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NICOTINAMIDE-ADENINE DINUCLEOTIDE INHIBITION OF PIG KIDNEY ALKALINE PHOSPHATASE

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Summary

1. The interaction of NAD^+, NADH and various nucleotide analogues with pig kidney alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1) has been investigated by kinetic means. Some inhibitors act uncompetitively whereas others markedly increase the slopes of double reciprocal plots suggesting they have some affinity for the free enzyme.

2. The compounds seem to bind to alkaline phosphatase through interactions of their bases with a relatively non-specific region of the enzyme, although it is likely that for those nucleotides having some affinity for the free enzyme there is some attraction between the pyrophosphate backbone and the active site.

3. From studies of the effect of NAD^+ and NADH on ATPase activity it was concluded that the substrate inhibition that is characteristic of the ATPase activity of alkaline phosphatase originates from binding of ATP to the site assumed to exist for NAD^+ and NADH. The potentiation of NAD^+-inhibition of ATPase activity by Mg^{2+} is probably a result of the depletion of $[ATP^{4-}]$ the true substrate. The depletion allows NAD^+ to compete more effectively for the active site.

4. Binding of NADH is favoured by protonation of an enzymic group with a pK of approx. 9.0 belonging possibly to a tyrosine residue or a zinc hydrate.

5. A large entropy decrease was found to accompany the binding of NAD^+ and NADH to alkaline phosphatase. This may be further evidence of an "induced-fit" mechanism previously suspected because of the synergistic inhibitory effects of adenosine and nicotinamide.

Introduction

In a previous report [1] we described the inhibition of pig kidney alkaline

phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1.) by NAD^+, NADH, nicotinamide and adenosine. Inhibition by NAD^+ resulted in double reciprocal plots that converged to the left of the vertical axis, i.e. the inhibition can be described as "mixed" [2] or non-competitive [3]. The other compounds acted as uncompetitive inhibitors. The inhibition patterns were discussed in terms of a model in which the uncompetitive agents have affinity only for an enzyme · substrate complex (or a phosphoryl-enzyme intermediate) whereas the non-competitive inhibitors have affinity for the free enzyme also [1].

A comparison of NAD^+ and NADH as inhibitors showed that the two forms of the coenzyme differed not only in type of inhibition, but also in potency; NADH being the most effective. In spite of these differences, the kinetic evidence seemed to favour the conclusion that the two forms of the coenzyme competed for the same binding region on alkaline phosphatase and that a conformational change in the enzyme protein may accompany the binding of these substances.

As the inhibition of alkaline phosphatase by NAD^+ and NADH could possibly be used for control purposes in vivo, (see [1] for an extensive discussion of this point) the inhibition has been studied further to gain an insight into the ways that these compounds interact with kidney alkaline phosphatase. The studies included experiments with coenzyme analogues and investigations of the effects of pH and temperature on inhibition.

The response of the ATPase activity associated with alkaline phosphatase to NAD^+ and NADH has also been investigated since substrate inhibition occurs when ATP is hydrolysed by pig kidney alkaline phosphatase, and the structural similarities of ATP, NAD^+ and NADH could mean that these compounds form inhibitory complexes by binding to a common site on the enzyme.

Experimental

Materials

ATP, NAD^+ (Grade III), NADH, coenzyme analogues, *N*-acetylimidazole and bovine serum albumin were purchased from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. The compounds were used without further purification. The ATP was of special grade and contained minimal amounts of Ca^{2+} and other bivalent cations. Disodium *p*-nitrophenyl phosphate was obtained from British Drug Houses, Poole, Dorset, U.K., and tetranitromethane from Ralph Emanuel, Wembley, U.K. All other chemicals were of the best grades available.

Alkaline phosphatase

The enzyme was purified from pig kidney by the methods described previously [4,11]. The product had a specific activity of 30 μmol product formed/min per mg of protein and this enzyme was used in most of the studies described below. The experiments in which ATPase activity was measured however, were conducted at a later stage of the investigation by which time the purification method had been improved to allow structural studies on the enzyme [11]. Thus the ATPase activity was studied with enzyme of a specific

activity of 100 μmol/min per mg protein. Purified enzyme in 0.05 M Tris · HCl buffer (pH 7.6) was stored at −15°C and diluted approx. 4-fold in bovine serum albumin solution (0.1% in water) on the day of each experiment. The diluted enzyme was kept at 0°C for the duration of the experiment.

Enzyme assay and determination of kinetic parameters

Hydrolysis of *p*-nitrophenyl phosphate at 30°C and pH 9.0 was followed by recording the increase in A_{400nm} resulting from the release of *p*-nitrophenol in a Perkin-Elmer 124 double beam spectrophotometer. The reaction mixtures of 3 ml contained either 0.05 M Tris · HCl buffer or 0.05 M ammediol · HCl (2-amino-2-methyl-1,3-propanediol · HCl) buffer (both at pH 9.0), either 1.0 mM *p*-nitrophenyl phosphate or a range of 10—100 μM substrate and 10 μl of diluted enzyme solution. When inhibitors were included in reaction mixtures, the pH values of the incubation media were checked to ensure that there was no deviation from pH 9.0 either before or during incubation. Under all conditions the amount of product formed increased linearly with time during the observation period. Enzymic activity (v) was calculated as μmol product formed/min per mg of enzyme protein.

The results of kinetic experiments were analysed by calculating a non-weighted "least squares" fit to the double reciprocal form of the Michaelis-Menten equation or, where appropriate, to the linear equation relating $1/v$ with inhibitor concentration [2]. Values for K_{is} and K_{ii} were obtained from the calculated fit and analysis of results from several experiments allowed mean values and standard deviations for kinetic constants to be determined. The terminology used to describe inhibition patterns is that of Cleland [3].

ATPase assay

The system was based on that already described [5,6] but with some modifications. Reaction volumes were of 1.0 ml and contained 0.05 M ammediol · HCl buffer (pH 9.0) plus ATP, NAD^+, NADH and $MgCl_2$ at the concentration indicated in Results. The pH of reaction mixtures was checked with a glass electrode. P_i released during 10 min at 30°C was determined by the method of Baginski et al. [7] with allowance for interference from NAD^+, NADH and ATP [6]. The enzyme was dialysed exhaustively against 0.05 M Tris · HCl buffer during preparation and passed through a column (25 cm × 2.5 cm) of Sephadex G-25 equilibrated in the same buffer prior to assay. All glassware used in the experiments was washed in 5% EDTA before use. These precautions were taken to remove as much extraneous bivalent cation as possible.

Effect of pH on inhibition

A series of buffers was prepared containing 0.1 M ammediol to cover the range of pH 8.0—10. The ionic strength of each was adjusted to 0.09 by the addition of NaCl. Portions of the buffers (1.5 ml) were included in 3 ml reaction mixtures containing 1 mM *p*-nitrophenyl phosphate and inhibitors at the concentrations indicated in Results. Thus the final values for total ammediol concentration and ionic strength were 0.05 and 0.045 M, respectively. The amount of inhibition at each pH was determined by comparison with inhibitor-

free controls. The pH of mixtures was determined before and after incubation using a glass electrode and no significant change was detected. The experiments were repeated at an ionic strength of 0.11.

Effect of temperature on inhibition

Inhibition by NAD^+ and NADH was studied between 20—35°C and kinetic parameters were determined at each temperature using the methods described above. The pH of reaction mixtures was adjusted to 9.0 at the temperature of the particular experiment. Even at the highest temperature the decomposition of NAD^+ at pH 9.0 during kinetic runs could be ignored as judged from rates of breakdown given by Lowry et al. [8].

Reaction with tetranitromethane

Tetranitromethane was dissolved in ethanol (95%) to give a solution containing 10% (v/v) and then 20 µl of this solution was incubated at 21°C with 130 µg of alkaline phosphatase in 0.2 ml of 0.05 M Tris · HCl buffer (pH 7.6). Suitable portions were removed for assay of residual activity with 1 mM *p*-nitrophenyl phosphate at various times up to 1 h. Controls containing 20 µl of 95% ethanol to replace the solution of tetranitromethane were treated in a similar manner. The experiment was repeated in the presence of 2.5 mM P_i.

Enzyme that had been treated with tetranitromethane in the absence of P_i for 30 min as described above was dialysed for a week at 4°C against several changes of 0.05 M Tris · HCl buffer (pH 7.6) containing 0.1 mM $MgCl_2$. A control sample treated with ethanol was dialysed similarly. Inhibition of the modified alkaline phosphatase by NAD^+ and NADH was then studied as described above. Samples were also subjected to electrophoresis on polyacrylamide gels at pH 8.0 [9] and in sodium dodecylsulphate-polyacrylamide gels [10]. Sample preparation and methods of detecting protein were as described in Ramasamy and Butterworth [11].

Reaction with N-acetylimidazole

In a reaction mixture of 0.2 ml, 0.24 mg of alkaline phosphatase was incubated at 21°C with 70 mM *N*-acetylimidazole. The mixture was buffered at pH 7.6 with 0.01 M Tris · HCl buffer. At various time intervals 10-µl portions were withdrawn into 0.1 ml of the 0.01 M Tris · HCl buffer and suitable amounts of this diluted solution were assayed for phosphatase activity at pH 9.0 and 30°C.

Reaction with iodine

The enzyme (0.12 mg protein) was incubated at 21°C with 20 µl of a 50 mM I_2 /200 mM KI solution, 4 µl of 0.125 M sodium acetate · acetic acid buffer (pH 5.2) plus 0.1 ml of 0.05 M Tris · HCl buffer (pH 7.6). The pH of the final mixture was 6.3. At various time intervals 5-µl samples were withdrawn into 0.1 ml of 0.01 M Tris · HCl buffer (pH 7.6) and the diluted enzyme was assayed for alkaline phosphatase activity.

Buffer solutions

The molarities of the various buffers refers to the total concentration of

Tris, ammediol or acetate present ignoring the distribution between different ionic forms present at a particular pH. The pH values were determined at 25° C except when specifically mentioned otherwise.

Results

Inhibition by coenzymes and analogues

Pig kidney alkaline phosphatase is inhibited by a large number of coenzyme analogues and related compounds. The inhibitors can be divided into two groups depending on whether an inhibitor resembles NADH in affecting only the intercept of double reciprocal plots (uncompetititve action) or resembles NAD^+ in affecting mainly the slope of such plots. The two kinds of inhibition are presented in Fig. 1 by *N,N*-diethylnicotinamide and thionicotinamide-NAD^+. For all the inhibitors tested the inhibition was "linear" as evidenced by secondary plots of slopes and intercepts against inhibitor concentration and plots of $1/v$ against inhibitor concentration [12]. The inhibition data obtained at pH 9.0 and 30° C are summarised in Table I. Some inhibitors e.g. NAD^+ and adenosine diphosphoribose changed the slope of double reciprocal plots and had an effect on intercepts also, i.e. they inhibited non-competitively. For others, e.g. thionicotinamide-NAD^+, the data do not allow an unambiguous choice to be made between a competitive and non-competitive action.

Changes in slope result from combination of a dead-end inhibitor with the free enzyme whereas changes in intercept alone, occur when the inhibitor binds to the enzyme · substrate complex or an intermediate, e.g. a phosphoryl-enzyme, generated during the reaction pathway [3]. It is interesting that in spite of much structural similarity between the inhibitors tested, some of them apparently have an affinity for the free enzyme whereas others do not.

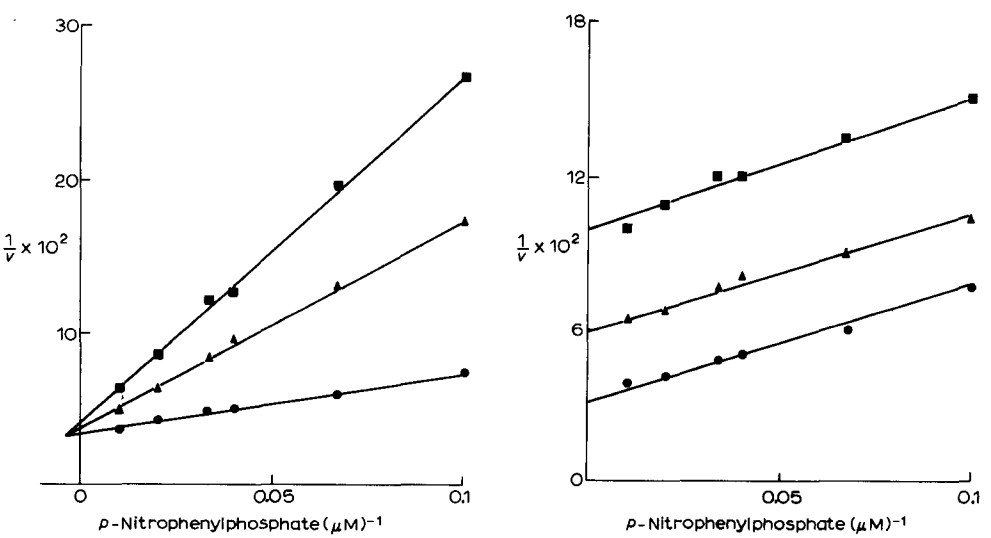


Fig. 1. Inhibition of *p*-nitrophenylphosphatase activity at 30° C and pH 9.0. The results are plotted in double-reciprocal form and inhibition constants K_{is} and K_{ii} were calculated from slopes and intercepts respectively of such plots. (a) Thionicotinamide-NAD^+ was present at concentrations of (mM): ●, zero; ▲, 0.25; ■, 0.5. (b) *N-N*-Diethylnicotinamide was present at concentrations of (mM): ●, zero; ▲, 10; ■, 25.

TABLE I

KINETIC DATA OBTAINED FOR THE INHIBITION OF PIG KIDNEY ALKALINE PHOSPHATASE

The values were obtained at 30°C and pH 9.0 in 0.05 M ammediol · HCl buffer with *p*-nitrophenyl phosphate as substrate. Where indicated 0.05 M Tris · HCl buffer replaced ammediol. K_{is} and K_{ii} are respectively "slope" and "intercept" inhibition constants derived from double reciprocal plots of initial velocities. Mean values ± S.D. are given for the constants together with the number of determinations. K_m values were 0.018 mM (Tris) and 0.012 mM (ammediol) under these conditions.

	K_{is} (mM)	K_{ii} (mM)
Inhibitors affecting intercept:		
6-Aminonicotamide	—	67.9 ± 18.7 (4)
3-Acetylpyridine	—	20.4 ± 5.0 (4)
N-N-Diethylnicotinamide	—	15.8 ± 3.6 (4)
N-Methylnicotinamide	—	31.9 ± 3.7 (4)
3-Aminopyridine	—	42.0 ± 3.0 (4)
Thionicotinamide	—	8.2 ± 1.5 (3)
Nicotinamide	—	18.9 ± 2.8 (4)
Nicotinamide	—	17.4 ± 3.4 (12) Tris
NADH	—	0.16 ± 0.06 (10) Tris
Inhibitors affecting slope:		
Deamino-NAD^+	1.06 ± 0.42	0.35 ± 0.04 (3)
Thionicotinamide-NAD^+	0.1 ± 0.01	2.23 ± 0.57 (4)
3-Acetyl-pyridine-NAD^+	0.92	4.38 (1)
Adenosine diphosphoribose	1.08 ± 0.09	2.61 ± 0.24 (3)
α-NAD^+	0.73	0.55 (1)
NAD^+	0.45 ± 0.08	2.91 ± 0.28 (4)
NAD^+	0.49 ± 0.11 (9) Tris	1.73 ± 0.41 (6) Tris

The results showed that the amide group linked through Position 3 in the pyridine ring seems to play little part in the binding of nicotinamide or NAD^+ to alkaline phosphatase. Rather similar findings have been reported for mammalian lactate dehydrogenase [13]. With kidney alkaline phosphatase, analogues of NAD^+ did not always cause parallel changes in K_{ii} and K_{is}. With deamino-NAD^+ for example, K_{ii} was larger and K_{is} smaller than the corresponding constants obtained for NAD^+. All of the analogues of NAD^+ increased the slope of double reciprocal plots and a change in configuration about C-1 of the ribose ring (as in α-NAD^+) did not prevent binding of the nucleotide. Methyl iodide nicotinamide seemed to be non-inhibitory. The slight inhibition of enzymic activity at relatively high levels of the analogue (0.5 M) was found on checking with similar concentrations of NaI and KI, likely to be attributable to I^-.

Inhibition of ATPase activity

The hydrolysis of ATP by alkaline phosphatase is characterised by considerable substrate inhibition that is not overcome by the addition of bivalent cations [5,6]. This inhibition makes kinetic studies difficult because data need to be collected at low substrate levels where the inhibition can be ignored, but where true initial velocities are difficult to determine because of substrate depletion. In the absence of a satisfactory "continuous" assay system for measuring true initial reaction rates, we decided to study inhibition by NAD^+ and NADH under conditions where substrate inhibition occurs but substrate depletion may be ignored.

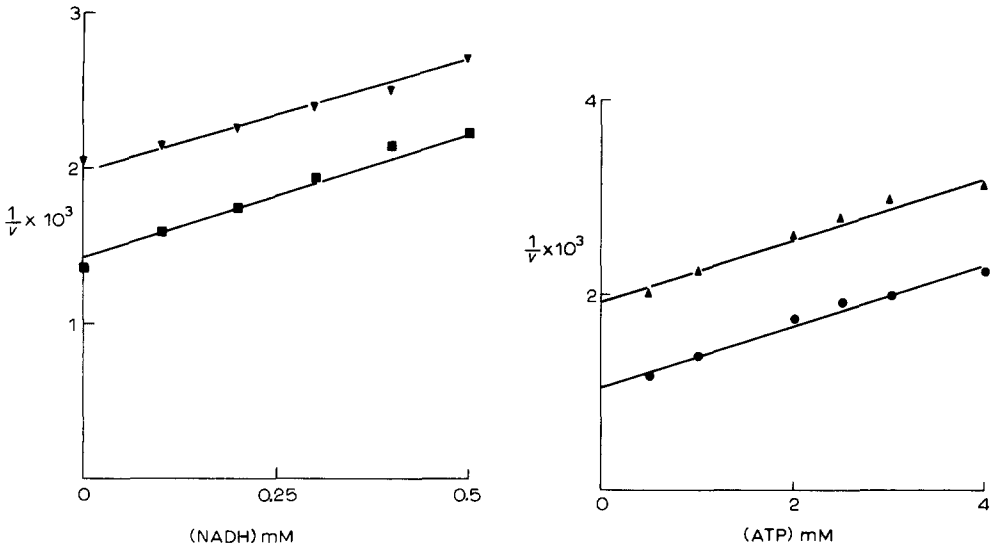
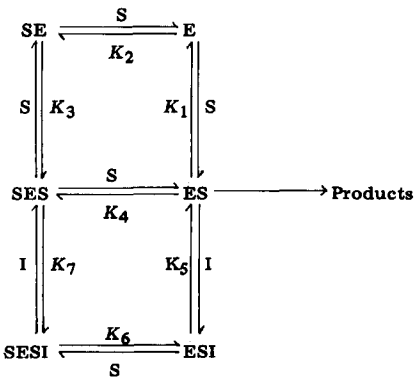


Fig. 2. Substrate inhibition of ATPase activity in the presence of NADH at 30°C and pH 9.0. (a) [ATP] was constant at 1 mM (■) and 4 mM (▼) while [NADH] was varied as shown. (b) [NADH] was constant at zero (●), and 0.3 mM (▲) while [ATP] was varied as shown. In both (a) and (b) the results are plotted in the form of $1/v$ against the concentration of variable inhibitor. The non-convergent plots suggest competition between NADH and ATP for one site on the enzyme.

Fig. 2 shows the effect of NADH on substrate inhibition of alkaline phosphatase by ATP and also the inhibition by NADH at different concentrations of ATP. Plots of $1/v$ against ATP concentration (at a fixed concentration of NADH) and of $1/v$ against NADH concentration (at fixed concentrations of ATP) both generated parallel lines. No $MgCl_2$ was added to reaction mixtures in these experiments. If the interactions between alkaline phosphatase, ATP and NADH are represented by Scheme I, the rate equation for the reaction,

SCHEME I

Inhibition by substrate in the presence of an unrelated inhibitor



It is assumed that I binds only to the enzyme · substrate complex whereas S may bind in its inhibitory mode both to free enzyme and the enzyme · substrate complex. Infinite values for K_6 and K_7 indicate exclusive binding of S or I to the inhibitor site.

assuming a rapid equilibrium mechanism, can be written:

$$\frac{1}{v} = \frac{[S]}{VK_4}\left(1 + \frac{[I]}{K_7}\right) + \frac{1}{V}\left(1 + \frac{K_1}{K_2} + \frac{K_1}{[S]} + \frac{[I]}{K_5}\right)$$

or alternatively in the form:

$$1/v = \frac{[I]}{V}\left(1/K_5 + \frac{[S]}{K_4K_7}\right) + \frac{1}{V}\left(1 + \frac{K_1}{K_2} + \frac{K_1}{[S]} + \frac{[S]}{K_4}\right)$$

Thus when [S] is large relative to K_1 plots of $1/v$ against [S] and plots of $1/v$ against [I] are linear. The slope of the S plots will be independent of [I] when K_7 is very large, i.e. when there is exclusive binding of S or I to the inhibitor site [14]. Similar arguments hold for $1/v$ against [I] plots in the presence of S. The parallel plots in Fig. 2 suggest that inhibition by ATP and NADH is mediated through a common binding site.

With a substrate concentration of 1 mM ATP, raising the concentration of $MgCl_2$ to 2 mM potentiated NAD^+ inhibition of ATPase activity 3—4-fold whereas NADH inhibition was affected very little. With *p*-nitrophenyl phosphate as substrate no detectable change in the inhibition by NAD^+ or NADH occurred when Mg^{2+} was included in reaction mixtures.

Effect of pH on inhibition

Inhibition by NADH, adenosine and nicotinamide decreases with increasing pH (Fig. 3) but between pH 8.0 and 10 there is little change in the amount

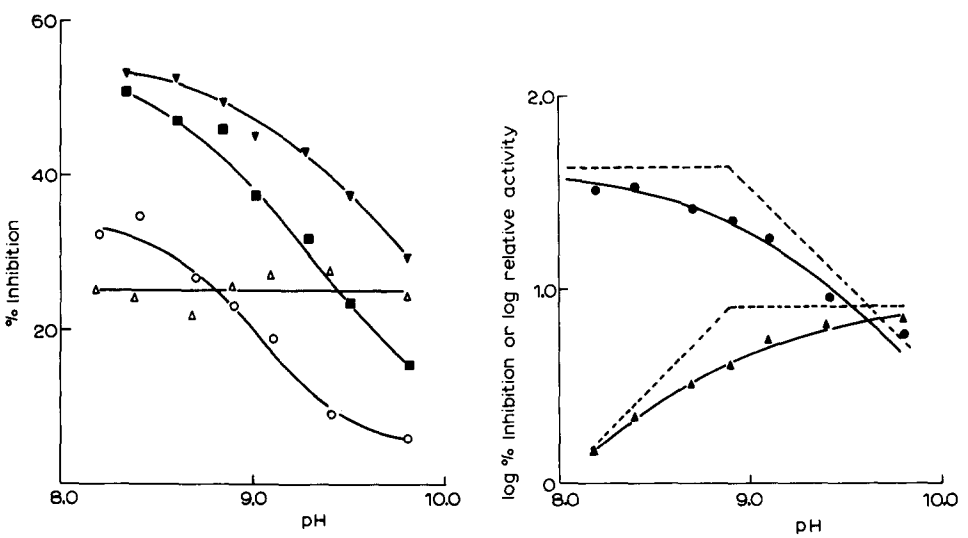


Fig. 3. Effect of pH on the inhibition of *p*-nitrophenylphosphatase activity by 0.1 mM NADH (○); 1.0 mM NAD^+ (△), 10 mM nicotinamide (▼) and 10 mM adenosine (■). For each experimental point the percent inhibition was calculated by comparison with a control lacking inhibitor. The substrate concentration was 1.0 mM throughout and the ionic strength was 0.11 in the NAD^+ and NADH experiments and 0.045 in those of nicotinamide and adenosine. (b) Logarithmic plot of NADH inhibition (●) and non-inhibited activity (▲) as a function of pH [2]. Measured velocities at 1.0 mM *p*-nitrophenylphosphate were assumed to approximate to maximum velocities at each pH. The relative units in which velocities are plotted were chosen for convenience of display within the same set of axes as the inhibition data. Calculated curves assuming a p*K* of 8.9 were fitted to the experimental points.

of inhibition caused by NAD^+. Raising the ionic strength from 0.045 to 0.11 did not change the inhibition patterns. The fall in inhibition by NADH and the other compounds suggested that binding is hindered by deprotonation of a group on the inhibitors or enzyme · substrate complex with a pK of approx. 8.8—9.2. From the variation of the initial reaction velocity of the non-inhibited enzyme, it was found that the enzyme behaved as if deprotonation of a group titrating at approx. pH 8.8 was also important in catalysis. The curves in Fig. 3b are calculated ones assuming that the pK of the important group is 8.9.

The inhibition of human placental and intestinal alkaline phosphatases by L-phenylalanine varies with pH in a more complex way than does NADH inhibition of kidney enzyme, but a fall in L-phenylalanine inhibition does occur with increasing pH and a group titrating at pH 9.7 has been suggested to have an important binding function [15].

Effect of temperature on inhibition by NAD^+ and NADH

Slope and, for NAD^+, intercept inhibition constants were found to increase with temperature. The enthalpy of binding was obtained from experimental data plotted according to the integrated form of the Van't Hoff equation assuming that the kinetically determined constants represented true dissociation constants. The free energy and entropy of binding were calculated using the Gibbs-Helmholtz equation. The values obtained for the various parameters are shown in Table II.

There is a marked decrease in entropy on binding of the inhibitors and a difference of between 24—50 e.u./mol between NAD^+ and NADH binding. The enthalpy and entropy of binding of L-phenylalanine to rat intestinal enzyme [16] and of L-tryptophan to human placental alkaline phosphatase [17] have also been found to be negative, but the entropy change of —28 J/°K per mol for L-phenylalanine binding [16] is considerably less than for binding of NAD^+ and NADH.

TABLE II

THERMODYNAMIC PARAMETERS FOR THE BINDING OF NAD^+ AND NADH TO PIG KIDNEY ALKALINE PHOSPHATASE

The parameters were calculated from the results of kinetic experiments at pH 9.0 with *p*-nitrophenyl phosphate as substrate in 0.05 M Tris · HCl buffer. Entropy units (e.u.) are expressed as J per °K. K_{is} and K_{ii} refer to "slope" and "intercept" inhibition constants respectively.

Inhibitor and binding constant	Temperature (°K)	Constant (M^{-1})	ΔH (J/mol × 10^{-3})	ΔG (J/mol × 10^{-3})	ΔS (e.u./mol)
NAD^+ ($1/K_{is}$)	298	3140	− 80.3	− 20.0	− 203
	303	2040		− 19.3	− 201
	309	1020		− 17.8	− 202
NAD^+ ($1/K_{ii}$)	298	1190	− 85.1	− 17.6	− 227
	303	575		− 16.0	− 228
	309	349		− 15.1	− 227
NADH ($1/K_{ii}$)	293	12830	− 75.5	− 23.1	− 179
	303	6210		− 22.0	− 177
	309	3050		− 20.6	− 178

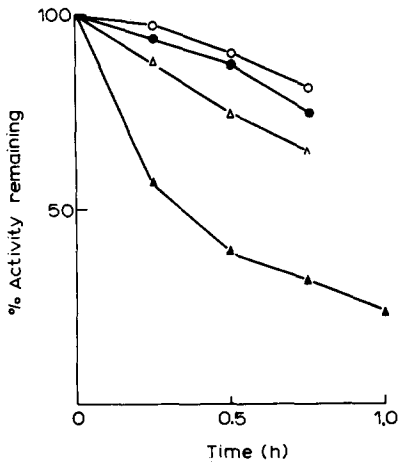


Fig. 4. Inactivation of alkaline phosphatase by incubation with tetranitromethane at 21°C and pH 7.6. The plots show activity remaining after treatment with tetranitromethane in the absence (▲) and presence (△) of 2.5 mM P_i. Also shown are the activities of controls incubated in the absence (●) and presence (○) of 2.5 mM P_i.

Chemical modification with tetranitromethane

Incubation of alkaline phosphatase with the reagent at 21°C caused a progressive loss in activity (Fig. 4). Only about 30% of the original activity remained after 45 min compared with 80% in controls incubated for a similar time without tetranitromethane. There was some protection against inactivation when P_i was present. Enzyme that had been treated for 1 h and which retained approx. 25% of the original activity migrated 5—10% faster on polyacrylamide-gel electrophoresis, but no extra bands arising from chemical modification were detectable. The modified enzyme migrated at the same rate as native enzyme on sodium dodecylsulphate-polyacrylamide gel electrophoresis [11]. No significant loss of activity or change in electrophoretic mobility was detected during iodination or acetylation.

After nitration, alkaline phosphatase seemed less sensitive to inhibition by NADH. Nitration did not change the K_m for *p*-nitrophenyl phosphate. A sample of treated enzyme with 26% of its original activity remaining had an inhibition constant for NADH double that of the control but there was no significant change in NAD^+ binding. With 16% active enzyme K_{ii} for NADH was increased approx. 10-fold. Thomas and Moss [15] found an increase in K_{ii} for L-phenylalanine acting on human placental phosphatase after nitration, but no equivalent change with intestinal enzyme.

Discussion

The inhibition studies with analogues of NAD(H) did not provide an absolute guide to the specificity requirements for binding of nucleotides to alkaline phosphatase, but the studies did reveal some interesting features. A change in configuration about C-1 of the ribose ring attached to the nicotinamide moiety (as in α-NAD^+) did not prevent binding. In fact, the change may result in some tightening of binding as indicated by the 5-fold decrease in K_{ii}

compared with NAD^+. Thus the orientation of the bases with respect to the ribose phosphate backbone seems relatively unimportant in inhibition and it is likely that the main anchorage to the enzyme is through interactions with the bases. This suggestion is supported by the findings that the free bases and their analogues are reasonably effective as inhibitors of pig kidney alkaline phosphatase. As ADP-ribose is a stronger inhibitor than adenosine however, the contribution by the ribose phosphate backbone to binding is obviously not negligible. The non-competitive rather than uncompetitive action of adenosine diphosphoribose could represent a degree of affinity of the pyrophosphate linkage for the active site of the enzyme so that some competition with substrate hydrolysis ensues. All of the analogues that increase the slope of double reciprocal plots probably share the property of possessing some affinity for the hydrolytic site.

Previously, we predicted that the substrate inhibition of alkaline phosphatase by ATP may arise through interactions analogous to those occurring between the enzyme and NADH [1]. Provided that the relatively simple interpretation of substrate inhibition shown in Scheme I holds, the experiments in which inhibitory amounts of both ATP and NADH were present do suggest that ATP and NADH can interact with the same site.

Since Mg^{2+} has no effect on the inhibition of orthophosphatase activity by NAD^+ and NADH, it is possible that the potentiation of NAD^+ inhibition of ATPase activity results from changes in the levels of ATP^{4-}, $MgATP^{2-}$ and Mg^{2+} rather than from a direct action of Mg^{2+} operating only when pyrophosphate substrates are being hydrolysed. From studies of the ATPase and pyrophosphatase activities of the enzyme [5,6,18] it has been proposed that Mg^{2+} affects such activities in at least two ways: (a) activating by binding to a specific stimulatory site [19]; (b) inhibiting by conversion of the actual substrate, ATP^{4-}, to the inactive $MgATP^{2-}$ complex (18). If NAD^+ does compete with ATP^{4-} for the active site, then the inhibitor would be expected to be much more effective at the depleted levels of ATP^{4-} when Mg^{2+} is present. The degree of inhibition by an uncompetitive inhibitor, e.g. NADH, lacking affinity for the free enzyme is less responsive to the concentration of substrate but does tend to decrease slightly as the concentration of substrate falls. Thus it is not surprising that NADH inhibition of ATPase activity is less dependent on the concentration of Mg^{2+}.

Protonation of a group on the enzyme or the inhibitors with a pK of 9.0 approx. seems important for binding, and for a decrease in catalytic activity. As NADH, adenosine and nicotinamide do not appear to share a group of such property, the group important for inhibition must be an enzymatic one. The group could be the hydroxyl of a tyrosine residue since nitration, which can lower the pK of this group in a protein molecule from 9.2 to 7.3 [20] has some effect on inhibition. Further attempts to implicate tyrosine however, through acetylation and iodination were unsuccessful. Based on nitration experiments, the suggestion has been made that tyrosine residues are important for the binding of L-phenylalanine to human placental and intestinal phosphatases [15]. It is possible however, that the group involved in inhibition by NADH is identical with that in catalysis and the inhibition ensues from a raised pK of this group in alkaline phosphatase. There are grounds for identifying the catalytic group in mammalian alkaline phosphatases with Zn^{2+} hydrate rather than

a tyrosine or lysine residue which titrate in a similar pH region [21]. A role for tyrosine in positioning inhibitors close to Zn^{2+} may still be important however. If the aromatic moieties in the inhibitors form non-polar interactions with the phenolic ring structure, nitration could interfere with binding by lowering the pK of the hydroxyl group so making tyrosine more polar.

The different response of NAD^+ inhibition to change of pH is difficult to explain unless it is assumed that there is an increased contribution to binding by electrostatic attraction between the nucleotide and a negative charge of an ionised residue generated at higher pH values. This might compensate to some extent for potentially less favourable conditions for inhibition.

The dissociation constants for the binding of NAD^+ and NADH to pig kidney alkaline phosphatase increase with temperature. Binding of the inhibitors is characterised by a decrease in both enthalpy and entropy. The entropy change is relatively large and is in the direction of a "tightening" of structure. Although the total entropy change will include contributions from electrostatic effects and from the juxtaposing or "unmixing" of the enzyme and ligand in the aqueous environment [22], the large change is probably indicative of a change in protein structure resulting in a constrained ESI ternary complex. A conformational change was predicted to account for the synergistic inhibitory effects of adenosine and nicotinamide [1]. It is envisaged that the binding of one of the pair of inhibitors induces the strained condition of the enzyme resulting in a lower free energy of binding for the other partner.

Although it is still uncertain whether alkaline phosphatase functions as an ATPase linked to the transport of Ca^{2+} [6,23,24,25], if such a role becomes firmly established, then the interactions between NAD^+, NADH, ATP and Mg^{2+} with alkaline phosphatase are likely to be very important in the physiology of the kidney cell.

Many enzymes of very diverse function seem to contain regions in their structure that bind nucleotide and nucleotide-like molecules and where it has been possible to compare their structure, the pockets have been found to have many structural features in common. The findings have led to speculation that the pockets represent structural regions that became established at a very early stage of enzyme evolution [26]. It is interesting to consider whether mammalian alkaline phosphatase is another example of an enzyme that retains the essential features of a binding domain established long ago.

References

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