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Dual nucleotide specificity of bovine glutamate dehydrogenase

The role of negative co-operativity

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The thionicotinamide analogues of NAD^+ and $NADP^+$ were shown to be good alternative coenzymes for bovine glutamate dehydrogenase, with similar affinity and approx. 40% of the maximum velocity obtained with the natural coenzymes. Both thionicotinamide analogues show non-linear Lineweaver–Burk plots, which with the natural coenzymes have been attributed to negative co-operativity. Since the reduced thionicotinamide analogues have an isosbestic point at 340nm and have an absorption maximum at 400nm, it is possible to monitor reduction of natural coenzyme and thionicotinamide analogue simultaneously by dual-wavelength spectroscopy. When glutamate dehydrogenase is presented with NAD^+ and thio-$NADP^+$ simultaneously, the enzyme oligomer senses saturation of its coenzyme-binding sites irrespective of the exact nature of the coenzyme and locks the oligomer into its highly saturated form even when low saturation of the monitored coenzyme is present. These experiments substantiate the suggestion that glutamate dehydrogenase shows negative co-operativity in its catalytically active form.

Bovine glutamate dehydrogenase (EC 1.4.1.3) is a hexameric enzyme with a mol.wt. of 320000 (Cassman & Schachman, 1971) and six apparently identical polypeptide chains (Smith *et al.*, 1970). Glutamate dehydrogenase shows complex initial-rate kinetics in the oxidative deamination reaction (Dalziel & Engel, 1968; Engel & Dalziel, 1969), and these have been interpreted in terms of negatively co-operative interactions between the subunits of the hexamer. Further evidence for negative co-operativity in glutamate dehydrogenase has come from studies of coenzyme binding (Dalziel & Egan, 1972; Melzi D'Eril & Dalziel, 1972) and coenzyme-induced conformational changes (Bell & Dalziel, 1973). All of these studies have been characterized by the observation that similar effects are seen with NAD^+ or $NADP^+$ as the coenzyme. Glutamate dehydrogenase is one of the few dehydrogenases that can utilize either coenzyme with similar efficacy. The only difference between the behaviour with these two coenzymes appears to be at the level of binding of the reduced coenzymes NAD(P)H, where there appears to be an additional binding site for NADH (Bell, 1974; Melzi D'Eril & Dalziel, 1973). The relationship between the utilization of NAD^+ and $NADP^+$ by glutamate dehydrogenase has not been studied.

In the present paper we report the results of experiments with the newly developed (Levy & Daouk, 1979) approach of dual-wavelength spectroscopy with thionicotinamide analogues of NAD^+ and $NADP^+$ in the presence of NAD^+ and $NADP^+$ to study the effects of simultaneous presentation of both coenzymes on the kinetic properties of glutamate dehydrogenase, with particular emphasis on the kinetic manifestations of the negative co-operativity exhibited by this enzyme.

Experimental

Bovine liver glutamate dehydrogenase was obtained as a crystalline suspension in $(NH_4)_2SO_4$ solution from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzyme was prepared by extensive dialysis against 0.1M-sodium phosphate buffer, pH7.0, containing 0.01mM-EDTA, and was assayed spectrophotometrically by using absorbance changes at 280nm and a specific absorption coefficient of 93 litre$\cdot g^{-1}\cdot cm^{-1}$ (Egan & Dalziel, 1971). All coenzymes, nucleotides and coenzyme analogues used in this study were obtained from Sigma Chemical Co. Rate measurements were made in 3ml cuvettes on a Cary model 219 spectrophotometer after addition of the appropriate enzyme concentration to the pre-equilibrated reaction mixture.

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Dual-wavelength assays in the presence of NAD^+ and thio-$NADP^+$ or of $NADP^+$ and thio-NAD^+ were based on the fact that the reduced thionicotinamide coenzyme analogues have an absorption maximum at 400nm and essentially no absorbance at 340nm, owing to an isosbestic point in the spectra of reduced and oxidized coenzymes (Stein *et al.*, 1963). The natural coenzymes show absorbance maxima at 340nm in the reduced form, with little absorbance at 400nm. Assays in the presence of both natural coenzymes and thionicotinamide analogues were performed in two ways. By making use of the wavelength-switching programme of the Cary spectrophotometers, absorbances were measured at 400nm for 10s, then at 340nm for 10s etc. Alternatively, values were collected at 400nm with one set of replicates, followed by collection of values at 340nm with a second set of replicates. Essentially identical results were obtained with either approach; however, under circumstances where rapid initial rates were measured the later techniques gave more accurate and reproducible results. Absorption coefficients for NAD(P)H were taken as $6.22\,mM^{-1}\cdot cm^{-1}$, $11.9\,mM^{-1}\cdot cm^{-1}$ for thio-NADH and $11.7\,mM^{-1}\cdot cm^{-1}$ for thio-NADPH (Stein *et al.*, 1963).

During the present experiments it became apparent that the small absorbance changes at 400nm during the reduction of the natural coenzymes did indeed interfere with the absorbance changes measured at 400nm when the thionicotinamide analogues were being monitored. Subsequently values collected at 400nm in the presence of both analogue and natural coenzyme were corrected for the contribution of the reduced natural coenzyme. This was done by making measurements on identical reaction mixtures at both 340nm and 400nm. The 400nm values can then be corrected for the contribution of reduced natural coenzyme, since the 340nm values measured only reduction of natural coenzyme, as there is an isosbestic point in the spectra of reduced and oxidized thionicotinamide coenzyme analogues at 340nm.

Results

Ability of thio-NAD(P)$^+$ analogues to function as coenzymes for glutamate dehydrogenase

Both thio-NAD^+ and thio-$NADP^+$ were tested as coenzymes in the oxidative deamination of glutamate. Shown in Fig. 1 are Lineweaver–Burk plots (Lineweaver & Burk, 1934), with either thio-NAD^+ or thio-$NADP^+$ as the varied substrate at pH 7.0 and with 50mM-glutamate as the second substrate. In both cases a concentration range of 0.01–0.4mM was used. The rates of production of reduced coenzyme analogues were monitored at 400nm. When 340nm was used with the thionicotinamide analogues no rates could be detected. With each of the coenzyme analogues, two distinct linear regions in the Lineweaver–Burk plots were observed. From these Lineweaver–Burk plots the apparent $V_{max.}$ for each analogue and two apparent K_m values for each analogue can be calculated. These values are given in Table 1, together with values for the natural coenzymes. The $V_{max.}$ values for the thionicotinamide analogues represent 40% of the value for NAD^+ and 38% of the value for $NADP^+$.

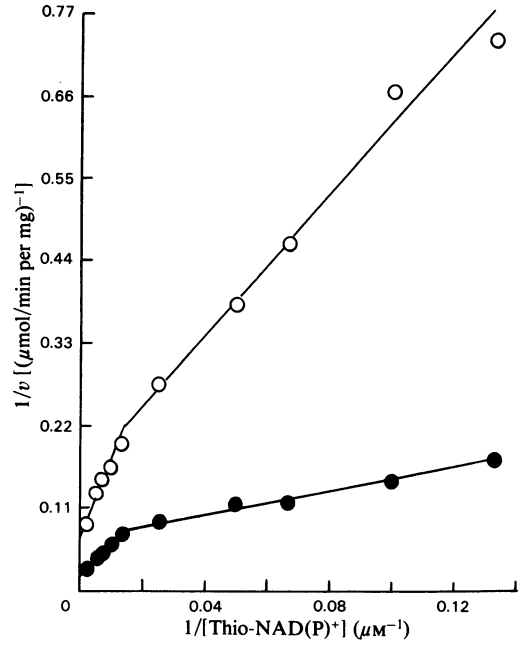

Fig. 1. *Thionicotinamide analogues as substrates for glutamate dehydrogenase*

Lineweaver–Burk plots with either thio-NAD^+ (●) or thio-$NADP^+$ (○) as the varied substrate are shown. Experiments were performed at 25°C with 0.1M-phosphate buffer, pH 7.0, in the presence of 10μM-EDTA and 50mM-glutamate. For full experimental details see the text.

We have also examined, over limited concentration ranges, the ability of NAD^+ or $NADP^+$ to inhibit reduction of its own thionicotinamide analogue. As shown in Fig. 2(*a*), both coenzymes are effective inhibitors of their own thionicotinamide analogues when the rate of reduction of the analogue is measured at 340nm, and each thionicotinamide analogue is an effective inhibitor against its parent compound (Fig. 2*b*). From these experiments apparent K_i values for both thionicotinamide analogues and the native coenzymes can be calculated. These are summarized in Table 1.

Table 1. *Apparent kinetic and inhibition constants for coenzymes and coenzyme analogues*
For explanation see the text. Standard errors were calculated by using standard procedures (Draper & Smith, 1968).

Coenzyme	Apparent $V_{max.}$ (μmol/min per mg of enzyme)	$K_{m\,(app.)}$ (μM)	$K_{I(app.)}$ (μM)
NAD^+	91.3 (±3%)	6 (±4%), 70 (±3%)	11 (±5%)
Thio-NAD^+	36.5 (±4%)	12 (±4%), 200 (±3%)	41 (±5%)
$NADP^+$	35.5 (±4%)	9 (±4%), 42 (±4%)	11.5 (±5)
Thio-$NADP^+$	13.5 (±4%)	27 (± 4%), 310 (±3%)	29 (±5%)

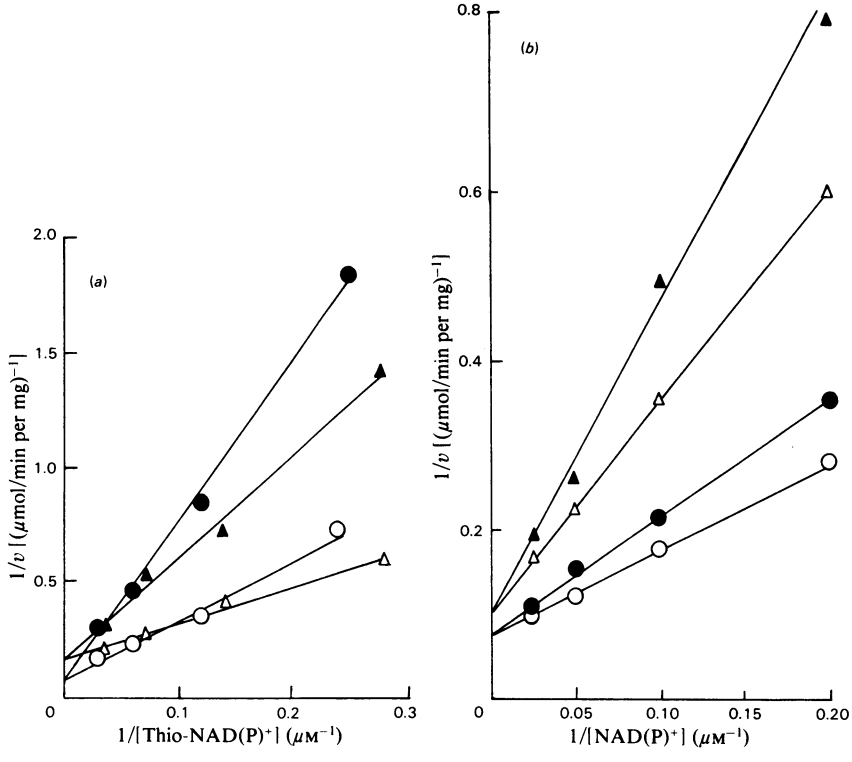

Fig. 2. *Inhibition by analogue of coenzyme utilization by glutamate dehydrogenase*
(*a*) Lineweaver–Burk plots with either thio-NAD^+ (○ and ●) or thio-$NADP^+$ (△ and ▲) as the varied substrate, in either the absence (○ and △) or the presence of 0.02mM-NAD^+ (●) or 0.02mM-$NADP^+$ (▲). (*b*) Lineweaver–Burk plots with either NAD^+ (○ and ●) or $NADP^+$ (△ and ▲) as the varied substrate, in either the absence (○ and △) or the presence of 0.017mM-thio-NAD^+ (●) or 0.014mM-thio-$NADP^+$ (▲). For full experimental details see the text.

Experiments in the presence of NAD^+ and thio-$NADP^+$ or in the presence of $NADP^+$ and thio-NAD^+

When the enzyme is simultaneously presented with NAD^+ and thio-$NADP^+$, the rate of each coenzyme can be monitored by making measurements at 340nm for the production of NADH and at 400nm for the production of thio-NADPH. Fig. 3(*a*) shows results of measurements at 340nm with NAD^+ as the varied substrate and a fixed concentration of thio-$NADP^+$ (0.25mM), with 50mM-glutamate. Quite clearly the $NADP^+$ analogue has two distinct effects on the Lineweaver–Burk plot. (1) It removes the indications of negative co-operativity seen in its absence. The Lineweaver–Burk plot with NAD^+ as the varied substrate is described by a

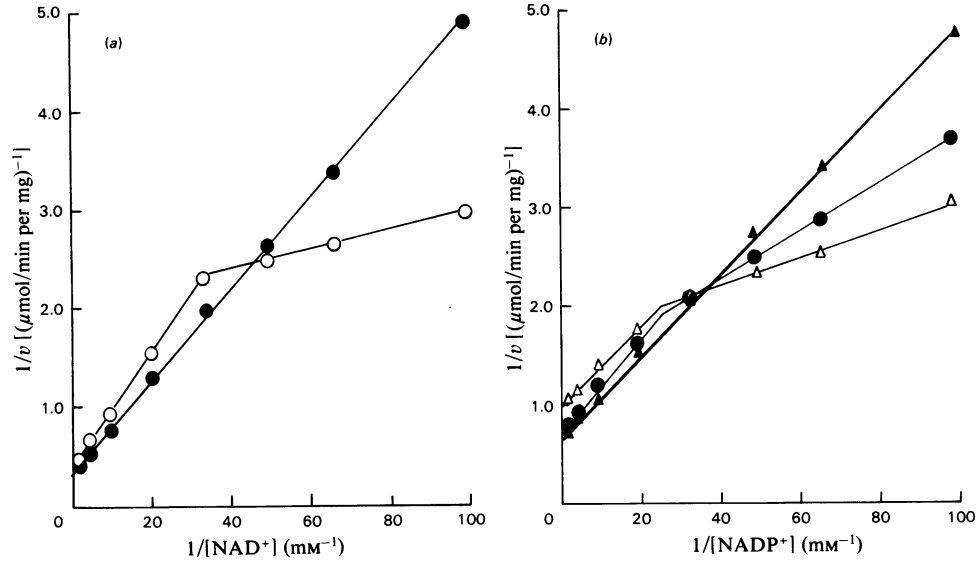

Fig. 3. *Experiments with NAD(P)H as the measured product of oxidative deamination of glutamate by glutamate dehydrogenase*

(*a*) Lineweaver–Burk plots with NAD^+ as the varied substrate in the presence (●) or absence (○) of 0.25 mM-thio-$NADP^+$. (*b*) Lineweaver–Burk plots with $NADP^+$ as the varied substrate in the presence (▲) or absence (△) of 0.25 mM-thio-NAD^+ and (●) in the presence of 0.01 mM-thio-NAD^+. Other conditions were as indicated in Fig. 1. For full experimental details see the text.

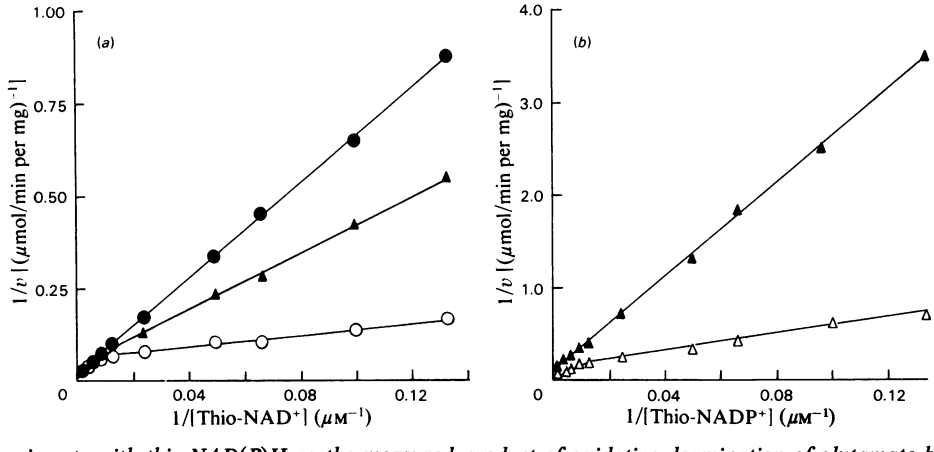

Fig. 4. *Experiments with thio-NAD(P)H as the measured product of oxidative deamination of glutamate by glutamate dehydrogenase*

(*a*) Lineweaver–Burk plots with thio-NAD^+ as the varied substrate in the presence (●) or absence (○) of 0.25 mM-$NADP^+$ and (▲) in the presence of 0.01 mM-$NADP^+$. (*b*) Lineweaver–Burk plots with thio-$NADP^+$ as the varied substrate in the presence (▲) or absence (△) of 0.25 mM-NAD^+. Other conditions were as indicated in Fig. 1. For full experimental details see the text.

single linear portion when the $NADP^+$ analogue is present. (2) As would be expected, the presence of the $NADP^+$ analogue inhibits the utilization of NAD^+ at low NAD^+ concentrations. However, quite unexpected is the activation of the reduction of NAD^+ by the $NADP^+$ analogue at high NAD^+ concentrations. When the coenzyme and analogue are reversed, i.e. with $NADP^+$ and thio-NAD^+, with reduction of $NADP^+$ being monitored at 340 nm, similar effects are observed (Fig. 3*b*). Also shown in

Fig. 3(*b*) are the effects of 0.01mM-thio-NAD^+, where a non-linear Lineweaver–Burk plot is observed.

When the experiment is reversed, and the production of reduced thionicotinamide coenzymes is followed, similar effects are observed upon the addition of the natural coenzymes. As shown in Fig. 4, addition of 0.25mM-NAD^+ to the reaction mixtures when reduced thio-$NADP^+$ is being monitored (Fig. 4*a*) results in a completely linear Lineweaver–Burk plot, showing inhibition at all analogue concentrations. When 0.25mM-$NADP^+$ is added to reaction mixtures with various thio-NAD^+ concentrations (Fig. 4*b*), similar effects are observed. When the enzyme is given a much lower concentration of the competing nucleotide, as shown in Fig. 4(*a*) with 0.01mM-$NADP^+$ instead of 0.25mM-$NADP^+$, inhibition is seen throughout the coenzyme concentration range of thio-NAD^+ and the Lineweaver–Burk plot retains its characteristic changes in slope.

Discussion

Quite clearly, the thionicotinamide analogues of NAD^+ and $NADP^+$ are good substrates with glutamate dehydrogenase. It has been shown with glucose 6-phosphate dehydrogenase (Levy & Daouk, 1979) that the thionicotinamide analogues of NAD^+ and $NADP^+$ will function with that enzyme also. As with the natural coenzymes, both thionicotinamide analogues show non-linear Lineweaver–Burk plots, and this has been ascribed to negative co-operativity in the glutamate dehydrogenase hexamer (Dalziel & Engel, 1968; Engel & Dalziel, 1969). Over the coenzyme concentration ranges used in the present study both the natural coenzymes and the thionicotinamide analogues show two linear regions in double-reciprocal plots with coenzyme or analogue as the varied substrate at a fixed glutamate concentration of 50mM. From these linear regions apparent K_m values for both natural and thionicotinamide analogues have been calculated. These are also given in Table 1. Comparison of these K_m values with the $K_{i(app.)}$ values for the appropriate coenzyme shown in Table 1 reveals internal consistency between the calculated numbers and the true competitive nature of the thionicotinamide coenzyme analogue and its parent natural coenzyme. As shown in Fig. 2, both analogues are indeed strict competitive inhibitors with their parent compounds. The reverse is also true. With this evidence that the thionicotinamide analogues accurately mimic the behaviour of the parent coenzymes it is possible to interpret the results obtained when both NAD^+ and thio-$NADP^+$ (or the reverse) are presented to the enzyme simultaneously. When this is done, monitoring the production of NADH or NADPH, as in Fig. 3, it seems that the simultaneous presence of both coenzymes mimics saturation of the oligomer with one coenzyme alone, and also causes quite a significant activation of the utilization of $NAD(P)^+$ at high concentrations. Similar results are seen when the enzyme is presented with both natural and thionicotinamide analogue and the reduction of the thionicotinamide analogue is monitored (Fig. 4). These results can be interpreted if one considers the extent of saturation of the enzyme by the coenzyme form whose reduction is not being followed in the particular experiment. For example, where the reduction of the thionicotinamide analogues is being followed, as in Fig. 4, we must consider the saturation of the enzyme by the natural coenzyme. Dissociation constants for NAD^+ binding to glutamate dehydrogenase in the presence of glutarate have previously been determined to be 0.006mM and 0.04mM (Dalziel & Egan, 1972). Therefore, when the rate of reduction of thio-$NADP^+$ is followed in the presence of 0.25mM-NAD^+ (Fig. 4*b*), we can calculate that the enzyme is already approx. 70% saturated with NAD^+ (Bell & Dalziel, 1973). On the other hand, in the presence of 0.01mM-NAD^+ the enzyme is only about 10% saturated with NAD^+. The results shown in Fig. 4 therefore indicate that, when the enzyme is more than half-saturated with NAD^+, the sites remaining available for reaction with thio-$NADP^+$ behave as though they were in a hexamer already more than half-saturated with coenzyme. When, however, the total saturation of the hexamer is less than half, as with low concentrations of the second coenzyme (Figs. 3*b* and 4*a*), the enzyme responds to saturation with the varied coenzyme in its normal manner, i.e. it shows the usual kinetic manifestations of negative co-operativity.

The activation seen at high varied coenzyme concentrations (Fig. 3) is most probably due to the fact that with the natural coenzymes more than the two linear regions of the Lineweaver–Burk plots studied in these experiments are observed. In fact, as the coenzyme concentration is increased, at least one more linear region is found (Engel & Dalziel, 1969). Since addition of the second type of coenzyme appears to lock the enzyme into its most saturated form, this activation is probably due to the fact that this higher coenzyme concentration region in the Lineweaver–Burk plot is characterized by a higher $V_{max.}$ than the region covered in these experiments, and that addition of the second coenzyme locks the enzyme into a form characterized by this higher $V_{max.}$.

It is apparent that, when the enzyme is presented with a mixture of coenzymes, it behaves as though it were presented with saturating concentrations of either alone. Although this conclusion is in many

ways unsurprising, it has great significance when discussed in relationship to the negative homotropic interactions exhibited by glutamate dehydrogenase. Since when measurements are made at 340nm only the reduction of NAD^+ and $NADP^+$ is being measured, it is apparent that saturation of the oligomers with, for example, thio-$NADP^+$ when NADH production is monitored causes a conformational change that affects the rate of NAD^+ reduction. Since NAD^+ and thio-$NADP^+$ cannot be present in the active site simultaneously, this indicates that the conformational change induced by thio-$NADP^+$ binding must occur across subunit interfaces within the oligomer. The converse is of course true when reduction of the thionicotinamide analogue is followed at 400nm. Although such conformational changes associated with negative co-operativity have been shown to occur previously (Bell & Dalziel, 1973), it should be pointed out that this previous demonstration of negative co-operativity in glutamate dehydrogenase involved the use of static measurements in the presence of the inactive glutamate analogue glutarate. The experiments reported in the present paper demonstrate directly for the first time the existence of negatively co-operative interactions occurring in the catalytically active form of glutamate dehydrogenase.

In conclusion, the thionicotinamide analogues of NAD^+ and $NADP^+$ have been shown to be suitable substrates for glutamate dehydrogenase. Both analogues show the characteristic indications of negative co-operativity in Lineweaver–Burk plots with coenzyme as the varied substrate, indicating that the analogues are sensitive to the same conformational changes that are thought to occur with the natural coenzymes (Dalziel & Engel, 1968; Engel & Dalziel, 1969; Bell & Dalziel, 1973). We have made use of these similarities in behaviour between the natural coenzymes and the thionicotinamide analogues to demonstrate unequivocally that glutamate dehydrogenase exhibits negative co-operativity in the catalytically active form of the enzyme.

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