Probing the active site of cytoplasmic aldehyde dehydrogenase with a chromophoric reporter group

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3,4-Dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one ('DMNB') reacts with cytoplasmic aldehyde dehydrogenase in a similar way to that previously observed with the structurally related *p*-nitrophenyl dimethylcarbamate, but provides a covalently linked *p*-nitrophenol-containing reporter group at the enzyme's active site. The pK_a of the enzyme-linked reporter group is much higher than that of free *p*-nitrophenol, which is consistent with its being in a very hydrophobic environment, or possibly one containing negative charge. Upon binding of NAD^+

to the modified enzyme, the pK_a falls dramatically, by about $4\frac{1}{2}$ pH units. This implies that under these conditions there is a positive charge near the *p*-nitrophenoxide moiety, perhaps that of the nicotinamide ring of NAD^+ . The modified enzyme binds NAD^+ very tightly; neither gel filtration nor dialysis is effective in separating them. However, the reporter group provides a convenient way of monitoring the displacement of this bound NAD^+ when $NADH$ is added.

INTRODUCTION

Since the pioneering work of Hempel et al. [1] with iodoacetamide, it has become well established that Cys-302 is the active-site nucleophile in the reactions of aldehyde dehydrogenase. This residue, the only conserved cysteine in all known aldehyde dehydrogenase sequences [2], has been shown to react with various active-site-directed reagents, including a bromoacetyl analogue of NAD^+ [3], a vinyl ketone [4], bromoacetophenone [5], 4-*trans*-(*NN*-dimethylamino)cinnamaldehyde and 4-*trans*-(*NN*-dimethylaminocinnamoyl)imidazole [6], and *p*-nitrophenyl dimethylcarbamate [7]. We reasoned that a cyclic analogue of the last compound, namely 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB), would react with Cys-302 in a similar way and would place at the active site a covalently linked *p*-nitrophenol moiety capable of acting as a reporter group (a concept originally developed by Burr and Koshland [8]). Accordingly, DMNB was synthesized and, in experiments with chymotrypsin, it was confirmed that the compound does indeed behave in the manner for which it was designed [9]. The present paper presents the results of experiments with DMNB and the cytoplasmic (or Class I) form of aldehyde dehydrogenase from sheep liver and, in the light of these results, discusses what may be the nature of the environment around the enzyme's active site.

EXPERIMENTAL

Materials

DMNB was synthesized as previously described [9]. Cytoplasmic

aldehyde dehydrogenase from sheep liver was purified as follows. [All buffers contained 0.3 mM EDTA and dithiothreitol (15 mg/l).] Fresh sheep liver was homogenized in 2 vol. of ice-cold 4 mM sodium phosphate buffer, pH 7.4, containing 0.25 M sucrose. The homogenate was centrifuged at 15000 *g* for 5 min; supernatant was transferred to smaller bottles and re-centrifuged at 27000 *g* for 30 min. Polyethylene glycol 8000 was added to the supernatant (10%, w/v) and the precipitate was spun down and discarded. Further polyethylene glycol (20%, w/v) was added to the supernatant; after centrifuging, the precipitate was redissolved in 10 mM Bistris buffer, pH 6.2, containing 5 mM NaCl. The solution was loaded on a column of DEAE-Iontosorb (ICS Identifikanci Systemy A.S., Prague, Czech Republic) equilibrated with the same buffer; the column was washed with this buffer until the A_{280} was less than 0.1. The pH of the eluting buffer was changed to 5.8 and the column was again washed until the A_{280} was less than 0.1. (This step removes the mitochondrial isoenzyme of aldehyde dehydrogenase.) Cytoplasmic aldehyde dehydrogenase was eluted by 30 mM sodium phosphate buffer, pH 4.8. After dialysis against 25 mM sodium phosphate buffer, pH 7.4, containing 15 mM NaCl, the enzyme was loaded on a column of *p*-hydroxyacetophenone affinity resin [10], equilibrated with the same buffer. The column was washed with this buffer until the A_{280} was less than 0.05. *p*-Hydroxyacetophenone (10 mM) was then added to the buffer in order to elute aldehyde dehydrogenase. The enzyme solution was concentrated by dialysis against 50 mM sodium phosphate buffer, pH 7.4, containing $(NH_4)_2SO_4$ (400 g/l). Precipitated enzyme was redissolved in a small volume of 50 mM sodium phosphate buffer, pH 7.4, dialysed to remove $(NH_4)_2SO_4$, and stored at $-20^\circ C$. The preparation showed a single protein band on polyacrylamide gels corresponding to the position of an aldehyde dehydrogenase activity stain. Successful removal of the mitochondrial isoenzyme was demonstrated by isoelectric focusing and activity staining.

Isolation of DMNB-labelled aldehyde dehydrogenase

Enzyme (approx. 90 μM , based on a molar mass of 212000 g/mol [11] and an $A_{280}^{1\%}$ value of 11.3 [12]) in 3 ml of 50 mM sodium phosphate buffer, pH 7.4, was mixed with DMNB in acetone

(75 μ l) to give a modifier concentration of 0.6 mM. The mixture was protected from light (to stop the spontaneous hydrolysis of DMNB reported previously [9]) and left at 25 °C for 2–3 h. The solution was then passed down a column of Bio-Gel P-6 (25 cm $\times$ 0.8 cm) at room temperature, eluted with 10 mM sodium phosphate buffer, pH 7.4, and the labelled enzyme was collected in a volume of 5–7 ml, well separated from excess modifier. In an otherwise identical experiment, the initial enzyme solution contained NAD⁺ (5 mM).

Denatured DMNB-labelled aldehyde dehydrogenase

Labelled enzyme solution (5.5 ml), as prepared above, was mixed with HClO₄ [55 μ l of a 75% (w/v) aq. solution], and the protein that precipitated was collected by brief centrifugation. The precipitate was washed two or three times with water by dispersal with a glass rod, centrifugation, and decantation, and finally the precipitate was dissolved in aq. 10 M urea (16 ml).

Determination of the pK_a of the *p*-nitrophenol group in DMNB-labelled aldehyde dehydrogenase

Samples of labelled enzyme (0.5 ml), prepared as above, were mixed with 1.5 ml of various buffers [13] and the absorbance spectrum from 300 to 550 nm was recorded at 20 °C with a Varian Cary 1 spectrophotometer. In measuring the amplitude of the *p*-nitrophenoxide peak, the A_{550} was taken as zero. In some cases after scanning, NAD⁺ or NADH was added as 10 μ l on a glass nail to give a concentration of 2 mM or 0.4 mM respectively, and the absorbance spectrum was re-scanned. With denatured enzyme, 1.5 ml samples were mixed with 0.5 ml of the various buffers. In all cases after recording the spectrum, the pH of each labelled enzyme solution was measured. The data were plotted by using Enzfitter [14] to compute the best theoretical titration curve, allowing the computer program to select the best-fit values for the pK_a and maximum and minimum absorbances. It was not assumed that the curves would necessarily tend to zero at low pH. Factors that would preclude this are the possible presence of denatured enzyme (as discussed below) and the small but finite absorbance of concentrated enzyme solutions in the 400–450 nm range.

Progress curve for reaction of DMNB with aldehyde dehydrogenase

A 3 ml solution of enzyme (approx. 12 μ M) in 50 mM sodium phosphate buffer, pH 7.4, containing NAD⁺ (2 mM) was mixed with DMNB in acetone (25 μ l) to give a modifier concentration of 0.2 mM. The A_{440} of this solution was monitored continuously at 25 °C. Samples (0.1 ml) were taken periodically from an identical mixture and used for the assay of remaining enzyme activity. This was determined in 50 mM sodium phosphate buffer, pH 7.4, 25 °C, containing NAD⁺ (1 mM) and acetaldehyde (1 mM) in a total volume of 3 ml. The fall in enzyme activity of a reaction mixture containing no NAD⁺ was monitored in a similar way.

Displacement of NAD⁺ from DMNB-labelled aldehyde dehydrogenase

A 2.5 ml solution of enzyme, labelled by DMNB in the absence of NAD⁺ as described above, was scanned from 300 to 550 nm before and after the addition of NAD⁺ (3.3 mg, giving a concentration of 2 mM). The solution was then dialysed at 4 °C for a total of 24 h against 4 $\times$ 4 litres of 10 mM sodium phosphate buffer, pH 7.4. The absorbance spectrum was re-recorded as a test of how efficient dialysis is in removing NAD⁺ from the

labelled enzyme. At this point 10 μ l of NADH solution was added (giving a concentration of 0.4 mM), and the absorbance spectrum was monitored periodically over a period of approx. 12 min as a test of the ability of NADH to displace tightly bound NAD⁺ from the labelled enzyme.

RESULTS

When cytoplasmic aldehyde dehydrogenase from sheep liver is incubated in the dark with DMNB at pH 7.4, the reaction mixture does not turn yellow. However, as shown in Figure 1, the activity of the enzyme does fall; this suggests that DMNB modifies the enzyme, but that the pK_a of the enzyme-linked *p*-nitrophenol moiety must be considerably higher than 7.4. Investigating the absorption spectrum of the modified enzyme over a

Figure 1 Reaction of cytoplasmic aldehyde dehydrogenase with DMNB

The dashed line shows the absorbance increase due to the formation of enzyme-linked *p*-nitrophenoxide when enzyme (12 μ M) reacts with DMNB (0.2 mM) in the presence of NAD⁺ (2 mM) at pH 7.4 and 25 °C. The corresponding residual enzymic activity is also shown (●). The ○ symbols show the decline in enzymic activity in a similar experiment without NAD⁺.

Figure 2 Ionization profiles of the DMNB-derived reporter group linked to aldehyde dehydrogenase

The maximal absorbance of the enzyme-linked *p*-nitrophenoxide group is plotted as a function of pH. ●, Undenatured enzyme (approx. 10 μ M) in the absence of NAD⁺ (best-fit parameters: pK_a , 9.75; A_{min} , 0.065; A_{max} , 0.244); ○, undenatured enzyme (approx. 10 μ M) in the presence of NAD⁺ (2 mM) (pK_a , 5.35; A_{min} , 0.0318; A_{max} , 0.327); □, denatured enzyme (approx. 10 μ M) (pK_a , 7.21; A_{min} , 0.011; A_{max} , 0.203).

Figure 3 Denaturation of DMNB-labelled aldehyde dehydrogenase at pH 11.6

Five spectra were recorded over a period of 10 min showing how the A_{433} progressively declines, whereas the A_{406} rises. This change is interpreted as a change in environment of the DMNB-derived reporter group as the enzyme denatures. The enzyme concentration was approx. 10 μM .

Figure 4 Ionization profile of the DMNB-derived reporter group after modification of the enzyme in the presence of NAD^+

Enzyme (90 μM) was modified by DMNB (0.6 mM) in the presence of NAD^+ (5 mM). The labelled enzyme was isolated by gel filtration and the absorbance of the enzyme-linked *p*-nitrophenoxide group was determined as a function of pH (pK_a , 5.0; $A_{\text{min.}}$, 0.0387; $A_{\text{max.}}$, 0.186). The labelled enzyme concentration in the scans was approx. 10 μM .

range of pH confirms that the pK_a is 9.75, as shown in Figure 2. This result is to be contrasted with the pK_a that is observed when the previously modified enzyme is denatured by HClO_4 and redissolved in urea solution (see Figure 2); the value of 7.21 is now virtually the same as that of free *p*-nitrophenol itself (7.15 [9]). From the maximal absorbance of the reporter group on the denatured enzyme (where the absorption coefficient is unlikely to be much perturbed from that of free *p*-nitrophenol) it is calculated that 1.35 modified groups per enzyme tetramer are present. From the shape of the 'undenatured' curve in Figure 2, it appears that a little of the enzyme may in fact have become denatured during the incubation and gel-filtration procedures, such that the absorbance at lower pH values is somewhat higher than would otherwise have been expected.

The $\lambda_{\text{max.}}$ of the enzyme-linked *p*-nitrophenoxide group is 433 nm and 414 nm before and after denaturation with HClO_4 /urea respectively. At very high pH, the value of 433 nm is not constant; for instance, at pH 11.6 it changes over a period of 10 min to a new value of 406 nm, as shown in Figure 3, presumably as the enzyme unfolds.

Figure 1 also shows that incubation of the enzyme with DMNB at pH 7.4 in the presence of NAD^+ , unlike in its absence, results in the slow development of yellowness ($\lambda_{\text{max.}}$ = 440–

Figure 5 Displacement of bound NAD^+ from DMNB-labelled aldehyde dehydrogenase

(a) The spectrum of DMNB-labelled aldehyde dehydrogenase (approx. 40 μM) at pH 7.4 is shown in the absence of NAD^+ (1) and in the presence of 2 mM NAD^+ (2). (b) The spectrum of DMNB-labelled aldehyde dehydrogenase at pH 7.4 is shown in the presence of 2 mM- NAD^+ (1) and again after exhaustive dialysis (2). (c) The upper curve is the spectrum of DMNB-labelled aldehyde dehydrogenase at pH 7.4 after the addition of NAD^+ (2 mM) and after subsequent exhaustive dialysis. The other curves show the progressive decline in the absorbance of enzyme-linked *p*-nitrophenoxide over a period of 12 min after addition of NADH (0.4 mM).

443 nm), and that the increase in absorbance is paralleled by loss of enzyme activity. (There was apparently a slight initial rise in activity, but this may have been an artefact of the incubation conditions and is probably not important.) Over most of the range of Figure 1, the activity loss approximates to a pseudo-first-order decay with a rate constant of about 0.5 h^{-1} . The increase in absorbance obeys pseudo-first-order kinetics well and has a rate constant of 0.47 h^{-1} . This observation suggests, of course, that the pK_a of the enzyme-linked *p*-nitrophenol group is lower in the presence of NAD^+ than in its absence, and the results shown in Figure 2 confirm this. Modification of aldehyde dehydrogenase by DMNB in the absence of NAD^+ , followed by the addition of NAD^+ to samples of the isolated labelled enzyme over a range of pH values, gives a pK_a that is very much lower (5.35) than that seen in the absence of NAD^+ .

It was predicted that modifying the enzyme with DMNB in the presence of NAD^+ , followed by gel filtration to remove NAD^+ and excess DMNB, would result in a pK_a curve like the right-hand one in Figure 2. However, experiment does not support this prediction; Figure 4 shows that the pK_a is still approx. 5, and the conclusion to be drawn from this is that passage of the labelled enzyme through Bio-Gel P6 is ineffective in removing enzyme-bound NAD^+ . (The shape of the curve in the high-pH region means perhaps that a small fraction of the enzyme has become NAD^+ -free.) This tight binding of NAD^+ to the labelled enzyme was confirmed by dialysis experiments. In Figure 5(a) the absorption spectrum of the reporter group at pH 7.4 is shown

Figure 6 Ionization profile of the DMNB-derived reporter group in the presence of NADH

The maximal absorbance of the enzyme-linked *p*-nitrophenoxide group is plotted as a function of pH. The labelled enzyme concentration in the scans was approx. 10 μ M. ●, In the absence of NADH (pK_a , 10.1; $A_{min.}$, 0.054; $A_{max.}$, 0.233); ○, in the presence of NADH (0.4 mM) (pK_a , 9.16; $A_{min.}$, 0.064; $A_{max.}$, 0.211).

both before the addition of NAD^+ (where the un-ionized *p*-nitrophenol group absorbs at approx. 330 nm) and after the addition of NAD^+ (where the ionized group absorbs at 440–443 nm). Figure 5(b) shows that the latter absorbance peak declines only minimally over the 24 h period of dialysis (with four changes of a large volume of buffer at pH 7.4). The subsequent addition of excess NAD^+ returns the absorption peak to its original size.

Unlike the major effect of NAD^+ , NADH causes only a small perturbation in the pK_a of the reporter group, as shown in Figure 6. In the absence of nucleotide, the pK_a was found to be 10.1, in fairly good agreement with the value of 9.75 found on a previous occasion (Figure 2), and in the presence of NADH the pK_a is 9.16. This result means that, over the pH range of approx. 7–8, modified enzyme is yellow when NAD^+ is bound to it, but colourless when NADH is bound. Figure 5(c) illustrates how these properties can be used to monitor the competition of the nucleotides for the modified enzyme's binding site. The upper curve is the absorbance of the reporter group after most of the initially added NAD^+ has been removed by prolonged dialysis; the lower curves show progressively the decrease in the absorbance as the tightly bound NAD^+ is displaced by NADH over a period of about 12 min.

DISCUSSION

Previous work with *p*-nitrophenyl dimethylcarbamate [7,15,16] led to the premise that DMNB would be an interesting 'reporter group' reagent for cytoplasmic aldehyde dehydrogenase; the results reported above have amply borne out this hope. Because of the very close structural similarity of the two compounds, we assume that DMNB, like *p*-nitrophenyl dimethylcarbamate, modifies residue Cys-302. The enzyme reacts with DMNB significantly faster than with *p*-nitrophenyl dimethylcarbamate in both the absence and the presence of NAD^+ ; in the latter case, the rate of reaction with *p*-nitrophenyl dimethylcarbamate is virtually zero [15]. As found previously with *p*-nitrophenyl esters,

carbonates and carbamates [15,17], the presence of the nucleotide decreases the acylation rate when DMNB reacts with the enzyme (Figure 1). Under these conditions, the observed increase in absorbance and loss of activity follow a similar time course; this is consistent with modification involving a catalytically active group. The stoichiometry of the modification (1.35 groups per tetramer) is similar to that of other processes studied with aldehyde dehydrogenase (e.g. reaction with disulfiram [18] and *p*-nitrophenyl dimethylcarbamate [7]), and approximates closer to 'half of the sites' reactivity than full reactivity of all four subunits. The acyl-enzyme produced by DMNB is stable and amenable to further study at leisure; there is no need to 'trap' it, for instance by drastic lowering of the pH or by precipitation of the protein. Observation of the spectrum of the enzyme-linked *p*-nitrophenoxide group provides a convenient method of monitoring changes that occur, for example, on denaturation (Figures 2 and 3), and on binding of nucleotides (Figures 2, 5 and 6). Use of this reporter group will allow many useful experiments to be performed, including, for instance, determination of the rate of binding and strength of binding of NAD^+ and NADH. The effect of other species that bind to aldehyde dehydrogenase (such as aldehydes, Mg^{2+} [19,20] and diethylstilboestrol [21]) could also be followed by using the DMNB-derived reporter group as the 'handle'.

At this stage, the most important results to emerge from the use of DMNB are shown in Figure 2. Clearly, the environment of the reporter group when bound to the undenatured enzyme is one which strongly disfavours the ionization of the *p*-nitrophenol moiety, whereas conversely in the modified enzyme- NAD^+ complex the negatively charged *p*-nitrophenoxide ion is greatly stabilized. The acidity of the *p*-nitrophenol is in fact tens of thousands of times greater in the presence of NAD^+ than in its absence. The obvious explanation for this is that the environment of the reporter group in the presence of NAD^+ contains positive charge(s). It has often been thought that NAD^+ causes a conformational change in the enzyme (for instance, NAD^+ must bind first before aldehyde can bind in the enzyme-catalysed reaction [22]), and the present result is certainly consistent with that idea. Perhaps the conformation of the enzyme changes on binding of NAD^+ , bringing a Lys, Arg or His residue close to the *p*-nitrophenoxide group. Alternatively, it may be the positive charge in the nicotinamide ring of NAD^+ itself that interacts to stabilize the negative charge of the *p*-nitrophenoxide group, as suggested in Scheme 1. Topologically, this is not an unreasonable proposal, since in the postulated mechanism of the enzyme-catalysed reaction [23], the thiohemiacetal group (which becomes the carbonyl group of the acyl-enzyme) must be positioned very

Scheme 1 Possible ionic interaction between enzyme-linked *p*-nitrophenoxide and enzyme-bound NAD^+

close to the 4-position of the nicotinamide ring (so as to enable transfer of the hydride ion). The facts that, first, NADH does not have the same huge effect on the pK_a of the reporter group that NAD^+ has, and secondly, that NAD^+ binds extremely tightly to the modified enzyme (as shown by the failure of gel filtration or dialysis to remove it; Figures 4 and 5) are consistent with the direct ionic interaction shown in Scheme 1. The idea that the binding sites for NAD^+ and DMNB are physically close agrees also with the fact that the former slows reaction of the enzyme with the latter, as mentioned above.

Two explanations present themselves for why the pK_a of the reporter group on the undenatured enzyme (in the absence of NAD^+) is so much higher than that observed after unfolding the protein. One is that the environment of the group contains negative charge(s) (which would destabilize the ionized form of *p*-nitrophenol in the same way that positive charge would stabilize it, as discussed above), and the other is that a very non-polar or hydrophobic region of the enzyme would likewise favour the neutral *p*-nitrophenol group over its ionized equivalent. Aldehyde dehydrogenase is inactivated by iodoacetamide, but not by iodoacetate [24], and by disulfiram, but not by a negatively charged analogue [25]. It does not use the negatively charged aldehyde glyoxylic acid as a substrate [26], nor do carboxylate ions (the product of aldehyde dehydrogenation) bind to the enzyme [22]. These observations suggest, but do not prove, the presence of negatively charged groups at the active site. A possible candidate for such a group is Glu-268, which according to Pietruszko et al. [27] exists in the enzyme as a 'naked anion', explaining its modification (along with Cys-302) by bromoaceto-phenone. These authors suggest a role for Glu-268 involving its removal of the proton from the active site's Cys-302, rendering it more nucleophilic towards the aldehyde substrate. In this scenario, however, Glu-268 then exists in the un-ionized form in the resulting acyl-enzyme, and if this is true for the DMNB-derived acyl-enzyme, the residue could not be responsible for the observed high pK_a value. Pietruszko et al. [27] go on to suggest a further role for Glu-268, namely in repelling the carboxylate product of aldehyde dehydrogenation from the active site, causing its dissociation. By the same token, it might be thought that such a negatively charged group would cause the DMNB-derived reporter group to twist away from its binding site into the surrounding solvent (in which case its pK_a would be nearer to 7). This is what has previously been suggested to happen in the case of DMNB and trypsin [9].

A hydrophobic active site was the explanation favoured by Hempel et al. [28] for observations such as the inactivation of aldehyde dehydrogenase by iodoacetamide, but not by iodoacetate. The presence of a hydrophobic binding region is also suggested by the effects of steroids and analogues (such as progesterone and diethylstilboestrol) on the activity of aldehyde dehydrogenase [21]. The same indication arises from the activity of the enzyme towards *p*-nitrophenyl ester substrates and from its binding to an acetophenone-linked affinity resin [10]. The enzyme has a broad substrate specificity and will catalyse the dehydrogenation of non-polar substrates such as long-chain aliphatic aldehydes, aromatic aldehydes and steroidal aldehydes. Recently it has become clear that a very important natural substrate of the cytoplasmic form of aldehyde dehydrogenase is retinal, the aldehyde form of vitamin A. Human cytosolic aldehyde dehydrogenase has an extremely high affinity for retinal ($K_m = 0.06 \mu M$) [29]. (The product, retinoic acid, binds to several receptors, and the complexes thus produced act as potent transcriptional regulators for the genes involved in cell differentiation and development of the embryo [30].) A hydrophobic milieu for the DMNB-derived reporter group in aldehyde de-

hydrogenase is therefore very plausible. Chymotrypsin, with its well-established hydrophobic binding pocket, has fairly similar pK_a values (before and after denaturation) after derivative formation with DMNB to those found in the present work [9].

Intriguingly, on the other hand, the λ_{max} of enzyme-linked *p*-nitrophenoxide is quite different in aldehyde dehydrogenase (433 nm) from what it is in chymotrypsin (395 nm). Superficially this might be taken as meaning that a polar negatively charged environment exists in aldehyde dehydrogenase, as that of chymotrypsin is known to be non-polar. A neutral *p*-nitrobenzenesulphonyl reporter group used with chymotrypsin exhibits a spectral shift to shorter wavelength in a hydrophobic environment [31]. The λ_{max} of the negative *p*-nitrophenoxide reporter group, however, is likely to be critically dependent not only on the overall polarity of its surroundings, but also on the presence or absence of hydrogen-bond donors such as water molecules and suitable amino acid side chains [32]. (In both chymotrypsin and aldehyde dehydrogenase, hydrolysis of the acyl-enzyme of course demands access of at least one water molecule to the active site.) The red shift in the spectrum of a *p*-nitrophenoxide-containing reporter group in ornithine transcarbamoylase was interpreted in terms of an environment that is a poorer hydrogen-bond donor than water [33]. We found that the λ_{max} of free *p*-nitrophenoxide varies over a wide range in different solvents, with no obvious trend being apparent. For example, dimethylformamide, acetonitrile and dimethyl sulphoxide are all aprotic solvents of very similar dipole moment, and yet in them the λ_{max} of *p*-nitrophenoxide varies widely (398, 418, 432 nm respectively).

At this stage, therefore, it seems difficult to decide conclusively whether a negative or hydrophobic environment for the DMNB-derived reporter group in aldehyde dehydrogenase is more likely, but at present we consider there is more definite evidence in favour of the latter. In the future, we aim to widen our study to include glyceraldehyde-3-phosphate dehydrogenase, the mitochondrial isoenzyme of aldehyde dehydrogenase, and site-specific mutants of cytoplasmic aldehyde dehydrogenase that we are currently preparing. For example, if DMNB still reacts with a mutant in which Glu-268 is changed to alanine, the resulting spectral properties may be very informative. Finally, when the tertiary structure of aldehyde dehydrogenase inevitably becomes known, the present results with DMNB will be clarified; it may even prove possible to crystallize the reporter-group-labelled enzyme and subject it directly to X-ray analysis.

We are grateful to Mr. Graham H. Freeman for assistance in the synthesis of DMNB, to Dr. N. Haggarty for synthesis of the affinity resin used in enzyme isolation, and to the New Zealand Lottery Grants Board for funding for the spectrophotometer used in this work.

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Received 27 September 1993/15 December 1993; accepted 24 December 1993