The Isocitrate Dehydrogenases of *Acinetobacter lwoffii*

STUDIES ON THE REGULATION OF A NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-LINKED ISOENZYME

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Of the two NADP-linked isocitrate dehydrogenases in *Acinetobacter lwoffii* the higher-molecular-weight form, isoenzyme-II, is reversibly stimulated sixfold by low concentrations of glyoxylate or pyruvate. Kinetic results indicate that this stimulation of activity involves both an increase in $V_{\max}$ and a decrease in the apparent K_m values for substrates, most markedly that for NADP⁺. Other changes brought about by glyoxylate or pyruvate include a shift in the pH optimum for activity and an increased stability to inactivation by heat or urea. Mixtures of glyoxylate plus oxaloacetate, known to inhibit isocitrate dehydrogenases from other organisms, produce inhibition of both *A. lwoffii* isoenzymes, and do not reflect the stimulatory specificity of glyoxylate for isoenzyme-II. Isoenzyme-II is also stimulated by AMP and ADP, but the activation by glyoxylate or pyruvate is shown to be quite independent of the adenylate activation. Differential desensitization of the enzyme by urea to the two types of activator further supports the view that the enzyme possesses two distinct allosteric regulatory sites. The metabolic significance of the activations is discussed.

A previous paper (Self & Weitzman, 1972) described the identification and separation of two isoenzymes of NADP-linked isocitrate dehydrogenase [*threo*-D₂-isocitrate-NADP oxidoreductase (decarboxylating), EC 1.1.1.42] from *Acinetobacter lwoffii*. It was also shown that the stimulation of isocitrate dehydrogenase activity in unpurified preparations by low concentrations of glyoxylate was due to the stimulation of only one of the isoenzymes, the higher-molecular-weight species, which we have termed isoenzyme-II. In another report the regulation of the same isoenzyme by adenylates was described (Parker & Weitzman, 1970). It was found that AMP and (to a lesser extent) ADP stimulated enzymic activity and that the activation of the enzyme showed the dependence on 'energy charge' to be expected for a catabolic enzyme (Atkinson, 1968).

In the present paper we describe in detail the nature of the stimulation by glyoxylate and pyruvate and the interaction of this effect with the adenylate activation.

Materials and Methods

Chemicals used were analytical grade or the purest grade available.

Measurements of isocitrate dehydrogenase activity and of the concentrations of NADP⁺ and isocitrate were made as previously described (Self & Weitzman, 1972). Unless otherwise stated, standard assay mixtures consisted of 20 mM-Tris-HCl buffer-10 mM-MgCl₂-1 mM-EDTA-0.2 mM-NADP⁺-4 mM-DL-

isocitrate, at pH 8.0, and reactions were initiated by the addition of enzyme. The action of effectors on enzyme activity was tested by including these compounds in the assay mixture before the addition of enzyme. 'Activation' of the enzyme is defined as the activity measured in the presence of the activator divided by the activity in its absence. For most of the work presented here, isoenzyme-II was prepared as described previously (Self & Weitzman, 1972). In addition, the isoenzyme was prepared by an alternative procedure (Parker & Weitzman, 1970) and this material was used in some experiments.

Results

Activation of the enzyme

The activity of isoenzyme-II is markedly stimulated by low concentrations of glyoxylate or pyruvate, an activation of sixfold being attained with either effector at a concentration of 0.1 mM under standard assay conditions. Fig. 1 shows the dependence of activation on effector concentration. These measurements, and most of the other experiments reported here, were carried out with Mg²⁺ satisfying the cation requirement of the enzyme. We have noted that the activity measured with Mg²⁺ is somewhat less than that with Mn²⁺ (Self & Weitzman, 1972). However, the activation produced by glyoxylate or pyruvate is greater with Mg²⁺ than with Mn²⁺. Kinetic studies with both metal ions are reported below.

Fig. 1. Dependence of activation on effector concentration

Assays were performed under standard conditions (see the text) with the inclusion of various concentrations of glyoxylate (●) or pyruvate (○).

The action of the effectors was shown to be reversible in the following way. A sample of isoenzyme-II was first assayed in the absence of any effector. To other portions of the same sample of enzyme were added separately glyoxylate and pyruvate, each to a concentration of 1 mM. Samples (0.2 ml) of each of these mixtures of enzyme plus effector were then taken into a 1.0 ml assay mixture and the activity was thereby determined at 0.2 mM effector concentration, i.e. when fully activated. Other reaction mixtures were set up without isocitrate but containing 0.22 mM-NADH. To these were added 0.2 ml samples of the mixtures of enzyme plus effector and a small amount of lactate dehydrogenase. The E_{340} was followed until no further decrease was produced, indicating complete reduction of the pyruvate or glyoxylate by the NADH and lactate dehydrogenase. The isocitrate dehydrogenase reaction was then initiated by the addition of isocitrate and the formation of NADPH followed in the usual way. The activity determined in each case corresponded precisely with that measured in the absence of effectors, thereby indicating the complete reversibility of the action of both glyoxylate and pyruvate.

The nature of the activation of isoenzyme-II by glyoxylate or pyruvate in terms of changes in the kinetic characteristics of the enzyme was next investigated. The effects of 0.2 mM-glyoxylate or -pyruvate on the substrate dependences of the isoenzyme were examined at 1 mM-NADP⁺ and various isocitrate concentrations or 4 mM-DL-isocitrate and various NADP⁺ concentrations. Double-reciprocal plots are presented in Figs. 2 and 3, showing that both glyoxylate and pyruvate increase $V_{\max}$, and lower the apparent K_m values for NADP⁺ and isocitrate. Apparent K_m values for NADP⁺ and isocitrate in the

Fig. 2. Effects of glyoxylate and pyruvate on the isocitrate dependence of enzyme activity

Assays were done in 0.1 M-Tris-HCl-10 mM-MgCl₂-1 mM-EDTA buffer, pH 8.0. The results are plotted in the form of double-reciprocal plots of enzyme activity (arbitrary units) against *threo*-D₅-isocitrate concentration in the absence of either effector (■), in the presence of 0.2 mM-glyoxylate (●) or in the presence of 0.2 mM-pyruvate (○). The concentration of NADP⁺ was kept at 1 mM.

Fig. 3. Effects of glyoxylate and pyruvate on the NADP⁺ dependence of enzyme activity

Assays were done in 0.1 M-Tris-HCl-10 mM-MgCl₂-1 mM-EDTA buffer, pH 8.0. The results are plotted in the form of double-reciprocal plots of enzyme activity (arbitrary units) against NADP⁺ concentration in the absence of either effector (■), in the presence of 0.2 mM-glyoxylate (●) or in the presence of 0.2 mM-pyruvate (○). The concentration of *threo*-D₅-isocitrate was kept at 2 mM.

absence and in the presence of glyoxylate or pyruvate, and with Mg^{2+} or Mn^{2+} as the metal cation, are presented in Table 1. The most striking alteration is that in the K_m value for $NADP^+$ in the presence of

Mg^{2+} . A 10-fold decrease in this value was produced by 0.2mM-glyoxylate or -pyruvate (from 0.31 to 0.03mM). This is in marked contrast to the changes caused by AMP and ADP. Both these nucleotides

Table 1. *Effects of glyoxylate and pyruvate on the apparent K_m values of isoenzyme-II*

Assays were carried out in 0.1M-Tris-HCl-1mM-EDTA buffer, pH8.0, with either 10mM- $MgCl_2$ or 10mM- $MnCl_2$. The dependence of activity on $NADP^+$ concentration was determined at 4mM-DL-isocitrate and the dependence on isocitrate at 1mM- $NADP^+$. Effectors were present at 0.2mM concentration.

Effector	Metal ion	Substrate	Apparent K_m (μM)
None	Mg^{2+}	$NADP^+$	310
Glyoxylate	Mg^{2+}	$NADP^+$	32
Pyruvate	Mg^{2+}	$NADP^+$	35
None	Mn^{2+}	$NADP^+$	100
Glyoxylate	Mn^{2+}	$NADP^+$	49
Pyruvate	Mn^{2+}	$NADP^+$	49
None	Mg^{2+}	<i>threo</i> -D ₅ -Isocitrate	17
Glyoxylate	Mg^{2+}	<i>threo</i> -D ₅ -Isocitrate	7.0
Pyruvate	Mg^{2+}	<i>threo</i> -D ₅ -Isocitrate	6.3
None	Mn^{2+}	<i>threo</i> -D ₅ -Isocitrate	13
Glyoxylate	Mn^{2+}	<i>threo</i> -D ₅ -Isocitrate	5.5
Pyruvate	Mn^{2+}	<i>threo</i> -D ₅ -Isocitrate	5.5

Fig. 4. *Effects of glyoxylate and pyruvate on the pH-dependence of enzyme activity*

Activities were measured in buffers of composition 20mM-Tris-HCl-10mM- $MgCl_2$ -1mM-EDTA, in the presence of 1mM- $NADP^+$ and 4mM-DL-isocitrate, and are expressed in arbitrary units. No effector present (■); with 0.2mM-glyoxylate (●); or with 0.2mM-pyruvate (○).

Fig. 5. Dependence of activation by glyoxylate, pyruvate and adenylates on pH

Assays were done in buffers of composition 20mM-Tris-HCl-10mM-MgCl₂-1 mM-EDTA, with 0.2mM-NADP⁺ and 2mM-DL-isocitrate. Activations were measured in the presence of 0.2mM-glyoxylate (●), 0.2mM-pyruvate (○), 1mM-AMP (■) or 1mM-ADP (▲).

stimulate activity but produce little change in K_m values (Parker & Weitzman, 1970).

Another alteration in the kinetic behaviour of isoenzyme-II caused by the effectors is seen in the pH-dependence of enzyme activity. The pH optimum (8) is shifted to pH 8.5-9 in the presence of 0.2mM-glyoxylate or -pyruvate (Fig. 4). This shift in the pH optimum is also reflected in an increase of activation by effectors with increase in pH. Fig. 5 shows the relationship between activation and pH for glyoxylate and pyruvate and also for AMP and ADP, which cause a similar shift in pH profile. It is noteworthy that in the presence of glyoxylate or pyruvate the kinetic behaviour of isoenzyme-II is very similar to that of isoenzyme-I. Thus the decrease in the K_m value for NADP⁺ to 0.03mM brings this parameter close to that for isoenzyme-I, i.e. 0.025mM, whereas the shift of pH profile produces an apparent optimum which is close to that for isoenzyme-I (Self & Weitzman, 1972).

Fig. 6. Effects of glyoxylate and pyruvate on the thermal inactivation of the enzyme

Enzyme activities were measured under standard conditions after various times of heating at 43°C in 20mM-Tris-HCl-10mM-MgCl₂-1 mM-EDTA buffer, pH 8.0. No effectors present (□); with 0.2mM-glyoxylate (●); with 0.2mM-pyruvate (○).

We have sought evidence for structural changes arising from the interaction of the enzyme with glyoxylate or pyruvate by examining the effects of these compounds on the sensitivities of isoenzyme-II to inactivation by heat and urea. Fig. 6 shows the effect of heating the enzyme at 43°C in the absence and in the presence of effectors; samples were removed at various times, cooled rapidly and subsequently assayed for activity. Both glyoxylate and pyruvate exert a protective effect on the thermal inactivation; the reason for the non-linear decay in their presence is not known. We have similarly shown that AMP and ADP also protect the enzyme against thermal inactivation (Parker & Weitzman, 1970).

The effect of urea was examined by including various concentrations of urea in the assay mixture and initiating the reaction with enzyme; no prior treatment of the enzyme with urea was carried out. Fig. 7 shows that 0.2mM-glyoxylate or -pyruvate afford some protection against urea inactivation.

We have tested a number of compounds of similar chemical structure to glyoxylate and pyruvate for any stimulatory effect on isoenzyme-II. The com-

Fig. 7. Effects of glyoxylate and pyruvate on the inactivation of the enzyme by urea

Enzyme activities were measured under standard conditions (see the text) but with the addition of various concentrations of urea. No effectors present (■); with 0.2mM-glyoxylate (●); with 0.2mM-pyruvate (○).

pounds were all tested at 0.2mM concentration, and the results are presented in Table 2. Of the compounds tested, only α -oxobutyrate and methyl and ethyl pyruvate showed appreciable stimulation. The stimulation by pyruvate esters was greater than could be accounted for by contaminating free pyruvate. Those compounds that are activators have the structure $R-CO-CO_2R'$ where R and R' may be H, CH_3 or C_2H_5 . R may apparently not be OH, CH_2CO_2H or $CH_2CH_2CO_2H$, since oxalate, oxaloacetate and α -oxoglutarate fail to produce any activation.

Concerted inhibition

It is worth emphasizing that the other isocitrate dehydrogenase (isoenzyme-I) present in *A. lwoffii* is completely unaffected by glyoxylate or pyruvate (Self & Weitzman, 1972). In view of this differential effect on the two isoenzymes we examined the action of a mixture of glyoxylate and oxaloacetate on their activities. Low concentrations of mixtures of glyoxylate and oxaloacetate have been found to inhibit isocitrate dehydrogenases from a variety of organisms

Table 2. Activation of isoenzyme-II by glyoxylate analogues

Assays were carried out in 0.1M-Tris-HCl-10mM-MgCl₂-1mM-EDTA buffer, pH8.0, with 0.2mM-NADP⁺ and 2mM-DL-isocitrate. The substances were all tested at 0.2mM concentration.

Compound	Activation
Acetyl phosphate	0
Carbamoyl phosphate	0
Glyoxal	0
Glycol	0
Glycollate	0
Oxalate	0
Oxaloacetate	0
α -Oxoglutarate	0
Ethyl acetoacetate	0
α -Oxobutyrate	4.6
Methyl pyruvate	5.7
Ethyl pyruvate	5.6

(Shiio & Ozaki, 1968; Hampton & Hanson, 1969; Marr & Weber, 1969b, 1971; Barrera & Jurtshuk, 1970; Charles, 1970). The sensitivity of our isoenzyme-II to glyoxylate stimulation suggested the possibility of a connexion between the two effects. However, it was found that both isoenzymes were inhibited by glyoxylate plus oxaloacetate, and no differential effect could be observed. Mixtures of oxaloacetate and pyruvate caused no inhibition of either isoenzyme, nor did oxaloacetate on its own produce any effect.

Allostery of the activations

The dissimilarity between the structures of glyoxylate and pyruvate on the one hand and of AMP and ADP on the other suggested that although both types of compound are functionally analogous in producing stimulation of enzymic activity they may well interact with the enzyme at physically distinct (allosteric) regions. With this in mind we examined the interaction between the two types of activators to see whether or not the effects they produced were independent and additive. Enzyme activities were determined at increasing AMP concentrations from 0 to 1mM, at which the activation was essentially maximal, and then at a fixed 1mM-AMP and with increasing concentrations of glyoxylate from 0 to 0.3mM. The results are shown in Fig. 8, and it is clear that the activations produced by AMP and glyoxylate are indeed independent and additive. Identical results were obtained with pyruvate in place of glyoxylate.

The independence of the two activation mechanisms led us to attempt the differential desensitization

Fig. 8. Additive nature of the glyoxylate and AMP activations

Enzyme activities (arbitrary units) were measured under standard conditions (see the text) first with increasing AMP concentrations from 0 to 1 mM (●), and then, at fixed 1 mM-AMP, with increasing glyoxylate concentrations from 0 to 0.3 mM (○).

of the enzyme to the two types of activator. In some preliminary experiments on the effect of NaCl on the thermal inactivation of isoenzyme-II we observed that 2–3 M-NaCl afforded considerable protection to the enzymic activity but not to its glyoxylate or pyruvate sensitivity. This observation suggested the non-identity of the active and glyoxylate/pyruvate sites. Treatment of the enzyme with 1 M-urea for 5 min followed by dilution into the assay mixture in the absence and in the presence of effectors gave the following results. Enzymic activity alone was unchanged as was also the activation by glyoxylate. The activation by AMP, on the other hand, was decreased from 5 to 2.3. When a similar experiment was carried out, but in which the enzyme was treated with 2.5 M-urea for 30 min, again no loss of activity was observed, but the AMP activation fell to 1.3 and the glyoxylate activation from 6 to 3.4. These results indicate that the enzyme may be desensitized selectively to its regulatory effectors.

The possibility that desensitization of the enzyme was accompanied by dissociation into subunits was tested by passing the enzyme through a column of Sephadex G-200 equilibrated with 5 M-urea. The enzyme was eluted from the column at the same point

as in the absence of urea. Although fully active, however, it was not activated by AMP. Thus desensitization to AMP appears not to have resulted in a gross dissociation of the enzyme.

Discussion

The results presented here and in a previous paper (Parker & Weitzman, 1970) indicate the complex nature of the regulatory responses of isoenzyme-II, one of two isoenzymes of NADP-linked isocitrate dehydrogenase in *A. lwoffii*. Isocitrate dehydrogenase is believed to play an important role in the regulation of the tricarboxylic acid cycle in various organisms. Most studies, however, have concentrated on the regulatory behaviour of the NAD-linked enzyme in eukaryotic cells. In these cases the isocitrate dehydrogenase has been shown to exhibit sigmoidicity in its rate-dependence on isocitrate concentration, and AMP or ADP have been found to act as powerful stimulators of enzyme activity (Hathaway & Atkinson, 1963; Sanwal *et al.*, 1964; Plaut, 1970). The NADP-linked isocitrate dehydrogenase also found in eukaryotic cells has not been assigned a role in metabolic regulation.

Although a few bacteria contain NAD-linked isocitrate dehydrogenase (Hathaway & Atkinson, 1963; Burchall *et al.*, 1964; Ragland *et al.*, 1966; Hampton & Hanson, 1969), most bacteria contain only the NADP-linked enzyme (Ragland *et al.*, 1966). Inhibition of NADP-linked isocitrate dehydrogenase by nucleotides has been reported (Marr & Weber, 1968, 1969a; Hampton & Hanson, 1969), but our finding of the nucleotide stimulation of isoenzyme-II of *A. lwoffii* (Parker & Weitzman, 1970) appears so far to be the only example of regulation of bacterial isocitrate dehydrogenase similar to that of the NAD-linked enzyme in higher organisms. There are significant differences between the *A. lwoffii* and eukaryotic systems, the most prominent being the absence of any apparent sigmoidicity in the dependence of *A. lwoffii* isoenzyme-II activity on isocitrate concentration. In this connexion it is noteworthy that although isoenzyme-II may be regulated by adenylates and glyoxylate or pyruvate, and in a manner that is probably allosteric, we have observed no sigmoidicity in the response to any of the substrates or effectors.

The activation of isoenzyme-II by low concentrations of glyoxylate or pyruvate appears to be a quite novel regulatory response and has no counterpart in the regulatory behaviour of eukaryotic NAD-linked isocitrate dehydrogenase. The stimulatory action of the effectors has been shown to be completely reversible and, like the adenylate regulation, is specific to isoenzyme-II. Some stimulation by glyoxylate or pyruvate of the isocitrate dehydrogenase activity in extracts of *Pseudomonas fluorescens* has been reported (Hampton & Hanson, 1969). However, we have

examined three strains of *Ps. fluorescens* but have been unable to detect any activation.

It is perhaps noteworthy that isoenzyme-II is a much larger enzyme than isoenzyme-I (Self & Weitzman, 1972), so that as in eukaryotic cells it is the larger enzyme which exhibits regulatory properties. That conformational rearrangements are associated with the stimulation of isoenzyme-II is supported by the finding of some protection against thermal inactivation by AMP and ADP (Parker & Weitzman, 1970), and against inactivation by both heat and urea by glyoxylate or pyruvate, as well as an alteration of the pH-dependence of activity brought about by all the effectors.

A likely mechanism for enzyme stimulation is the interaction of the activator with the enzyme at an allosteric site accompanied by conformation-mediated 'improvement' of the active site. Moreover, diverse activators would be expected to interact at independent sites, each being allosteric with respect to the active site. Our suggestion that such is indeed the case in the regulation of isoenzyme-II is based on several lines of evidence.

(i) Treatment of the enzyme with urea differentially affected the AMP response, the glyoxylate/pyruvate response and the catalytic activity.

(ii) The activations produced by AMP and glyoxylate/pyruvate are additive. The stimulatory ability of each type of effector alone is expressed undiminished in their joint presence.

(iii) Although there is a functional similarity between the two effector types there is a marked kinetic distinction. Whereas the activation by glyoxylate or pyruvate results in a considerable decrease in the K_m value for NADP⁺, the activation by AMP does not alter this parameter.

We therefore conclude that isoenzyme-II possesses two distinct regulatory sites. Interactions of these sites with their specific effectors lead to conformational and kinetic reorganization resulting in stimulation of enzymic activity.

Finally, let us consider the possible metabolic significance of these activation phenomena. The AMP/ADP effects may be related to the cellular control of energy metabolism. The relative concentrations of the adenine nucleotides provide a measure of the energy state or 'energy charge' (Atkinson, 1968) of the cell, AMP and ADP being 'low-energy' signals. The stimulation of isoenzyme-II by AMP and ADP might lead to enhanced tricarboxylic acid-cycle activity, resulting in phosphorylation and a decrease of the AMP/ADP concentration. The method of examining rate-dependence on energy charge proposed by Atkinson (1968) has been applied to isoenzyme-II and has shown the expected relationship (Parker & Weitzman, 1970).

The metabolic significance of the activation by glyoxylate or pyruvate is not so immediately obvious.

Isocitrate stands at a metabolic branch-point, and may be metabolized to α -oxoglutarate by isocitrate dehydrogenase or to glyoxylate and succinate by isocitrate lyase. A balance must be achieved between these alternatives. The stimulation of isocitrate dehydrogenase by glyoxylate, itself a product of the other branch, might contribute to the maintenance of such a balance, achieving an appropriate partitioning of isocitrate between the two pathways.

The stimulation of isoenzyme-II by pyruvate may be an example of 'precursor' (Sanwal, 1970) or 'feed-forward' (Shen & Atkinson, 1970) activation, in which a metabolite stimulates an enzyme further ahead of it in a metabolic pathway.

The double sensitivity of isoenzyme-II, to adenylate control on the one hand and to glyoxylate/pyruvate stimulation on the other, may permit the activity of the enzyme to respond both to the energy-producing and biosynthetic roles of the tricarboxylic acid cycle. The complexity of regulatory sensitivity of isocitrate dehydrogenase may reflect its key position in this important metabolic pathway.

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