The carbanion of nitroethane is an inhibitor of, and not a substrate for, flavocytochrome $b_2$ [L- (+)-lactate dehydrogenase]

Roger GENET* and Florence LEDERER†
Institut National de la Santé et de la Recherche Médicale Unité 25, Centre National de la Recherche Scientifique Unité Associée 122, Hôpital Necker, 161 rue de Sèvres, 75743 Paris Cedex 15, France

Although nitroethane does not bind to the active site of flavocytochrome $b_2$, its anion, ethane nitronate, behaves as a competitive inhibitor, with a $K_i$ of 2.2 mM. No electron transfer can be detected between the nitronate and the enzyme, in contrast with the observations of other workers on D-amino acid oxidase. Propionate is a competitive inhibitor, with a $K_i$ of 28 mM. The significance of these results with respect to the proposed carbanion mechanism and the putative existence of a covalent enzyme-substrate intermediate is discussed.

INTRODUCTION

A number of flavoenzymes catalyse the oxidation of $\alpha$-hydroxy and $\alpha$-amino acids. The chemical mechanism of the reaction is generally believed to involve abstraction of the substrate $\alpha$-hydrogen as a first step. Electrons would then be transferred to the flavin, possibly after the formation of a covalent bond between substrate C-2 and flavin N-5 (for reviews, see [1–5]).

Flavocytochrome $b_2$ (EC 1.1.2.3) is an atypical member of this class of enzymes insofar as it contains stoichiometric amounts of FMN and protohaem IX [6]. It catalyses the oxidation of lactate to pyruvate at the expense of monoelectronic acceptors such as ferricyanide and cytochrome $c$. The structure of the tetrameric protein from baker’s yeast (Saccharomyces cerevisiae) has been determined and refined to 0.24 nm (2.4 Å) resolution [7,8]. There exists for this enzyme an important body of evidence in favour of the carbanion mechanism [10–12], and the active-site chains have been ascribed a catalytic role in terms of this mechanism [12].

Nevertheless, neither solution studies nor the crystal structure have provided any clues about the way in which electrons are transferred from the carbanion to the flavin in flavocytochrome $b_2$ [12]. The generally proposed possibilities are the successive transfer of two single electrons [4] or transfer through an intermediate covalent adduct between the carbanion carbon and flavin N-5 [1]. We have addressed this problem by studying the enzyme behaviour towards nitroethane and ethane nitronate.

The nitroethane function has two interesting properties. Firstly, the nitro group is considered to be isosteric with a carboxylate group. Secondly, the hydrogen atoms on the adjacent carbon atom have acidic properties, with $pK_a$ values usually in the range pH 8–10 [13], and the similarity to carboxylate is increased upon ionization of nitroalkanes to the nitronate state (Scheme 1).

The first pieces of evidence in favour of a catalytically competent covalent adduct in flavinchemistry were published by Porter et al. [14,15]. Those authors showed that ethane nitronate was a substrate for D-amino acid oxidase (EC 1.4.3.3) and could trap a covalent intermediate with cyanide. Subsequently the same authors confirmed the existence of a covalent intermediate with the use of 1-chloro-1-nitroethane [16]. Since nitroethane differs no more greatly from lactate than from alanine (a substrate for D-amino acid oxidase), it appeared to constitute a promising tool in the study of the flavocytochrome $b_2$ chemical mechanism.

MATERIALS AND METHODS

Flavocytochrome $b_2$ (intact form) was purified from freeze-dried commercial baker’s yeast (Lesaffre, 59701 Marcq, France) as described by Jacq & Lederer [17] and stored as a 70 % satd-(NH$_4$)$_2$SO$_4$ precipitate at 4 °C in lactate-containing standard buffer [0.1 M-sodium/potassium phosphate buffer (pH 7)/1 mM-EDTA] in the presence of 0.58 mM-phenylmethanesulphonyl fluoride. Working solutions were prepared from the suspension by dialysis in order to eliminate salts and lactate.

Ethane nitronate was formed immediately before use by equilibration with a molar equivalent of 1.0 M-KOH. A half-life of 4.5 min was determined for the anion in the standard buffer by monitoring either the pH variation of the reaction medium or the disappearance of absorbance at 240 nm ($\Delta\varepsilon_{240} = 3500 \text{ m}^{-1}\cdot\text{cm}^{-1}$). This half-life value was sufficient for measurement of initial rates in enzymic assays. Kinetic studies were carried out in the standard

* On leave from the Service de Biochimie, Département de Biologie, Commissariat à l’Energie Atomique (Saclay), 91191 Gif-sur-Yvette Cedex, France.
† To whom correspondence and reprint requests should be sent.

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buffer at 30 °C. Ferricyanide (1 mM) was the acceptor for assays in the absence of ethane nitronate. In its presence, cytochrome c (170 μM) was used as the acceptor because ethane nitronate alone was found to give rise to a slow ferricyanide consumption. Flavin difference spectra were recorded at 30 °C, as described in [18], with a Cary 2200 or a Cary 118 CX spectrophotometer.

RESULTS

Ethane nitronate is a competitive inhibitor of, not a substrate for, flavocytochrome b₂

When the enzyme was incubated with nitroethane (up to 50 mM) or its anion (up to 100 mM) in the presence of ferricyanide or ferricytochrome c, no reduction of the acceptors was observed. Under our experimental conditions it would have been possible to detect a reaction rate of 0.02 s⁻¹, i.e. 2 x 10⁴-fold less than the kcat, at saturating lactate concentration. After contact with high concentrations of ethane nitronate under turnover conditions, the enzyme was found to be still fully active upon dilution.

By contrast, in the presence of L-lactate, ethane nitronate behaved as a competitive inhibitor with a Kᵢ value of 2.25 ± 0.14 mM (Fig. 1). Nitroethane was not a competitive inhibitor. Although it decreased the enzymic activity somewhat, no intersecting lines could be obtained in a Dixon plot. The phenomenon appeared to be independent of the substrate concentration (about 15 % inhibition at 50 mM-nitroethane and about 65 % at 340 mM, with lactate concentrations ranging from 0.5 mM to 2 mM). This can probably be ascribed to a solvent effect.

Fig. 1. Inhibition of L-lactate oxidation by ethane nitronate

Experimental conditions are given in the Materials and methods section. The nitronate concentrations were as follows: ○, 0 mM; ●, 2.04 mM; ∙, 6.08 mM; □, 10.11 mM; □, 15.08 mM; ▲, 20 mM; △, 29.68 mM; and +, 39.16 mM. The total enzyme concentration, E₄, was 2.2 nM.

Fig. 2. Spectral perturbation of the flavin chromophore by ethane nitronate

The general experimental conditions are given in the Materials and methods section. — (a and b), 9 μM enzyme versus 9 μM enzyme + 100 μM-sulphite; ——, same conditions with 23 mM-ethane nitronate (a) or 17 mM-nitroethane (b) added. The spectrum shown in (a) was completed within 2 min after ligand addition in order to keep as low as possible the concentration of nitroethane arising from the nitronate protonation. A difference spectrum with 12 mM-KOH added (pH 8.2) (not shown) indicated that the perturbation in (a) is indeed due to ethane nitronate and not to a pH effect (reagent reprotonation). Spectra were corrected for dilution. The trough in (b) occurs at the oxidized haem absorbance maximum (413 nm); it probably arises from a solvent perturbation of the haem chromophore.
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Ethane nitronate binding induces a perturbation of the flavin spectrum

A previous study had shown the possibility of observing the flavin chromophore in flavocytochrome $b_2$ by measuring the difference between oxidized enzyme and an enzyme–sulphite complex, which has a reduced-type spectrum [18]. Fig. 2(a) shows that ethane nitronate binding induces a spectral perturbation of the flavin chromophore. In the long-wavelength band, a bathochromic shift from 454 to 464 nm is observed as well as a decrease in intensity. Ligands such as D-lactate and oxalate were found, by contrast, to have a hyperchromic effect and to induce a more marked effect on the fine structure [18]. Whatever the origin of these effects, the results suggest that ethane nitronate indeed binds at the active site and, more importantly, they confirm that no electron transfer takes place from ethane nitronate to flavin.

By contrast, addition of nitroethane led only to the disappearance of fine structure, without any bathochromic or hypochromic shift (Fig. 2b), in agreement with the notion that the effect of nitroethane is essentially attributable to an alteration of the solvent polarity.

Contribution of the lactate α-hydroxy group to substrate binding

Since nitroethane lacks an alcohol group, we tried to evaluate the importance of this hydroxy function in substrate binding. Propionate, a lactate analogue lacking the hydroxy group, had been found to be a weak inhibitor of flavocytochrome $b_2$ [19]. In our hands propionate behaved as a competitive inhibitor of lactate, with a $K_d$ of $28 \pm 1$ mM (Fig. 3).

**DISCUSSION**

Ethane nitronate, a transition-state analogue for the enzyme?

The work described above demonstrates a remarkable difference between nitroethane and its anion. The former gives no sign of specific binding, whereas the latter presents a reasonable affinity for the active site. Considering the chemical mechanism postulated for the enzymic reaction, this transformation of nitroethane upon ionization can be ascribed to the steric and ionic similarity between ethane nitronate and the postulated intermediate carbanion. It is thus tempting to consider ethane nitronate as a transition-state analogue for flavocytochrome $b_2$. Its affinity might at first sight appear too weak for such a contention. For enzymes like fumarase, aspartase and aconitase (for a review, see [13]), which are thought to form a carbanion adjacent to a carboxylate function during catalysis, the affinities of the respective nitronates are often larger by several orders of magnitude than those of both the un-ionized nitro analogue and the substrate [14]. The affinity of ethane nitronate for flavocytochrome $b_2$ is also clearly much larger than that of nitroethane, but the value of its dissociation constant lies in the range observed for normal substrates and competitive inhibitors.

However, nitroethane is not as exact a substrate analogue for flavocytochrome $b_2$ as are the nitro substrates of the enzymes mentioned above: it lacks the lactate hydroxy group and its hydrogen-bonding potential. The relative affinities of lactate (its $K_n$ value, 0.4 mM, is thought to lie close to the $K_n$ value [20,21]) and of propionate ($K_d = 28$ mM) indicate the alcohol function could contribute on the order of 10.5 kJ/mol in binding energy. In the postulated mechanism it hydrogen-bonds to the oxygen atom of Tyr-254 in the Michaelis complex [12]. A hypothetical 1-hydroxy-1-nitroethane carbanion would be expected to bind with a $K_n$ of, atmost, (0.4/28)·2.2 mM (= 30 $\mu$M). Since, in addition, the nitro group does not appear to be such a good carboxylate substitute for flavocytochrome $b_2$ (cf. propionate and nitroethane), considering ethane nitronate as a transition-state analogue for flavocytochrome $b_2$ appears a reasonable hypothesis. Alternatively, it might be considered that ethane nitronate is, rather, a product analogue, in view of the trigonal character of C-1 in the nitrate (Scheme 1). This possibility appears, however, less likely, since pyruvate presents an affinity no better than that of ethane nitronate ($K_{pyruvate} = 3$ mM [18]), whereas its oxo (‘keto’) oxygen provides a hydrogen-bonding possibility (probably to Tyr-254) unavailable to the nitronate.

The second piece of mechanistic information provided by the present work deals with the ionization state of the bound substrate carboxy group. A proton adjacent to a $-CO_2^–$ group is thought to have a $pK_a$ above 20. Therefore enzymologists are usually loath to think that a protein side chain could abstract a proton from a carbon adjacent to a carboxylate. The fact that, in the crystal structure, the lactate carboxy oxygen atoms interact both with Arg-376 and Tyr-143 was naturally interpreted as indicating an ionized carboxylate [12]. The binding of ethane nitronate supports the idea of a transition state where the carbanion electrons are delocalized towards the carboxylate, as in the nitrate (Scheme 1). This is in keeping with the slow protonation of the carbanion when the enzyme is working in the reverse direction [11]. Does the lack of affinity of nitroethane for flavocytochrome $b_2$ indicate that lactate binds with its carboxy group protonated? The idea does not seem reasonable: why should lactate pick up a proton upon binding to the protein only to lose it again when going over to the transition state? On the contrary, since the net charge of the nitro group is zero, one might suggest that the negative character of its oxygen atoms is

**Fig. 3. Inhibition of lactate oxidation by propionate**

Experimental conditions are given in the Materials and methods section. Lactate concentrations are shown against the curves. The total enzyme concentration, $E_t$, was 12.5 nM.
insufficient to afford satisfactory neutralization of the Arg-376 charge. In conclusion, it appears that the active site of flavocytochrome \( \beta_2 \) [9,12] provides an example of how an enzyme can dissipate the electrostatic potential of a carboxylate by hydrogen-bonding and ion-pairing, so that \( \alpha \)-carbanion formation becomes energetically feasible.

**Why is there no redox reaction between ethane nitrate and flavocytochrome \( \beta_2 \)?**

The final point to be discussed is the lack of electron transfer between ethane nitrate and the flavin. The reason why no transfer occurs is unclear. It can be suggested that the carbanion is not oriented correctly for lack of the hydrogen bond to the lactate hydroxy group. However, the deletion of the \( \alpha \)-amino group in a \( \beta \)-amino acid oxidase substrate would reasonably be expected to entail the same consequence as the hydroxy-group deletion for flavocytochrome \( \beta_2 \). The divergent behaviour of the two enzymes can hardly be ascribed to a redox-potential difference, since the flavin in \( \beta \)-amino acid oxidase has a lower potential than that of flavocytochrome \( \beta_2 \) [22,23]. Furthermore, one should also consider that nitroethane was reported not to be a substrate either for long-chain-hydroxy-acid oxidase [24] and that 1-chloro-1-nitroethane, which behaves as a suicide substrate toward \( \beta \)-amino acid oxidase by acylating flavin N-5, failed to inactivate \( \alpha \)-amino acid oxidase, lactate oxidase and glucose oxidase [13]. The question may thus be ‘why does ethane nitrate react with \( \beta \)-amino acid oxidase?’ rather than ‘why is it unreactive as electron donor to flavocytochrome \( \beta_2 \)?’

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**REFERENCES**


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