# FORMATE AS AN INHIBITOR OF CYTOCHROME c OXIDASE

Peter Nicholls [+]

Institute of Biochemistry, University of Odense,

DK-5000 Odense, Denmark

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SUMMARY: Formate inhibits cytochrome c oxidase with a $K_i$ between 5 and 30 mM at pH 7.4 (depending on assay conditions). The formate binding site is accessible in the fully oxidized ($a^{3+}$ $a_3^{3+}$) and partially reduced ($a^{2+}$ $a_3^{3+}$) states, but not in the fully reduced ($a^{2+}$ $a_3^{2+}$ state). Azide competes with formate for the binding site. Formate induces a blue shift in the Soret peak of fully oxidized enzyme. The rate of formate binding, and the apparent affinity ($1/K_i$), increase as the pH is diminished, suggesting that HCOOH is the bound species.

Four reversible terminal inhibitors of the respiratory chain reacting with ferric cytochrome $a_3$ have been identified, cyanide, azide, sulphide, and fluoride (see ref. 1). Of these cyanide and sulphide react slowly and dissociate very slowly, while azide induces a marked shift in the spectrum of reduced cytochrome a. Other ferric haemoproteins, including peroxidase and catalase, do not show any such anomalous behaviour towards these ligands. But the comparative principles developed by Keilin lead us to expect some analogies between inhibitor binding by the terminal oxidase and by the soluble enzymes.

Formate is both an inhibitor and a hydrogen donor for catalase (2), with which it reacts rapidly and reversibly. The

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[+] Present address: Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada

formate-catalase complex is a high spin one, while the corresponding cyanide and sulphide complexes are low spin, and the catalase-azide complex is of a mixed type. An attempt was therefore made to inhibit mammalian cytochrome c oxidase with formate. The results obtained indicate that formic acid is bound at the sixth coordination position of ferric haem iron in cytochrome $a_3$, forming a high spin ferric complex not readily reducible by electrons from cytochrome c.

MATERIALS AND METHODS: Submitochondrial particles of the Keilin-Hartree type were prepared as previously described (3). Maximal cytochrome c oxidase activity was elicited by 1% deoxy cholate treatment, according to Smith and Camerino (4). Cytochrome $aa_3$ was isolated according to van Buuren's modification (5) of the method of Fowler et al. (6).

Spectrophotometry was carried out with either a Hitachi Perkin-Elmer 356 dual wavelength instrument or a Cary 118C recording spectrophotometer. Oxygen electrode measurements were made using a Radiometer electrode with appropriate amplifier and recorder.

Formate was added either as sodium formate (J.T. Baker 'Analyzed') or ammonium formate solution (Hopkin & Williams' 'AnalaR'). Cytochrome c was Sigma Type VI (horse). Ascorbic acid was neutralized with KOH and stored as a frozen solution.

Cytochrome $aa_3$ content is obtained using $\Delta E$, 605-630 nm, reduced-oxidized, equal to 27 $mM^{-1}$ $cm^{-1}$.

RESULTS AND DISCUSSION: Fig. 1 illustrates the kind of result obtained when formate is added to an aerobic steady-state mixture of ascorbate, cytochrome c oxidase-containing particles in buffer. As described previously (7), the amount of active enzyme (E) present following the addition of inhibitor is given by:

$$[E]_t/[E]_o = [c^{3+}]_t/[c^{2+}]_t \times [c^{2+}]_o/[c^{3+}]_o \qquad (1)$$

where the subscripts 'o' and 't' indicate the concentrations of the indicated component at time zero and time t respectively. The addition of 7.25 mM formate to the mixture changes the steady-state from 39% to 55% reduction, indicating almost 50%

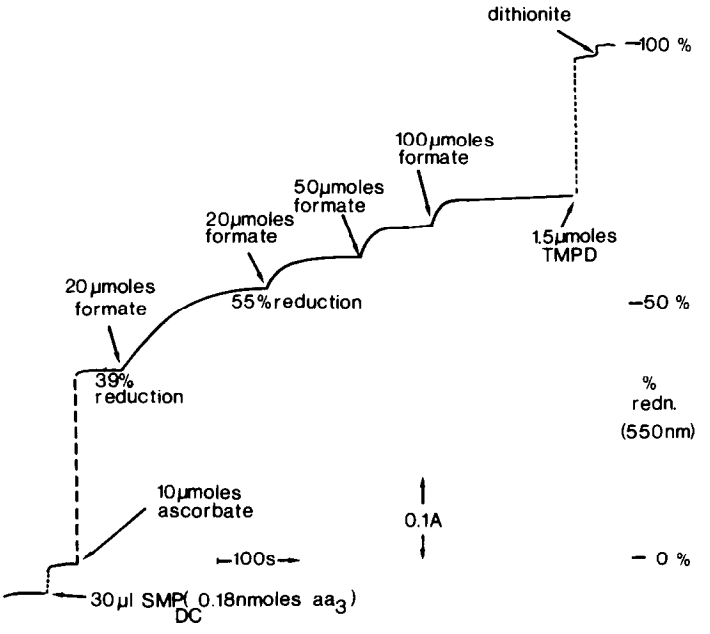


Fig. 1. Inhibition of cytochrome c oxidase activity by formate. 2.7 ml of a solution containing 30 µM cytochrome c in 67 mM phosphate pH 7.4 buffer at 26°. Additions of 30 µl deoxy cholate treated submitochondrial particles (SMPDC) and ascorbate as indicated. TMPD indicates N,N,N'N'-tetramethyl-p-phenylene-diamine. Absorbance measured at 550 nm.

net inhibition of the enzyme. The inhibition at this pH takes a few minutes to complete, but at lower pH values the effect is much faster. Addition of formate some minutes before initiation of activity with ascorbate abolishes this delay in reaching the final inhibited state. Analogous results are obtained using the conventional oxygen electrode technique, but with somewhat higher apparent $K_i$ values.

Fig. 2 shows that similar slow inhibition patterns are seen with intact respiratory chain systems oxidizing succinate. The slow binding of formate permits the analysis of events occurring after the initial anaerobiosis (point a). If oxygen is added back (bubbling air) the steady-state returns to its initial value (b) and formate binding begins again until anaerobiosis sets in (c).

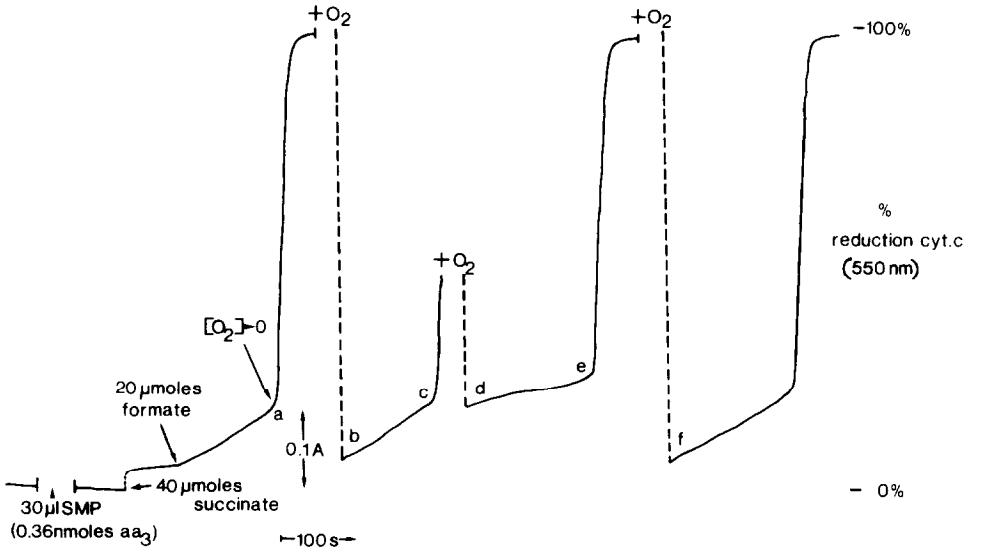


Fig. 2. Inhibition of succinate oxidase activity by formate. 2.7 ml of a cytochrome c solution were used as in Fig. 1. Addition of 30 µl intact submitochondrial particles (SMP) and succinate as indicated. Anaerobiosis begins at points (a), (c) and (e). Reaeration shows formate dissociation at points (b) and (f) but not at (d) (see text). Absorbance measured at 550 nm.

If oxygen is now admitted without delay, it is evident that the system is still inhibited (d) and the inhibited state persists until anaerobiosis occurs again (e). Allowing full reduction of the system to occur before oxygenation for the third time reverses the formate inhibition (point f). Evidently the formate binding site goes slowly reduced at anaerobiosis, and formate is released.

Fig. 3 supports the idea that azide and formate are competing for a common binding site. If azide is added to an uninhibited enzyme system (e.g. the control treated with acetate instead of formate) the new inhibited steady-state is attained immediately with no subsequent change until anaerobiosis occurs. But if formate is added first, giving rise to the formate-inhibited state (a), subsequent addition of azide produces an immediate high level

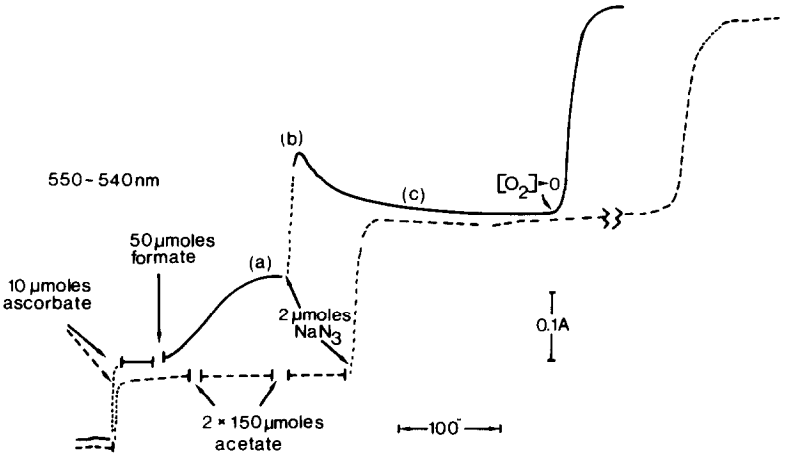


Fig. 3. Displacement of formate by azide. 2.7 ml of a solution at 29°C containing 30 µM cytochrome c, 0.4 µM $aa_3$ in the form of submitochondrial particles, and 67 mM phosphate buffer pH 7.4 were used. Additions of ascorbate and inhibitors as indicated. Either formate (———) or acetate (------) addition preceded azide addition. The inhibition by formate (point a) is increased by azide (point b) but subsequent formate dissociation restores partial activity (point c). Absorbance measured at 550-540 nm.

of inhibition (b), followed by a relaxation to the final steady level (c). This phenomenon is apparently a consequence of the faster equilibration between enzyme and azide than between enzyme and formate. 18 mM formate induces about 75% inhibition of the enzyme ($K_i \simeq 6$ mM); 0.7 mM azide induces 84% inhibition of the enzyme ($K_i \simeq 0.13$ mM). Immediately after azide addition, therefore, only 4% of enzyme remains free (21% being azide-bound and 75% formate-bound). Finally, however, 11% of free enzyme appears (leaving 56% azide-bound and 33% formate-bound).

The rate of formate dissociation estimated from such experiments is close to that obtained from the product $k_{on} \times K_i$. Table I compares the rate and equilibrium constants for the binding of cyanide, sulphide, azide, and formate to cytochrome $aa_3$. Binding rates actually obtained with formate of 2 $M^{-1}s^{-1}$ at pH 7.4, and 20 $M^{-1}s^{-1}$ at pH 6.3, and $K_i$ values of 8 mM at pH 7.4, and 1 to

2 mM at pH 6.3, are given in this table recalculated in terms of the concentration of free formic acid. Association rates of cyanide (7), sulphide (8), and formate are similar, but formate has a larger $K_i$ because it dissociates at least twenty times as rapidly as the two 'low-spin' ligands.

Spectroscopic studies, to be reported in more detail elsewhere, show that formate induces a blue shift in the Soret peak of fully oxidized cytochrome $\underline{aa}_3$, suggesting a low to high spin state transition. Unlike azide and sulphide (8), formate does not cause any well-defined shifts in the reduced steady-state spectrum, the α-band attributed to cytochrome $\underline{a}$ remaining close to 605 nm.

Formate has a further potential advantage over azide and cyanide. Its rates of binding and dissociation in isolated

Table I. Comparison of formate inhibition of cytochrome oxidase with that by cyanide, sulphide and azide.

| | Inhibitor | | | |
|---|---|---|---|---|
| | HCN | $H_2S$ | $HN_3$ | HCOOH |
| $K_i$ ⁂ (μM) | 0.1 | 0.025 | 0.15 | 2.0 |
| $k_{on}$ ⁂ ($M^{-1}s^{-1}$) | $5x10^3$ | $4x10^4$ | $2x10^6$ | $8x10^3$ |
| $k_{off}$ ($s^{-1}$) | $5x10^{-4}$ | $10^{-3}$ | 0.3 | 0.016 |
| pK | 9.1 | 7.0 | 4.7 | 3.7 |

⁂ All values refer to undissociated acid, using the indicated pK value to calculate its concentration at pH 7.4, the usual experimental pH. 25°C, 67 mM potassium phosphate buffer, mammalian cytochrome $\underline{c}$ oxidase acting on cytochrome $\underline{c}$.

systems, coupled with its low pK (Table I) make it a possible topological probe for cytochrome $a_3$ in intact mitochondria. Experiments to test this last possibility are under way.

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