The purification and some properties of the Mg^{2+}-activated cytosolic aldehyde dehydrogenase of *Saccharomyces cerevisiae*

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A purification procedure has been developed for the cytosolic aldehyde dehydrogenase of *Saccharomyces cerevisiae* that yields homogeneous enzyme. The enzyme seems to be a tetramer of identical 58 kDa subunits. The enzyme reaction is strongly stimulated by Mg^{2+} at low $NADP^+$ concentrations but there is no absolute requirement for bivalent cations. The kinetics of the reaction have been studied in the presence and absence of $MgCl_2$. $NADP^+$ binding studies of the quenching of protein fluorescence in the presence and absence of $MgCl_2$ show that the effect of Mg^{2+} is to increase the affinity of the enzyme for $NADP^+$ by approx. 100-fold. $NADP^+$ binding causes a slow conformational change in the enzyme and converts the enzyme from the inactive or low-activity form in which it is isolated into the fully active form. This conformational change seems to explain the marked lag-phases seen in enzyme assays. The enzyme is strongly inhibited by disulfiram and pyridoxal 5-phosphate.

INTRODUCTION

Two principal aldehyde dehydrogenase isoenzymes have been described for *Saccharomyces cerevisiae*. The first is located in the mitochondria and is activated by K^+ ions, the second is cytosolic and activated by Mg^{2+} ions [1]. It is believed that the mitochondrial enzyme functions under aerobic conditions in the oxidation of ethanol. The cytosolic enzyme, although present under aerobic conditions, functions in fermentative metabolism in a pathway generating acetyl-CoA from pyruvate [2].

The mitochondrial enzyme has been fairly well studied and various isolation procedures have been published [3,4], but a homogeneous and undegraded preparation was not obtained until the procedure of Bostian and Betts [5] was developed, which involved the use of a protease inhibitor (PMSF) and very rapid processing of extracts. By contrast the Mg^{2+}-activated yeast enzyme has received little attention. A partial purification procedure has been available for many years [6,7] but there was no indication of the state of homogeneity of the final product and the enzyme was only poorly characterized. Work in this laboratory is currently concerned with acetyl-CoA production by yeast under fermentative conditions. As the Mg^{2+}-activated aldehyde dehydrogenase is thought to be a key enzyme in this process [2] it was important to obtain an homogeneous enzyme preparation and study some of its properties. The work done forms the subject matter of this paper.

EXPERIMENTAL

Materials

$NADP^+$ (sodium salt) and disulfiram (tetraethylthioperoxydicarbonic diamide) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of A.R. grade whenever available, from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Solutions of acetaldehyde were made up daily from 1 M stock solutions (kept frozen), which were prepared from freshly distilled acetaldehyde. Fresh bakers' yeast was donated by Mauri Products Ltd., Hull, North Humberside, U.K.

Methods

Dialysis tubing was boiled in 10 mM EDTA, pH 7.0, and washed with water before use. All solutions were prepared with deionized water.

Protein concentrations

These were measured by the method of Bradford [8], with BSA as standard.

Enzyme assays

These were conducted at 25 °C in a filter fluorimeter of a design described by Dalziel [9]. The mixture contained, in a total volume of 4 ml: Hepes buffer, pH 7.5, 200 μmol; $NADP^+$, 0.055 μmol; acetaldehyde, 0.5 μmol; and $MgCl_2$, 15 μmol. For routine assays the reaction was initiated by adding enzyme. The unit of enzyme activity was defined as that amount catalysing the formation of 1 μmol of NADPH per min in the above assay. Initial-rate measurements for kinetic studies were performed in 50 mM Hepes, pH 7.5, at 25 °C in the same fluorimeter.

Electrophoresis

Under denaturing conditions this was performed on 10 % (w/v) slab gels with a discontinuous buffer system [10,11]. The molecular mass markers were carbonic anhydrase (29 kDa), yeast alcohol dehydrogenase (35 kDa), ovalbumin (45 kDa), BSA (66 kDa) and phosphorylase (97.4 kDa). For non-denaturing conditions electrophoresis was conducted on 7 % (w/v) slab gels with a high-pH discontinuous buffer system [11]. After electrophoresis was complete the gels were divided in half and stained for protein with Coomassie Blue, or for activity. The activity stain contained, in 25 ml of 50 mM Hepes buffer, pH 7.5: NAD^+, 24 mg; phenazine methosulphate, 0.25 mg; Nitro Blue Tetrazolium, 6 mg; acetaldehyde, 1.2 μmol; $MgCl_2$, 90 μmol.

Molecular mass determination by gel filtration

This was performed on a 0.8 cm × 30 cm G3000SW Tokohaas column in conjunction with an LKB HPLC/FPLC system. Samples (0.05 ml) were processed with a flow rate of 0.5 ml/min.

Abbreviation used: DTT, dithiothreitol.

The effluent was monitored at 280 nm. The molecular mass markers were ovalbumin (45 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

Fluorescence spectra and titrations

These were obtained or performed at room temperature in 4 ml, 0.5 ml or 0.2 ml cuvettes in a Perkin-Elmer LS-5B Luminescence Spectrometer.

Inhibition by disulfiram

For these experiments the enzyme preparation was dialysed overnight against several changes of 50 mM Hepes buffer, pH 7.5, to remove dithiothreitol (DTT), which was used in storage of the enzyme. The dialysed enzyme was completely stable at 0 °C for 24 h after removal of the DTT.

Enzyme purification

Unless stated otherwise operations were all conducted at 0–4 °C. Fresh yeast (1 kg) was suspended in 2.5 litres of 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT, and disrupted by continuous passage (15 min) through a type KDL Dyno-Mill bead beater (Willy A. Bachofen, AG Maschinfabrik, Basel, Switzerland). Cell breakage was essentially complete after this time. Immediately after disruption, PMSF (40 mM in methanol) was added to give a final concentration of 1 mM. Cell debris was removed by centrifugation to yield the cell extract.

Ammonium sulphate (351 g/l) was added and after 15 min the precipitate was removed by centrifugation and rejected. A further 165 g/l ammonium sulphate was added and after 15 min the pellet was collected by centrifugation and dissolved in a minimum volume of 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT. The sample was then heated to 58 °C for 15 min and after rapid cooling the precipitate was removed by centrifugation and rejected. The supernatant was dialysed exhaustively against 10 mM sodium phosphate, pH 7.0, containing 1 mM DTT.

The dialysed sample was centrifuged to remove insoluble material and then 90 ml of 0.2 M sodium phosphate, pH 6.0, was added, followed by 100 ml of a 30 mg/ml suspension of calcium phosphate gel. The pink pellet was removed after 5 min by centrifugation and discarded. The supernatant was treated with ammonium sulphate (516 g/l) and after 15 min the precipitate was collected and redissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT. The sample was then dialysed exhaustively against the same buffer.

The dialysed enzyme was applied to a 15 cm × 4.2 cm column of DEAE-Sephacel CL-6B equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and after being washed with 500 ml of this buffer the column was developed with a linear gradient of 0–250 mM NaCl in 500 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT. Large quantities of protein were eluted before the enzyme-bearing fractions, which appeared between one-half and two-thirds of the way through the gradient. The fractions of the highest specific activity were combined, dialysed against 40 mM sodium phosphate buffer, pH 6.0, containing 1 mM DTT, and then applied at room temperature (18 °C) to a 12 cm × 1.5 cm hydroxyapatite (Bio-Gel HTP) column equilibrated with the same buffer. The column was developed with a linear gradient of 40–350 mM sodium phosphate buffer, pH 6, in 100 ml of 1 mM DTT. The gradient eluted substantial quantities of inactive protein. The enzyme was eluted in a sharp band by applying 0.4 M sodium phosphate, pH 7.0, containing 1 mM DTT directly to the column. The purified enzyme was dialysed against 50 mM Hepes, pH 7.0, containing 1 mM DTT, and stored as a 5 mg/ml solution in a liquid-N_2 container.

Table 1 Purification of Mg^{2+}-activated aldehyde dehydrogenase

The values relate to the processing of 1 kg of fresh bakers' yeast.

Step	Volume (ml)	Activity (units)	Protein (g)	Specific activity (units/mg)	Yield (%)
Extract	3000	6900	131	0.054	100
$(NH_4)_2SO_4$ precipitation	350	4500	37.5	0.12	65
Heat	380	4100	14.6	0.28	59
$Ca_3(PO_4)_2$ precipitation	470	3700	9.3	0.4	54
DEAE-Sepharose	70	2200	0.76	2.9	32
Hydroxyapatite	30	1500	0.063	24	22

RESULTS AND DISCUSSION

Preliminary attempts to purify the enzyme by using the method of Seegmiller [6,7] were unsuccessful. Very poor yields of enzyme were obtained and the specific activities of the best fractions were very low. A new purification procedure was developed, for which the progress of a typical purification of the enzyme is summarized in Table 1. This shows an overall purification of 450-fold with a yield of 20%. The hydroxyapatite column step was crucial to the success of the procedure. Various alternatives were tried at this stage in preliminary attempts to establish the purification method and all were relatively ineffective. Thus gel filtration on Sephacryl G-300 HR at pH 7.0, ion-exchange chromatography on an Econopac Q cartridge (BioRad) at pH 6.4 and 7.5 with a salt gradient, and attempted affinity chromatography on $NADP^+$–Sepharose or Reactive Red 120 agarose at pH 7.5, with or without $MgCl_2$ added to buffers, increased the specific activity by 1.5–2.0-fold at most. When enzyme of the highest purity was required for analytical gel electrophoresis or sequence work, a final step on TSK G-3000 SW was effected by using an LKB FPLC system. This removed material of lower molecular mass (3–5%) that was sometimes present. When due allowance is made for the difference in standard assay conditions and for the different ways in which the enzyme unit was defined it was clear that the specific activity of the final product described here (24 units/mg) was approx. 9.7-fold higher than that described by Seegmiller [6].

The purified enzyme dissolved in either 50 mM Hepes buffer, pH 7.5, or 20 mM sodium phosphate buffer, pH 7.0, could be stored in liquid N_2 for several weeks without loss of activity. The A_{280}/A_{260} value was 1.73, suggesting that the preparation contained no significant amount of bound nucleotide. Examination of the fluorescence emission spectrum revealed no trace of bound reduced nucleotide cofactor; this contrasts somewhat with sheep liver cytosolic aldehyde dehydrogenase [12]. PAGE of the purified enzyme on 10% gels in 0.1% SDS [10] showed only one sharp protein-staining band with a molecular mass of 58 kDa. This was the value used to calculate enzyme subunit concentrations in subsequent experiments. There was no evidence for proteolytic digestion of the enzyme, which can be a serious problem with the yeast K^+-activated enzyme [6]. Electrophoresis on 7% polyacrylamide gels under non-denaturing conditions showed only one heavy protein-staining band three-quarters of the way through the gel. This corresponded exactly to the band appearing on activity staining. No other protein bands appeared, but there was significant streaking back from the main band. The activity

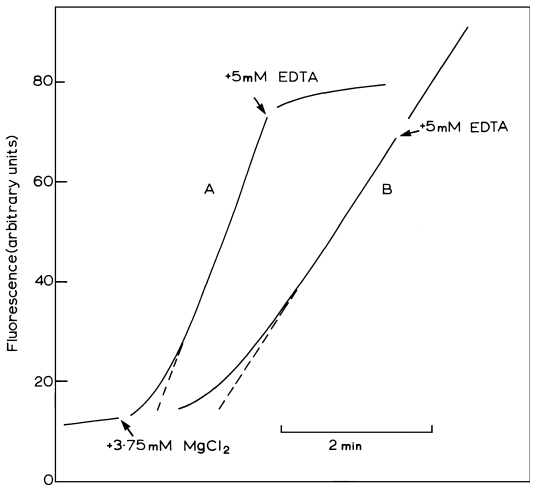

Figure 1 Progress curves from enzyme assays at pH 7.5 and 25 °C

(**A**) Standard enzyme activity assay. The reaction was initiated by adding 0.019 units of enzyme to a mixture containing 14 μM $NADP^+$ and 125 μM acetaldehyde. Other additions were made as indicated. (**B**) The reaction was initiated by adding 0.019 units of enzyme to a mixture containing 140 μM $NADP^+$ and 125 μM acetaldehyde.

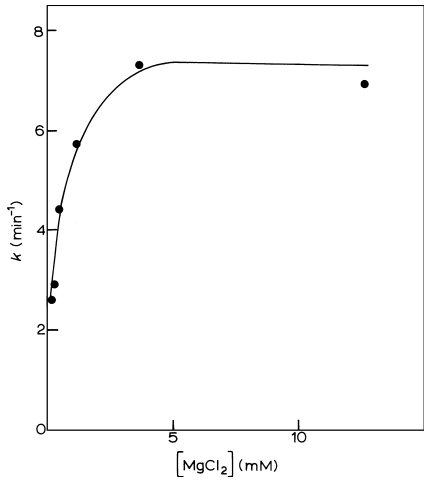

Figure 2 The effect of $MgCl_2$ on the lag phases in enzyme assays at pH 7.5 and 25 °C

The lag phases such as that seen in curve **A** of Figure 1 were analysed by the method of Dalziel et al. [16]. For these experiments the reaction was initiated by adding enzyme to the complete reaction mixture (i.e. including $MgCl_2$).

stain was, by contrast, sharp. The streaking was almost certainly due to the instability of the enzyme at pH values above 8.0. Tests showed that whereas the enzyme was completely stable in dilute solution for 4 h at pH 4.8 and pH 7.0, at pH 8.0 the half-life was approx. 1 h. Gel filtration of the purified enzyme on a calibrated TSK G-3000 SW column at pH 7.5 showed a sharp symmetrical peak with a molecular mass of 190 ± 25 kDa. These combined data suggest a tetrameric structure for the enzyme as for the yeast K^+-activated enzyme [6] and indeed for many other aldehyde dehydrogenases. It may be noted here that preliminary sequence work with the purified enzyme has shown that the N-terminal amino acid is threonine. The first 15 amino acids were sequenced and a clean clear sequence was established. This result formed part of another project, but it showed that the enzyme preparation was highly pure and suggests that the four enzyme subunits are of identical sequence.

Assays of the enzyme were characterized by a lag phase that lasted from 30 s to 1 min (Figure 1). This is a phenomenon that has been described for the sheep mitochondrial enzyme [13] and the yeast K^+-activated enzyme [14]. For the sheep mitochondrial enzyme the length of the lag phase was concentration dependent, suggesting dissociation of the tetramer into a more active dimer [15]. This was not the case here. Fluorimetric and spectrophotometric assays over a 10-fold range of enzyme concentration showed the same length of lag phase and the same specific rate at the end of the lag. The Mg^{2+}-activated enzyme seemed to be more similar to the K^+-activated yeast enzyme. Thus analysis of the data [16] yielded an apparent first-order rate constant for the process that varied with the $MgCl_2$ concentration (Figure 2) in a way similar to the K^+-activated enzyme with KCl [14]. It may be noted that the $MgCl_2$ effect was fully reversible. Thus addition of excess EDTA abolished the activation within 10–20 s (Figure 1). The lag phase could be abolished by preincubation of the enzyme with $MgCl_2$ and $NADP^+$ for 1–2 min at 25 °C before acetaldehyde was added to initiate the reaction. No other combination of substrate, $NADP^+$ and $MgCl_2$ could do this. This characteristic is similar to, but not identical with, the K^+-activated yeast enzyme. In that case preincubation with KCl alone was sufficient to abolish the lag phase [14].

Although the enzyme is activated by $MgCl_2$, this compound was not absolutely required for activity. Thus, as Figure 1 shows, even under the conditions of the standard assay there was a low rate (about 3 % of the $MgCl_2$-activated rate) when no $MgCl_2$ was present. This low rate was not affected by adding excess EDTA. When the $NADP^+$ concentration was raised 10-fold, the $MgCl_2$-independent rate was greatly increased (Figure 1), was insensitive to excess EDTA and showed a lag phase, somewhat longer in duration, but otherwise similar to the Mg^{2+}-activated case. The insensitivity to EDTA showed that this activity was not due to trace metal contamination of the reagents.

The lag phases in assays raised a problem in the measurement of initial rates. In the experiments described below, reactions were initiated by adding acetaldehyde after preincubating the enzyme in the assay mixture for 3 min with $NADP^+$ and $MgCl_2$, when the latter was included, or with $NADP^+$ alone when no $MgCl_2$ was to be added. These precautions abolished the lag phases and initial rates could be measured straightforwardly.

Detailed kinetic studies of the enzyme at 25 °C and pH 7.5 showed that the characteristics of the reactions were quite different in the presence and absence of $MgCl_2$. In the presence of 19 mM $MgCl_2$, which preliminary experiments showed to be saturating, double-reciprocal plots were linear (Figure 3) and the data conformed to an equation of the type:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_{NADP^+}}{[NADP^+]} + \frac{\phi_{acetaldehyde}}{[acetaldehyde]} + \frac{\phi_{NADP^+.acetaldehyde}}{[NADP^+][acetaldehyde]} \quad (1)$$

where v_0 is the initial rate, [E] the enzyme (subunit) concentration and ϕ_0 etc. are the initial rate parameters [17]. In the absence of $MgCl_2$, on the other hand, the reciprocal plots were convex towards the abscissa (Figure 4). This arose because the plots of $v_0/[E]$ against $[NADP^+]$ were sigmoidal, perhaps indicating a positive interaction between $NADP^+$ binding sites. When acetaldehyde was the varied substrate, reciprocal plots were linear within experimental error. The change in character of the kinetics on removal of $MgCl_2$ made it difficult to compare the results. However, at high $NADP^+$ concentrations (over 150 μM), the

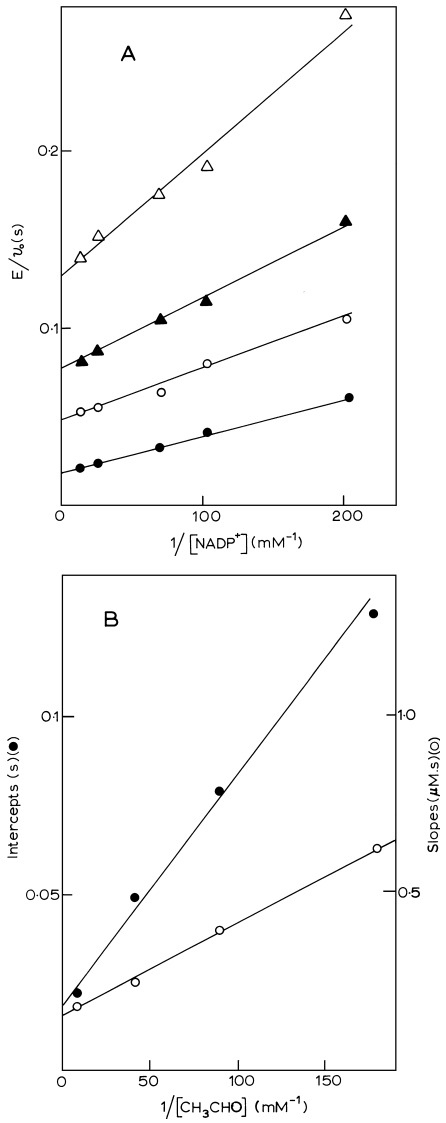

Figure 3 Initial rates of acetaldehyde oxidation at pH 7.5 and 25 °C in the presence of 19 mM $MgCl_2$

(**A**) Primary plots show the variation of the reciprocal of the specific initial rate with the reciprocal of the $NADP^+$ concentration at different acetaldehyde concentrations. The acetaldehyde concentrations (μM) were: ●, 106; ○, 24; ▲, 11.2; △, 5.6. (**B**) Secondary plots showing the variation of the intercepts (●) and slopes (○) of (**A**) with the reciprocal of the acetaldehyde concentration.

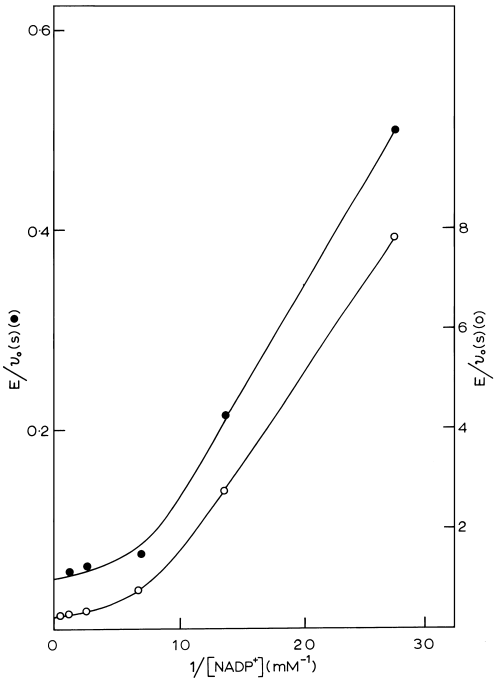

Figure 4 Initial rates of acetaldehyde oxidation at pH 7.5 and 25 °C in the absence of $MgCl_2$

The plot shows variation of the reciprocal of the specific initial rate with the recriprocal of the $NADP^+$ concentration at two acetaldehyde concentrations. The acetaldehyde concentrations (μM) were: ●, 1200; ○, 30.

Table 2 Initial rate parameters for the oxidation of acetaldehyde at pH 7.5 and 25 °C by aldehyde dehydrogenase

The initial rate parameters were obtained as described in the text. Two complete experiments were performed for both $[MgCl_2] = 0$ and $[MgCl_2] = 19$ mM. Values of apparent $K_{m,acetaldehyde} = 35\ \mu M$ and apparent $K_{m,NADP^+} = 14\ \mu M$ were obtained by Seegmiller [6], with 14 mM $MgCl_2$.

$[MgCl_2]$ (mM) ...	0	19
ϕ_0 (s)	0.045 ($\pm$0.004)	0.016 ($\pm$0.0025)
ϕ_{NADP^+} (μM·s)	3.0 ($\pm$0.15)	0.13 ($\pm$0.25)
$\phi_{acetaldehyde}$ (μM·s)	4.5 ($\pm$0.45)	0.9 ($\pm$0.15)
$\phi_{NADP^+.acetaldehyde}$ (μM^2·s)	1260 ($\pm$60)	3.2 ($\pm$0.64)
$\phi_{NADP^+}/\phi_0 = K_{m,NADP^+}$ (μM)	66	8.7
$\phi_{acetaldehyde}/\phi_0 = K_{m,acetaldehyde}$ (μM)	100	60
$1/\phi_0 = V_{max}$ (s^{-1})	22	63
$\phi_{NADP^+.acetaldehyde}/\phi_{acetaldehyde} = K_{NADP^+}$ (μM)	280	3.6

reciprocal plots in the absence of $MgCl_2$ approached linearity and the data conformed reasonably well to eqn. (1). Analysis of the data on this basis allowed some comparisons, and these appear in Table 2. The reciprocal plots also approached linearity at low $NADP^+$ concentrations (less than 60 μM), but in these cases the lines extrapolated to below the origin.

The data of Table 2 indicate that the initial rate parameters were increased in the absence of $MgCl_2$ with a 390-fold increase in $\phi_{NADP^+.acetaldehyde}$. Interestingly V_{max} ($1/\phi_0$) was only approx. one-third in the absence of $MgCl_2$, suggesting that Mg^{2+} might not have a large effect on the catalytic step of the reaction. NAD(P)-linked dehydrogenases commonly function by using a compulsory ternary-complex mechanism in which the coenzyme is the first substrate to combine. For such a mechanism, $\phi_{NADP^+.acetaldehyde}/\phi_{acetaldehyde} = K_{NADP^+}$, where K_{NADP^+} is the dissociation constant of the E–$NADP^+$ complex [17]. On this basis the present data indicated that the affinity of the enzyme for $NADP^+$ was strengthened approx. 70-fold [K_{NADP^+} 3.6 μM (19 mM $MgCl_2$); $K_d = 280\ \mu M$ (no $MgCl_2$)] by adding $MgCl_2$. More direct evidence on this point is given below. The K_m for $NADP^+$ (ϕ_{NADP^+}/ϕ_0) was increased 7-fold in the absence of $MgCl_2$.

Mixing 6 μM enzyme with 12 μM NADPH, in the presence or absence of 5 mM $MgCl_2$, gave no significant enhancement of the fluorescence above that of NADPH alone at the same concen-

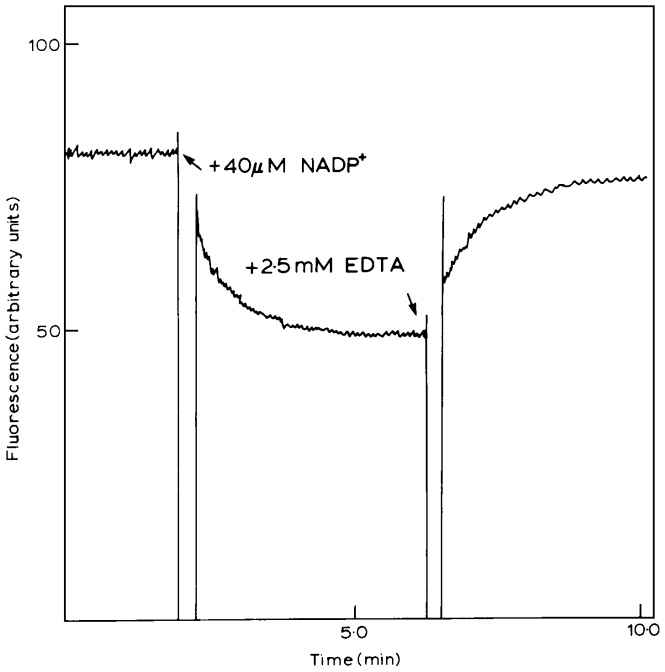

Figure 5 Quenching of the fluorescence of aldehyde dehydrogenase by $NADP^+$

The initial mixture contained 35 μM enzyme subunits and 1.6 mM $MgCl_2$ in 50 mM Hepes, pH 7.5, at 18 °C. Additions were made as indicated. The wavelength settings were: excitation, 303 nm; emission, 340 nm with 5 nm slits.

tration. Either the enzyme bound the reduced cofactor very weakly indeed by comparison with mammalian aldehyde dehydrogenases or there is no fluorescence enhancement on binding. The K^+-activated yeast enzyme gave only a rather weak interaction with NADH when studied in this way [14]. As an alternative approach to the study of coenzyme binding, the effect of $NADP^+$ on protein fluorescence has been studied. Some results are shown in Figure 5. The exciting wavelength (303 nm) was chosen to minimize inner filtering effects from absorbance of the exciting radiation by $NADP^+$. It was clear that $NADP^+$ binding occurred and was accompanied by fluorescence quenching. It was also clear that the effect was not instantaneous but was established over a 1–2 min period. The slow change suggested that a conformational change occurred after coenzyme binding. The fluorescence quenching consequent upon $NADP^+$ binding occurred either in the presence or absence of $MgCl_2$ but, as with enzyme assays (Figure 1), much higher concentrations of $NADP^+$ were required for the same effect in the absence of $MgCl_2$ (see below). Again, as with the activity assays, the Mg^{2+} effect could be reversed by adding excess EDTA. The EDTA effect was also quite slow, requiring about the same time (1–2 min) for completion. Presumably the relaxation of the enzyme into the native form is also slow. These experiments suggested that the principal effect of $MgCl_2$ was to strengthen the binding of the cofactor by stabilizing the enzyme–coenzyme complex.

It seemed likely that the conformational change observed in the $NADP^+$-binding studies was at the root of the activation seen in enzyme assays (Figure 1). In an attempt to pursue this idea further the kinetics of protein fluorescence quenching were analysed and compared with the kinetics of activation seen in assays. The fluorescence quenching followed apparently first-order kinetics, as does the activation process, and the results are

Table 3 Kinetic analysis of the activation of reaction progress curves and of the quenching of protein fluorescence on addition of $NADP^+$ to mixtures containing enzyme

Experiments were conducted at 16 °C in 50 mM Hepes buffer, pH 8.0, with or without 4 mM $MgCl_2$. For the enzyme assay the concentration of $NADP^+$ was 275 μM, that of acetaldehyde 125 μM and that of the enzyme subunits was 10 nM. For fluorescence quenching, $NADP^+$ and enzyme subunits were present at 550 and 10 μM respectively. Values of k_{app} were obtained as described in the text.

Method	k_{app} (min^{-1})	
	Without Mg^{2+}	With Mg^{2+}
Enzyme assay	2.6	10
Fluorescence quenching	2.4	5.6

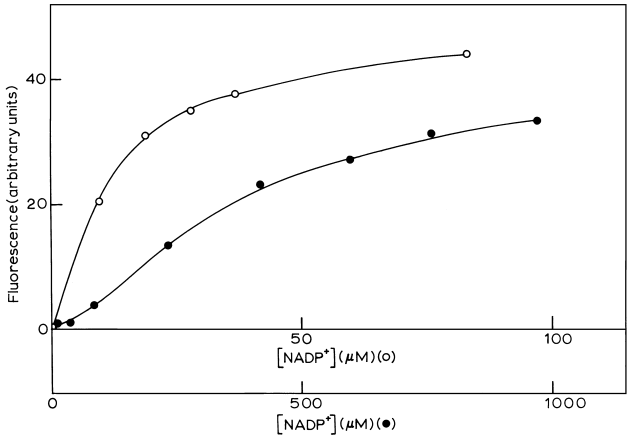

Figure 6 $NADP^+$ binding to aldehyde dehydrogenase studied by fluorescence quenching

The initial mixture contained 35 μM Hepes, pH 7.5, at 18 °C. Additions of $NADP^+$ were made to mixtures containing either no $MgCl_2$ (●) or 16 mM $MgCl_2$ (○) and the resulting change in fluorescence was noted. The wavelength settings were as for Figure 5.

recorded in Table 3. The enzyme concentrations in the two sets of experiments were necessarily very different but, as indicated above, the length of the lag phase in activity assays seemed to be independent of the enzyme concentration. It was therefore clear that the two processes in Table 3 have similar (4 mM $MgCl_2$) or identical time courses (no $MgCl_2$), consistent with their arising from the same event.

The extent of protein fluorescence quenching on binding $NADP^+$ was used to study the strength of $NADP^+$ binding in the presence and absence of $MgCl_2$. Results are shown in Figure 6 where the changes in fluorescence (i.e. ΔF at 3 min after mixing) at each $NADP^+$ concentration are plotted against the concentration of $NADP^+$ added. In the presence of 16 mM $MgCl_2$ the plot seemed to be hyperbolic, and when these and other data were analysed by the method of Stinson and Holbrook [18] the data indicated 1.9 binding sites per tetramer with a dissociation constant of 3 ± 1 μM. The analysis assumes that the extent of quenching is proportional to saturation. The facts that the enzyme undergoes a conformational change after binding (see above) and that, at least in the absence of $MgCl_2$, the enzyme also exhibits non-hyperbolic kinetic properties might bring this assumption into question.

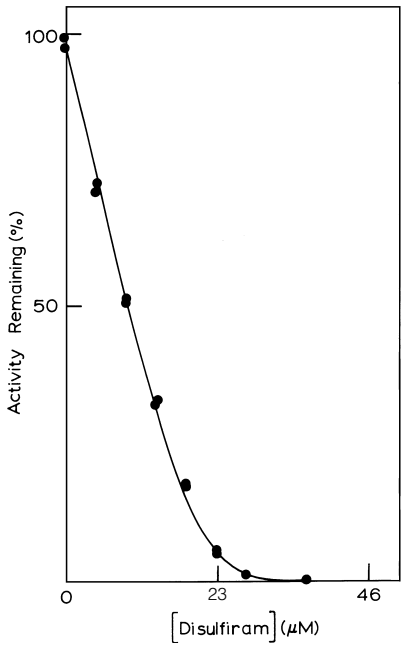

Figure 7 Inhibition of the enzyme activity by disulfiram

Disulfiram at different concentrations was added to solutions containing enzyme (39.7 μM subunits) in 50 mM Hepes, pH 7.5. The mixtures were kept at 0 °C for 30 min, after which samples were removed for assay.

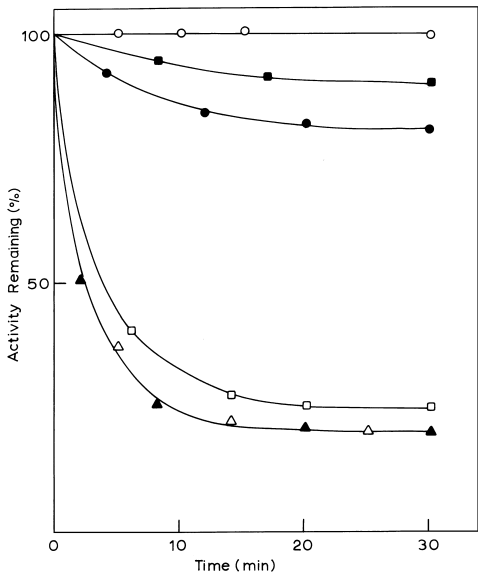

Figure 8 Inhibition of the enzyme by pyridoxal 5-phosphate

Pyridoxal 5-phosphate (1.5 mM final concentration) was added to solutions containing enzyme (2 μM subunits) in 50 mM Hepes, pH 7.5. The mixtures were kept at 25 °C and samples were removed at selected times for assay. Conditions for individual experiments were: ■, 0.18 mM $NADP^+$ + 7 mM $MgCl_2$; ●, 1.8 mM $NADP^+$; □, 0.18 mM $NADP^+$; ▲, 4 mM $MgCl_2$ + 10 mM acetaldehyde; △, no addition. A control (○) was also performed to which no pyridoxal 5-phosphate added.

In the absence of $MgCl_2$ the fluorescence quenching data (Figure 6) suggested that the binding of $NADP^+$ was much weaker; so much so that even with concentrations of 1 mM $NADP^+$ saturation was not achieved. If it was assumed that saturation with $NADP^+$ in the absence of $MgCl_2$ would produce the same total quenching of fluorescence as saturation with $NADP^+$ in the presence of 16 mM $MgCl_2$ (45 %), the half-saturation point of the titration suggested a dissociation constant of 300–350 μM, approx. 100-fold larger than with $MgCl_2$ present. The same reservations apply to the assumptions about fluorescence quenching and saturation as were raised earlier. Nevertheless, the estimates for the dissociation constants from the data of Figure 6 (K_d 3 μM, $[MgCl_2] = 16$ mM; K_d 350 μM, $[MgCl_2] = 0$) seem to agree quite well with the values derived from the kinetic experiments (K_d 3.6 μM, $[MgCl_2] = 19$ mM; K_d 280 μM, $[MgCl_2] = 0$). A further interesting feature of the binding experiments in the absence of $MgCl_2$ was that the binding isotherm appeared to be slightly sigmoidal. The effect was weak but was observed each time the titration was attempted. The effect might well be correlated with the sigmoidal plots of rate against $NADP^+$ concentration seen in the kinetic studies performed in the absence of $MgCl_2$. Presumably it was positive interactions between the $NADP^+$ binding sites that gave rise to the kinetic effects.

The enzyme was subject to potent inhibition by disulfiram, which is a well-known inhibitor of mammalian cytosolic aldehyde dehydrogenase. Figure 7 shows the effect of a 30 min incubation of enzyme with stoichiometric quantities of disulfiram of 0 °C on the activity of the enzyme. The initial slope indicates 2.0 sites per tetramer reacting with the reagent. This is a very similar result to that found with sheep liver cytosolic aldehyde dehydrogenase under similar conditions [19]. The result also agreed with the results of $NADP^+$ titrations in the presence of $MgCl_2$ in indicating 2 active sites per tetramer.

Inhibition by disulfiram was extremely rapid. Addition of 2.5 μM disulfiram to an incomplete assay mixture containing acetaldehyde, $MgCl_2$ and enzyme (3.9 nM) 30 s before addition of $NADP^+$ to initiate the reaction rendered the enzyme essentially inactive (less than 1 % residual activity). Addition of 1 mM DTT to the assay resulted in a rapid (approx. 10 s) and full recovery of activity. In contrast, addition of 2.5 μM disulfiram to an incomplete assay containing $NADP^+$, $MgCl_2$ and enzyme with initiation by adding acetaldehyde 20 s later showed different behaviour. The enzyme was initially almost completely protected from the inhibitor and inhibition was progressive, taking about 5 min to be fully established. Addition of 2.5 μM disulfiram to an assay already in progress showed exactly the same kind of behaviour. Clearly the enzyme was protected by $NADP^+/MgCl_2$, and the further addition of acetaldehyde, generating a catalytic cycle (involving perhaps an acyl thioester intermediate), did not confer greater stability. Perhaps the conformational change after $NADP^+$ binding is the crucial event here. Supporting this hypothesis is the fact that addition of $NADP^+$ at 20-fold higher concentration (275 μM) than for normal assays to incomplete assay mixtures containing enzyme (3.9 nM) but no $MgCl_2$ provided substantial (30 %) protection for the enzyme when 2.5 μM disulfiram was added 30 s before the addition of acetaldehyde and $MgCl_2$ to initiate the reaction.

The enzyme was also susceptible to inhibition by pyridoxal 5-phosphate. The results of mixing 2 μM enzyme subunits with this reagent in 50 mM Hepes, pH 7.5, are shown in Figure 8. The activity fell to approx. 20 % of the initial value after 15 min and was stable thereafter. Protection was obtained by adding 0.18 mM $NADP^+$ and 7 mM $MgCl_2$ or by adding 1.8 mM

$NADP^+$ alone, but none by adding 4 mM $MgCl_2$ or 10 mM acetaldehyde either separately or together. The results were very similar to those seen, most notably with glutamate dehydrogenase [20], but with other dehydrogenases as well [20–22]. Analogy with these systems suggests that the reagent reacted with an active centre residue (probably lysine) in a reversible manner indicated by the following scheme, where Pyr represents pyridoxal:

$$E + \text{Pyr-P} \rightleftharpoons \text{E–Pyr-P} \rightleftharpoons \text{E-Pyr-P}$$

The first complex is a Michaelis complex, which is converted into the covalently bound complex by Schiff base formation. As the Michaelis complex could be present in significant concentrations even at high reagent concentrations and as it might rapidly dissociate on dilution into an enzyme assay, a significant proportion of enzyme activity could be resistant to inhibition. That this analysis also applied to aldehyde dehydrogenase was confirmed by the finding that addition of $NaBH_4$ 30 min after mixing enzyme with pyridoxal 5-phosphate, followed by dialysis and a second treatment, resulted in a further loss of 80% of the activity (i.e. the same proportion as before) that survived the first treatment.

A point of interest is that disulfiram and pyridoxal 5-phosphate inhibited the enzyme quite independently. Enzyme in 50 mM Hepes, pH 7.5, was inactivated by excess disulfiram and then subsequently treated with 1.5 mM pyridoxal 5-phosphate for 30 min. At the end of this period, disulfiram inhibition was relieved by adding 1 mM DTT. Only 20% of the original activity could be recovered, i.e. the same level of activity that would have been observed by treatment with pyridoxal 5-phosphate alone.

Conclusion

Yeast cytosolic aldehyde dehydrogenase was active without added $MgCl_2$ but, as Figures 3 and 4 and Table 2 show, the kinetics of the reaction were greatly altered by $MgCl_2$. The activation was particularly marked at low $NADP^+$ concentrations. The rate in the standard assay was increased approx. 30-fold on addition of 4 mM $MgCl_2$, whereas the true V_{max} was increased only 3-fold (Table 2). It is important to know what role the metal ion plays in the mechanism of the reaction. The binding studies (Figure 6) suggested that Mg^{2+} increased the affinity of the enzyme for $NADP^+$ by approx. 100-fold. This was probably the principal effect of Mg^{2+}. It is true that $NADP^+$ binding produced a conformational change in the enzyme that was important in developing full enzymic activity, but this change also took place in the absence of $MgCl_2$ when the $NADP^+$ concentration was raised to much higher levels. It is not envisaged that the $NADP^+$ binds to the enzyme as an $NADP^+$–Mg^{2+} complex. The dissociation constant for this complex is about 20 mM [23] and the kinetic effects of $MgCl_2$ on aldehyde dehydrogenase seemed to be fully saturated by this reagent at 5 mM.

For the sheep liver cytosolic aldehyde dehydrogenase $MgCl_2$ is a powerful inhibitor of activity and is so because it severely decreases the dissociation rate of NADH from the terminal rate-limiting E–NADH complex [24,25]. In consequence the affinity of the enzyme for NADH is markedly enhanced by Mg^{2+}. This has provided the basis for active-site titrations with NADH in the presence of Mg^{2+} [26]. At this stage it is not clear whether $MgCl_2$ affects the affinity of the yeast enzyme for NADPH. However, the facts that no enhancement of fluorescence of NADPH was observed in the presence of 5 mM $MgCl_2$ and that $MgCl_2$ is an activator and not an inhibitor of the enzyme suggests that the NADPH binding to the enzyme is not stabilized to any significant extent.

Although the sheep liver cytosolic enzyme is inhibited by $MgCl_2$, at least one of the internal steps of the mechanism is accelerated by it [26]. Stopped-flow measurements suggested that the thioester intermediate (E–S-CO-CH_3·NADH), formed directly after hydride transfer, is hydrolysed approx. 3-fold more rapidly when $MgCl_2$ is present. It is possible that the same effect occurs with the yeast enzyme. The V_{max} was increased approx. 3-fold in the presence of $MgCl_2$ (Table 2). At this stage it is not clear what is the rate-limiting step of the reaction. The susceptibility of the enzyme to disulfiram seems to be consistent with the formation of a thioester intermediate as part of the mechanism.

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