Site-directed Inhibition of *Haemophilus influenzae* Malate Dehydrogenase

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Previous studies of *Haemophilus* organisms documented the importance of an NAD⁺-dependent malate dehydrogenase in the incomplete tricarboxylic acid cycle present in these organisms. Selective interactions occurring at the coenzyme and substrate binding sites of a purified *Haemophilus influenzae* malate dehydrogenase were investigated. Coenzyme-competitive inhibition by adenosine derivatives demonstrated the presence of regions in the coenzyme binding site that interacted with the adenosine and pyrophosphate moieties of the coenzyme. Positive chainlength effects in the coenzyme-competitive inhibition by aliphatic carboxylic acids indicated the presence of a hydrophobic region at this site that was close to the pyrophosphate region. Seven analogues of NAD⁺ that were structurally altered in either the pyridine or purine ring were evaluated as selective inhibitors of the enzyme. The three most effective inhibitors of the purified malate dehydrogenase inhibited the growth of *H. influenzae* when the organism was grown on a limiting concentration of NAD⁺.

INTRODUCTION

Haemophilus influenzae is representative of a number of *Haemophilus* species that exhibit unique growth requirements for intact NAD⁺ (V-factor) and haemin (X-factor). *H. influenzae* type b is the primary cause of bacterial meningitis in infants and is responsible for 8000 cases annually in the USA (Smith, 1979). Treatment of *H. influenzae* infections has relied upon the use of ampicillin and chloramphenicol; however, a continuing increase in strains resistant to these antibiotics has been observed (Gunn *et al.*, 1974; Syriopoulou *et al.*, 1978). The inability of V-factor-requiring organisms to synthesize NAD⁺ from low molecular mass precursor molecules utilized by other bacterial systems suggested the possible presence of processes of NAD⁺ metabolism unique to these organisms, the inhibition of which might provide an alternative approach to controlling the growth of these organisms. Recent studies of the utilization of externally added intact NAD⁺ for growth of *H. influenzae* revealed the presence of a periplasmic nucleotide pyrophosphatase responsible for the hydrolysis of NAD⁺, providing nicotinamide mononucleotide (NMN) for the intracellular resynthesis of NAD⁺ (Kahn & Anderson, 1986). The 3-acetylpyridine and thionicotinamide analogues of NAD⁺ were demonstrated to function as substrates for the purified pyrophosphatase and to support growth of the organism. 3-Aminopyridine adenine dinucleotide, also a substrate for this enzyme, was an extremely potent inhibitor of growth of the organism. An effective inhibition of growth by ADP correlated well with the selective inhibition of the nucleotide pyrophosphatase; however, the mechanism of growth inhibition by the aminopyridine analogue of NAD⁺ remained unclear. The ability of several NAD⁺ analogues to support growth prompted a study of the intracellular functioning of these derivatives as coenzymes for important cytosolic dehydrogenases. Malate dehydrogenase (EC 1.1.1.37), recently implicated in an incomplete tricarboxylic acid cycle in *Haemophilus* organisms (Tuyau *et al.*, 1984), was purified to electrophoretic homogeneity (Yoon & Anderson,

Abbreviation: NMN, nicotinamide mononucleotide.

1988). Kinetic studies indicated an ordered bi-bi mechanism and showed that the 3-acetylpyridine and thionicotinamide analogues of NAD^+ served as coenzymes. In the present study, selective inhibition of the purified malate dehydrogenase was investigated in attempts to determine interactions of importance in the binding of ligands at the coenzyme and substrate binding sites of the enzyme. Since NAD^+ is a limiting factor in the growth of *H. influenzae*, selective inhibition of the utilization of NAD^+ by dehydrogenases, in conjunction with inhibition of the periplasmic nucleotide pyrophosphatase, could enhance the inhibition of the growth of the organism.

METHODS

Materials. The *H. influenzae* malate dehydrogenase was purified according to Yoon & Anderson (1988). All mono- and dinucleotides were purchased from Sigma except 3-aminopyridine adenine dinucleotide, pyridine adenine dinucleotide, 3-pyridylcarbinol adenine dinucleotide, 3-pyridylacetonitrile adenine dinucleotide and 3-methylpyridine adenine dinucleotide, which were prepared by published procedures (Yost & Anderson, 1982; Anderson *et al.*, 1986). Straight chain aliphatic carboxylic acids were obtained from Eastman. Other hydroxy acids, oxo acids and dicarboxylic acids were purchased from Sigma. Procion Blue HB was obtained from Aldrich. N^1 -Methyl- and N^1 -octylnicotinamide chlorides were synthesized as previously reported (Anderson *et al.*, 1965).

Methods. Initial velocities of the enzyme-catalysed reduction of NAD^+ were determined at 23 °C in 1.0 ml reaction mixtures containing 45 mM-glycine/NaOH buffer, pH 9.6. Reactions were initiated by the addition of enzyme, and formation of NADH was monitored spectrophotometrically at 340 nm. Multiple inhibition studies using two different competitive inhibitors (Yonetani & Theorell, 1964) were carried out as described previously (Anderson & Reynolds, 1965). Spectrophotometric measurements were made on a Beckman Acta MVI recording spectrophotometer.

Studies to investigate the ability of various compounds to inhibit growth of *H. influenzae* were carried out in Brain Heart Infusion medium as described previously (Kahn & Anderson, 1986). Several concentrations of inhibitor were studied at 37 °C in 50 ml of medium containing $0.1 \mu\text{g NAD}^+ \text{ml}^{-1}$. Growth rates were determined turbidimetrically in the presence and absence of inhibitors.

RESULTS

Inhibitors of *H. influenzae* malate dehydrogenase were studied by varying the concentration of the coenzyme, NAD^+ , or the substrate, malate. The type of inhibition and inhibitor dissociation constants were determined through double-reciprocal plotting methods (Rudolph & Fromm, 1979) using several fixed concentrations of the inhibitor.

Inhibition of *H. influenzae* malate dehydrogenase by adenosine 5'-diphosphate (ADP) (Fig. 1) is representative of the inhibition observed with adenosine derivatives and structural analogues of NAD^+ . The inhibition by ADP was studied at 6 mM-malate and the concentration of NAD^+ varied from 40 μM to 270 μM . Pure coenzyme-competitive inhibition was observed and a K_i value of 0.85 mM was determined. The K_i values for the four adenosine derivatives studied are listed in Table 1. Under the same experimental conditions, seven structural analogues of NAD^+ were evaluated as coenzyme-competitive inhibitors and the respective K_i values are listed in Table 1. Although the adenosine derivatives, representing fragments of the NAD^+ molecule, were inhibitory, no inhibition was observed at 100 mM concentrations of nicotinamide derivatives, such as N^1 -methylnicotinamide chloride and N^1 -octylnicotinamide chloride. Procion Blue HB, the ligand used effectively for affinity chromatography in the purification of the malate dehydrogenase (Yoon & Anderson, 1988), was a very effective coenzyme-competitive inhibitor, exhibiting a K_i value of 0.2 μM .

The more effective binding of AMP and ADP relative to adenosine, attributable to the additional electrostatic interactions of these negatively-charged nucleotides, prompted the study of other negatively-charged molecules such as straight chain aliphatic carboxylic acids. Coenzyme-competitive inhibition was observed with carboxylic acids varying in chainlength from acetic to octanoic acid (Table 2). A mixed type inhibition was observed with nonanoic and decanoic acids. Inhibition by the carboxylic acids increased with increasing chainlength and a linear relationship between the logarithm of $1/K_i$ versus the number of carbons in the alkyl sidechains was observed. In contrast to the inhibition observed with the negatively-charged

Fig. 1. Coenzyme-competitive inhibition of malate dehydrogenase by ADP. Reaction mixtures (1.0 ml) contained 45 mM-glycine/NaOH buffer, pH 9.6, 6 mM-malate, 50 ng enzyme and NAD^+ , with the four different concentrations of ADP indicated.

Table 1. Coenzyme-competitive inhibition of *H. influenzae* malate dehydrogenase

Inhibitor	K_i (mM)
Adenosine	12.7
Adenosine 5'-monophosphate (AMP)	1.1
Adenosine 5'-diphosphate (ADP)	0.85
Adenosine 5'-diphosphoribose	0.05
Nicotinamide 1, N^6 -ethenoadenine dinucleotide	5.50
Pyridylacetonitrile adenine dinucleotide	3.48
3-Aminopyridine adenine dinucleotide	2.47
Pyridine adenine dinucleotide	0.79
Nicotinic acid adenine dinucleotide	0.58
3-Methylpyridine adenine dinucleotide	0.14
3-Pyridylcarbinol adenine dinucleotide	0.02
Procion Blue HB	0.0002

Table 2. Inhibition of *H. influenzae* malate dehydrogenase by carboxylic acids

Inhibitor	K_i (mM)	Type of inhibition
Acetic acid	258	Coenzyme-competitive
Propionic acid	174	
Butyric acid	146	
Pentanoic acid	87	
Hexanoic acid	60	
Heptanoic acid	29	
Octanoic acid	21	
Nonanoic acid	15	Mixed
Decanoic acid	8	

butyric acid ($K_i = 146$ mM), no inhibition was observed with 200 mM-butanol. The original assumption, that coenzyme-competitive inhibition by carboxylic acids could occur through interactions of the negatively-charged carboxylate groups with a region at the coenzyme binding site that interacted with AMP and ADP, was investigated further using multiple inhibition analysis (Yonetani & Theorell, 1964). Pairs of competitive inhibitors were studied for simultaneous binding or mutual exclusion at the coenzyme binding site. An inhibitor interaction

Fig. 2. Multiple inhibition of malate dehydrogenase by AMP and hexanoic acid. Reaction mixtures (1.0 ml) contained 45 mM-glycine/NaOH buffer, pH 9.6, 6 mM-malate, 50 ng enzyme, 54 μ M-NAD⁺ and AMP (0.1–0.4 mM) at the four different concentrations of hexanoic acid indicated.

Table 3. Substrate-competitive inhibition of *H. influenzae* malate dehydrogenase

Inhibitor	K_i (mM)
Fumaric acid	18.0
Succinic acid	15.4
2-Hydroxyglutaric acid	14.9
Malonic acid	12.7
Hydroxymalonic acid	1.6
2-Oxoglutaric acid	0.025
2-Hydroxybutyric acid	—
Pyruvic acid	—
D-Lactic acid	—
L-Lactic acid	—

—, No inhibition at 20 mM.

constant, α , which is a measure of the interactions existing between inhibitor₁ (I_1) and inhibitor₂ (I_2) in an EI_1I_2 complex can be calculated from the plot of v_0/v_i against $[I_1]$. Multiple inhibition by the inhibitor pair composed of AMP and hexanoic acid resulted in a parallel line relationship (Fig. 2), indicating an α value of infinity. Thus, these two inhibitors mutually exclude one another from binding to the coenzyme binding site. A parallel line relationship was also observed when hexanoic acid was paired with ADP. Any inhibitor pair composed of just adenosine derivatives, as expected, also exhibited mutual exclusion as coenzyme-competitive inhibitors.

H. influenzae malate dehydrogenase was also inhibited effectively by structural analogues of the substrate, malate. Substrate-competitive inhibition was observed in 1.0 ml reaction mixtures containing 45 mM-glycine/NaOH buffer, pH 9.6, 0.54 mM-NAD⁺ and malate varied from 60 to 400 μ M. K_i values obtained for the derivatives studied are listed in Table 3. Hydroxy- and oxomonocarboxylic acids were not inhibitory at 20 mM concentration. Of the dicarboxylic acids studied, 2-oxoglutarate was the most effective, exhibiting a K_i value of 25 μ M. Although effectively bound, 2-oxoglutarate did not serve as a substrate.

Inhibitors of the purified malate dehydrogenase were analysed for their ability to inhibit the growth of the organism in the presence of NAD⁺. Inhibition of growth by 3-pyridylcarbinol adenine dinucleotide, 2-oxoglutarate and Procion Blue HB was investigated at concentrations five times the K_i values observed in the inhibition of malate dehydrogenase by these compounds. All three compounds decreased the growth rate of the organism, by 76%, 62% and 84%, respectively.

DISCUSSION

Since several *Haemophilus* organisms exhibit an unusual growth requirement for external NAD⁺, selective inhibition of enzymic processes involved in the acquisition and intracellular utilization of NAD⁺ could have a pronounced effect on the growth of these organisms. The design of effective inhibitors of NAD⁺-dependent enzymes may be facilitated by determining the specific interactions of importance in the dinucleotide binding processes of these enzymes. Recent site-mapping studies of the *H. influenzae* periplasmic nucleotide pyrophosphatase (Kahn & Anderson, 1986) led to the recognition of several effective inhibitors of this important NAD⁺-processing enzyme. Of special interest was the correlation between ADP inhibition of the nucleotide pyrophosphatase and ADP inhibition of growth of the organism.

The coenzyme-competitive inhibition observed with carboxylic acids in the present study provided the opportunity to probe the NAD⁺ binding site for the presence of a hydrophobic region near the pyrophosphate region. The positive chainlength effects observed in the binding of carboxylic acids (Table 2) are consistent with a facilitation of binding of these compounds through non-polar interactions at the coenzyme binding site. Multiple inhibition analysis (Fig. 2) demonstrated that hexanoic acid and ADP interact with a common region at the NAD⁺ binding site. The presence of a hydrophobic region provides information of importance for the further design of effective inhibitors of this enzyme. The coenzyme-competitive inhibition of dehydrogenases by compounds having no obvious structural analogy to the coenzyme molecule can be explained on the basis of interactions of these compounds with different regions existing in the coenzyme binding site. In the case of the *Haemophilus* malate dehydrogenase, the presence of a hydrophobic region adjacent to the pyrophosphate region of the NAD⁺ binding site provides the necessary two-point interactions for the selective binding of aliphatic carboxylic acids. Without prior knowledge of the spatial arrangement of regions at this binding site, coenzyme-competitive inhibition by carboxylic acids would not have been anticipated. The competitive binding of fatty acids and alkylammonium ions to the NAD⁺ binding site of rabbit muscle L- α -glycerophosphate dehydrogenase was likewise attributed to selective interactions of these compounds with three spatially related regions at this coenzyme binding site (Kim & Anderson, 1968*a*, 1969).

In coenzyme-competitive inhibition studies (Table 1), the relatively poor binding of the ethenoadenine analogue was consistent with previous studies (Yoon & Anderson, 1988) demonstrating that structural alterations in the purine portion of the dinucleotide reduced the effectiveness of these derivatives to serve as coenzymes. 3-Aminopyridine adenine dinucleotide likewise exhibited relatively poor binding, which indicates that the very potent inhibition of growth of *H. influenzae* by this analogue (Kahn & Anderson, 1986) cannot be explained on the basis of interactions with the malate dehydrogenase. Of the NAD⁺ analogues studied, 3-pyridylcarbinol adenine dinucleotide was the most effective inhibitor. The hydrogen-bonding possibilities of the 3-carbinol substituent may enhance the binding of this derivative. The dye Procion Blue HB was by far the most effective inhibitor studied, having a K_i value of 0.2 μ M.

In studies of substrate-competitive inhibition by structural analogues of malate (Table 3), 2-oxoglutarate was the most effective inhibitor, being 64 times better than the next best inhibitor, hydroxymalonic acid. The better inhibition by the oxoacid is consistent with the previous observation that the K_m for malate is 22-fold higher than that of the oxo substrate, oxalacetate.

Although selective interactions with the pyridinium moiety of the coenzyme are to be expected, the lack of effective binding of *N*¹-alkylnicotinamide chlorides suggests that such interactions are not available in the absence of the ADP-ribose portion of the coenzyme. Such observations would be consistent with a conformational change induced by the binding of the adenosine half of the coenzyme molecule. The absence of direct binding of *N*¹-alkylnicotinamide chlorides was observed previously (Anderson & Reynolds, 1966) with beef heart and rabbit muscle lactate dehydrogenases and yeast glucose-6-phosphate dehydrogenase; in contrast, yeast alcohol dehydrogenase (Anderson *et al.*, 1965), rabbit muscle glycerophosphate dehydrogenase (Kim & Anderson, 1968*b*) and bovine liver glutamic dehydrogenase (Anderson & Reynolds, 1966) bind these pyridinium derivatives in a direct and selective fashion.

The three most effective inhibitors of the purified malate dehydrogenase were observed to

inhibit growth of the organism. At present, growth inhibition by 2-oxoglutarate and 3-pyridylcarbinol adenine dinucleotide cannot be directly correlated with intracellular interactions with the malate dehydrogenase since the metabolic fate of these compounds is unknown. As a substrate for the periplasmic nucleotide pyrophosphatase, the 3-pyridylcarbinol analogue would, upon hydrolysis, yield the 3-pyridylcarbinol mononucleotide; however, this mononucleotide may not serve as a substrate for the intracellular NMN:ATP adenylyltransferase. The substrate specificity of the adenylyltransferase has yet to be determined. Of the three inhibitors tested, Procion Blue HB was the most effective growth inhibitor, exhibiting 84% inhibition at 1 μM . The expected inability of this large polar molecule to diffuse into the cells suggests that the growth inhibition is probably due to inhibition of the periplasmic nucleotide pyrophosphatase. This is supported by the fact that affinity chromatography on Matrex Blue gel was a very effective step in the purification of the pyrophosphatase (Kahn & Anderson, 1986). The previously reported inhibition of growth of *H. influenzae* by 3-aminopyridine adenine dinucleotide (Kahn & Anderson, 1986) was confirmed in the present study. Although this analogue remains the most potent inhibitor of growth of the organism, exhibiting 40% inhibition at 0.1 μM , the specific process involved in this inhibition remains to be identified.

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