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NICKEL-INDUCED SUBSTRATE INHIBITION OF BOVINE LIVER GLUTAMATE DEHYDROGENASE

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The effects of nickel ions on reductive amination and oxidative deamination activities of bovine liver glutamate dehydrogenase (GDH) were examined kinetically by UV spectroscopy, at 27°C, using 50 mM Tris, pH 7.8, containing 0.1 M NaCl. Kinetic analysis of the data obtained by varying NADH concentration indicated strong inhibition, presumably due to binding of the coenzyme to the regulatory site. In contrast, almost no inhibition was observed in the forward reaction. The fact that nickel ions have the capacity to enhance binding of NADH to the enzyme was confirmed by an electrochemical method using a modified glassy carbon electrode. Use of NADPH instead of NADH showed only a weak substrate inhibition, presumably related to lower affinity of NADPH for binding to the regulatory site. Lineweaver–Burk plots with respect to α-ketoglutarate and ammonium ions indicated substrate and competitive inhibition patterns in the presence of nickel ions, respectively. ADP at 0.2 mM concentration protected inhibition caused by nickel.

These observations are explained in terms of formation of a nickel–NADH complex with a higher affinity for binding to the regulatory site in GDH, as compared with the situation where nickel is not present. Such effects may be important for regulation of GDH and other NADH-utilizing enzymes.

Keywords: Bovine liver glutamate dehydrogenase; α-Ketoglutarate inhibition; NADH inhibition; Substrate inhibition; NADH–metal ion association

Abbreviations: GDH, Glutamate dehydrogenase; NADH, Nicotinamide adenine dinucleotide reduced; NADPH, Nicotinamide adenine dinucleotide phosphate reduced; NAD^+, Nicotinamide adenine dinucleotide; ADP, Adenosine-5′-diphosphate; GTP, Guanosine-5′-triphosphate

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INTRODUCTION

GDH [L-glutamate: NAD-(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme, composed of six identical subunits,[1,2] with a molecular weight of 336,000 Da.[3] Mammalian GDH is involved in integration of amino acid metabolism via the tricarboxylic acid cycle. Also, it has a significant role in controlling the levels of ammonia and glutamate, an ubiquitous neurotransmitter within the central nervous system.[4] Bovine liver GDH is a pyridine nucleotide enzyme which catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonia and is subject to extensive allosteric control.[5] The most well described effectors are ADP (which enhances activity) and GTP (which decreases it). NAD$^+$, NADH, and NADPH can also alter the enzyme's activity, as can hormones such as estradiol, progesterone, testosterone, thyroxin and Δ^4-androstene-13,17-dione[6] as well as diethylstilbestrol.

GDH was initially considered to be a zinc-dependent metallodehydrogenase.[7] However, subsequent works have shown that the enzyme is fully active in the absence of zinc ions[8] and its activity is suppressed in the presence of zinc ions.[9–11] Although many reports exist which describe the effects of anions on bovine liver GDH,[12–17] only few studies have been reported on the effects of cations.[18–20] In the present paper we report on the effects of nickel(II) ions on the catalytic activity, kinetic parameters and NADH binding, of bovine liver GDH.

MATERIALS AND METHODS

Materials

Bovine liver GDH was obtained as a solution in 50% glycerol from Sigma. β-NADH, β-NAD$^+$, β-NADPH, α-ketoglutarate and L-glutamate were also purchased from Sigma. NH_4Cl, $NiCl_2$, $CoCl_2$, $CuCl_2$ and chlorogenic acid were obtained from Merck. ADP was purchased from Boehringer Mannheim (Germany). All other materials were of analytical grade and solutions were made in double-distilled water. The solutions of ADP, NADH, NAD$^+$ and NADPH were prepared freshly and their concentrations were determined spectrophotometrically using the absorption coefficients: $\varepsilon_{340} = 6.22\,mM^{-1}\,cm^{-1}$ for both NADH and NADPH, $\varepsilon_{259} = 15.8\,mM^{-1}\,cm^{-1}$ for ADP and $\varepsilon_{260} = 17.6\,mM^{-1}\,cm^{-1}$ for NAD$^+$. Fifty mM Tris, pH 7.8 containing 0.1 M NaCl was used as buffer for determination of catalytic activities.

Methods

GDH Assays

Activity in the reductive amination reaction was determined from the decrease in absorption at 340 nm following the oxidation of NAD(P)H at 27°C using a Shimadzu UV-3100 spectrophotometer with jacketed cell holders. Its temperature was regulated by an external thermostated water circulator within ±0.05°C. The assay mixture normally contained 5 mM α-ketoglutarate, 50 mM NH_4Cl and 0.1 mM NAD(P)H except where stated otherwise. The enzymatic reaction was started by addition of 7 µl of diluted enzyme (in 50 mM Tris, pH 7.8 containing 0.1 M NaCl) and the final volume was 1040 µl. For the oxidative deamination reaction, the increase in absorption at 340 nm corresponding to reduction of NAD^+ was followed. In this case, the concentrations of L-glutamate and NAD^+ were 10 and 0.2 mM, respectively. Other conditions were the same as for the reverse reaction. Every experiment was repeated three times.

Cyclic Voltammetry

In order to investigate the effect of nickel ions on binding of NADH to GDH, a glassy carbon electrode modified by chlorogenic acid was used.[21] Electrochemical experiments were carried out using a 746 VA electrochemical analyzer equipped with a 747 VA stand three-electrode cell both from Metrohm (Switzerland). This configuration contained a modified glassy carbon working electrode, a platinum-wire auxiliary electrode and a silver–silver chloride (with 3.0 M potassium chloride) electrode serving as the reference electrode. The potential scan rate was 25 mV/s for all the electrochemical experiments carried out.

RESULTS AND DISCUSSION

The effects of Ni^{2+} on the oxidative deamination and reductive amination reactions catalyzed by GDH were examined at pH 7.8. As can be seen from Figure 1, Ni^{2+} at a concentration of 200 µM inhibited the reductive amination activity of GDH while at the same concentration of Ni^{2+}, practically no inhibition could be seen in the oxidative deamination reaction.

In order to investigate the effects of Ni^{2+} on the kinetic behavior of GDH, the rate of enzymatic reaction at various substrate concentrations was measured. Figure 2 shows double reciprocal plots of enzymatic rate using various concentrations of NADH. As indicated, Ni^{2+} decreases

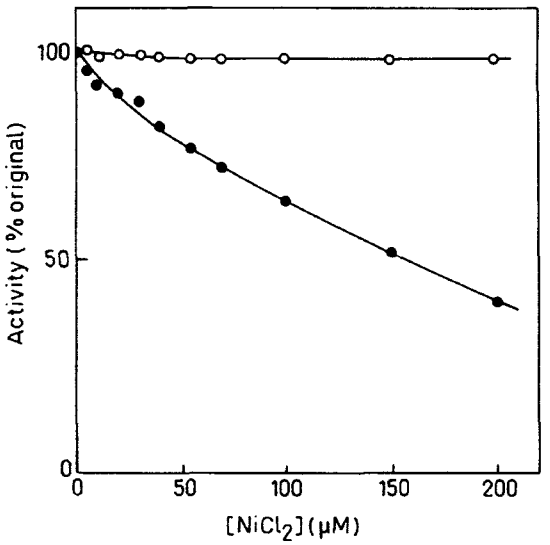

FIGURE 1 The effects of Ni^{2+} on the oxidative deamination (○) and reductive amination (●) reactions catalyzed by GDH at pH 7.8 in 50 mM Tris containing 0.1 M NaCl. The concentrations of substrates in the assay mixture for the oxidative deamination reaction were 10 and 0.2 mM for L-glutamate and NAD^+, and in the reductive amination reaction were 5, 50 and 0.1 mM for α-ketoglutarate, NH_4Cl and NADH, respectively. The assays were performed at 27°C.

V_{max} and K_m for NADH (see Table I). It is well established that there are two distinct binding sites for NADH on each GDH subunit.[22-24] Since NADH has different affinities for binding to these two sites,[25,26] the reduced coenzyme first binds to site I (active site) and only at high NADH concentrations (greater than 0.1 mM) does binding to site II (non-active or regulatory site) occur.[26,27] In the presence of nickel, this is shifted to a lower concentration, so that extensive inhibition is observed at NADH concentrations greater than 0.04 mM (Figure 2). To investigate whether this effect is specific for nickel experiments were repeated using $CuCl_2$ and $CoCl_2$ (Figure 3). No substrate inhibition was observed in corresponding plots and only uncompetitive inhibition occurred in the presence of these heavy metal ions. The effects of these metal ions on the kinetic parameters of the enzymatic reaction catalyzed by GDH are listed in Table I.

Bovine liver GDH is capable of utilizing both NADH and NADPH with similar rates.[5] NADPH can also bind to the regulatory site.[25] The effects of nickel(II) ions on the enzymatic reaction rates were examined using NADPH as the reduced coenzyme. The results are shown in Figure 4 and Table I. It is obvious that no substrate inhibition occurs with 100 μM $NiCl_2$ but in the presence of 200 μM $NiCl_2$, very weak substrate inhibition

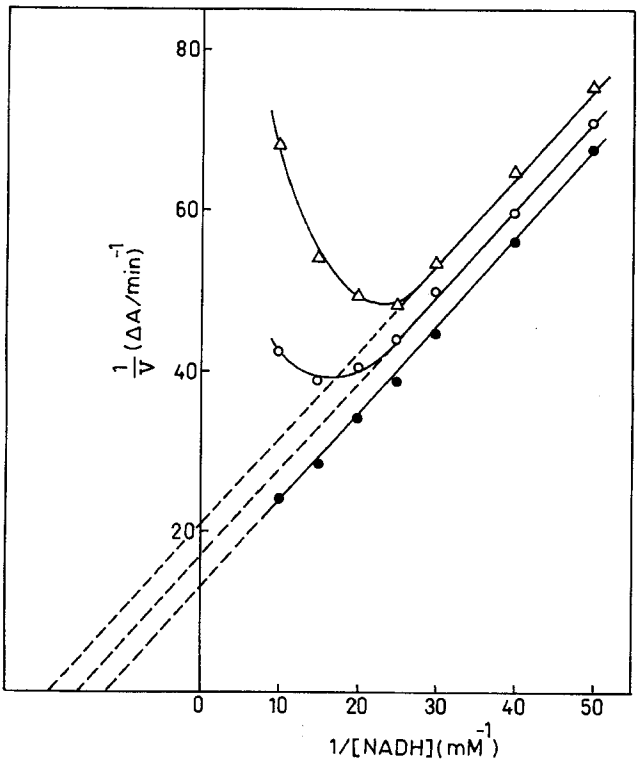

FIGURE 2 Double reciprocal plots of reductive amination reaction using various concentrations of NADH. The concentrations of α-ketoglutarate and NH_4Cl were 5 and 50 mM, respectively. No $NiCl_2$ present (●) in the presence of 100 μM $NiCl_2$ (○) and in the presence of 200 μM $NiCl_2$ (△). Other conditions are as described in legend to Figure 1.

TABLE I Kinetic parameters for the interaction between GDH and metal ions. For more details see text

Salt	*Concentration* (μM)	*Variable substrate*	K_m (mM)	V_{max} ($\Delta A\,min^{-1}$)
$NiCl_2$	0	NADH	0.083	0.0769
$NiCl_2$	100	NADH	0.064	0.0588
$NiCl_2$	200	NADH	0.053	0.0482
$CoCl_2$	100	NADH	0.067	0.0615
$CoCl_2$	200	NADH	0.056	0.0513
$CuCl_2$	100	NADH	0.061	0.0555
$CuCl_2$	200	NADH	0.048	0.0440
$NiCl_2$	0	NADPH	0.077	0.0526
$NiCl_2$	100	NADPH	0.049	0.0328
$NiCl_2$	200	NADPH	0.036	0.0238
$NiCl_2$	0	α-ketoglutarate	0.85	0.0476
$NiCl_2$	100	α-ketoglutarate	0.62	0.0333
$NiCl_2$	200	α-ketoglutarate	0.48	0.0263
$NiCl_2$	0	NH_4Cl	26.3	0.0625
$NiCl_2$	100	NH_4Cl	83.3	0.0625
$NiCl_2$	200	NH_4Cl	166.7	0.0625

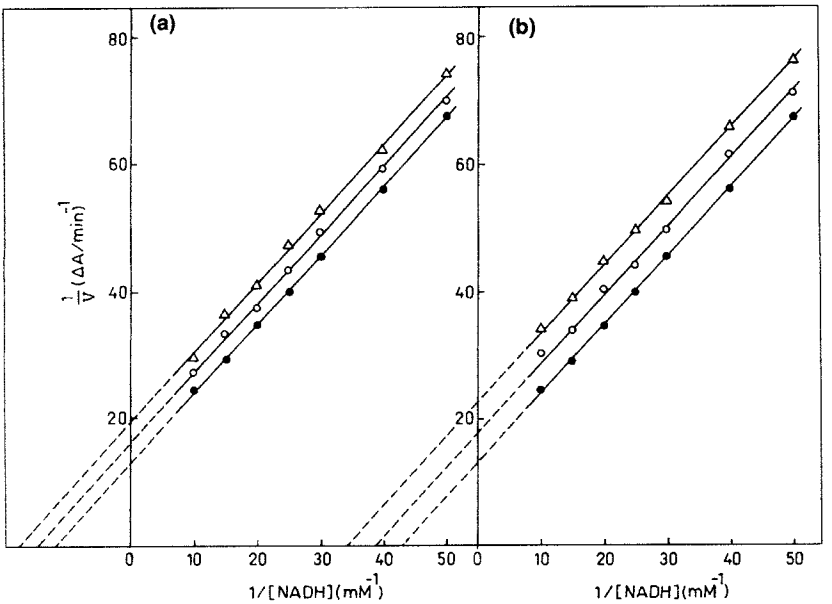

FIGURE 3 Double reciprocal plots of reductive amination reaction using various concentrations of NADH. (a) No $CoCl_2$ present (●) in the presence of 100 μM $CoCl_2$ (○); and in the presence of 200 μM $CoCl_2$ (△). (b) No $CuCl_2$ present (●) in the presence of 100 μM $CuCl_2$ (○) and in the presence of 200 μM $CuCl_2$ (△). Other conditions are as described in legend to Figure 2.

is observed (in comparison to Figure 2). Although, there is no remarkable difference in affinities between NADH and NADPH for binding to the active site, there is an approximately ten-fold higher affinity for NADH related to NADPH for the regulatory site.[26] Such differences may explain the different extents of substrate inhibition observed for NADH and NADPH in the presence of nickel(II) ions.

The double reciprocal plots of enzymatic rates using various concentrations of α-ketoglutarate and NH_4Cl in the absence and presence of nickel(II) ions are shown in Figures 5 and 6, respectively. The changes in kinetic parameters are also listed in Table I. It can be seen (Figure 5) that the presence of nickel(II) ions induce Ni^{2+}-concentration dependent substrate inhibition with respect to α-ketoglutarate. It has been reported that binding of NADH to the regulatory site enhances binding of α-ketoglutarate to the coenzyme–enzyme complex which results in substrate inhibition by α-ketoglutarate.[28,29] Thus, it seems that Ni^{2+} enhances association of NADH with the regulatory site and thereby enhances substrate inhibition by α-ketoglutarate. It has been shown that the reductive

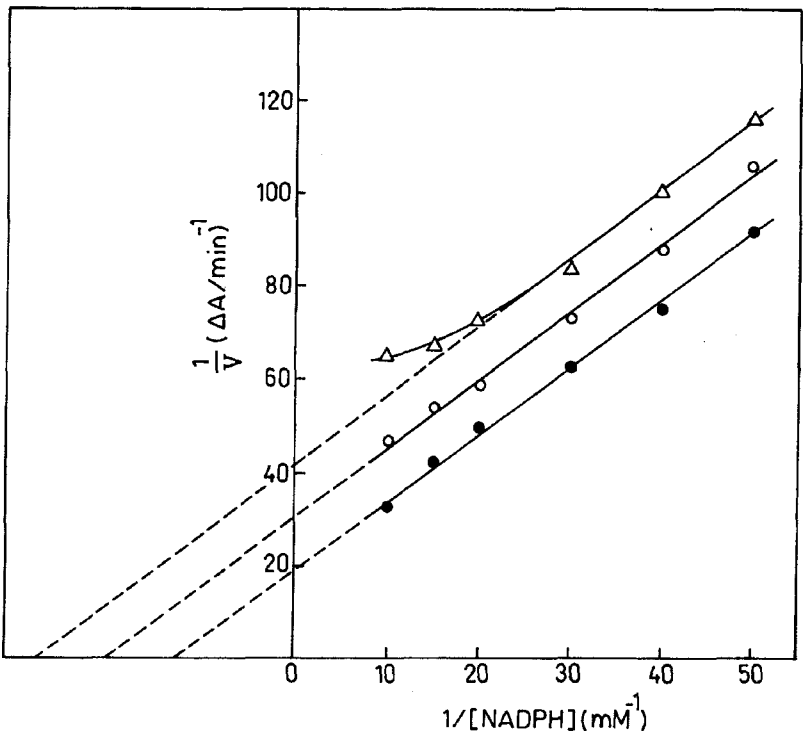

FIGURE 4 Double reciprocal plots of reductive amination reaction using various concentrations of NADPH. No $NiCl_2$ present (●) in the presence of 100 μM $NiCl_2$ (○) and in the presence of 200 μM $NiCl_2$ (△). Additional information is provided in legend to Figure 2.

amination reaction of GDH is ordered[29,30] suggesting that binding of NADH to the active site of GDH is required for subsequent binding of α-ketoglutarate to the binary active complex. Accordingly, the observed decrease in K_m for α-ketoglutarate (Figure 5) may be explained in terms of enhancement in NADH binding (*vide supra*).

The presence of nickel(II) ions also results in an increase in K_m for NH_4^+ without affecting V_{max}, which is the characteristic of competitive inhibition as shown in Figure 6 and Table I. Whereas, there is no structural similarity between NH_4^+ and Ni^{2+}, the sign of the charge and the size of these two types of ions may be worthy of consideration. While Ni^{2+} has two positive charges, ammonium ion has only one positive charge. On the other hand, comparison of their ionic radii in the crystalline state reveals that the ionic radius of NH_4^+ is two times greater than that of Ni^{2+} (ionic radii of NH_4^+ and Ni^{2+} are 1.43 and 0.69 Å, respectively[31]). Since the relatively high density charge on Ni^{2+} induces more hydration as compared to NH_4^+, the two

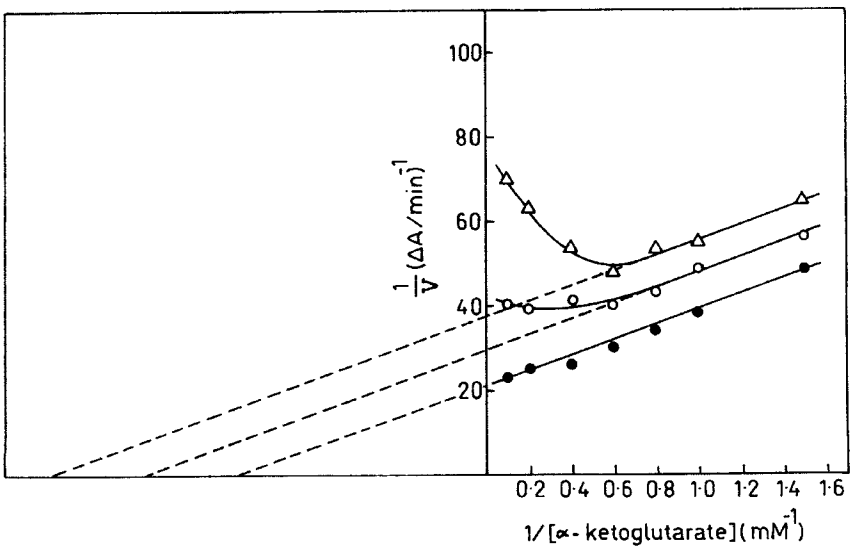

FIGURE 5 Double reciprocal plots of reductive amination reaction using various concentrations of α-ketoglutarate. No $NiCl_2$ present (●) in the presence of 100 μM $NiCl_2$ (○) and in the presence of 200 μM $NiCl_2$ (△). The concentrations of NH_4Cl and NADH were 50 and 0.1 mM, respectively.

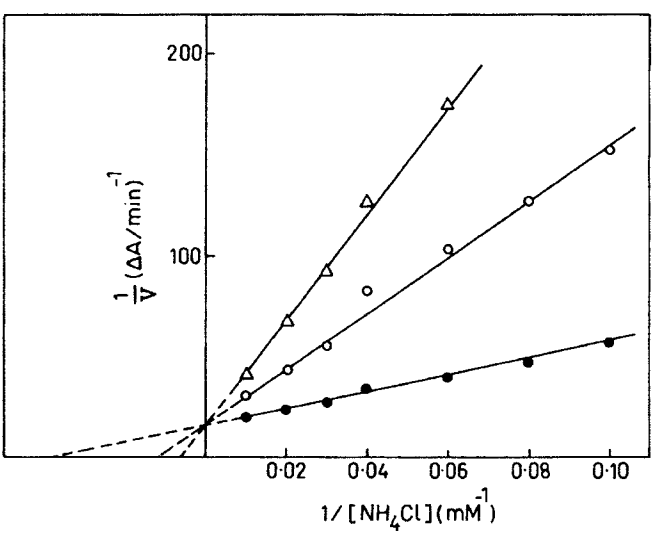

FIGURE 6 Double reciprocal plots of reductive amination using various concentrations of NH_4Cl. No $NiCl_2$ present (●) in the presence of 100 μM $NiCl_2$ (○) and in the presence of 200 μM $NiCl_2$ (△). The concentrations of α-ketoglutarate and NADH were 5 and 0.1 mM, respectively.

ions may have comparable hydrated radii and accordingly, hydrated nickel(II) ion may mimic hydrated NH_4^+ as a positively charged sphere and thus become accommodated in the active site of the enzyme.

The effect of ADP on inhibition of GDH (reductive amination) by Ni^{2+} is shown in Figure 7. ADP is an allosteric activator of the enzyme which exerts its activating effect by competition with NADH for binding to the regulatory site.[26] As can be observed in Figure 7, ADP enhances the reductive amination reaction of GDH in the absence and presence of various concentrations of Ni^{2+} and lowers the extent of inhibition from 60% (in the absence of ADP) to about 14% (in the presence of 200 μM ADP). Such a decrease in the amount of inhibition caused by ADP clearly confirms that interaction between Ni^{2+} and NADH is responsible for enhancement of NADH binding to the regulatory site.

To further confirm that nickel(II) ions enhance binding of NADH to GDH, an independent electrochemical method was employed. A glassy

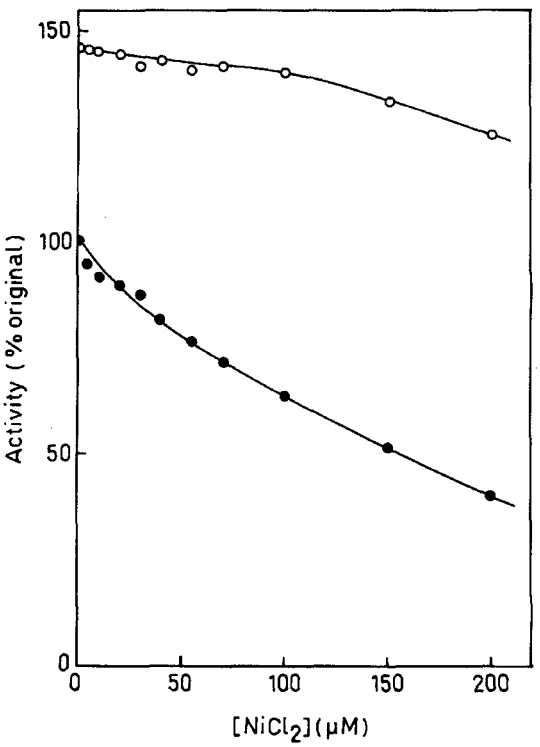

FIGURE 7 The effects of $NiCl_2$ on GDH in the reductive amination reaction in the absence and presence of ADP. No ADP present (●) In the presence of 200 μM ADP (○). Other conditions are the same as described for legend to Figure 1.

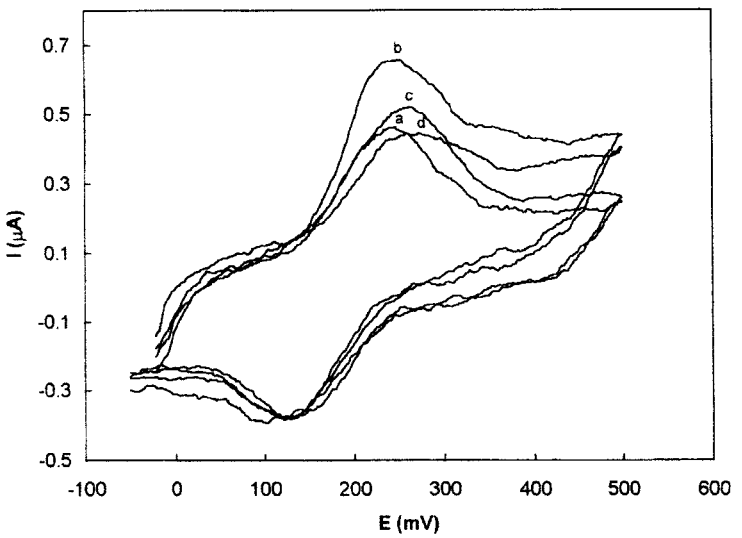

FIGURE 8 Cyclic voltammograms of chlorogenic acid modified glassy carbon electrode in 50 mM Tris buffer (pH 7.8) containing 0.1 M NaCl. The potential scan rate was 25 mV/s. (a) Only buffer; (b) 0.1 mM NADH in buffer solution; (c) 0.1 mM NADH + 0.1 mg/ml enzyme; (d) the same as "c" + 200 μM $NiCl_2$.

carbon electrode modified by chlorogenic acid was used as an NADH sensitive electrode.[21] The results of cyclic voltammetric experiments are shown in Figure 8. Comparison of curves "a" (buffer alone) and "b" (0.1 mM NADH in buffer solution) shows that the current corresponding to anodic peak of curve "b" is more than the anodic peak of curve "a" which shows the electrocatalytic effect of the modified electrode toward NADH oxidation. Also, comparison of curves "b" (0.1 mM NADH) and "c" (0.1 mM NADH + 0.1 mg/ml GDH) reveals that the peak current is significantly decreased in the presence of GDH and the peak potential is also shifted to more positive values. We ascribe these effects to variations in binding of NADH to the enzyme, causing a more difficult oxidation of NADH. Addition of 200 μM $NiCl_2$ to the test solution (the same as "c"; 0.1 mM NADH + 0.1 mg/ml GDH) exhibits the voltammogram illustrated as curve "d" in Figure 8. It is obvious that in the presence of Ni^{2+}, NADH oxidation becomes more and more difficult which appears as a decrease in peak current and a minor shift of peak potential to a more positive value with respect to the case where Ni^{2+} is absent. Indeed, such an effect clearly suggests that the binding of NADH to the enzyme is reinforced in the presence of nickel(II) ions.

The kinetic and electrochemical experiments taken together show that in the presence of nickel(II) ions, more binding of NADH to GDH occurs. Accordingly, this additional binding is ascribed to the non-active or regulatory site of the enzyme as judged by substrate inhibition. Similar results have been reported with a high concentration of Mg^{2+} (2 mM).[20] We also examined the effect of 200 μM $MgCl_2$ on the reductive amination reaction of GDH but no effect was observed at this concentration (data not shown). Therefore, it seems that Ni^{2+} is a more potent inducer of substrate inhibition with respect to GDH than Mg^{2+}. It may be reasonable to propose that complex formation between NADH and nickel(II) ion(s) proceeds via an ionic interaction between the pyrophosphate group of NADH and Ni^{2+} as well as the formation of coordination bonds between Ni^{2+} and the non-bonded electrons of the nitrogen atoms of NADH. As evident in Figure 1, Ni^{2+} has practically no effect on the oxidative deamination reaction of GDH. Since in the oxidized coenzyme (NAD^+), the nitrogen atom of the nicotinamide ring has lost its non-bonded electrons and hence is unable to coordinate the Ni^{2+} due to the resulting electric repulsion force of the acquired positive net charge, as well as its inability to coordinate may prevent complex formation.

In conclusion, it may be suggested that complex formation between NADH and metal ions such as nickel may have a regulatory effect on the catalytic activity of GDH. This may also be true for other dehydrogenases, which utilize NADH as the reduced coenzyme.

Acknowledgment

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