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THERMAL INACTIVATION OF ALCOHOL DEHYDROGENASES IN THE PRESENCE OF NAD+ OR NADP+

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SUMMARY

1. Alcohol dehydrogenase from yeast or liver (alcohol:NAD+ oxidoreductase, EC 1.1.1.1) was heated at 43° in sodium pyrophosphate buffer (pH 8.8) with various concentrations of NAD+ or NADP+, prior to assay.

2. Recovery of enzyme was concentration-dependent above 1.5 mM NAD+ or NADP+. Recovery reached zero at 3.5 mM NAD+ or NADP+ for the yeast enzyme and at 4.3 mM NAD+ or 3.3 mM NADP+ for the liver enzyme.

3. Cysteine or bovine serum albumin (most effective at 0.8 and 0.03 mg/ml), respectively) had some protective action against thermal inactivation of these enzymes.

4. A mechanism of enzyme inactivation is suggested that involves allosteric binding of NAD+ or NADP+ at these concentrations.

INTRODUCTION

A variety of contradictory reports have appeared on the effect of substrates, including co-factors such as NAD+, as stabilizers or destabilizers for their respective enzymes *in vitro*. GRISOLIA[1] has published a comprehensive review of both types of effect. JOLY[2], also BERNHARD AND ROSSI[3], have reviewed the best known effect, which is the stabilization of an enzyme by its substrate. In 1959 GRISOLIA AND JOYCE[4] published a brief note in which a curious loss of yeast alcohol dehydrogenase (alcohol: NAD+ oxidoreductase, EC 1.1.1.1) activity was seen when the preincubation medium (Tris–HCl buffer pH 7.4) at 43°, contained some NAD+.

Work by YONETANI AND THEORELL[5] on liver alcohol dehydrogenase showed the opposite effect. These workers reported that some stabilization of this enzyme occurred during preincubation at 75° (pH 7.0) with in fact quite low concentrations of NAD+ (0.04 mM) relative to other studies. We find no stabilization at this concentration of NAD+ under our conditions of preincubation (43°, pH 8.8), but observe a concentration-dependent inactivation of alcohol dehydrogenase by NAD+ or NADP+.

MATERIALS AND METHODS

Crystalline yeast alcohol dehydrogenase (activity about 160 units/mg) and crystalline horse liver alcohol dehydrogenase (activity about 2 units/mg) were purchased from Miles-Seravac, Maidenhead, Great Britain. NAD^+ (98% purity) was purchased from The Boehringer Corporation (London) and $NADP^+$ from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Heat treatment (i.e. preincubation), prior to assay, of alcohol dehydrogenases with NAD^+ or $NADP^+$

The assay mixture (see below), complete except for the ethanol, was incubated at 43° for 20 min with the yeast enzyme and 50 min for the liver enzyme (*cf.* BRAND *et al.*[6]). This resulted in about 40% recovery of each enzyme in the absence of NAD^+ or $NADP^+$. One investigation on the liver enzyme involved prior incubation for 30 min, and here the recovery was 75%. The mixture was cooled to 30° before the addition of ethanol and the absorbance measurements.

Assay of alcohol dehydrogenase activity

The assay mixture, at 30°, contained: 1.94 ml 10 mM (or 100 mM) sodium pyrophosphate buffer (pH 8.8), 0.3 ml 50% (v/v) absolute ethanol in water, and 0.66 ml of 2.25 mM NAD^+ solution. This gives a final concentration of at least 0.5 mM NAD^+ so as to give maximum initial rate. Thus higher NAD^+ concentrations after some of the prior heat-treatment studies causes no further increase in this rate. The enzyme solution, 1 μg in 0.1 ml 10 mM potassium phosphate buffer (pH 7.5) for the yeast enzyme and 70 μg for the liver enzyme, was added to start the reaction and the increase (linear) in absorbance at 340 nm was recorded at 15 sec intervals for 120 sec. Samples were read against the appropriate blank *i.e.* same NAD^+ or $NADP^+$ concentration. The effect on the assay of the enzyme-stabilisers, at the concentration used in heat treatment was investigated.

NAD^+ and $NADP^+$ concentrations employed were in the range 0–4 mM. Since NAD^+ is sold as the free acid, the quantity of buffer used must be sufficient to ensure no significant pH change. Control experiments were done by heat treatment at 30°, where no enzyme loss occurs.

Use of enzyme stabilizers

Preincubation was done in the additional presence of the determined optimal concentrations of two stabilisers found for these enzymes, cysteine 0.8 mg/ml (for thiol protection) and bovine serum albumin 0.03 mg/ml (for general protective action–including conformation).

RESULTS

Fig. 1 shows the effect of NAD^+ on the yeast alcohol dehydrogenase, alone and in the presence of the enzyme stabilizers. Fig. 2 shows the corresponding effect of NAD^+ and stabilisers with the liver alcohol dehydrogenase; Fig. 3 shows the effect of $NADP^+$ on the yeast and liver enzymes. NAD^+ concentrations above 1.5 mM promote rapid inactivation of the yeast enzyme leading to zero recovery of enzyme

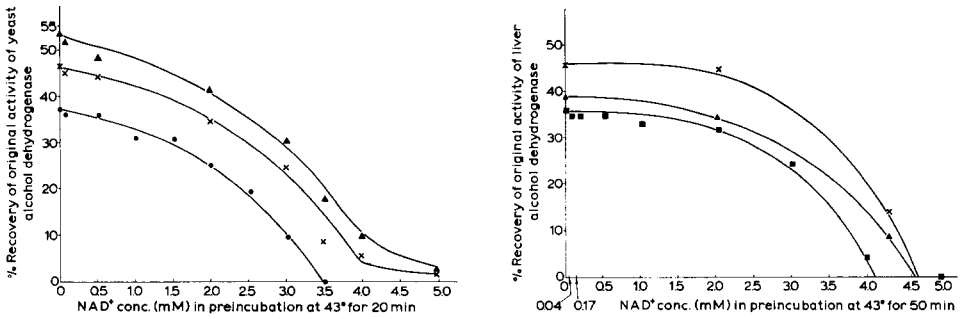

Fig. 1. Effect of NAD+ concentration during preincubation at 43° for 20 min on yeast alcohol dehydrogenase recovery. In addition to NAD+, during preincubation, (a) cysteine was present at 0.8 mg/ml (▲—▲); (b) bovine serum albumin was present at 0.03 mg/ml (×—×); (c) no stabiliser added (●—●).

Fig. 2. Effect of NAD+ concentration during preincubation for 50 min at 43° on liver alcohol dehydrogenase recovery. In addition to NAD+, during preincubation, (a) cysteine was present at 0.8 mg/ml (▲—▲); (b) bovine serum albumin was present at 0.03 mg/ml (×—×); (c) no stabiliser added (■—■).

at 3.5 mM NAD+. For the liver enzyme, NAD+ concentrations above 2.0 mM also promote rapid inactivation leading to zero recovery at the same level of NAD+ (3.5 mM).

The enzyme stabilisers show a fairly constant effect throughout the NAD+ range employed. The stabilizer values are shown after subtraction of a small correction due to some activator effect (1 to 7%) of the stabilisers in the assays themselves, since all these components of the preincubation mixture are present in the assay. For 3.5 mM NAD+ with yeast enzyme (normally zero recovery here) 13% recovery was obtained with cysteine and 6% with bovine serum albumin. For the liver enzyme and 4.3 mM NAD+ (normally zero recovery here) recoveries were, with cysteine 7% and with bovine serum albumin 14%. No stabilization effect was found at 0.04 mM NAD+ for our liver alcohol dehydrogenase preparation, under these particular conditions of heat treatment (contrast ref. 5).

NADP+ has a very similar effect to NAD+ on both enzyme preparations, with

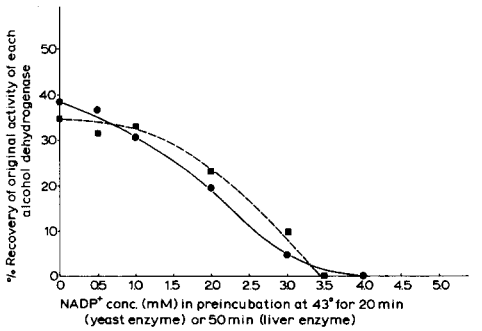

Fig. 3. Effect of NADP+ concentration during preincubation for 20 min or 50 min, respectively, at 43°, on yeast and liver alcohol dehydrogenase recovery (no stabiliser added). ●—●, yeast enzyme; ■---■, liver enzyme.

zero recovery concentration of 3.5 mM for yeast enzyme (as for NAD^+) and 3.3 mM for liver enzyme (NAD^+ value was 4.3 mM). $NADP^+$ therefore has an equal effect with NAD^+ on yeast enzyme but a more marked effect than NAD^+ on the liver enzyme (the latter is therefore the weakest effect found).

In control experiments, no significant loss of enzymes was seen at 30° with either NAD^+ or $NADP^+$, even at 4 mM, so the promoted denaturation clearly is very temperature dependent. Similar results were obtained using 10 mM or 100 mM pyrophosphate buffer. Another purchased sample of the yeast enzyme (Boehringer crystals in $(NH_4)_2SO_4$ suspension), showed an identical zero recovery level of NAD^+, and increasing the concentration (× 100) of the yeast enzyme for preincubation had no additional effect. No inhibitors of the enzymes were produced by heating these coenzymes in the buffer alone.

DISCUSSION

It is clear that the rate of heat denaturation of yeast and liver alcohol dehydrogenase at 43° is greatly increased at high concentrations of NAD^+ (or $NADP^+$). The small sparing action of the stabilisers, cysteine and bovine serum albumin, is largely independent of NAD^+ concentration. This result suggests that the mechanism of enzyme inactivation is similar throughout the NAD^+ concentration range. It seems probable therefore that high concentrations of NAD^+ accelerate the normal heat destruction process.

The effects of cysteine and bovine serum albumin are fairly similar, although their relative effectiveness is different for the two enzymes. Cysteine would be expected to protect thiol groups, and perhaps the particularly reactive essential thiol of the active centre. Bovine serum albumin, however, could protect the enzymes from conformational change, including dissociation to give the subunits of the enzymes. This effect of cysteine is of particular interest in relation to the work of BUHNER AND SUND[7]. They postulated the existence of intermediates in the denaturation of yeast alcohol dehydrogenase prior to dissociation into its four subunits. Cysteine was found to be only slightly effective in the regeneration of active enzyme from aged inactive samples. We found a 7% increase in assay value in the presence of cysteine, in addition to the 13% sparing effect in heat denaturation (our corresponding values for the liver enzyme were 3% and 7%).

It is of considerable interest that $NADP^+$ has a similar effect to NAD^+ with both enzymes. $NADP^+$ does not act as a co-factor for the yeast enzyme, but does have some activity with liver alcohol dehydrogenase[8]. In its normal co-factor role, NAD^+ has been shown by RABIN AND WHITEHEAD[9] to bind to an especially reactive thiol group in yeast alcohol dehydrogenase, similarly to the binding of NAD^+ to liver alcohol dehydrogenase[10]. NAD^+ therefore can protect these enzymes against thiol-attacking reagents such as iodoacetamide[11,12]. NAD^+ can also protect yeast alcohol dehydrogenase against denaturation by acid[13].

Those observations are in agreement with the usual reports of protection or stabilization of enzymes by their substrates[2,3]. Our results show the reverse effect, one explanation for which could be the NAD^+ ($NADP^+$)-promoted oxidation of the active-site thiol group. Moreover, it cannot be ruled out that breakdown products of these coenzymes are responsible for the inactivation of the enzyme. Thus deamino-

NAD^+ (ref. 14) can bind to yeast alcohol dehydrogenase and inactivate it, so it is possible that traces of this compound are responsible for our NAD^+ effect at the concentrations used, despite any competition from NAD^+ itself.

More likely explanations involve conformational changes. Thus, a conformational change may be involved even during normal binding of NAD^+ to an essential thiol of liver alcohol dehydrogenase (REYNOLDS *et al.*[15]). Unfavourable conformational changes, perhaps including dissociation to subunits, is not unlikely during long heat-treatment of these enzymes at 43°, with these concentrations of NAD^+ or $NADP^+$. However, the effectiveness of both these substances on the two enzymes may suggest that an allosteric action occurs, especially because of the concentration dependence of the thermal inactivation.

Thus 50% recovery, of the activity obtained after heating in the absence of NAD^+ or $NADP^+$, was found at about 2.5 mM NAD^+ or $NADP^+$. This suggests a K_D of this order for some other binding site present, in view of the fact that the catalytic site of alcohol dehydrogenases show a much lower K_m for these coenzymes. For example, the K_m for NAD^+ and liver alcohol dehydrogenase was reported by THEORELL AND MCKINLEY-MCKEE[16] to be 51 μM at pH 8. Clear evidence for other such additional binding sites has been published by WEINER[17]. Each molecule of liver alcohol dehydrogenase (two subunits per molecule) bound 7 or 8 molecules of a spin-labelled coenzyme analogue that is an inhibitor of this enzyme competitive with NAD^+. This was shown by electron paramagnetic resonance binding studies, which allowed the detection of 2 strong ($K_D = 17 \pm 8\,\mu$M) and 5 or 6 somewhat weaker sites ($K_D = 75 \pm 9\,\mu$M) in metal-free phosphate buffer at pH 6.0. Also, NAD^+ has been found to quench the fluorescence of NADH bound to liver alcohol dehydrogenase[16].

Thus the thermal inactivation of alcohol dehydrogenases promoted by NAD^+ or $NADP^+$ preparations could be due to a conformational change caused by allosteric binding of these coenzymes, with relatively weak affinity for that site.

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