Biochimica et Biophysica Acta, 452 (1976) 302—309

BBA 67986

MECHANISM OF THE INHIBITORY EFFECT OF GLYOXYLATE PLUS OXALOACETATE AND OXALOMALATE ON THE NADP-SPECIFIC ISOCITRATE DEHYDROGENASE

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(Received May 18th, 1976)

Summary

The effects of glyoxylate plus oxaloacetate and of oxalomalate on the NADP-linked isocitrate dehydrogenase (*threo*-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating, EC 1.1.1.42) from pig heart have been studied with steady state methods as well as with stopped flow technique. When equimolar mixtures of glyoxylate and oxaloacetate were premixed for different lengths of time prior to addition to the assay mixture, the extent of inhibition increased with the premixing time. The results indicated that the inhibition by glyoxylate plus oxaloacetate is caused by a compound formed in a reversible interaction between the two components.

Glyoxylate plus oxaloacetate and oxalomalate affected the enzyme in at least three different ways. They inhibited the enzyme in a reaction competitive with regard to the substrate isocitrate. This inhibition needed a certain time to be fully expressed. The time lag could be eliminated by premixing of the enzyme and inhibitor with NADP plus metal ion. Secondly, if the enzyme is premixed with NADP plus metal ions, a time lag occurs before the reaction rate approaches a constant value after initiation of the reaction with isocitrate. The inhibitors were found to enhance this effect of NADP plus metal ions on the enzyme. Thirdly, it has previously been shown that the enzyme can be activated by metal complexing agents. Glyoxylate plus oxaloacetate as well as oxalomalate are able to form complexes with metal ions and were found to cause an initial activation of the enzyme under certain assay conditions. The controversy regarding the mechanism of action of the above inhibitors on the enzyme is probably due to the fact that they affect the enzyme in several different ways.

Introduction

NADP-linked isocitrate dehydrogenases (*threo*-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) from eukaryotic and prokaryotic

organisms are strongly inhibited by mixtures of glyoxylate and oxaloacetate [1—15]. Addition of one of the compounds alone has usually only a negligible effect on the enzyme activity. It is not established whether the inhibition is caused by a concerted action of the two compounds [3,4] or by a condensation product e.g. oxalomalate or γ-hydroxy α-ketoglutaric acid [1,2,14,15].

The mechanism of inhibition is not understood. Kinetic experiments have shown that the inhibition by glyoxylate plus oxaloacetate as well as by oxalomalate is competitive with regard to isocitrate [3,5,15]. The fact that there is no simple structural analogy between isocitrate and the inhibitors [6], and the observation that the inhibition by glyoxylate plus oxaloacetate requires some minutes to be fully expressed [2,7,13], suggest that the inhibition is not caused by simple competition for the active site. This conclusion is furthermore supported by the observation [8] that the enzyme can be modified by SH reagents in such a way that the inhibition by glyoxylate plus oxaloacetate is lost while the enzyme activity is unaffected.

Isocitrate dehydrogenases need metal ions as cofactor, and Colman [16] has suggested that isocitrate complexed with metal ions is the "true" substrate for the enzyme and not isocitrate itself. Since glyoxylate and oxaloacetate as well as oxalomalate form complexes with metal ions in a similar way as the substrate isocitrate, the possibility exists that metal ions are involved in the inhibitory effect. We have recently found [17,18] that metal chelating agents may under certain conditions enhance the enzyme activity by a factor of more than 3 and remove the time lag found at low metal ion concentration before the absorption increases linearly with time. On the basis of this new information we have reinvestigated the mechanism of the inhibition by glyoxylate plus oxaloacetate and by oxalomalate by steady state kinetic methods as well as by stopped flow technique.

Materials and Methods

Materials. The NADP-dependent isocitrate dehydrogenase from pig heart, oxaloacetate and NADP were obtained from Boehringer, Mannheim GmbH, Germany. The enzyme was purified as described by Colman [19]. DL-Isocitrate (trisodium salt), glyoxylate, oxalomalate and EGTA (ethyleneglycol-bis(β-aminoethylether)) were purchased from Sigma Chem. Co., St. Louis, Mo.

Assay of enzyme activity. The activity was measured from the increase in absorption at 340 nm upon reduction of NADP. All activity measurements were made at 25°C in 20 mM phosphate buffer, pH 8.0). The steady state kinetic studies were carried out with a Gilford Model 2400 recording spectrophotometer. A Durrum stopped flow spectrophotometer (model D-100) was used in the presteady state kinetic experiments. Substrates and cofactor concentrations are given in the legends to the figures.

Glyoxylate and oxaloacetate. The compounds were dissolved in 40 mM phosphate buffer (pH 8.0) immediately before the start of the experiments. Both compounds were found to be stable under these conditions. Where indicated, the two compounds were premixed at equal concentrations and kept at room temperature for a certain period of time prior to the measurement of their inhibitory effect on the enzyme.

Treatment of data. The changes in transmission in the stopped flow experiments were displayed on a Tektronix storage oscilloscope. Photographs of the curves were taken with a Polaroid camera. The change in transmission was converted to absorbance and the data fitted to third order curves by the least square method [20]. The reaction rate was determined from the derivative of the curve. A Hewlett Packard calculator with an external XY-plotter was used.

Results

The fact that some authors [2,7,13] find that it takes some minutes before the inhibition by glyoxylate and oxaloacetate is fully expressed, while others [4] do not observe any time lag, suggests that the lag may depend on the experimental conditions. Since it has been claimed [1,2,14,15] that the inhibition by glyoxylate plus oxaloacetate is caused by a compound formed by interaction of the two substances, the possibility was considered that the lag is affected by mixing of glyoxylate and oxaloacetate prior to addition to the assay mixture. Fig. 1A shows that the lag time before the inhibition is fully expressed decreases with increasing mixing time of glyoxylate and oxaloacetate. Thus, when the two compounds had been premixed for 20 min prior to addition to the assay mixture, the time-absorbance curve becomes linear

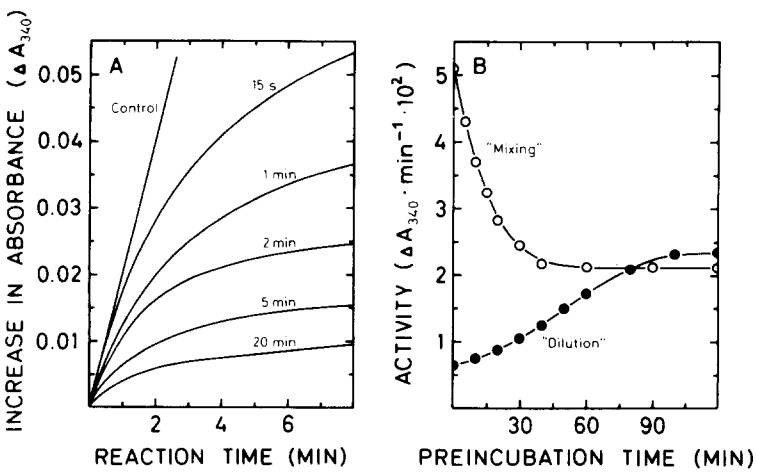

Fig. 1. Effect of premixing of glyoxylate and oxaloacetate on the inhibition of isocitrate dehydrogenase. (A) Increase in absorbance at 340 nm as a function of reaction time. Glyoxylate (10 mM) and oxaloacetate (10 mM) were preincubated at room temperature for the time indicated before addition to the assay mixture, and the reaction was initiated by addition of the enzyme. The final concentration in the assay mixture was 0.17 mM both for glyoxylate and oxaloacetate (B). Enzyme activity in the presence of glyoxylate and oxaloacetate which had been subjected to different treatments prior to addition to the assay mixture. Solutions containing glyoxylate and oxaloacetate in a final concentration of 1 mM of each were made (1) by mixing of the two compounds (curve marked "mixing") or (2) by diluting a concentrated mixture containing 10 mM of each components with buffer, just prior to the initiation of the experiment. The concentrated mixture had been premixed for 30 min prior to the dilution. The inhibitory effect was determined as a function of time after mixing or dilution. The final concentration of glyoxylate and oxaloacetate in the assay mixture was in all cases 8 nM. The assay mixture contained 0.3 mM isocitrate, 0.05 mM NADP and 0.3 mM $MgCl_2$.

after approximately 3—4 min, while for premixing times of less than 1 min, more than 8 min were needed before the time absorption curve becomes linear, i.e. the inhibition is fully expressed. It should be stressed that in all cases the time-absorption curves eventually become linear. The results show, moreover, that the extent of inhibition increases with the mixing time of glyoxylate and oxaloacetate.

Before studying in more detail the factors influencing the lag before the inhibition is fully expressed, further experiments on the effect of premixing on the extent of inhibition were performed. Fig. 1B shows that the inhibitory effect of the equimolar mixture of glyoxylate and oxaloacetate increases strongly during the first 30 min after mixing, and subsequently approaches a constant value (see curve marked "mixing"). On the other hand, if a concentrated solution of glyoxylate and oxaloacetate (10 mM of each) mixed for 30 min (until maximum inhibitory effect was obtained) was diluted 10 times with buffer, the inhibitory effect of this mixture decreases with time (curve marked "dilution"). In the experiments shown in Fig. 1B the final concentration during the preincubation both in the mixing and in the dilution experiments was the same (1 mM of both glyoxylate and oxaloacetate). The activity was in all experiments calculated from the increase in absorption occurring after the time absorption curve had become linear. It is apparent that the inhibitory effect of the two mixtures approaches the same value both in the mixing and in the dilution experiments after approximately 90 min. These results thus support the previous view that the inhibition by glyoxylate and oxaloacetate is caused by a compound formed from glyoxylate and oxaloacetate [1,2,14,15]. Moreover, the inhibitory compound is formed in a reversible reaction.

Further information on the factors influencing the time lag before the inhibition by glyoxylate and oxaloacetate is fully expressed was sought in experiments using stopped flow spectroscopy. The experiments shown in Fig. 2 have been carried out both with glyoxylate plus oxaloacetate as well as with oxalomalate. However, since the effect of glyoxylate and oxaloacetate depends strongly on the premixing conditions the experiments are more difficult to perform, and the data obtained with oxalomalate are presented. It should be stressed that the results with mixtures of glyoxylate and oxaloacetate corresponds closely to those shown for oxalomalate.

A small lag time is observed in the stopped flow experiments before the absorption started to increase linearly with time. This lag [17] is considerably greater when the enzyme is premixed with NADP and Mg^{2+} (Fig. 2B) than when premixed with isocitrate and Mg^{2+} (Fig. 2A) prior to initiation of the reaction. Interestingly, when oxalomalate was mixed with the enzyme at the same time as the reaction was started, the lag time disappeared. Thus, under these conditions oxalomalate activated the enzyme immediately after initiation of the reaction. The slope of the absorption curve obtained in the presence of oxalomalate levelled off after a short time, and the inhibition was fully expressed after approximately one min. On the other hand, if the enzyme was premixed with NADP and Mg^{2+} together with oxalomalate the inhibition was fully expressed when the reaction was initiated by addition of isocitrate (Fig. 2B). With all other combinations of substrate, co-factor and

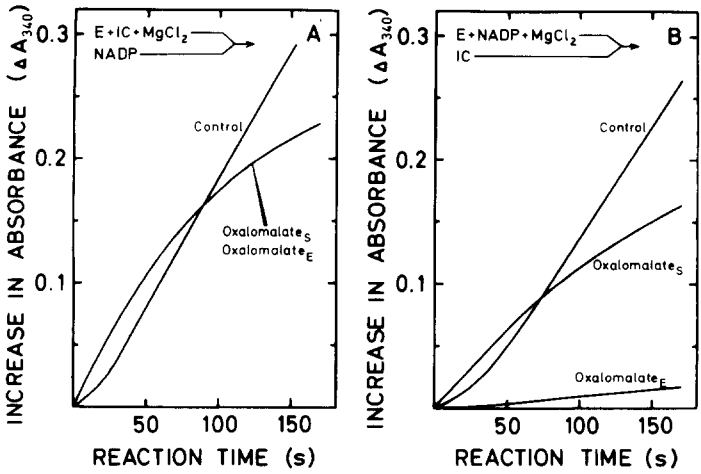

Fig. 2. Effect of different premixing conditions on the inhibition by oxalomalate. (A) Increase in absorption as a function of time after initiation of the reaction by addition of NADP. The enzyme was preincubated with isocitrate and $MgCl_2$. Oxalomalate was present as indicated. (B) Increase in absorption as a function of time after initiation of the reaction by addition of isocitrate. The enzyme was preincubated with NADP and $MgCl_2$. Oxalomalate was present as indicated. The experiments were carried out with stopped flow spectrophotometry. The subscript to oxalomalate in the text on the figures indicate whether the inhibitor was present together with the enzyme (E) or with the substrate used to initiate the reaction (S). The final concentrations were: 0.9 mM isocitrate, 0.05 mM NADP and 0.03 mM $MgCl_2$ and 2 mM oxalomalate where indicated.

co-enzyme it was found that a lag time was needed before the inhibition was fully expressed independently of whether oxalomalate was premixed with the enzyme or not. From these experiments it can be concluded that the inhibitor reacts with the enzyme in a relatively slow reaction and that this reaction requires both NADP and metal ion.

One possible interpretation of the above results is that the inhibitor is converted to a second, more potent inhibitor by the enzyme in a reaction requiring NADP plus metal ions. In order to test this hypothesis, additional enzyme was added to an assay mixture after the inhibition had been fully expressed (Fig. 3A). Glyoxylate plus oxaloacetate were used in this experiment. It is apparent that a new lag occurs after addition of more enzyme before the inhibition is fully expressed again. Moreover, the results show in agreement with the data in Fig. 2B, that the lag disappears if the inhibitors are preincubated with enzyme, NADP and metal ions prior to initiation of the reaction with isocitrate. Preincubation of the inhibitors with enzyme, isocitrate and metal ions had no effect on the lag time.

Since glyoxylate plus oxaloacetate as well as oxalomalate can form complexes with metal ions, the effect of the metal concentration on the inhibition was studied. Fig. 3B shows that the inhibition increases with increasing Mg^{2+} concentration. These results thus show that the inhibition cannot be caused by a competition with isocitrate for the metal ions. Ruffo et al. [2] have previously studied the effect of Mn^{2+} and Mg^{2+} on the inhibition. They found in contrast to our results that the inhibition patterns with Mg^{2+} were different for oxalomalate and mixtures of glyoxylate plus oxaloacetate.

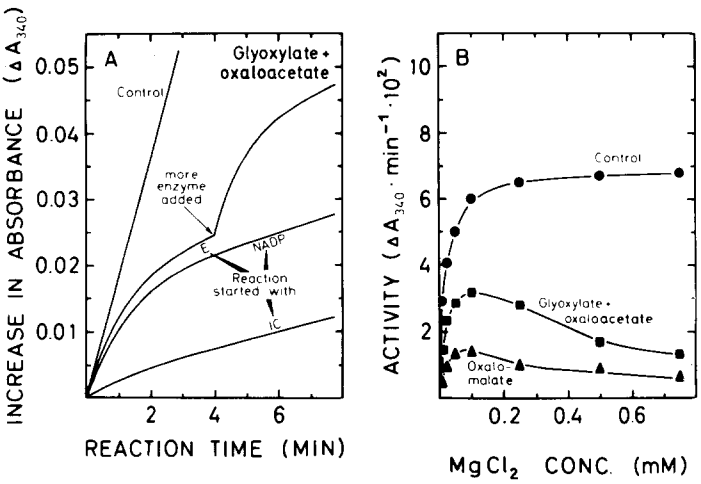

Fig. 3. (A) Increase in absorbance as a function of reaction time in the absence and presence of glyoxylate plus oxaloacetate. Aliquots of glyoxylate plus oxaloacetate were added to the assay mixture 15 s prior to the initiation of the reaction by addition of enzyme, isocitrate or NADP. When the reaction was initiated by the enzyme, an equal amount of additional enzyme was added after 4 min. The concentration of $MgCl_2$ was 0.3 mM. (B) Enzyme activity as a function of $MgCl_2$ concentration. The activity was measured in the absence of any inhibitors as well as in the presence of glyoxylate plus oxaloacetate (4 nm) of each, and oxalomalate (0.25 mM). The glyoxylate plus oxaloacetate had in all experiments been premixed in a concentration of 10 mM for 30 min. The assay mixture contained 0.3 mM isocitrate and 0.05 mM NADP.

We have previously found [17,18] that metal chelating agents remove the time lag and increases the enzyme activity. In Fig. 4 the effect of ethyleneglycol-bis(β-aminoethylether) *N,N'*-tetraacetic acid (EGTA) on the inhibition by different concentrations of oxalomalate was studied. Oxalomalate was in all experiments preincubated with the enzyme together with NADP and metal ions. The results in the absence of the activator, EGTA, is shown in Fig. 4A. The lag increases with increasing concentrations of oxalomalate. In fact, there is a linear relationship between the lag time before the absorption increases linearly with time, and the concentration of oxalomalate (insert). We have previously obtained evidence [17] that NADP plus metal ions transform the enzyme to a nonactive state. The lag time observed is in part explained on this basis. Apparently, oxalomalate enhances the effect of NADP and Mg^{2+} in transforming the enzyme. Consequently, the time needed for isocitrate to activate the enzyme will increase with increasing concentrations of oxalomalate.

EGTA prevents the induction of the non-active state by NADP plus metal ions [18]. In the presence of EGTA (Fig. 4B) no lag is observed (compare curves marked No EGTA and Control), and the inhibitory effect of oxalomalate is fully expressed from the start of the reaction. Interestingly, oxalomalate inhibited the enzyme to a considerably larger extent in the presence of EGTA (insert, data taken both from panel A and B). Thus, it is apparent that 50% inhibition in the absence of EGTA required 2—3 times higher oxalomalate concentration than in the presence of EGTA.

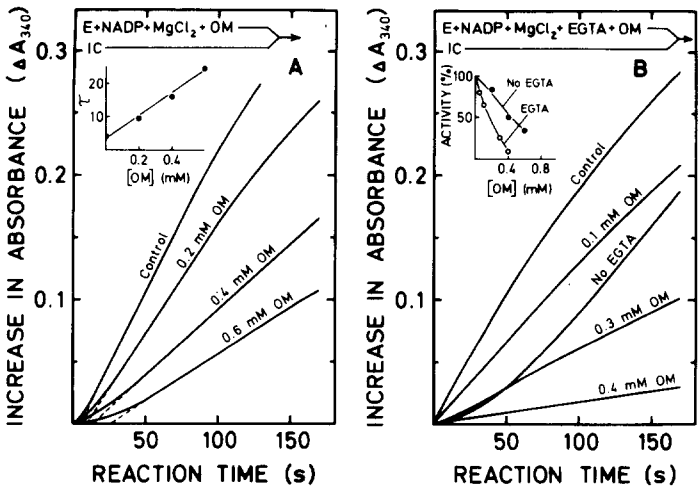

Fig. 4. The inhibitory effect of oxalomalate. The increase in absorbance as a function of time after mixing was determined in the absence (A) and in the presence of EGTA (B). The enzyme was in all experiments preincubated with NADP and $MgCl_2$. Oxalomalate and EGTA were present together with the enzyme as indicated. The reaction was initiated by the addition of isocitrate. The insert in Panel A shows the lag time as a function of oxalomalate concentration. The lag time was determined by extrapolation of the curve to zero change in absorption as indicated. The insert in Panel B shows the inhibition as a function of the oxalomalate concentration in the absence and presence of EGTA (data taken both from Panel A and B). The experiments were carried out with the stopped flow spectrophotometer. The final concentrations were: 0.3 mM isocitrate, 0.05 mM NADP, 0.03 mM $MgCl_2$ and 0.03 mM EGTA where indicated. Since EGTA activates the enzyme, less enzyme protein was used in the experiments shown in Panel B than in those in Panel A.

Discussion

The present results indicate that the inhibition of the NADP-dependent isocitrate dehydrogenase by glyoxylate plus oxaloacetate and by oxalomalate are caused by interaction of the inhibitors with a site different from the binding site of the substrate, and that the allosteric inhibition is mediated through a relatively slow change in the conformation of the protein. The results support the view [1,2,14,15] that the inhibition by glyoxylate plus oxaloacetate is caused by a complex or condensation product formed between the two substances. In all experiments it was found that oxalomalate which is formed upon mixing of glyoxylate and oxaloacetate [1,14,15] showed the same inhibition pattern as mixtures of glyoxylate plus oxaloacetate. It is not possible from the present experiments to decide whether the condensation product responsible for the inhibition upon mixing of glyoxylate and oxaloacetate is oxalomalate or another compound.

Previous authors [3,5,15] have found that glyoxylate plus oxaloacetate as well as oxalomalate inhibit the enzyme in a competitive manner with regard to isocitrate, and in a noncompetitive manner with regard to NADP. Our results confirm this (data not presented). On this basis it has been claimed that the inhibition is non-allosteric. The present results do not support this conclusion. Thus, the inhibition needs a considerable time to be fully expressed unless the inhibitors are preincubated with the enzyme together with NADP plus metal

ions. Secondly, we have previously shown [18] that citrate which competes with isocitrate for the active site inhibits the enzyme to the same extent independently of whether the activator EGTA is present or not. EGTA itself has only a slight effect on K_m for isocitrate [18]. If oxalomalate inhibited the enzyme by competing for the active site the same inhibition pattern would be expected as found for citrate. However, the present results show that oxalomalate inhibits the enzyme considerably more efficiently when the enzyme is activated by the presence of EGTA than in the absence of EGTA.

The results show that glyoxylate plus oxaloacetate and oxalomalate affects the enzyme in several ways. As discussed above they inhibit the enzyme in a competitive manner with regard to isocitrate. Secondly, the inhibitors increases the lag time found when the enzyme is preincubated with NADP plus metal ions prior to initiation of the reaction with isocitrate. We have previously found that NADP and metal ions convert the enzyme to a non-active state [17] and apparently the inhibitors enhance this effect. Thirdly, due to the ability of the inhibitors to form complexes with metal ions they are able to activate the enzyme in a similar way as previously shown for metal chelating agents [18]. The fact that the enzyme is affected in several different ways by the inhibitors is probably the reason for the controversy concerning the mechanism of inhibition by glyoxylate plus oxaloacetate.

Acknowledgements

This work was supported by The Norwegian Research Council for Science and the Humanities. The author is indepted to Dr. T. Sanner for helpful discussions. The able technical assistance of Mrs. Astri Nordahl and Mrs. Margareth Skogland is gratefully acknowledged.

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