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ACTION OF SULPHITE ON PLANT MALATE DEHYDROGENASE

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Key Word Index—*Spinacia oleracea*, Chenopodiaceae; spinach; *Zea mays*; Gramineae maize; malate dehydrogenase, NAD- and NADP-dependent; SO_3^{2-}-inhibition; change in MW and conformation by SO_3^{2-}.

Abstract—SO_3^{2-} acts on NAD- and NADP-dependent malate dehydrogenase in several ways. Firstly, SO_3^{2-} favours the appearance of low MW species (65000 and 39000 daltons) in Sephadex gel chromatography. Secondly, the enzyme form which is obtained by gel chromatography with dithioerythritol plus nucleotide cofactor is changed in the presence of SO_3^{2-}. This is indicated by the appearance of a linear reaction (instead of curvilinear) and by the abolition of the biphasic sigmoidal kinetics on varying substrate and cofactor concentrations. SO_3^{2-} causes the loss of negative cooperativity at low substrate or cofactor concentrations. Thus the inhibition of initial velocity at high substrate or cofactor concentrations is even more marked than at lower ones. Thirdly, SO_3^{2-} strongly reduces the activity in substrate saturating conditions.

INTRODUCTION

As SHOWN in the preceding paper, the MW of the NAD-dependent malate dehydrogenase (E.C. 1.1.1.37) from spinach leaves is regulated by ions and the cosubstrate.[1] Changes in reaction rate and in substrate and cofactor kinetics indicate different enzyme forms which may represent different conformations. The same is true for the NADP-dependent enzyme.

A MW species of 127000 daltons, obtained by gel chromatography of the NAD-dependent enzyme with dithioerythritol (DTE) + NADH, has a special interest: at non-saturating substrate concentrations its reaction rate is non-linear and its kinetics with respect to substrate and cofactor concentrations respectively are characterized by two slopes with positive cooperative substrate interaction, separated by an intermediary plateau of negative cooperativity.[2]

The activity of heart muscle malate dehydrogenase is strongly inhibited by sulphite.[3,4] This is caused by the formation of a sulphite-NAD addition compound which exerts a strong inhibitory action.[3] Since SO_2, entering the plant through the stomates, is initially dissolved during the formation of HSO_3^-/SO_3^{2-},[5] the action of SO_3^{2-} on both plant malate dehydrogenases was investigated as a model reaction of SO_2 action.

The K_i-values of SO_3^{2-} for phosphoenolpyruvate carboxylase of *Zea mays* are high:[6]

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[1] ZIEGLER, I. (1974) *Phytochemistry*, **13**, 2405.
[2] LEVITZKI, A. and KOSHLAND, Jr. D. E. (1967) *Proc. Nat. Acad. Sci. U.S.* **62**, 1121.
[3] PFLEIDERER, G., JUCKEL, D. and WIELAND, T. H. (1956) *Biochem. Z.* **328**, 187.
[4] PFLEIDERER, G. and HOHNHOLZ, E. (1959) *Biochem. Z.* **331**, 245.
[5] ZIEGLER, I. (1972) *Planta* **105**, 155.
[6] ZIEGLER, I. (1973) *Phytochemistry* **12**, 1027.

84·5 mM with respect to Mg^{2+}; 27 mM with respect to HCO_3^-; no inhibition at all with respect to PEP at concentrations up to 10 mM SO_3^{2-}. Since the action of PEP-carboxylase in plants possessing the C_4-dicarboxylic acid pathway of photosynthesis is followed by that of NAD- and NADP-dependent malate dehydrogenase,[7] it is of interest to know whether the further metabolism of CO_2 in the presence of SO_3^{2-} is impaired due to a marked sensitivity of malate dehydrogenase to this anion.

RESULTS

The action of SO_3^{2-} *on the activity at substrate saturating conditions*

In agreement with the findings of Johnson and Hatch[8] incubation of the 50–60% ammonium sulfate fraction with DTE (0·8 mg/ml; 60 min at 33°) causes a 2–4-fold increase in the activity of the NADP-dependent malate dehydrogenase, whereas the activity of the NAD-dependent enzyme remains essentially unchanged. Figure 1 demonstrates the decrease in activity of the NAD- and NADP-dependent malate dehydrogenase in substrate saturating conditions, when SO_3^{2-} is present in the assay medium. In all of the three experiments performed, incubation with DTE has no effect on the sensitivity of the NAD-dependent enzyme. At SO_3^{2-} concentrations of 0·2–0·3 mM, the non-activated, NADP-dependent enzyme is less impaired. All enzyme forms maintain 10–15% of their activity even up to concentrations of 5 mM SO_3^{2-}.

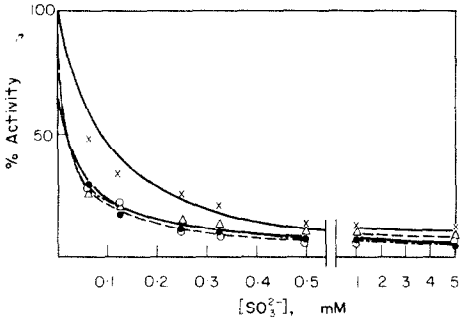

FIG. 1. EFFECT OF SO_3^{2-}, PRESENT IN THE ASSAY MEDIUM, ON THE ACTIVITY OF NAD- AND NADP-DEPENDENT MALATE DEHYDROGENASE IN SUBSTRATE SATURATING CONDITIONS.
The reaction was started by addition of the enzyme (crude homogenate of *Zea mays*): × ··· × NADP-dependent malate dehydrogenase, without prior incubation with DTE; ○ —○ NADP-dependent malate dehydrogenase, with prior incubation with DTE; ● ·-·-● NAD-dependent malate dehydrogenase, without prior incubation with DTE; △ – △ NAD-dependent malate dehydrogenase, with prior incubation with DTE.

In Table 1 the action of SO_3^{2-} is not referred to the percentage of the normal activity, but to the actual turnover rates. It demonstrates that the remaining activity is the same whether the enzyme is preincubated with DTE or not. Since preincubation increases the activity of the NADP-dependent enzyme, a lower percentage of inactivation with the non-activated enzyme results.

[7] HATCH, M. D. and SLACK, C. R. (1970) *Ann. Rev. Plant Physiol.* **21**, 141.
[8] JOHNSON, M. S. and HATCH, M. D. (1970) *Biochem. J.* **119**, 273.

The action of SO_3^{2-} on the formation of different MW forms; their characterization by reaction rate and substrate kinetics

Gel chromatography and addition of SO_3^{2-} to the solvent system, but not to the subsequently used assay medium, were employed to study the action of SO_3^{2-} on the formation of different MW species. Gel chromatography of the 60–75% ammonium sulphate fraction of spinach with 50 mM Tris buffer (pH 8) + DTE (0·8 mg/ml) + NADH (0·05 mM) results in only one MW species of 127000 daltons. Addition of 0·25 mM SO_3^{2-} to this solvent system causes the formation of additional MW forms. The correspond to those obtained by the action of K^+ ions, which favour disaggregation of the enzyme.[1]

TABLE 1. ACTIVITY OF NAD- AND NADP-DEPENDENT MALATE DEHYDROGENASE WITHOUT AND WITH PRIOR INCUBATION WITH DTE AT VARIOUS SULPHITE CONCENTRATIONS

	NAD-dependent malate dehydrogenase ($\Delta\epsilon$ 340 nm/mg protein/hr)		NADP-dependent malate dehydrogenase ($\Delta\epsilon$ 340 nm/mg protein/hr)	
	Without DTE incubation	After DTE incubation	Without DTE incubation	After DTE incubation
No SO_3^{2-}	900	970	400	800
0·067 mM SO_3^{2-}	210	215	220	220
0·5 mM SO_3^{2-}	110	110	70	70

Addition of 0·25 mM SO_3^{2-} to the solvent system containing 50 mM Tris buffer (pH 8) + 0·1 mM KCl produces similar results, as does prior incubation of the enzyme with DTE.[1] In both cases the formation of a malate dehydrogenase of low MW (39000 daltons) is favoured.

The 127000 daltons MW form, stabilized by the addition of NADH to the solvent system, is characterized by a non-linear reaction rate at low substrate concentrations and by complex, sigmoidal kinetics with respect to changing substrate and cosubstrate concentrations (see Figs. 1 and 2 in [1]). Though after addition of 0·25 mM SO_3^{2-} this MW species still predominates, its reaction rate at low substrate concentration and its substrate kinetics are changed. They approach those obtained by chromatography with Mg^{2+} or K^+ ions without addition of NADH (see Figs. 1 and 4 in [1]) or by prior dilution of the enzyme (see Fig. 6 in [1]). The substrate kinetics of the MW forms obtained by addition of 0·25 mM SO_3^{2-} to the K^+ or Mg^{2+} containing solvent system show no difference after statistical evaluation to those obtained without SO_3^{2-} (see Fig. 4 in [1]).

It should be emphasized that the presence of 0·25 mM SO_3^{2-} in the chromatographic solvent system but its absence in the assay medium, caused no or only very slight (< 10%) inhibition of total activity of the fractions obtained. The enzyme is only changed in its MW, its reaction rate, and its substrate kinetics. This indicates a change in its conformation.

The direct action of SO_3^{2-} on activity, reaction rate and substrate kinetics

In the previous section the indirect effect of SO_3^{2-} in addition to the chromatographic system was studied. Addition of SO_3^{2-} to the assay medium, however, enables the direct effect of this ion on activity, reaction rate and substrate kinetics to be tested.

In addition to chromatography with the nucleotide cosubstrate, incubation with DTE

and the subsequent presence of 0·1 mM NADH or NADPH in the assay medium also results in a non-linear reaction rate and biphasic substrate kinetics with both the NAD- and the NADP-dependent enzyme.[1] In contrast to the NAD-dependent malate dehydrogenase, however, the NADP-dependent enzyme concomitantly shows a 2–4-fold increase in activity. For both effects it makes no difference whether the corresponding ammonium sulfate fraction (60–75% saturation) or the crude homogenate is employed.

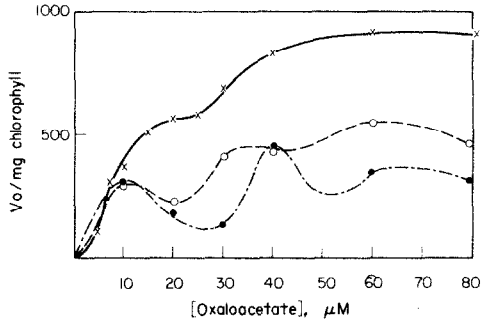

FIG. 2. INITIAL VELOCITY OF THE NADP-DEPENDENT MALATE DEHYDROGENASE AT 0·1 mM NADH VS. INCREASING SUBSTRATE CONCENTRATIONS, AFTER INCUBATION OF THE EXTRACT OF *Zea mays* WITH DTE. The reaction was started by addition of the enzyme: × ····· × without SO_3^{2-}; ○–––– ○ 0·125 mM SO_3^{2-} in the assay medium; ● · · · ● 0·250 mM SO_3^{2-} in the assay medium.

Thus, for example, the effect of SO_3^{2-} on the kinetics of the NADP-dependent enzyme at varied substrate and cosubstrate concentrations in the crude homogenate and in the ammonium sulfate fraction is given (Figs. 2 and 3). In both examples which were repeated

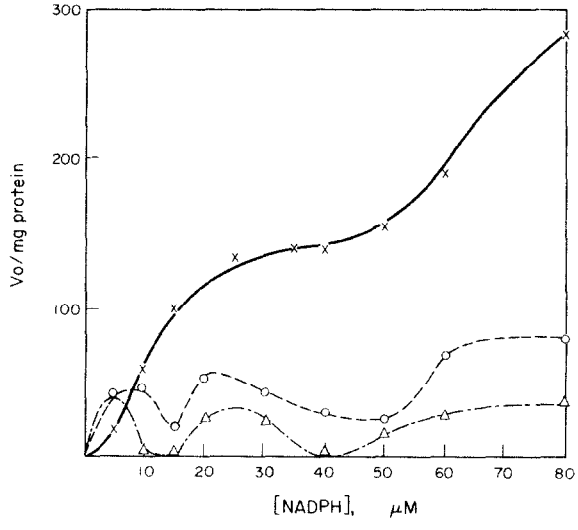

FIG. 3. INITIAL VELOCITY OF THE NADP-DEPENDENT MALATE DEHYDROGENASE AT 0·1 mM OXALOACETATE VS. INCREASING COFACTOR CONCENTRATIONS, AFTER INCUBATION OF THE AMMONIUM SULPHATE FRACTION WITH DTE.
The reaction was started by addition of the enzyme: × ····· × without SO_3^{2-}; ○ ····· ○ 0·125 mM SO_3^{2-} in the assay medium; △ · · · △ 0·250 mM SO_3^{2-} in the assay medium.

4 times, the same type of kinetics were obtained. Due to the less refined enzyme used, the absolute values differed. Firstly, a strong inhibitory action of SO_3^{2-} is observed when it is present in the assay medium. The percentage of inhibition increases with increasing substrate or cofactor concentration. Secondly, the curvilinear reaction rate at non-saturating substrate concentrations becomes linear and the kinetics with respect to substrate or cofactor concentrations are changed. This favours the idea that the enzyme is not only inhibited, but by the action of SO_3^{2-} its conformation is also changed, as it was after chromatography in the presence of SO_3^{2-}. Consequently, the only slight inhibitory action or even "stimulatory" effects of SO_3^{2-} at low substrate concentrations can be explained: the negative cooperativity of the original conformation[1] at such concentrations is no longer found.

Low MW species are obtained if the enzyme is first diluted with the buffer of the assay system and the reaction is then started by the addition of NADH + oxaloacetate. These seemingly undergo conformational changes if the substrate concentration is varied (see Fig. 6 in [1]). SO_3^{2-} added to the assay system strongly reduces their activity, but it does not further alter their substrate kinetics at concentrations up to 0·1 mM SO_3^{2-}. They essentially correspond to those which were obtained by the action of sulphite on the enzyme, previously incubated with DTE (see Figs. 2 and 3).

DISCUSSION

The experiments described show that both the NAD- and the activated NADP-dependent malate dehydrogenase are similarly affected by SO_3^{2-} in two ways. Firstly, a strong inhibition takes place. It is evident that the usual method for the determination of the K_i value cannot be used in this instance; the half-maximum inhibition at about 0·75 mM and 0·25 mM SO_3^{2-} respectively shows that the sensitivity of both malate dehydrogenase is much greater than that of ribulosediphosphate carboxylase and of phosphoenolpyruvate carboxylase.[5,6] The fact that a residual activity of 10–15% even at high SO_3^{2-} concentrations is maintained, whether or not the enzyme was preincubated with DTE (and thus, in the case of the NADP-dependent enzyme, activated) indicates that the action of SO_3^{2-} results in an enzyme form of reduced activity which, however, cannot be further inhibited.

Secondly, SO_3^{2-} causes a splitting of the aggregated malate dehydrogenase. The conformation which is characterized by a non-linear reaction rate at low substrate concentrations and by biphasic substrate and cosubstrate kinetics is thereby destroyed. It is already known that SO_3^{2-} causes a decomposition of disulfide proteins through the cleavage of S–S bonds in polypeptide chains.[9] Such a decomposition may take place here. An effect of SO_3^{2-} on the conformation and thus on the regulatory properties of an enzyme is possibly a quite general phenomenon. SO_2 fumigation of pea seedlings changes the pattern of glutamate dehydrogenase conformers obtained after polyacrylamide gel electrophoresis, thus indicating a change in the quaternary and tertiary structures of the enzyme.[10] Whereas the SO_2 in this case was applied *in vivo*, the present investigations are *in vitro* experiments and thus the pH value could be controlled and kept absolutely constant. Therefore the speculations of Pahlich[10] that the conformational changes are only due to local acidification are doubtful; rather, a more specific action of SO_3^{2-} on the enzyme's structure through the splitting of S–S bonds must be considered.

[9] Bersin, Th. (1950) *Adv. Enzymology* **10**, 223.
[10] Pahlich, E. (1972) *Planta* **104**, 78.

Inhibition studies on PEP-carboxylase and NAD-malate dehydrogenase in extracts of *Zea mays* have shown that malate formation is not impaired by 10 mM SO_3^{2-}, since PEP-carboxylase is relatively insensitive towards SO_3^{2-}, but is clearly the rate limiting step of the reaction sequence. As a consequence, even at 85% inhibition of malate dehydrogenase enough of the enzyme activity remains to enable the metabolism of the oxaloacetate occurring through the action of PEP-carboxylase.[6] The extent to which CO_2-fixation in plants with the C_4-dicarboxylic acid pathway of photosynthesis is affected, however, is also dependent on the fact that SO_3^{2-} causes strong conformational changes with both malate dehydrogenases. This results, for instance, in an even more pronounced inhibition at higher substrate or cofactor concentrations than at lower ones.

EXPERIMENTAL

Extraction of fully grown leaves of *Spinacia oleracea* and *Zea mays* and the subsequent ammonium sulfate fractionation of the extracts was carried out as described.[1] In extracts of spinach the bulk of NAD-MDH was found between 60–75% $(NH_4)_2SO_4$ saturation. In *Zea mays* the fraction of 50–60% saturation contained 98% of the NADP-dependent and 63% of the NAD-dependent activity.

Sephadex G-200 superfine gel chromatography, spectrophotometric determination of activity and calculation of initial velocity are also described.[1]

As indicated in [5] dissolution of either Na_2SO_3 or $Na_2S_2O_5$ at pH 7·9 yields an equilibrium of HSO_3^-/SO_3^{2-} of about 1:9·8. For the experiments described here, Na_2SO_3 was used. It should be emphasized that at all sulphite concentrations used, the pH was held constant. If not otherwise indicated, the sulphite was added to the test cuvette containing oxaloacetate + NADH and the reaction was started by addition of the enzyme. Chlorophyll determinations were made according to Arnon.[11]

[11] ARNON, D. I. (1949) *Plant Physiol.* **24**, 1.