Fast and Slow Inhibitors of Ox Heart Succinate Dehydrogenase: Action of Oxaloacetate and its Fluoro Analogues

L. S. KAPPEN,* J. D. BRODIE and P. NICHOLLS


The potent inhibition of succinate dehydrogenase by oxaloacetate involves a lag period during which the degree of inhibition gradually increases. Reversal of inhibition by excess of succinate also occurs slowly. Wojtczak et al. (1969) suggest that oxaloacetate induces a reversible conformation change in the enzyme, whereas Zeylemaker et al. (1969) propose the rapid formation of an initial EI complex followed by its slow conversion into a second, tighter, EI’ complex. Most recently, Vinogradov et al. (1972) have implicated an enzyme thiol group and suggest thiosemiacetal formation as the slow step. The interconversion may thus involve changes in enzyme chemistry or conformation as well as the keto–enol tautomerism of oxaloacetate. We used fluorinated inhibitor analogues to study this problem, since introduction of one fluorine atom into oxaloacetate decreases enolization (Hellerman et al., 1960; Kun et al., 1958) and enolization is precluded in the case of difluoro-oxaloacetate. The enol form of oxaloacetate is approximately isosteric with monofluoromaleate, and the effects of halo-substituted maleates were therefore also examined.

Succinate dehydrogenase, obtained from Keilin–Hartree ox heart submitochondrial particles by the method of King (1967), was stored in liquid N₂. Monofluoro-oxaloacetic acid and difluoro-oxaloacetic acid were prepared as described by Pattison et al. (1956) and Pattison (1959), fluoromaleic acid was prepared as described by Raasch et al. (1959), chloro- and bromo-substituted maleic acids were prepared by anhydride hydrolysis; all acids were recrystallized from nitromethane. Dehydrogenase activity was measured by dichlorophenol-indophenol reduction with phenazine methosulphate as intermediate electron carrier. Inhibition rates were obtained by adding enzyme to otherwise complete reaction mixtures, and equilibrium inhibition was obtained by incubating enzyme with inhibitor and succinate before starting the reaction with phenazine methosulphate. Traces of oxaloacetate were determined fluorimetrically, with malate dehydrogenase and NADH. As reported (Wojtczak et al., 1969; Zeylemaker et al., 1969), oxaloacetate inhibition increased with time. The final inhibition was competitive, and \( K_i \) (oxaloacetate) was between 0.1 and 0.2 \( \mu M \) and \( K_m \) (succinate) was 0.2–0.5 \( \mu M \). Monofluoro-oxaloacetate and difluoro-oxaloacetate showed a similar slow inhibitory effect, as did fluorofumarate and fluoromaleate. Fumarate, maleate, chloromaleate and bromomaleate gave an immediate inhibition that did not increase with time (as with the classical inhibitor malonate). Addition of excess of succinate reversed the inhibition in all cases. A marked lag was again observed with the oxaloacetates. Inhibition constants calculated from double-reciprocal plots are listed in Table 1. Monofluoro-oxaloacetate has a much larger \( K_i \) than oxaloacetate. This confirms the report by Hellerman et al. (1960), and suggests that oxaloacetate inhibition may involve the enol form, diminished in concentration on the substitution of hydrogen by fluorine. Difluoro-oxaloacetate is a still weaker inhibitor. Difference spectra of the enzyme in the presence and in the absence of these inhibitors have shown that all three give complexes of the oxaloacetate type, whereas all the ‘fast’ inhibitors give complexes of the malonate type (Tober et al., 1970).

Fluorofumarate and fluoromaleate also behave as ‘slow’ inhibitors, but inhibition is attributable to other factors. Oxaloacetate can be produced from fluorofumarate by traces of fumarase in the dehydrogenase preparation (Tober et al., 1970). Fluoromaleate as prepared also forms oxaloacetate; and 0.01% oxaloacetate induces an appreciable

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Table 1. Inhibition of ox heart succinate dehydrogenase by fluoro analogues of oxaloacetate and maleate

$k_{in}$: calculated from the rate of inhibition by oxaloacetate according to:

$$k_{in} = \frac{k}{(K_m + s)} \cdot \frac{[\text{oxaloacetate}]}{K_m}$$

where $k$ is the first-order rate (Fig. 1).

$k_{off}$: calculated from the rate of reactivation by succinate according to:

$$k_{off} = \frac{1}{\text{lag} \ ' \ \text{time}}.$$

$K_i$: obtained by direct equilibration in the presence of inhibitor and succinate (competitive).

Conditions were as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pattern</th>
<th>Spectral type†</th>
<th>$K_i$ (µM)</th>
<th>$k_{on}$ (m$^{-1}$·s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$k_{off}/k_{on}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxaloacetate</td>
<td>Slow</td>
<td>II</td>
<td>0.15</td>
<td>$4 \times 10^4$</td>
<td>$5 \times 10^{-3}$</td>
<td>0.13</td>
</tr>
<tr>
<td>Fluoro-oxaloacetate</td>
<td>Slow</td>
<td>II</td>
<td>9.0</td>
<td>$10^3$</td>
<td>$4 \times 10^{-3}$</td>
<td>~4.0</td>
</tr>
<tr>
<td>Malonate</td>
<td>Fast</td>
<td>I</td>
<td>1400</td>
<td>5</td>
<td>$6 \times 10^{-3}$</td>
<td>~1200</td>
</tr>
<tr>
<td>Maleate</td>
<td>Fast</td>
<td>I</td>
<td>2300</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fluoromaleate</td>
<td>Fast‡</td>
<td>I‡</td>
<td>2700</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chloromaleate</td>
<td>Fast</td>
<td>I</td>
<td>3700</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bromomaleate</td>
<td>Fast</td>
<td>I</td>
<td>9600</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Malonate</td>
<td>Fast</td>
<td>I</td>
<td>4.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* At inhibitor concentrations giving 50–90% inhibition.
† Type I spectrum: like that induced by malonate (peak at 510nm in difference spectrum); type II spectrum: like that induced by oxaloacetate (broad absorption in the range 530–600nm in difference spectrum).
‡ When the contribution due to contaminant oxaloacetate is eliminated (see the text).

inhibition. But, if final traces of oxaloacetate are removed by heating at pH 7.8 (100°C for 5 min), the resulting fluoromaleate gives a high $K_i$ (approx. 2.7 nm) and shows no inhibition increase with time. A time-course of inhibition by oxaloacetate is shown in Fig. 1. If such exponential curves, representing inhibitor binding, are replotted in semi-logarithmic form, straight lines are then obtained, as in Fig. 1(b). Similar results were obtained with the two fluoro analogues. Table 1 lists the apparent association and dissociation rate constants. Variation in $K_i$ largely reflects different rates of binding and not of dissociation.

The rate of oxaloacetate inhibition is similar to that reported by Wojtczak et al. (1969), in the catalytic system, and by Zeylemaker et al. (1969), who used direct spectrophotometric measurement of binding. It is larger than the previously estimated rate of binding of stoichiometric quantities of oxaloacetate in the absence of succinate (Zeylemaker et al., 1969) and also larger than the rate obtained with submitochondrial particles (Brodie & Nicholls, 1970). Indeed, it approaches the rate of succinate binding (Tober et al., 1970). The rate of release of inhibition (approx. 0.005 s$^{-1}$) is similar to that observed by Wojtczak et al. (1969). If the enol form of oxaloacetate were the initially reactive form, the marked decrease in reactivity of the monofluoro- and difluoro-substituted analogues would be expected. However, the slow dissociation cannot be attributed to keto–enol interconversion, as it also characterizes the release of difluoro-oxaloacetate inhibition. The latter compound cannot exist in the enol form. We believe that a preferred initial reaction of the enzyme with the enol form of oxaloacetate is followed by either an intramolecular protein conformation change or by the thiosemiacetal reaction proposed by Vinogradov et al. (1972). Differences in the reactivity of the soluble and membrane-bound enzymes may be associated with different enzyme conformations or thiol accessibilities in the two environments. Like Wojtczak et al. (1969), we have been unable
Fig. 1. Time-course of the inhibition of ox heart succinate dehydrogenase by oxaloacetate

The incubation mixture contained (final concentrations) 0.1 M-sodium–potassium phosphate buffer, pH 7.8, 1.3 mM-KCN, 83 μM-dichlorophenol-indophenol, 330 μM-phenazine methosulphate, 2 mM-succinate, 10 μM-oxaloacetic acid and approx. 36 μg of enzyme added to 3 ml final volume at 26°C. Measurements were made at 610 nm. (a) Rate of dichlorophenol-indophenol reduction (ε: ΔE610/min) at various times; (b) the same data replotted as log(rate–final rate) versus time.

t+ ~ 6

Whether or not the rate constants are such as to permit such a separation may be a fairly subtle function of the preparation of enzyme employed.

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The Effect of Ischaemic Limb Injury on the Rate of Utilization of Ketone Bodies in the Post-Absorptive Rat in vivo

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The early stages of the response to a severe injury in the post-absorptive rat in a 20°C environment are characterized by a fall in body temperature, with lower rates of whole-body O\textsubscript{2} consumption and of glucose and free fatty acid oxidation (Stoner, 1970). After one such injury, bilateral hind-limb ischaemia, the concentration of ketone bodies in the liver rose, apparently because a higher proportion of the free fatty acids utilized were converted into them (Barton, 1971). This raised the question whether there was a corresponding increase in the rates of utilization of ketone bodies by extrahepatic tissues.

The rates of utilization can be written in the form \( k \cdot V \cdot C \), where \( C \) is the blood concentration and \( k \cdot V \) is a proportionality constant. Experiments in control rats with \( \beta \)-hydroxy[\textsuperscript{14}C]butyrate and [\textsuperscript{14}C]acetoacetate showed that for both substrates \( k \cdot V \) was independent of concentration, so that the rate of utilization was proportional to concentration, and values of \( k \cdot V \) could be calculated (Barton, 1972). For acetoacetate \( k \cdot V \) \((20.9 \pm 0.7, \text{ s.e.m., } \text{ml/min}^{-1} \cdot 100\text{g}^{-1})\) was much higher than for \( \beta \)-hydroxybutyrate \((5.8 \pm 0.3\text{ml/min}^{-1} \cdot 100\text{g}^{-1})\). Multiplied by their respective concentrations these values gave a total rate of utilization of ketone bodies of \(2.5 \pm 1.6\text{(1.6–3.9, s.d. range) } \mu\text{mol} \cdot \text{min}^{-1} \cdot 100\text{g}^{-1}\); the large standard deviation is due to the variability of the concentrations. The mean rate is equivalent to about 7\% of the whole-body O\textsubscript{2} consumption at 20°C (Stoner, 1969), assuming complete oxidation.

After hind-limb ischaemia, the rates of utilization of \( \beta \)-hydroxybutyrate and acetoacetate were still proportional to their blood concentrations, and the values of \( k \cdot V \) were respectively \(12.1 \pm 0.4\) and \(4.0 \pm 0.2\text{ml/min}^{-1} \cdot 100\text{g}^{-1}\). Since \( k \cdot V \) is a complex coefficient, these values should not be directly compared with those in the controls. Owing to an increase in the mean concentrations of both \( \beta \)-hydroxybutyrate and acetoacetate, their total rate of utilization rose to \(3.4 \pm 3.5\text{(2.3–5.1, s.d. range) } \mu\text{mol} \cdot \text{min}^{-1} \cdot 100\text{g}^{-1}\), in contrast with the decreased rates of glucose and free fatty acid oxidation. This is equivalent to an average of 15\% of the O\textsubscript{2} consumption, which falls by 44\% in these rats (Stoner, 1969).

These results emphasize that at these relatively low concentrations utilization of ketone bodies is a passive process and that its rate is determined by concentration.