Interactions of nicotinamide–adenine dinucleotide phosphate analogues and fragments with pigeon liver malic enzyme

Synergistic effect between the nicotinamide and adenine moieties

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The structural requirements of the NADP⁺ molecule as a coenzyme in the oxidative decarboxylation reaction catalysed by pigeon liver malic enzyme were studied by kinetic and fluorimetric analyses with various NADP⁺ analogues and fragments. The substrate L-malate had little effect on the nucleotide binding. Etheno-NADP⁺, 3-acetylpyridine–adenine dinucleotide phosphate, and nicotinamide–hypoxanthine dinucleotide phosphate act as alternative coenzymes for the enzyme. Their kinetic parameters were similar to that of NADP⁺. Thionicotinamide–adenine dinucleotide phosphate, 3-aminopyridine–adenine dinucleotide phosphate, 5'-adenylyl imidodiphosphate, nicotinamide–adenine dinucleotide 3'-phosphate and NAD⁺ act as inhibitors for the enzyme. The first two were competitive with respect to NADP⁺ and non-competitive with respect to L-malate; the other inhibitors were non-competitive with NADP⁺. All NADP⁺ fragments were inhibitory to the enzyme, with a wide range of affinity, depending on the presence or absence of a 2'-phosphate group. Compounds with this group bind to the enzyme 2–3 orders of magnitude more tightly than those without this group. Only compounds with this group were competitive inhibitors with respect to NADP⁺. We conclude that the 2'-phosphate group is crucial for the nucleotide binding of this enzyme, whereas the carboxamide carbonyl group of the nicotinamide moiety is important for the coenzyme activity. There is a strong synergistic effect between the binding of the nicotinamide and adenosine moieties of the nucleotide molecule.

INTRODUCTION

Malic enzyme [malate dehydrogenase (oxaloacetate-decarboxylating) (NADP⁺), EC 1.1.1.40] is a bifunctional enzyme. In the presence of bivalent cations it catalyses the oxidative decarboxylation of L-malate to give CO₂ and pyruvate, with concomitant reduction of NADP⁺ to NADPH (Ochoa *et al.*, 1947, 1948). In animal cells, malic enzyme may have various metabolic roles (for review see Frenkel, 1975). In the cytosol, it may function to provide NADPH for the biosynthesis of fatty acids or other NADPH-dependent anabolic reactions. In mitochondria, synthesis of L-malate by the reverse reaction may serve as an anaplerotic reaction of the tricarboxylic acid cycle. It may also serve as an important route for the total combustion of the tricarboxylic acid-cycle intermediates. More recently, mitochondrial malic enzyme has been correlated with the metabolism and regulation of glutamine, which is the major energy source for tumour cells (cf. Saucer & Dauthy, 1978; Moreadith & Lehninger, 1984).

Malic enzyme from pigeon liver has been studied extensively (for review see Hsu, 1982). A random order of addition for Mn²⁺ and NADP⁺ has been proposed. L-Malate binds last in the reaction, after both Mn²⁺ and NADP⁺ (Hsu & Pry, 1980). The products are released in the order CO₂, pyruvate and NADPH (Hsu *et al.*, 1967;

Schimerlik *et al.*, 1977). The release of NADPH is the rate-limiting step (Schimerlik *et al.*, 1977; Pry & Hsu, 1980). On the basis of kinetic measurements, Schimerlik & Cleland (1977b) proposed that two amino acid residues, with pK_a values of 9.3 and 5.3 respectively, were involved in the NADP⁺ binding. Our chemical modification studies (Chang & Hsu, 1977; Chang & Huang, 1979; Chang *et al.*, 1982) suggested that these residues were histidine and lysine respectively. Substrate (L-malate) specificity of malic enzyme has been studied by Schimerlik & Cleland (1977a). The nucleotide binding has also been studied by Hsu and colleagues, using equilibrium-dialysis and fluorimetric-titration techniques (Hsu & Lardy, 1967b; Pry & Hsu, 1980). However, information on the detailed structural requirements for the nucleotide binding and reaction for this enzyme is still lacking.

In the present work, fluorescence quenching and steady-state kinetic techniques were used to investigate the pyridine nucleotide specificity of pigeon liver malic enzyme. Studies on the binding of NADP⁺ fragments to the enzyme showed that binding of the nicotinamide and adenine moieties of the NADP⁺ molecule was positively co-operative. The 2'-phosphate group was found to be important for the NADP⁺ binding, whereas the carboxamide carbonyl group of the pyridine ring was essential for activity.

Abbreviations used: ϵ -NADP⁺, nicotinamide–1,N⁶-ethenoadenine dinucleotide phosphate; NHDP⁺, nicotinamide–hypoxanthine dinucleotide phosphate; APADP⁺, 3-acetylpyridine–adenine dinucleotide phosphate; SNADP⁺, thionicotinamide–adenine dinucleotide phosphate; AADP⁺, 3-aminopyridine–adenine dinucleotide phosphate; [β -imidol]ATP, 5'-adenylyl imidodiphosphate (adenosine 5'-[β -imidol]triphosphate); 3'-NADP⁺, nicotinamide–adenine dinucleotide 3'-phosphate; 2',5'-ADP, adenosine 2',5'-bisphosphate; MNA, N⁶-methylnicotinamide.

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MATERIALS AND METHODS

Materials

NADP⁺, ϵ -NADP⁺, NHDP⁺, APADP⁺, SNADP⁺, AADP⁺, 3'-NADP⁺, NAD⁺, [$\beta\gamma$ -imido]ATP, nicotinamide, MNA, NMN, 2'-AMP, 5'-AMP, 5'-ADP and 2',5'-ADP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The purity of NADP⁺ and its analogues was checked in a Pharmacia fast-protein-liquid-chromatography system with a Polyanion SI HR5/5 column (50 mm $\times$ 5 mm internal diam.) as described by Hansson (1983). All NADP⁺ analogues were shown to be free from NADP⁺ contamination before the experiments were done.

Other chemicals were all of reagent grade and obtained from either Sigma Chemical Co. or E. Merck (Darmstadt, Germany). Distilled deionized water was used throughout this work.

Enzyme preparation

Malic enzyme was purified from pigeon liver by our published procedure (Chang & Chang, 1982). The purified enzyme was routinely checked for purity by polyacrylamide-gel electrophoresis. Protein concentration was determined spectrophotometrically at 278 nm (Hsu & Lardy, 1967a).

Enzyme assay

Malic enzyme activity was assayed at 30 °C as described by Hsu & Lardy (1967a). The formation of NADPH was monitored continuously at 340 nm in a Gilford 2600 spectrophotometer. In a standard assay, the reaction mixture contained 66.7 mM-triethanolamine/HCl buffer, pH 7.4, 0.5 mM-sodium L-malate, pH 7.4, 4 mM-MnCl₂, 0.21 mM-NADP⁺ and an appropriate amount of enzyme in a total volume of 1 ml.

In the kinetic experiments, when nucleotide was used as the varied-concentration substrate, the assay was performed in a 10 cm cuvette compartment. Cylindrical cuvettes with a total volume of 30 ml and 10 cm light-path were used. The formation of NADPH was linear with time at least for several minutes, and the slope of the graphical plot was taken as the reaction rate. A molar absorption coefficient of $6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for NADPH was used for calculation.

Kinetic studies

Initial-velocity studies were performed by varying the concentrations of NADP⁺ or its analogue from 0.86 to $3.36 \mu\text{M}$ at substrate (L-malate) concentrations of 61–191 μM . Concentrations of the other components in the assay mixture were held constant.

Inhibition studies were performed in a similar manner, with NADP⁺ or L-malate as the varied-concentration substrate. The pyridine nucleotide inhibitor was held at several fixed concentrations around its inhibition constant.

Double-inhibition studies were performed with the L-malate concentration kept at 0.5 mM and the NADP⁺ concentration at 210 μM . Two compounds, one containing the nicotinamide end and the other containing the adenine end of the dinucleotide, were added to the reaction mixture separately or in combination. I_{50} was calculated as the concentration where half of the original enzyme activity was inhibited.

Fluorescence studies

Fluorescence-quenching studies were performed in a Farrand system 3 thermoregulated spectrofluorimeter as described previously (Chang & Lee, 1984). An excitation wavelength of 295 nm and emission wavelength of 328 nm were used. At the concentrations used in fluorescence titration, the absorbance of NADP⁺ at the excitation wavelength was not detectable, thus no correction was necessary for the inner-filter effect. However, substantial absorption was observed for SNADP⁺, ϵ -NADP⁺, and AADP⁺. The absorption of these quencher molecules at 295 nm was corrected as described by Lehrer & Leavis (1978):

$$F_{\text{corr.}} = F_{\text{obs.}} \cdot 10^{\Delta A/2} \quad (1)$$

where $\Delta A/2$ is the increase in absorbance at the centre of the cuvette caused by the addition of the quencher. The absorption was much lower than 0.2 A , and thus the above simple correction equation should be valid (Birdsall *et al.*, 1983). All reported values were the final concentrations. The fluorescence titration was performed with free enzyme, or in the presence of L-malate (0.25 mM), tartronate (0.25 mM) or Mn²⁺ (2 mM), or in various combinations. Quenching by AADP⁺ was performed in a similar manner.

Data processing

When NADP⁺ analogue was used as the coenzyme, reciprocal initial velocities were plotted versus reciprocal substrate concentrations. Data were fitted to eqn. (2):

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

where K_a , K_b and K_{ia} are Michaelis constants for A and B and dissociation constant for A respectively. A and B are reactant concentrations, A for NADP⁺ or its analogue and B for L-malate. V denotes the maximum velocity of the reaction.

When NADP⁺ analogue was tested as an inhibitor for the enzymic reaction, initial-velocity data exhibiting linear competitive or non-competitive inhibition were fitted to eqn. (3) or (4) respectively:

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (3)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (4)$$

where I is the inhibitor concentration. K_{is} and K_{ii} are the apparent inhibition constants associated with the effect of the inhibitor on the slope (K_{is}) and intercept (K_{ii}) of the double-reciprocal plots respectively (Cleland, 1963).

Initial-velocity data for double-inhibition experiments were fitted to eqn. (5):

$$v = \frac{VA}{K_a \left(1 + \frac{I_1}{K_{i1}} + \frac{I_2}{K_{i2}} + \frac{I_1 I_2}{\alpha K_{i1} K_{i2}} \right) + A} \quad (5)$$

where I_1 and I_2 represent the concentrations of two inhibitors. K_{i1} and K_{i2} denote the inhibition constants for I_1 and I_2 respectively. α represents an interaction coefficient. v_0/v_i instead of $1/v$ was plotted against I_1 or I_2 (Yonetani, 1982).

The dissociation constant (K_d) for NADP⁺ with malic enzyme was calculated from fluorescence-quenching data

Fig. 1. Quenching of pigeon liver malic enzyme fluorescence by NADP^+

The enzyme (9.1 $\mu\text{g}/\text{ml}$) in 0.67 M-triethanolamine/HCl buffer, pH 7.4, was excited at 295 nm, and fluorescence at 328 nm was determined with successive additions of NADP^+ . Additions in the cuvette: curve a, none; curve b, 0.25 mM-tartronate; curve c, 0.25 mM-L-malate; curve d, 2 mM- MnCl_2 ; curve e, 0.25 mM-tartronate plus 2 mM- MnCl_2 . (a) Stern-Volmer plot; (b) Lehrer (1971) plot; (c) double-reciprocal plot.

as described by Ward (1985). Fluorescence-quenching data were also fitted to eqn. (6) for calculation of various quenching parameters (Lehrer & Leavis, 1978):

$$F_0/\Delta F = 1/(f_a K_{sv} Q) + 1/f_a \quad (6)$$

where $\Delta F = F_0 - F$. K_{sv} is the dynamic quenching constant, f_a is the fractional maximum accessible protein fluorescence, and Q is the quencher concentration. Experimental data were fitted to eqn. (6) by the least-squares method by using the computer program written by R. S. Chang (Chang & Chang, 1987)

RESULTS

Quenching of the enzyme intrinsic fluorescence by titration with NADP^+

When a protein is excited at 295 nm, its emission spectrum represents the tryptophan fluorescence, because the tyrosine contribution is negligible at this wavelength (cf. Kahan *et al.*, 1986). The emission spectrum of the native malic enzyme at pH 7.4 and 30 °C shows that the enzyme fluoresces at 328 nm, with a shoulder at 340 nm.

Table 1. Fluorescence parameters of NADP^+ for pigeon liver malic enzyme

Values shown were from a single representative experiment.

Additions	K_d (μM)	f_a	k_{sv}
None	26.3	0.031	0.037
Tartronate (0.25 mM)	35.7	0.058	0.026
L-Malate (0.25 mM)	35.7	0.046	0.026
Mn^{2+} (2 mM)	44.4	0.070	0.025
Tartronate (0.25 mM) + Mn^{2+} (2 mM)	52.6	0.104	0.018

Addition of NADP^+ caused quenching of the fluorescence, without altering the spectrum. Titration of the enzyme with NADP^+ gave a downward Stern-Volmer plot (Fig. 1a). A Lehrer (1971) plot (based on eqn. 6) was thus used to analyse the data (Fig. 1b). The fluorescence parameters are summarized in Table 1. The results indicate that tryptophan residues are inaccessible to

Fig. 2. Arrhenius plot for the NADP⁺ quenching of pigeon liver malic enzyme fluorescence

The experimental procedure was as described in Fig. 1 legend. Enzyme concentrations were 3.8, 4.6, 3.8 and 18.5 $\mu\text{g/ml}$ respectively for lines a, b, c and d. Line a was obtained in the presence of 0.25 mM-L-malate.

NADP⁺. Both the K_{sv} and the f_a values were very small. No static quenching was assumed. The dissociation constant of NADP⁺, calculated from the plot of $1/\Delta F$ versus $1/[\text{NADP}^+]$, was approx. 26 μM (Fig. 1c). Malate, tartronate (a substrate analogue) or Mn^{2+} increased the NADP⁺ dissociation constant (Table 1) without changing the fluorescence spectra. The affinity of NADP⁺ for the enzyme decreased with increasing temperature, yielding a $\Delta H_{\text{dissociation}}$ value of 75.6 $\text{kJ}\cdot\text{mol}^{-1}$, which was not affected by the L-malate binding (Fig. 2).

It should be pointed out that the slight quenching (approx. 3%) accompanying coenzyme binding prevents the use of the present method to measure the NADP⁺ binding constant precisely. Various NADP⁺ analogues were also tested for quenching of the enzyme intrinsic fluorescence. Among the analogues, SNADP⁺, AADP⁺

and ϵ -NADP⁺ gave respectively 31%, 21% and 17% quenching of protein fluorescence. The first two analogues were not reduced by malic enzyme, and were found to be competitive inhibitors with respect to NADP⁺ (see below). Serious self-absorption of SNADP⁺ prevents its practical use. AADP⁺ was thus chosen to investigate the binding of the enzyme-nucleotide binary complex and enzyme-nucleotide-malate ternary complex. The K_d , f_a and K_{sv} for AADP⁺ were found to be $1.84 \pm 0.6 \mu\text{M}$, 0.043 ± 0.01 and 0.91 ± 0.3 respectively. The increase in the nucleotide-binding constant by L-malate was confirmed. In the presence of 0.25 mM-L-malate, K_d for AADP⁺ increased to $4.10 \pm 3.4 \mu\text{M}$.

Initial-velocity studies with NADP⁺ analogues as alternative coenzyme

ϵ -NADP⁺, NHDP⁺ and APADP⁺ were found to be good alternative coenzymes for the malic enzyme-catalysed reaction. They exhibit apparent V and K_m values similar to those for the natural coenzyme, and gave an intersecting type of initial-velocity patterns. The pattern obtained with ϵ -NADP⁺ is illustrated in Fig. 3. Table 2 lists the various kinetic parameters that were obtained by fitting the data to eqn. (2).

3'-NADP⁺ was a poor coenzyme, with V of $6.75 \pm 0.35 \mu\text{mol/min per mg}$, which is only 31% that for NADP⁺. NAD⁺, SNADP⁺ or AADP⁺ showed no detectable coenzyme activity.

Inhibition patterns of NADP⁺ analogues

SNADP⁺ and AADP⁺ were found to be strong inhibitors of the enzyme reaction with NADP⁺ and L-malate as substrates. The inhibition patterns were linear competitive with respect to NADP⁺, and linear non-competitive with respect to L-malate, for both analogues (Table 3). A typical result obtained with SNADP⁺ is shown in Fig. 4.

Both NAD⁺ and 3'-NADP⁺ were poor inhibitors, with K_i values 2–3 orders of magnitude larger than those for the analogues with a 2'-phosphate group. The inhibition

Fig. 3. Initial velocity of pigeon liver malic enzyme with ϵ -NADP⁺ as an alternative coenzyme

(a) The varied-concentration substrate was L-malate and the fixed-concentration substrate was ϵ -NADP⁺. From bottom to top the concentrations of ϵ -NADP were 3.36, 2.52, 1.68 and 0.86 μM respectively. (b) The varied-concentration substrate was ϵ -NADP⁺ and the fixed-concentration substrate was L-malate. From bottom to top the concentrations of L-malate were 0.191, 0.126, 0.093 and 0.061 mM respectively.

Table 2. Kinetic parameters for the reaction catalysed by pigeon liver malic enzyme with different pyridine nucleotides as the coenzymeResults are weighted averages $\pm$ S.E.M. calculated from two or three experiments.

Nucleotide	Kinetic parameter				
	V (μ mol/min per mg)	K_{ia} (μ M)	K_a (μ M)	K_b (μ M)	K_{ia}/K_a
NADP ⁺	21.3 $\pm$ 0.2	0.96*	1.57 $\pm$ 0.7 (1.4)*	82 $\pm$ 1 (86)*	0.61
ϵ -NADP ⁺	(26 $\pm$ 3)†	(5.9 $\pm$ 0.5)†	(4.1 $\pm$ 0.4)†	(290 $\pm$ 20)†	
NHDP ⁺	23.3 $\pm$ 0.4	1.09 $\pm$ 0.2	1.59 $\pm$ 0.2	125	0.68
APADP ⁺	23.9 $\pm$ 1.4	0.84 $\pm$ 0.02	0.92 $\pm$ 0.01	95 $\pm$ 4.5	0.91
	36.6 $\pm$ 1.2	3.46 $\pm$ 1.2	5.03 $\pm$ 0.6	300 $\pm$ 200	0.69

* From Hsu *et al.* (1967).

† From Schimerlik & Cleland (1977a).

Table 3. Inhibition patterns for the dead-end inhibitors of pigeon liver malic enzyme

L-Malate and NADP⁺ were held at 0.5 mM and 96.3 μ M respectively when used as the fixed substrate. In all assays Mn²⁺ concentration was 4 mM. Abbreviations: C, competitive; NC, non-competitive. K_{is} and K_{ii} were obtained from the slope and intercept replots respectively of the double-reciprocal plots. Values shown are averages $\pm$ S.E.M. of two or three experiments.

Varied-concentration substrate	Fixed-concentration substrate	Inhibitor	Inhibition pattern	Apparent inhibition constant	
				K_{is} (μ M)	K_{ii} (μ M)
NADP ⁺	L-Malate	SNADP ⁺	C	2.9 $\pm$ 0.6	
L-Malate	NADP ⁺	SNADP ⁺	NC	3.9 $\pm$ 0.1	6.8 $\pm$ 2.3
NADP ⁺	L-Malate	AADP ⁺	C	2.0 $\pm$ 0.3	
L-Malate	NADP ⁺	AADP ⁺	NC	26.2 $\pm$ 5.3	29.7 $\pm$ 4.7
NADP ⁺	L-Malate	[β -Imido]ATP	NC	640 $\pm$ 100	3000 $\pm$ 200
NADP ⁺	L-Malate	3'-NADP ⁺	NC	2300	1950
NADP ⁺	L-Malate	NAD ⁺	NC	1650	1500

was non-competitive with respect to NADP⁺ for both analogues (Table 3).

Synergistic inhibition of malic enzyme-catalysed reaction by NADP⁺ fragments

Fragments of NADP⁺ were divided into two categories: 5'-AMP, 2'-AMP, 2',5'-ADP and 5'-ADP were the group A fragments, containing the adenine end; nicotinamide, MNA and NMN were the group B fragments, containing the nicotinamide end. All these fragments were inhibitory to the malic enzyme. Those with the 2'-phosphate group were competitive, and the others non-competitive, with respect to NADP⁺. Group A and group B compounds exhibited strong positive co-operativity when used together. NMN did not show any observable inhibition until the concentration reached 9 mM. No inhibition was observed with 5'-AMP up to 4 mM. However, when 4 mM-AMP and 9 mM-NMN were used in combination, over 80% enzymic activity was inhibited (Fig. 5). The calculated α value was about 0.045 ± 0.015 , indicating a positive interaction between AMP and NMN in the enzyme-AMP-NMN ternary complex. The plot of v_0/v_i versus I_1 or $(I_1 + I_2)$ was not linear; the above α value was calculated from the initial linear portion. No attempts

were made to calculate the α value for the other combinations, where similar synergistic effects were observed. The results are summarized in Table 4. However, no synergistic effect between AMP and ribose 5'-phosphate was detectable (Fig. 6), indicating that the synergistic effect was due to the interactions between the nicotinamide and the adenosine parts. The I_{50} for ribose 5'-phosphate was near 190 mM.

DISCUSSION

The kinetic mechanism of malic enzyme requires an obligatory binding order. The initial-velocity data in this paper indicate that APADP⁺, ϵ -NADP⁺ and NHDP⁺ are alternative coenzymes for this enzyme and conform to the same sequential kinetic mechanism. L-Malate has little effect on the binding of pyridine nucleotides, as reflected in the ratio of the values for K_{ia} and K_a (Table 2). These results are in accordance with the fluorescence-quenching data (Table 1), where the ratio of K_d for the enzyme-NADP⁺ binary complex to that for the enzyme-NADP⁺-malate ternary complex was found to be 0.74. The greater quenching of the enzyme by AADP⁺ may be explained by its higher dynamic quenching constant

Fig. 4. Inhibition of pigeon liver malic enzyme by SNADP⁺

(a) Competitive inhibition with respect to NADP⁺. From top to bottom the concentrations of SNADP⁺ were 5.9, 4.2, 2.5 and 0 μM respectively. (b) Non-competitive inhibition with respect to L-malate. From top to bottom the concentrations of SNADP⁺ were 4.2, 2.5, 0.84 and 0 μM respectively.

Fig. 5. Synergistic inhibition of pigeon liver malic enzyme by NMN and 5'-AMP

Inhibition of the enzyme activity by various concentrations of 5'-AMP in the absence (○ and ●) or in the presence (□ and ■), of 9 mM-NMN. The results from separate experiments are represented by ○ and □ and by ● and ■.

(K_{sv}), which is an order of magnitude higher than that for NADP⁺.

Similar relative maximum velocities observed for ϵ -NADP⁺, NHDP⁺ and NADP⁺ (Table 2) indicate that an intact adenine structure is not essential for activity. On the contrary, structural changes at the nicotinamide part drastically change the coenzyme activity. The carbonyl group of the nicotinamide carboxamide must be essential in the hydride-transfer reaction, since removal of the carbonyl group (giving AADP⁺) or replacement of the carbonyl oxygen with a sulphur atom (giving SNADP⁺) totally abolished the coenzyme activity. For a pyridine nucleotide coenzyme to be reduced, a carbonium ion resonance structure was presumably to be involved in the transition state for

nucleophilic attack by the reductant (for review see Grau, 1982). At the 3-position of the pyridine ring, a group with electron-withdrawing power sufficient to activate the 4-position can result in a coenzymically active analogue (for review see Anderson, 1982). APADP⁺ has an acetyl group which is more electron-withdrawing than the carboxamide group, and, indeed, higher activity was found for this analogue. SNADP⁺ has a similar oxidation potential (−285 mV) to NADP⁺ (−320 mV). However, malic enzyme was unable to utilize SNADP⁺. The larger thioamide group must create sufficient steric hindrance to interfere with its proper orientation. SNADP⁺ was found to be inactive in the reaction catalysed by dihydrofolate reductase. The reason was attributed to the difference in the mode of binding of SNADP⁺ and NADP⁺ (Hyde *et al.*, 1980a,b). Feeney *et al.* (1983) suggested that the thionicotinamide moiety of SNADP⁺ did not have any specific interaction with the dihydrofolate reductase, but rather extended out into solution. Both SNADP⁺ and AADP⁺ were found to be strong inhibitors for malic enzyme, with inhibition constants in the micromolar range. The binding of these analogues with the enzyme thus seems not to be impaired.

The ribose moiety of the nicotinamide mononucleotide part was not essential for activity, since periodate-oxidized NADP⁺, which has the ribose ring opened to give a 2',3'-dialdehyde derivative, was active in the malic enzyme reaction with apparent K_m 30 ± 2 μM (Chang & Huang, 1979).

Pigeon liver cytosolic malic enzyme is specific for NADP⁺; NAD⁺ was inactive. With the 2'-phosphate group moved to the 3'-position, its activity was drastically decreased. In all analogues and fragments tested, compounds with a 2'-phosphate group gave K_m or K_i values in the micromolar range. Those without this group were all found to be inhibitory; however, they are non-competitive with respect to both NADP⁺ and L-malate. The K_i values were 2–3 orders of magnitude larger than those for the nucleotides with the 2'-phosphate group. The non-competitive nature with respect to NADP⁺ suggested that allowance must be made for an additional interaction of these inhibitors

Table 4. Synergistic inhibition of pigeon liver malic enzyme by NADP⁺ fragments

Abbreviations: NC, non-competitive; C, competitive. I_{50} was the inhibitor concentration that decreased V by 50%. Numbers in parentheses represent the times the experiment was repeated where results are given as averages $\pm$ S.E.M.

Inhibitor 1			Inhibitor 2		I_{50} for inhibitor 1 (mM)
Identity	K_i (mM)	Inhibition	Identity	Concn. (mM)	
MNA	58	NC	None	—	215 $\pm$ 73 (6)
			5'-ADP	0.2	125
				2.0	18 $\pm$ 5 (2)
				4.0	4
				4.2	2
			2',5'-ADP	0.05	155
				0.15	150
				0.2	125
			5'-AMP	1.0	85
			2'-AMP	1.0	75
NMN	7.1	NC	None	—	8.8 $\pm$ 3.2 (2)
			5'-AMP	1	4.2
				4	2.0
			2'-AMP	1	3.6
5'-AMP	3.8	NC	None	—	4.9 $\pm$ 0.1 (3)
2'-AMP	0.035	C	NMN	9	1.8 $\pm$ 0.2 (3)
			None	—	2.4
5'-ADP	4.2	NC	MNA	48	1.7
				119	< 0.1
			None	—	3.6 $\pm$ 0.6 (5)
			MNA	70	3.4 $\pm$ 0.3 (2)
2',5'-ADP	0.006	C	NMN	2	1.8
			None	—	1.2
			MNA	44	0.9
				76	0.3

Fig. 6. Inhibition of pigeon liver malic enzyme by 5'-AMP and ribose 5'-phosphate

Experimental conditions were the same as for Fig. 5. ○, 5'-AMP only; ●, 5'-AMP plus 9 mM-ribose 5'-phosphate; □, 5'-AMP plus 9 mM-NMN.

with another enzyme form. The 2'-phosphate group has been found to be essential for nucleotide binding for many NADP⁺-specific enzymes (cf. Mas & Colman, 1984; Stone *et al.*, 1984).

We found strong interactions between the adenine and nicotinamide parts of NADP⁺. Synergistic effects are

characteristic of a sequential response. The response of the nucleotide site of malic enzyme may be proposed as follows: for binding of the NADP⁺ molecule to the enzyme, the 2'-phosphate group of the adenosine makes a significant contribution to the binding energy. The functional role of the adenosine part is like a handle to facilitate the binding of the reactive nicotinamide part, which is poorly bound by itself. It is not necessary for the nicotinamide and adenosine parts to be covalently joined to give interactions. However, an intact dinucleotide structure is required for reduction to take place, since we have found that NMN or MNA cannot serve as a coenzyme for malic enzyme, with or without the help of adenosine. These results indicate that there are direct interactions among the different parts of the NADP⁺ molecules.

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