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INHIBITION OF SUCCINATE DEHYDROGENASE BY NITROACETATE AND BY THE TOXIC ANTIBIOTIC NITRAMINOACETATE

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SUMMARY

Nitraminoacetate and the nitronate of nitroacetate are effective inhibitors of beef heart succinate dehydrogenase, respectively binding about 17 and 6.6 times more tightly than succinate at pH 8.0 and 25° as judged by the K_m/K_i ratios. Unlike the case of the suicide inactivator 3-nitropropionate, these compounds are freely reversible inhibitors. The toxicity of nitraminoacetate, an antibiotic elaborated by Streptomyces noursei, may be secondary to the inhibition of the Krebs cycle at the succinate dehydrogenase reaction, and the nitramino group may prove useful as a carboxylate analog for other enzymes.

INTRODUCTION

Experiments from this laboratory have shown that nitro analogs of carboxylic acids may act as interesting enzyme substrates or inhibitors (1-3). For instance, 2-nitropropane (which is isoelectronic with the alanine zwitterion) is a good substrate for the flavoenzyme D-amino acid oxidase. In contrast to the oxidation of D-alanine, the mechanism of oxidation of nitroalkanes by the oxidase is understood in detail because an electrophilic flavin-substrate covalent adduct which is an intermediate can be trapped with nucleophiles such as cyanide (4). Nitroalkanes are, then, either substrates or inactivators of that enzyme, depending on experimental conditions. In another flavin-dependent reaction, mitochondrial succinate dehydrogenase is irreversibly and stoichiometrically inactivated by 3-nitropropionic acid, a succinate analog which is the toxic product of certain poisonous plants and microorganisms (2,5). Nitropropionate functions as a "suicide substrate" in that case. It is prob-

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ably oxidized to a reactive nitro olefin by the dehydrogenase, although several plausible mechanisms can be envisioned for this interesting phenomenon. Nitropropionate is also a remarkably potent reversible inhibitor of other enzymes such as fumarase and aspartase (6). Nitroalkanes are carbon acids (Figure 1), and the nitronate species formed upon deprotonation of nitropropionate bears strong analogy to carbanionic species which occur in the 2,3-elimination reactions catalyzed by fumarase and aspartase. The dianion of nitropropionate thus functions as a "transition state analog" in those cases and is bound to those enzymes with extraordinary affinity because of its electronic resemblance to high-energy species stabilized within the enzyme active sites during catalysis of the ordinary reactions (6). Succinate dehydrogenase shares the ability to catalyze 2,3-elimination reactions (such as the anaerobic elimination of HF from 3,3-difluorosuccinate) (7) and probably abstracts one or both hydrogen atoms as protons from succinate in the dehydrogenation reaction (2). Before irreversible inactivation, the nitronate of nitropropionate forms an affined but reversible complex at the active site of the dehydrogenase, and resemblance to carbanionic transition state species may thus also account for the high affinity of succinate dehydrogenase for nitropropionate. The use of nitronates other than that of nitropropionate as transition state analogs for enzymes generating carbanionic species from carboxylic substrates has been generalized to other cases such as that of aconitase (8). Furthermore, the chemistry of the nitro group is rich, and there are diverse reactions by which suitable nitro compounds may prove to react with enzymes. For another example, pyridoxal phosphate-dependent enzymes which act on glutamate catalyze the condensation of 5-nitronorvaline with the formyl group of the coenzyme to form a stable six-membered cyclic adduct (Alston, in preparation).

Nitraminoacetic acid is a toxic antibiotic elaborated by Streptomyces noursei (9). It is a diacid with pK_a values of 2.8 and 6.6 (9). We were prompted to examine its effect on succinate dehydrogenase and other enzymes because of the obvious chemical similarity to nitropropionic acid (Figure 1). Plau-

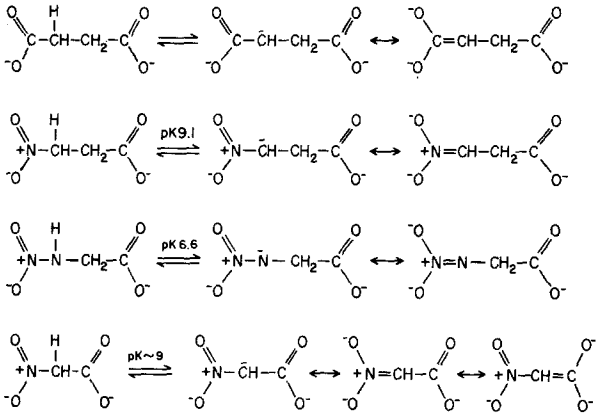

Figure 1. Steric and electronic similarity of succinate, nitropropionate, nitraminoacetate, and nitroacetate. Succinate is depicted as ionizing to a carbanionic species which may occur in the reaction catalyzed by succinate dehydrogenase.

sible mechanisms may be envisioned by which the compound may act as a suicide inactivator, transition state analog, or substrate for the dehydrogenase. This communication also reports our findings with nitroacetate, a malonate analog for which reactions with the dehydrogenase may similarly be anticipated.

MATERIALS AND METHODS

Nitraminoacetic acid was prepared by the action of anhydrous nitric acid on the ethyl ester of N-(carbethoxy)glycine followed by hydrolysis of the protecting groups (9,10). The dipotassium salt of nitroacetic acid was synthesized by the action of concentrated aqueous potassium hydroxide on nitromethane (11,12) with explosion precautions. Both products were obtained as white needles and recrystallized (9,12) several times before use. Because of the facile decarboxylation of the monovalent nitroacetate ion (13), stock solutions of dipotassium nitroacetate were kept at 0° and used within 8 h of preparation.

Electron transport particles containing succinate dehydrogenase were prepared from beef heart mitochondria (14). In order to fully activate the dehydrogenase, the particles were incubated at a protein concentration of 10 mg/ml at 25° in a solution containing 1.0 mM malonate, 250 mM sucrose and 5.0 mM Tris chloride, pH 7.4, for 45 min (15). The particles were then centrifugally washed at 0° with the same buffer lacking the malonate. Other enzymes were obtained from the Sigma Chemical Company.

Succinate dehydrogenase activity was measured by following the oxygen consumption of electron transport particles by means of a Clark-type oxygen electrode from Yellow Springs Instruments. Aspartase and fumarase activities were measured by following the absorbance of fumarate at 240 nm in either a Cary 15 or Gilford model 222A recording spectrophotometer. Reprotonation of the methinyl group of the nitroacetate dianion was followed by the decay in the ultraviolet absorbance due to the nitronate (6).

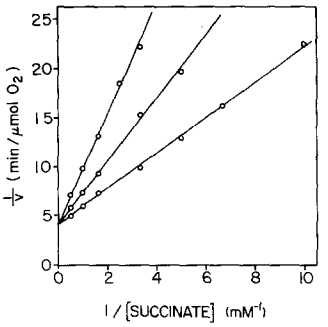

Figure 2. Competitive inhibition of succinoxidase by nitraminoacetate and by nitroacetate at pH 8.0 and 25° in 100 mM Tris chloride buffer. Succinate concentration is varied in the absence and in the presence of each inhibitor (.050 mM). The K_i values from experiments at several inhibitor concentrations are .026 mM for nitraminoacetate and .068 mM for the nitroacetate dianion.

RESULTS

Both nitraminoacetate and the dianion of nitroacetate are effective inhibitors of the oxidation of succinate by electron transport particles from beef heart. The inhibitors must act by binding to succinate dehydrogenase because the inhibition is fully competitive with respect to succinate (Figure 2). Furthermore, the nitro compounds do not inhibit the oxidation of NADH by these particles, and nitronates unrelated to succinate (nitromethane, nitroethane, 1- and 2-nitropropane anions) do not inhibit. Under the conditions described in Figure 2, the K_i values are .026 mM for nitroacetate and .068 mM for the nitroacetate dianion. By comparison, the K_m value which we obtain for succinate is .45 mM, and the K_i value for malonate is .0086 mM under these conditions.

Unlike the irreversible inactivator nitropropionate (2), these nitro compounds are freely reversible inhibitors of the dehydrogenase. When dipotassium nitroacetate is added to particles respiring with succinate as substrate, the respiratory rate instantly decreases to an inhibited level which then accelerates back to the uninhibited level with the same first-order rate constant at which the nitroacetate dianion reprotonates on carbon, namely 0.15 min^{-1} under the conditions of Figure 2. This observation indicates that only the dianionic form of nitroacetate is significantly inhibitory and that

the inhibition is rapidly reversible. The decarboxylation of the nitroacetate monoanion occurs more slowly with a rate constant of about 0.03 min^{-1} (13). The nitraminoacetic acid is stable under the experimental conditions (9) and is a rapidly equilibrating diacid. The extent of its inhibition is the same whether it is added to the dehydrogenase 15 min before the substrate or shortly after the substrate is added. The nitro compounds do not function, detectably, as substrates for the dehydrogenase. At saturating levels, their rate of oxidation must be less than 0.01% of that of succinate.

DISCUSSION

Although nitraminoacetate is one of the few competitive inhibitors known to bind more tightly than succinate to succinate dehydrogenase, the K_m/K_i ratio of only 17 does not convincingly support the hypothesis that nitraminoacetate binds to the dehydrogenase as an analog of a rate-limiting transition state species. The use of nitramino compounds in addition to nitronates (6,8) as transition state analogs is attractive because of the greater acidity of the nitramino group compared to the nitroalkyl group (Figure 1). Under physiological conditions in which a pharmacologically useful transition state inhibitor would be required to act, nitramino compounds are predominantly ionized. Consequently, we have also compared nitraminoacetate with 3-nitropropionate as a reversible inhibitor of fumarase (from porcine heart) and aspartase (from Bacterium cadaveris). Surprisingly, the nitraminoacetate is only a weak inhibitor of these enzymes which are exquisitely sensitive to the nitronate form of nitropropionate (6). At pH 7.0 and 25° in 50 mM potassium phosphate buffer, the K_m/K_i ratio for the binding of malate and divalent nitropropionate to fumarase is 380, whereas nitraminoacetate binds less than half as tightly as malate. At pH 6.9 and 25° in 100 mM Tris acetate buffer also containing 10 mM potassium chloride and 2.0 mM magnesium sulfate, the K_m/K_i ratio for the binding of aspartate and divalent nitropropionate to aspartase is 520 whereas divalent nitraminoacetate binds only 2.1 times as well as aspartate.

It is interesting to suppose that the toxic nitramine is metabolically reduced in a two-electron process to a diazonium type of alkylating agent. However, we observed no bleaching of the ultraviolet absorbance of either the nitramino group (9) or the reduced pyridine nucleotide when the nitroaminoacetate and NADH or NADPH were incubated with malate dehydrogenase (porcine heart mitochondrial and cytosolic isozymes), malic enzyme (chicken liver), succinate semialdehyde dehydrogenase (Pseudomonas fluorescens), or succinate dehydrogenase (reduced via the electron transport chain from NADH dehydrogenase in the presence of antimycin A) in 100 mM potassium phosphate buffer at pH 7.4 and 25°.

Although nitraminoacetate does not inhibit isolated succinate dehydrogenase quite as well as malonate, the nitramine is more toxic, the oral LD_{50} for mice reported to be 40 mg/kg (9). In view of the rapid metabolism of malonate (16), however, inhibition of the Krebs cycle at the succinate dehydrogenase reaction may well account for the toxicity of the antibiotic nitramine.

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