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The Nitro Group as a Masked Electrophile in Covalent Enzyme Inhibition

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Supporting Information Placeholder

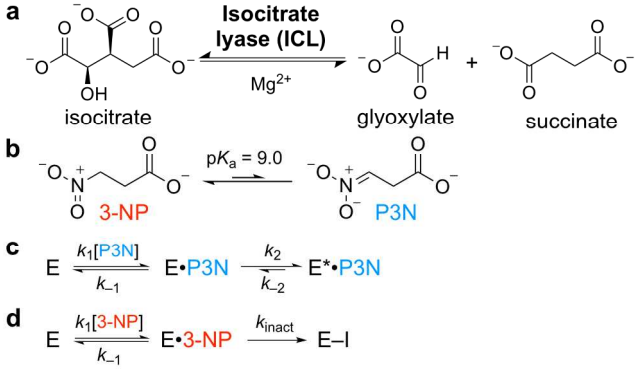
ABSTRACT: We report the unprecedented reaction between a nitroalkane and an active-site cysteine residue to yield a thiohydroximate adduct. Structural and kinetic evidence suggests the nitro group is activated by conversion to its nitronic acid tautomer within the active site. The nitro group, therefore, shows promise as a masked electrophile in the design of covalent inhibitors targeting binding pockets with appropriately placed cysteine and general acid residues.

Recent years have seen renewed interest in the development of inhibitors that function by covalently modifying their targets. To avoid the obvious potential for toxicity resulting from off-target reactions, so-called targeted covalent inhibitors are designed with a weakly electrophilic group that reacts selectively with a nucleophilic residue on the target only upon binding and proper positioning of the reactive functionalities.[2, 3] Novel electrophilic functional groups with low intrinsic reactivity are therefore highly desirable for this rapidly developing inhibitor design strategy.

In the current study, we report the previously unrealized ability of a nitroalkane to form a covalent adduct with a protein without the assistance of cofactors. This discovery stemmed from an investigation of isocitrate lyase (ICL), a drug target essential for the survival of *Mycobacterium tuberculosis* during the latent stage of infection. Whereas most organisms depend on carbohydrates as a carbon source, *M. tuberculosis* is capable of subsisting on acetate and fatty acids within the hypoxic confines of macrophages by utilizing the glyoxylate shunt.[4] As the first of two steps in the shunt, ICL catalyzes the reversible retro-aldol cleavage of the tricarboxylic acid cycle intermediate isocitrate into succinate and glyoxylate (**Scheme 1a**); malate synthase subsequently converts glyoxylate to L-malate, which, together with succinate, is ultimately converted to oxaloacetate for gluconeogenesis. Due to its absence in humans, ICL is a desirable target for inhibitor design.

3-Nitropropionate (3-NP), an analogue of succinate, bearing a nitro group in place of one of the carboxylates, is a potent time-dependent inhibitor of ICL.[5, 6] Like other nitroalkanes, 3-NP (pK_a = 9.0[7]) exists partially as its conjugate base propionate-3-nitronate (P3N) in aqueous solution near neutral pH (**Scheme 1b**). Taking advantage of the slow rate of proton transfer to/from 3-NP,[7] Schloss and Cleland reported that the onset of inhibition of *Pseudomonas indigofera* ICL was accelerated as the inhibitor was converted from the free acid to fully ionized form by increasing the pH in pre-incubated samples.[5] This prompted the authors to conclude that P3N is the true form of the inhibitor and that the onset of inhibition is the result of the slow formation of a tight binary complex (**Scheme 1c**). While our previous studies demonstrated time-dependent inhibition also occurs with the *M. tuberculosis* enzyme, we observed a large inverse solvent isotope effect (i.e., the onset of inhibition was twice as fast in D_2O) that cannot be explained by this mechanism.[6] Additionally puzzling is that after complete inhibition of *P. indigofera* ICL by pre-incubation with 3-NP, jump-dilution into assay mixtures containing excess succinate, which competes with 3-NP, resulted in recovery of only 25% of the uninhibited activity.[5]

Scheme 1. ICL reaction and inhibition by 3-NP.



To address these inconsistencies, we measured inhibition kinetics with *M. tuberculosis* ICL using 3-NP or P3N at pH 7.5. Inhibition by 3-NP, initiated by addition of ICL, was monitored spectrophotometrically in the isocitrate-synthesis direction by following the reduction of $NADP^+$ by isocitrate

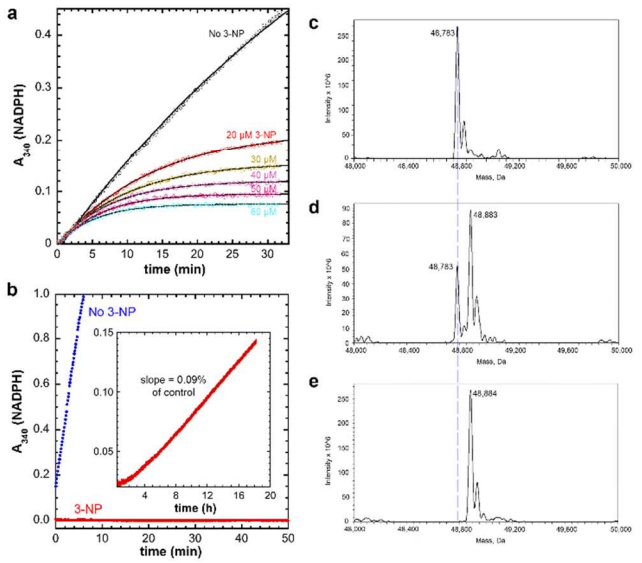


Figure 1. Kinetic and mass characterization of ICL inhibition by 3-NP. (a) Isocitrate dehydrogenase-coupled assay of ICL activity at pH 7.5 in the presence of 20–60 μM 3-NP. Curves are least-squares fits to an exponential equation (see SI). (b) Recovery of ICL activity upon 3000-fold dilution after 1 h pre-incubation of 0.5 mM ICL with 1 mM glyoxylate and either 1 mM 3-NP (red) or no inhibitor (blue). Inset: expansion of the red curve over an extended period. (c)–(e) Deconvoluted ESIMS spectra of ICL after 8 min of incubation (c) alone, (d) with 3-NP, or (e) with 3-NP and glyoxylate. The dashed line is centered on the mass of unmodified ICL.

dehydrogenase. Inhibition by P3N required rapid mixing of the fully ionized inhibitor (prepared in 0.1 M NaOH) and succinate with the rest of the assay components buffered at pH 7.5, in order to minimize the extent of reprotonation back to 3-NP. Reprotonation monitored spectrophotometrically (**SI Figure S1**) showed that using 10 mM potassium phosphate buffer, <10% of the total P3N would have been converted to 3-NP by the end of the assay. Both inhibitors demonstrated slow-onset inhibition (**Figure 1a** and **SI Figure S2a**), though it was not possible to distinguish whether any residual steady-state rate remained after prolonged incubations. To assess the reversibility of inhibition, we therefore conducted jump-dilution experiments similar to those by Schloss and Cleland.[5] Consistent with their findings but to a much greater extreme, we observed very limited recovery of activity (~0.09%) over the course of 20 h, compared to a control that had not been pre-incubated with inhibitor (**Figure S2b** and **SI Figure S2b**). Inclusion of 5 mM DTT had no noticeable effect on the activity.

The unexpected failure of ICL to recover most of its activity led us to hypothesize that it was due to irreversible formation of a covalent complex. To investigate this possibility, we performed electrospray ionization mass spectrometry on *M. tuberculosis* ICL. To our surprise, the protein's mass increased from 48,783 ± 1 to 48,884 ± 1 Da in the presence of 3-NP (**Figure 1c,d**). Although extended incubation periods led to complete modification (**SI Figure S3**), the mass increase was accelerated by the presence of glyoxylate (**Figure 1e**), indicative of synergistic binding of the inhibitor. This increase of 101 ± 1 Da is 18 less than the molecular weight of 3-NP, suggesting that covalent modification of ICL may be accompanied by loss of a molecule of water.

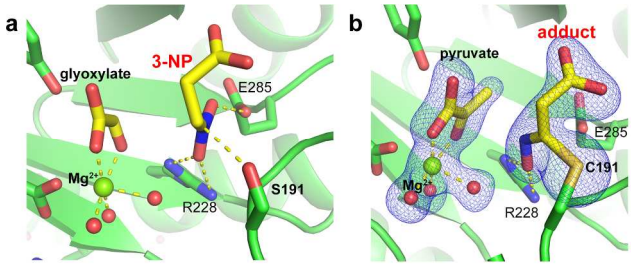
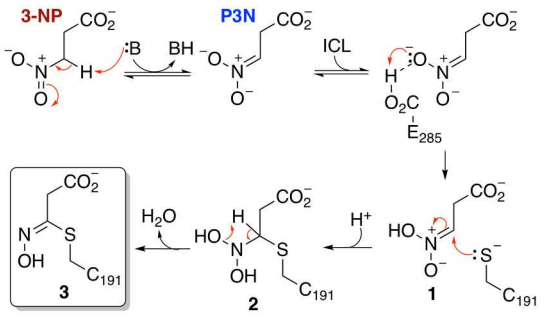


Figure 2. ICL–3-NP structures. (a) C191S•Mg^{2+}•glyoxylate•3-NP from PDB 1F8I1.[1] (b) Simulated annealing omit map countered at 3.0 σ for the thiohydroximate adduct obtained from wild-type ICL, Mg^{2+}, and 3-NP (PDB 6C4A).

Unequivocal identification of the protein modification was obtained by X-ray crystallography. Interestingly, Sharma et al. published the structure of the C191S mutant of *M. tuberculosis* ICL bound with Mg^{2+}, glyoxylate, and 3-NP, in which no covalent modification was observed (**Figure 2a**).[1] While no explanation for mutation of the active-site residue was provided, it is possible that the covalent modification of the wild-type enzyme interfered with crystallographic analysis under their conditions. Although the pseudo-C_2 symmetry of the inhibitor prevents definitive assignment of its orientation in this complex, the depiction in **Figure 2a** is compatible with formation of a covalent adduct (vide infra). Of note in this structure is the 3.0 Å distance between the hydroxyl group of Ser191 and C-3 of the inhibitor. Additionally, Arg228 and Glu285 make contacts with the oxygen atoms of the nitro group. Glu285 is believed to exist in the acid form in order to serve as a hydrogen-bond donor and to avoid electrostatic repulsion, especially when succinate is bound in place of 3-NP. In the presence of Mg^{2+} and 3-NP, wild-type ICL formed a covalent adduct between the sulfur of Cys191 and C-3 (**Figure 2b**). The trigonal planar geometry at C-3 is consistent with a thiohydroximate with Z stereochemistry and inconsistent with a nitroso tautomer. This adduct, which lacks one of the oxygen atoms present in the nitro group, agrees with the observed increase in mass. Pyruvate, present endogenously during protein expression, was found coordinated to Mg^{2+} in place of glyoxylate, as it has been shown to do previously.[8] Soaking the crystals with glyoxylate resulted in displacement of the bound pyruvate at three of the eight crystallographically independent active sites and a nearly identical structure (PDB 6C4C).

Based on the covalent nature of inhibition, we performed additional analysis of the kinetic data presented earlier (**Figure 1a**), fitting each curve to an exponential function. Plots of the observed rate constants (k_{obs}) vs. [3-NP] or [P3N] were linear (**SI Figure S4**), with slopes equal to the respective second-order rate constant for inactivation (k_{inact}/K_I). While this pattern is consistent with a single-step binding process, it is implausible considering the chemical transformation that must occur. We therefore favor a minimal two-step scheme (**Scheme 1d**) in which the initial equilibrium is far from saturation over all concentrations of inhibitor tested (i.e., [inhibitor] << k_{-1}/k_1). Global fitting of the data gave k_{inact}/K_I values of 270 ± 50 and 26,000 ± 3,000 M^{-1} s^{-1}, respectively, for 3-NP and P3N in 10 mM potassium phosphate (pH 7.5). The second-order rate constant for 3-NP is about fivefold higher than that for 2-*C*-vinyl-D-isocitrate, a mechanism-based inactivator that modifies Cys191 via an enzyme-generated Michael acceptor.[9] The value is also comparable to

Scheme 2. Proposed Mechanism for Thiohydroximate Formation[a]



[a] B = buffer.

the value of 1050 M^{-1} s^{-1} reported for the *P. indigofera* enzyme at pH 8,[5] considering there is about threefold more P3N present at equilibrium at that pH. That 25% of the uninhibited activity was recovered in jump-dilution experiments with this enzyme, in contrast to *M. tuberculosis* ICL, may indicate a partitioning of the bound inhibitor between covalent and non-covalent complexes that differs between the orthologues.

In light of the covalent nature of inhibition and the ~100-fold faster rate of inactivation compared to 3-NP, we propose that P3N is an intermediate in the formation of the thiohydroximate adduct. However, considering that the thermodynamic ratio of the two inhibitory species at pH 7.5 is only ~30, it is evident that conversion of 3-NP to P3N during inactivation is partially rate limiting. Consistent with this, we observed catalysis by buffer, both by anionic (HEPES) and cationic (imidazole) buffers (phosphate could not be tested due to its limited solubility in the presence of Mg^{2+}). Both buffers exhibited hyperbolic concentration dependence (**SI Figure S5**), consistent with a transition in rate-limiting step from 3-NP ionization to a subsequent process involved in covalent adduct formation.

A mechanism is suggested for this unprecedented protein modification, in which P3N is an intermediate, formed by deprotonation of 3-NP (**Scheme 2**). In this scheme, buffer catalyzes deprotonation of 3-NP to yield P3N, which binds to ICL. Although P3N is *nucleophilic*, protonation of one of the nitronate oxygens, presumably by Glu285, would lead to nitronic acid **1**, which is *electrophilic* and readily reacts with the neighboring cysteine residue to yield the initial covalent species **2**. The sulfhydryl most likely attacks as the thiolate, consistent with the inverse solvent isotope effect we observed previously.[6] Protonation of the other nitronate oxygen, possibly by Arg228, may occur prior to or following C–S bond formation. Dehydration of **2** via either an E_i-type elimination (shown) or stepwise process via a nitroso intermediate then results in formation of the stable thiohydroximate adduct **3**. This mechanism, which parallels that proposed for reaction of nitroalkanes with thiosilanes,[10] differs greatly from that employed in inhibition of succinate dehydrogenase by 3-NP, which instead uses its flavin cofactor to oxidize the inhibitor to a nitroalkene, which serves as a Michael acceptor.[11]

Although rare, thiohydroximates have been observed biologically as intermediates in the synthesis of some phosphonate natural products[12] and glucosinolates.[13] Interestingly, in formation of the latter, a thiohydroximate has also been proposed to result from reaction of a nitronic acid with a thiol nucleophile, presumably glutathione;[13] however, in this case, the nitronic acid is formed by oxidation of an aldoxime rather than tautomerization of a nitroalkane,[14] as demonstrated here.

The results of this study bring to question whether nitro groups can function more generally to modify other proteins (enzymes or receptors) by placement in regions of known ligands proximal to (1) an acidic residue and (2) a nucleophilic group (e.g., cysteine). As such, they could function as masked warheads for targeted covalent inhibition.[2, 3] A limitation of using nitro compounds as drugs, however, is their potential for toxicity, most commonly via metabolic reduction to reactive nitroso and hydroxylamine derivatives. While this is a valid concern that must be investigated early in the drug discovery process, there are a number of examples that are pharmaceutically active and pose no serious safety risks.[15]

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: This material is available free of charge *via* the Internet

Experimental details, table of X-ray data collection and refinement statistics, kinetics plots, time-dependent mass spectra

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Notes

The authors declare no competing financial interests.

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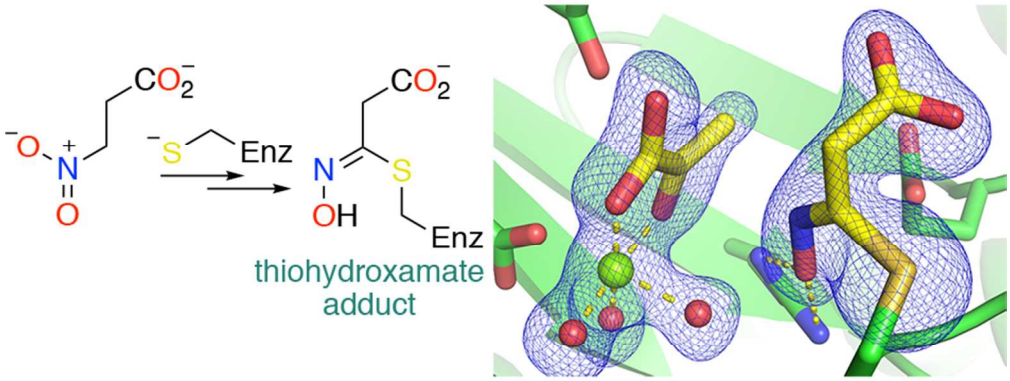

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