrate complex must be associated with the active site alone, whereas the second ionizing group is associated with both regulatory and active sites.

The results presented in this paper illustrate the importance of the pH at which the NAD-specific isocitrate dehydrogenase operates within the mitochondrion in consideration of the possible regulatory role of the enzyme in the tricarboxylic acid cycle. The same conclusion was drawn from the study of the binding characteristics of citrate and isocitrate (Cox & Davies, 1969). In the absence of adequate data on the intramitochondrial pH the regulatory role of the NAD-specific isocitrate dehydrogenase must remain speculative. However, it is clear that the enzyme may be of increasing significance in regulation with increasing pH, especially above pH 8.0, when the affinity of the enzyme for all ligands becomes markedly diminished.

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