BBA 65875

OXALOACETATE INHIBITION OF ACONITATE HYDRATASE

JOHN S. BRITTEN

Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032 (U.S.A.)

(Received November 14th, 1968)

SUMMARY

An oxaloacetate–Fe^{2+} complex has been found to be a potent competitive inhibitor of aconitate hydratase. The requirement for both oxaloacetate and Fe^{2+} in this inhibition appears to be absolute.

INTRODUCTION

Cis-aconitate hydratase (aconitase, EC 4.2.1.3) has not been characterized in the detail accorded many other Krebs-cycle enzymes. This relative neglect of part of the central machinery of energy metabolism is due to the instability of the enzyme. Reasonably stable preparations are purified only 30-fold[1] and reports of higher purification (approx. 100-fold) emphasize that the purified enzyme loses activity in a matter of a few days[2]. At present, work with aconitate hydratase must be conducted with relatively crude preparations. Yet even crude preparations containing the known stabilizing factors, citrate, Fe^{2+}, and a reducing agent[3], exhibit loss of activity over the course of hours on incubation at 25–38°. In attempts to minimize the instability of crude aconitate hydratase it was noted that the loss of enzymatic activity was much more pronounced in the presence of 0.04 M fumarate, maleate, D-malate, or L-malate than it was in the presence of 0.04 M 2-ketoglutatate, succinate, or pyruvate. Because of the relative delay in onset of effect of the inhibitory acids and differences in their relative effect at various pH values in the range 6–9, it appeared likely that some common metabolic product of the inhibitory acids was responsible for the observed inhibition. Oxaloacetate was thought to be the most likely suspect, and so a detailed investigation of the effect of oxaloacetate on *cis*-aconitate hydratase was begun. A pronounced inhibition by oxaloacetate was discovered. This inhibition depended on the simultaneous presence of both oxaloacetate and Fe^{2+}. No other divalent cation (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}) resulted in similar inhibition of aconitate hydratase in the presence of oxaloacetate.

METHODS AND MATERIALS

Citrate was estimated by the method of McARDLE[4], and protein by the biuret method of GORNALL *et al.*[5]. Aconitate hydratase was used in two forms. 'Crude aconitate hydratase' was an homogenate of frozen pig heart obtained according to step one in the method of MORRISON[1]. 'Purified aconitate hydratase' was a partially purified preparation obtained by the absorption of crude enzyme on a CM-cellulose column previously equilibrated with 0.002 M Tris–citrate buffer (pH 6.5). After washing the column with this same buffer until the effluent was clear of protein, the enzyme was eluted in batch fashion by washing with 0.03 M Tris–citrate buffer (pH 6.5). The eluate, containing 2–3 mg protein per ml, was fractionated and concentrated by $(NH_4)_2SO_4$ precipitation. The protein remaining in solution after addition of 46 g $(NH_4)_2SO_4$ per 100 ml of solution and precipitated by an additional 8.6 g $(NH_4)_2SO_4$ per 100 ml of solution was collected by centrifugation, taken up in a small amount of 0.02 M Tris–acetate (pH 7.4) and dialyzed against a solution of 0.5 mM $Fe(NH_4SO_4)_2$, 4 mM sodium citrate, and 14 mM mercaptoethanol in 0.02 M Tris–HCl buffer (pH 7.4) and stored in that buffer until used. In this way a 12–15-fold purification was obtained.

Aconitate hydratase was activated and assayed according to MORRISON[1]. Initial velocities of the enzyme catalyzed conversion of *cis*-aconitate to citrate were recorded in μmoles citrate produced per min per mg protein of enzyme preparation. Great care was taken to insure that the rate of citrate production was linear with respect to time and protein concentration in calculation of initial velocities.

Inhibition data are presented graphically in double-reciprocal LINEWEAVER–BURK plots[6], of the initial velocity of the aconitate hydratase catalyzed conversion of *cis*-aconitate to citrate against the *cis*-aconitate concentration initially present.

RESULTS

The inhibition of purified aconitate hydratase in the presence of oxaloacetate and Fe^{2+} is shown in Fig. 1. Very little inhibition was observed with oxaloacetate or with Fe^{2+} alone, but a pronounced competitive inhibition was found when both were present. At a fixed oxaloacetate concentration the inhibition increased with increased $[Fe^{2+}]$, and at a fixed $[Fe^{2+}]$ inhibition increased with increased oxaloacetate. This type of inhibition could also be demonstrated in the absence of the gluconate and mercaptoethanol which were added to hold the iron salt in solution and in the proper oxidation state. Because activated aconitate hydratase was used in these experiments, at least 10 μM Fe^{2+} was present in all reactions[1]. If unactivated aconitate hydratase was employed, the concentration of Fe^{2+} could be reduced to 0.1 μM. At this lower $[Fe^{2+}]$ no inhibition by oxaloacetate alone could be detected, while the addition of Fe^{2+} resulted in the expected enzyme activation[7]. Together oxaloacetate and Fe^{2+} inhibited unactivated aconitate hydratase in a manner similar to that shown in Fig. 1.

Fe^{2+} is known to promote the decarboxylation of oxaloacetate to pyruvate and CO_2 (ref. 8). But inhibition of aconitate hydratase does not occur in the presence of Fe^{2+} and pyruvate, nor does Fe^{2+} inhibit when aconitate hydratase is assayed in a Krebs bicarbonate buffer. In order to assess the structural specificity of oxaloacetate in the oxaloacetate–Fe^{2+} interaction involved in the inhibition of aconitate hydratase, a number of other carboxylic acids were examined for similar effects. No comparable

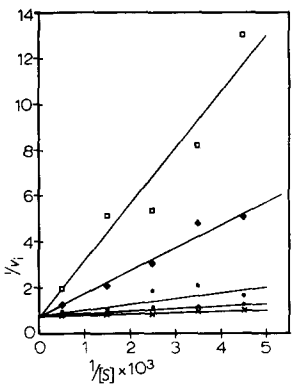


Fig. 1. Variation in Lineweaver–Burk plots of aconitase activity with [Fe^{2+}] and [oxaloacetate]. v_1 in μmoles citrate produced per min per mg enzyme protein and [S] in M sodium *cis*-aconitate. The 5-ml reaction mixture contained sodium *cis*-aconitate as indicated, Tris–acetate (1 mmole, pH 7.4), activated enzyme (0.024 mg protein), cysteine (1 μmole), $Fe(NH_4SO_4)_2$ (0.05 μmole), Na gluconate (11 μmoles), mercaptoethanol (71.4 μmoles) either alone (×), with sodium oxaloacetate (5 μmoles) (○), with $Fe(NH_4SO_4)_2$ (11 μmoles) (●), with sodium oxaloacetate (5 μmoles) and $Fe(NH_4SO_4)_2$ (11 μmoles) (◆), or with sodium oxaloacetate (10 μmoles) and $Fe(NH_4SO_4)_2$ (11 μmoles) (□). Incubation was at 38° for 15 min.

inhibition was found with other 2-keto acids, 3-keto acids, 2-hydroxy acids, Krebs cycle intermediates, Fe^{2+} chelators, or any other compound tested. In particular, the nonenolizable analogue of oxaloacetate, 3,3′-dimethyloxaloacetate, failed to produce any inhibition.

In order to examine the specificity of Fe^{2+} in the inhibition of aconitate-hydratase activity by oxaloacetate and Fe^{2+}, the effect of other divalent cations was examined. Several complications were anticipated. Divalent cations might be expected to compete in some fashion with Fe^{2+} in the role that ion plays in aconitate-hydratase activity. Some divalent cations might inactivate sulfhydryl groups known to be essential to enzymatic activity[9,10]. Further, standardization of oxaloacetate concentrations in the presence of different divalent cations is difficult, as the rate of oxaloacetate decarboxylation is controlled in part by the particular divalent cation present[8]. These obstacles seem to allow only crude relative comparisons of the effect of various oxaloacetate–M^{2+} complexes on aconitate-hydratase activity. To provide a base line for these observations, the effect of various divalent cations on aconitate hydratase was determined. Simultaneously the effect of both cation and oxaloacetate was assessed. In each case the data were analyzed in the form of Lineweaver–Burk plots and the maximal initial velocity (v_{max}) and the apparent Michaelis–Menten constant (K_m'), together with estimates of errors of these quantities, were determined by the method of least squares. The results are given in Table I. To facilitate comparisons of the results of inhibition by oxaloacetate–M^{2+} to that with oxaloacetate alone, the ratio of the dissociation constant of the enzyme–inhibitor complex to the concentration of inhibitor, $K_i/[I]$, was calculated for each ion according to the equation

$$K_m' = K_m (1 + [I]/K_i) \quad (1)$$

Since the use of this equation presupposes the existence of competitive inhibition,

TABLE I

MICHAELIS–MENTEN CONSTANT, K_m', AND MAXIMAL INITIAL VELOCITY, v_{max}, OF ACONITASE IN THE PRESENCE OF VARIOUS SALTS ALONE AND TOGETHER WITH OXALOACETATE

The constants were determined by the analysis of Lineweaver–Burk plots by the method of least squares. For each curve the experiment was conducted according to the procedure given in Fig. 1. This data has been used to calculate the ratio of inhibitor–enzyme dissociation constant to inhibitor concn., $K_i/[I]$. v_{max} is expressed in μmoles citrate per min per mg protein.

Salt (μmoles)	*Alone*		*Oxaloacetate (5 μmoles)*		*Calculated*
	$K_m' \times 10^4$	v_{max}	$K_m' \times 10^4$	v_{max}	$K_i/[I]$
—	0.6 ± 0.1	1.00 ± 0.02	1.7 ± 0.1	1.14 ± 0.04	0.55 ± 0.11
$CaCl_2$ (10)	0.8 ± 0.1	1.16 ± 0.04	1.9 ± 0.6	1.02 ± 0.17	0.72 ± 0.41
$CoSO_4$ (10)	0.4 ± 0.1	0.96 ± 0.03	1.3 ± 0.1	1.17 ± 0.03	0.44 ± 0.13
$Fe(NH_4SO_4)_2$ (10)	0.75 ± 0.01	1.25 ± 0.01	27.0 ± 3.0	1.5 ± 0.2	0.029 ± 0.003
$MgSO_4$ (10)	0.84 ± 0.01	1.19 ± 0.01	1.5 ± 0.1	1.16 ± 0.04	1.27 ± 0.19
$MnSO_4$ (10)	0.9 ± 0.4	1.0 ± 0.1	2.2 ± 0.1	1.14 ± 0.01	0.69 ± 0.39
$NiSO_4$ (10)	0.72 ± 0.02	1.18 ± 0.03	0.98 ± 0.02	1.17 ± 0.03	2.77 ± 0.31
$ZnSO_4$ (10)	4.3 ± 0.2	0.83 ± 0.03	5.6 ± 0.7	0.59 ± 0.06	3.3 ± 1.9
—	0.86 ± 0.04	0.98 ± 0.01	1.3 ± 0.3	1.02 ± 0.07	2.0 ± 1.3
$CdSO_4$ (2)	2.1 ± 0.4	0.21 ± 0.02	1.9 ± 0.1	0.20 ± 0.01	— —
$CuSO_4$ (2)	2.1 ± 0.1	0.46 ± 0.01	2.8 ± 0.3	0.42 ± 0.03	3.0 ± 1.4
$Fe(NH_4SO_4)_2$ (2)	0.9 ± 0.04	1.00 ± 0.01	1.71 ± 0.3	1.06 ± 0.08	1.1 ± 0.4
$PbAc_2$ (2)	1.1 ± 0.2	0.59 ± 0.02	1.3 ± 0.1	0.57 ± 0.02	5.5 ± 6.2
$ZnSO_4$ (2)	1.7 ± 0.1	0.89 ± 0.03	2.2 ± 0.2	0.72 ± 0.04	3.4 ± 1.5

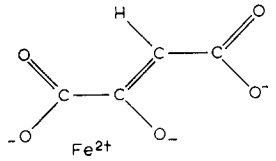
application to the case of Zn^{2+} does not appear valid. For the other cations, however, the calculations show that, except in the case of Fe^{2+} and Co^{2+}, the effect of oxaloacetate–M^{2+} is to give less inhibition of aconitate hydratase than does oxaloacetate alone. Moreover, the difference between $K_i/[I]$ for Co^{2+} and for no added ion is not statistically significant. Fe^{2+} is unique among these cations in the inhibitory effect on aconitate hydratase in the presence of oxaloacetate.

It should be emphasized that all of the preceding studies have involved the action of oxaloacetate on aconitate hydratase in the presence of substrate (*cis*-aconitate). If aconitase is exposed to oxaloacetate without substrate being present, a different type of inhibition is found. Very small concentrations suffice to inhibit enzyme activity totally. Further, the inhibition so produced persists even after the level of detectable oxaloacetate determined by the method of KALNITSKY AND TAPLEY[12] falls to zero. Aconitate hydratase, inactivated by oxaloacetate in this fashion, can not be reactivated either by exhaustive dialysis against the citrate–Fe^{2+}–mercaptoethanol buffer used in enzyme preparation, or by the activation procedure of MORRISON[1].

DISCUSSION

The evidence presented here suggests that an oxaloacetate–Fe^{2+} complex is a powerful competitive inhibitor of aconitate hydratase. Oxaloacetate–M^{2+} complexes of this type are known to inhibit citrate lyase (citrate oxaloacetate-lyase, EC 4.1.3.6), another enzyme of citrate metabolism[13,14]. Like aconitate hydratase, citrate lyase requires a divalent metal ion. It is tempting to assume that in each case the oxalo-

acetate–M^{2+} complex approximates the configuration of the enzyme–substrate normally present. The evidence presented suggests that both negative charges are necessary in the formation or action of the oxaloacetate–Fe^{2+} complex in the inhibition of aconitate hydratase. It also appears necessary that the keto group be able to enolize, although the absence of inhibition by dimethyloxaloacetate might be due to steric interference by the two methyl groups. These suggested requirements are the same as those found in the formation of the oxaloacetate–M^{2+} complexes involved in the M^{2+}-catalyzed decarboxylation of oxaloacetate. Consequently a structure of the type



might be anticipated as the inhibitor. This structure bears an intriguing resemblance to the 'bound' carbonium ion intermediate proposed by SPEYER AND DICKMAN[15] in the action of aconitate hydratase.

Superficially it would appear unlikely that the inhibition of aconitate hydratase by oxaloacetate in the presence of divalent cation is a significant factor in the control of oxidative respiration. Any oxaloacetate so involved would be rapidly decarboxylated and thus inactivated as an inhibitor. While oxaloacetate inhibition could be maintained by a steady production of the compound, respiratory regulation by oxaloacetate seems less likely by this route than by inhibition of the succinate dehydrogenase system[16,17]. However, the studies of SIMPSON[18,19] on the renal disposition of citrate are interesting in this regard. He has established that elevated serum bicarbonate leads to increased citrate excretion in the dog, and that increased pCO_2 leads to increased citrate levels and decreased citrate utilization in both slices of rabbit kidney and in preparations of rabbit kidney mitochondria. To a lesser degree, decreased utilization of 2-ketoglutarate was noted in rabbit kidney mitochondrial preparations under elevated pCO_2. This effect of CO_2 was most closely related to the intracellular HCO_3^- concentration and did not appear to be mediated by pH changes.

It is possible that the pCO_2 regulates the steady state of oxaloacetate in these systems by way of the various CO_2 fixation reactions and that the oxaloacetate in turn regulates the activity of aconitate hydratase and, perhaps, the succinate-dehydrogenase system. Elevated pCO_2 would then be expected to increase oxaloacetate levels, decrease aconitate-hydratase activity and so lead to the observed elevation of citrate levels and decreased citrate utilization. Oxaloacetate inhibition of the succinate dehydrogenase system might be expected to lead to the observed decrease in 2-ketoglutarate utilization and this would also tend to decrease citrate utilization. However, it should be emphasized that the relationship of oxaloacetate to these physiological observations remains to be established.

REFERENCES

1 J. F. MORRISON, *Biochem. J.*, 56 (1954) 99.
2 M. J. PALMER, *Biochem. J.*, 89 (1963) 98P.

3 S. R. DICKMAN AND A. A. CLOUTIER, *J. Biol. Chem.*, 188 (1951) 379.
4 B. MCARDLE, *Biochem. J.*, 60 (1955) 647.
5 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
6 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
7 J. F. MORRISON, *Biochem. J.*, 58 (1954) 685.
8 J. F. SPECK, *J. Biol. Chem.*, 178 (1949) 315.
9 H. A. KREBS AND L. V. EGGLESTON, *Biochem. J.*, 38 (1944) 426.
10 J. F. MORRISON, quoted by S. R. DICKMAN, *The Enzymes*, Vol. 5, Academic Press, New York, 1961, p. 435.
11 M. DIXON AND E. C. WEBB, *The Enzymes*, 2nd ed., Academic Press, New York, 1964, p. 318.
12 G. KALNITSKY AND D. F. TAPLEY, *Biochem. J.*, 70 (1958) 28.
13 S. DAGLEY AND E. A. DAWES, *Biochim. Biophys. Acta*, 17 (1955) 177.
14 R. EISENTHAL, S. S. TATE AND S. P. DATTA, *Biochim. Biophys. Acta*, 128 (1966) 155.
15 J. F. SPEYER AND S. R. DICKMAN, *J. Biol. Chem.*, 220 (1956) 193.
16 E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 87 (1963) 639.
17 W. P. ZEYLEMAKER AND E. C. SLATER, *Biochim. Biophys. Acta*, 132 (1967) 210.
18 D. P. SIMPSON, *Am. J. Physiol.*, 206 (1964) 883.
19 D. P. SIMPSON, *J. Clin. Invest.*, 46 (1967) 225.