Electrochemical study of the effect of ADP and AMP on the kinetics of glutamate dehydrogenase

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Abstract

A chronoamperometric method based on the 'diffusion' layer concept of the convective system was used to assay the glutamate dehydrogenase (GLDH) activity. Once the reaction was initiated by adding the enzyme GLDH into a well-stirred nicotinamide adenine dinucleotide NADH, coenzyme solution, the steady-state oxidation limiting current of NADH would decrease linearly in a short time. The major advantage of this method is that it directly indicates the continuous in-situ change of the coenzyme concentration, thus, the real initial reaction rate of enzyme-catalyzed reaction, \( V_0 \), can be determined. Using this method, the effect of adenosine-5'-monophosphate (AMP) and adenosine-5'-diphosphate (ADP) on the GLDH activity has been monitored. The results showed that ADP and AMP could increase the activity of GLDH. This activation mechanism was proposed by the voltammetric study.

Keywords: Chronoamperometry; Voltammetry; Glutamate dehydrogenase; Enzyme activity; Adenosine-5'-monophosphate; Adenosine-5'-diphosphate

1. Introduction

The enzyme glutamate dehydrogenase [L-glutamate: NAD(P)+ oxidoreductase (deaminating), EC 1.4.1.3], which is localized exclusively within mitochondria [1], has been extensively studied in terms of structure and kinetics [2] because it can provide a link between carbohydrate and nitrogen metabolism [3,4]. A number of potential regulators of glutamate dehydrogenase (GLDH), such as GTP [5], leucine [6], ADP [7], metal ions [4,8], and a large variety of anions [9,10], have been studied on the oxidation deamination reaction of glutamate. But so far, few studies have been carried out to examine regulator effects on GLDH in the direction of the reductive amination reaction of \( \alpha \)-Ketoglutarate (\( \alpha \)-KG) [7]. In the process of reductive amination of \( \alpha \)-KG catalyzed by GLDH, the NADH was oxidized to NAD\(^+\). The major problem of the enzyme assay is how to measure the initial reaction rate, \( V_0 \). In literature, the measurement of the enzyme-catalytic rate was usually carried out by monitoring the absorbance changes of NADH at 350 nm. However, in the conventional spectrophotometric method there exists a lag-phase [11]. In our previous work, we found that the chronoamperometric technique was suitable for assaying the GLDH activity in a well-stirred mixture solution. With this method, the initial rate can be measured [12]. The theoretical treatment of this method is based on a 'diffusion' layer approach described by Bard and Faulkner [13]. In this model, it is assumed that convection maintains uniform concentration in all species that are equal to the bulk values up to a certain distance, i.e., the 'diffusion' layer thickness (\( \delta_0 \)) at the electrode fed by convective transfer; within the layer, \( 0 \leq \chi \leq \delta_0 \), no solution moves and the mass transfer takes place by diffusion. The general equation for the flux of species \( O \) for one-dimensional diffusion and convection is expressed as follows [13]:

\[
\frac{\partial c_O}{\partial y} = \left( \frac{\partial c_O}{\partial y} \right)_{y=0} \int_0^\chi v_y \frac{D_O}{D} dy
\]

and

\[
\int_0^{c_O} \chi \frac{d c_O}{\partial y} = \left( \frac{\partial c_O}{\partial y} \right)_{y=0} \int_{c_O}^\chi d y \int_0^\gamma v_y \frac{D_O}{D} dy
\]

where \( c_O \) is the bulk concentration of species \( O \), \( D_O \) is the diffusion coefficient of species \( O \), and \( v_y \) is the
magnitude of solution velocity in the $y$ direction. If all the experimental conditions except the bulk concentration of $O$ are fixed, the second part in Eq. (1) will be a constant. Defining $P = \int_0^\infty \frac{df_0}{d\psi}(\psi)/\left(D_0\right)d\psi$, the limiting current will be,

$$i_l = nFAD_0 \left( \frac{\partial c_0}{\partial y} \right)_{\psi=0} = \frac{nFAD_0}{P} c_0$$

(2)

here, the value of $P$ depends on the stirring rate, diffusion coefficient, and viscosity. Under these fixed conditions, it is clear that the limiting current expressed in Eq. (2) will be proportional to the bulk concentration.

In this work, the chronoamperometric method was successfully used to study the effect of ADP and AMP on the initial kinetics of the reductive amination reaction of $\alpha$-KG. The voltammetric study was also performed to propose the mechanism of the regulator activation.

2. Experimental

2.1. Instrumentation

Chronoamperometric and cyclic voltammetric experiments were performed with an EG&G PAR (Princeton Applied Research) Model 273 potentiostat/galvanostat and a Model 270 electrochemical software in a three-electrode cell. In the Chronoamperometric experiment, a glassy carbon electrode (GCE) polished with 0.06-$\mu$m alumina slurry and sonicated in fresh water was used as working electrode. The saturated calomel electrode (SCE) and platinum wire electrodes were used as the reference and counter electrodes, respectively. The cyclic voltammetric measurements were performed with Model 303 (PAR) hanging mercury drop electrode (HMDE). The initial kinetic study of GLDH was carried out in 50 mmol/l Tris buffer solution ($\text{pH} = 8.0$), which contained quantitative NADH, 10 mmol/l $\text{NH}_4\text{Cl}$, and 2 mmol/l $\alpha$-KG. The mixture solution was 5 ml and stirred at $\omega = 200$ $\text{s}^{-1}$. All the measurements were carried out at 25 $\pm$ 0.1$^\circ$C and the solutions were previously de-aerated with high-purity nitrogen for at least 15 min.

2.2. Chemicals

Bovine liver GLDH, with a sub unit $M_r = 56\,000$, was obtained from Boehringer Mannheim (Germany) as lyophilized and was used directly. NAD$^+$ and NADH (purity 98%) were purchased from Sigma. $\alpha$-KG (biochemical reagent) was the product of Microbe Institute, the Chinese Academy of Sciences. ADP and AMP ($> 99\%$, HPLC) was obtained from Fluka (Switzerland). Other chemicals were of analytical-reagent grade. All solutions used in this work were prepared with triply distilled water.

3. Results and discussion

3.1. Electrochemical indication of the enzyme-catalyzed reaction

The advantage of hydrodynamic methods involving convective mass transport of reactants and products is that a steady state is attained rather quickly and measurements can be made with high precision. Since the rates of mass transfer in the convective system are much larger than the rates of diffusion, the ‘diffusion’ layer thickness $\delta_0$ is much thinner than the thickness of the real diffusion-layer in a stationary solution. In this way, the reactant in the bulk solution can reach the electrode surface very quickly. Therefore, the chronoamperometric technique can be used to indicate the continuous in-situ changes of the coenzyme concentration without a lag-phase.

The cyclic voltammogram recorded at a GCE in 0.3 mmol/l NADH + 10 mmol/l $\text{NH}_4\text{Cl}$ + 2 mmol/l $\alpha$-KG + 50 mmol/l Tris buffer ($\text{pH} = 8.0$) showed that the peak potential of NADH oxidation was about 0.75 V (vs. SCE). Therefore, in chronoamperometric experiments, the potential that stepped from 0.20 to 0.80 V can assure that the experiment was performed in the limiting current region. Under this condition, the chronoamperometric experiment progress of NADH in a well-stirred assay mixture was shown in Fig. 1 (Curve I). It was clear that the anodic current of NADH could quickly reach a steady state ($i_{1a}$) after potential stepped from 0.20 to 0.80 V (indicated as $a \rightarrow b$). The steady state current ($i_{1a}$) was proportional to its corresponding concentration ($c_{\text{NADH}}$) in the range $1 \times 10^{-5}$–$6 \times 10^{-4}$ mol/l. After adding GLDH to the NADH mixture solution, NADH could stoichiometrically be oxidized. Thus, the rate of glutamate formation can be measured by the decrease in the NADH concentration, i.e., $v = -dc_{\text{NADH}}/dt$. In a short time span after the start of

![Fig. 1. Progress curves of reductive amination reaction catalyzed by GLDH in a well-stirred assay mixture: 10 mmol/l $\text{NH}_4\text{Cl}$ + 2 mmol/l $\alpha$-KG + 0.1 mmol/l NADH in 50 mmol/l Tris buffer solution, with final volume of 5 ml, pH = 8.0. Reaction was started by addition of 10 µl of GLDH (10 mg/ml) as indicated by an arrow. (I) no ADP; (II) 80 µmol/l ADP.](image)
the enzyme-catalyzed reaction, the current $i_{1\text{lab}}$ would decrease linearly with time (indicated as $c \rightarrow d$). Thus, the initial rate $V_0$ of enzyme-catalyzed reaction could be measured from the initial slope of the progress curve:

$$V_0 = -\frac{d(c_{i,NADH}/dt)}{c_{i,NADH} - c_0} = \frac{c_0(1 - i_i/i_0)/(t - t_0)}$$

Here, $i_i$ and $i_0$ are marked as in Fig. 1.

3.2. Effect of ADP and AMP on the initial kinetics of the glutamate formation

The chronoamperometric method can be used to study the initial kinetic of the reductive amination reaction in the presence or absence of ADP and AMP. The result was shown in Fig. 1 (curve II). Comparing the chronoamperometric curve I (without ADP) and curve II (with ADP), it can be seen that the ADP could promote the enzyme-catalyzed reaction as an activator in the glutamate formation by increasing the initial rate $V_0$ of the reductive amination. A similar activation was observed when the effect of AMP on the reductive amination reaction was studied.

In order to further investigate the activation mechanism of ADP and AMP, the effects of ADP and AMP on Lineweaver–Burk plots [14] with NADH as the varied substrate have been studied. The plot of reciprocal initial rate $(1/V_0)$ vs. the reciprocal of NADH concentration $(1/c_{\text{NADH}})$ was shown in Fig. 2. From the slopes and intercepts, the maximum initial reaction rate $V_{\text{max}}$, and the Michaelis–Menten constants $K_m$ for NADH in the presence or absence of ADP and AMP can be obtained. The results showed that when ADP or AMP was present in the mixture substrate, the value of $V_{\text{max}}$ would increase from 56 ± 4 to 107 ± 9 μmol/l min⁻¹ (for ADP) and from 56 ± 4 to 97 ± 7 μmol/l min⁻¹ (for AMP), and $K_m$ would also increase from 93 ± 5 to 121 ± 10 μmol/l (for ADP) and from 93 ± 5 to 120 ± 13 μmol/l (for AMP).

Here, the standard deviations are based on the data from six independent experiments. The significant increase in $V_{\text{max}}$ expressed that both ADP and AMP had activation effect on the enzyme activity.

As shown in Fig. 3, the activating effects by ADP and AMP on the enzyme activity were dependent on their concentrations. When the concentration of ADP was below 0.4 mmol/l, the activation of ADP would be increased with the increase of concentration. After this concentration point (0.4 mmol/l), the activation has reached a platform region. However, for AMP, the activation reached the platform region earlier, at about 0.2 mmol/l. Here, the determination error of $V/V_0$ was less than 3%.

3.3. Voltammetric behavior of NAD⁺ and NAD⁺–GLDH complex

During the glutamate formation, the NADH was oxidized to NAD⁺, then NAD⁺ immediately combined with GLDH to form an abortive complex NAD⁺–GLDH [15,16]. Therefore, the voltammetric method was carried out to study the formation process of the NAD⁺–GLDH complex on mercury electrode. Fig. 4 gave the cyclic voltammograms of NAD⁺ and NAD⁺–GLDH complex. We found that the NAD⁺ was reduced at approximately −0.95 V (vs. Ag/AgCl) without the respective anodic progress. The peak current $I_{\text{p}}$ depends linearly on the square root of the scan rate $v$, as expected in a process controlled by diffusion. According to the previous work by Bojarska et al. [17], the reduction peak would correspond to a one-electron reduction process of NAD⁺, i.e., NAD⁺ + e⁻ → NAD⁺. After adding GLDH to the solution, the cathodic peak potential of NAD⁺ would negatively shift from −0.95 to −1.17 V. The potential shift clearly indicates that NAD⁺ can easily complex with GLDH and the shifted peak will be due to the reduction of the NAD⁺–GLDH complex. In the meanwhile, the reduction peak potential...
Fig. 4. Cyclic voltammograms of (a) 0.3 mmol/l NAD⁺; (b) 0.3 mmol/l NAD⁺ + 0.4 mg GLDH, in 50 mmol/l Tris buffer solution with final volume of 5 ml (pH = 8.0). Equilibrium time: 10 s; scan rate: 50 mV/s.

Fig. 5. Natural drop times for a DME in (a) 50 mmol/l Tris buffer, (b) 50 mmol/l Tris + 0.3 mmol/l NAD⁺, (c) 50 mmol/l Tris + 0.4 mg GLDH, (d) 50 mmol/l Tris + 0.3 mmol/l NAD⁺ + 0.4 mg GLDH, with final volume of 5 ml, pH = 8.0.

$E_{p2}$ of NAD⁺–GLDH complex will have a slight negative shift as the scan rate $v$ increased, and the peak current $I_{p2}$ increases proportionally with the scan rate $v$. The longer the accumulation time, the larger the $I_{p2}$ values before the adsorptive equilibrium is reached. These behaviors are consistent with an irreversible surface electrochemical reaction with the reactant strongly adsorbed on the surface of the electrode [18]. Thus, the whole electrode process of NAD⁺ and the NAD⁺–GLDH complex can be described as Scheme 1.

3.4. Certificate of the adsorption of GLDH and NAD⁺–GLDH complex by electrocapillary curves

In order to examine the adsorption of GLDH and NAD⁺–GLDH complex on mercury electrode, the natural drop time of a dropping mercury electrode (DME) was measured as a function of potential in 50 mmol/l Tris buffer solution (pH = 8.0). The results of electrocapillary curves are shown in Fig. 5. Between $-1.4$ and $-1.6$ V, the drop times are changed very little by the addition of NAD⁺, GLDH or NAD⁺–GLDH complex. However, when the potential is more positive than $-1.4$ V, there is a definite decrease in the drop time in the presence of GLDH or NAD⁺–GLDH complex. Thus, GLDH and NAD⁺–GLDH complex appear to be adsorbed on mercury electrode at potentials more positive than $-1.4$ V. From Fig. 5 we also found that the adsorption of NAD⁺–GLDH complex is stronger than that of GLDH. This may be contributed to the conformational change of GLDH when NAD⁺ binds to the enzyme [19].

3.5. Activation mechanism of ADP and AMP on GLDH

As mentioned in Section 3.3. above, when GLDH was added to the NAD⁺ solution, the reduction peak potential of NAD⁺ would shift from $-0.95$ to $-1.17$ V. This was due to the formation of NAD⁺–GLDH complex. However, when either ADP or AMP was added to the GLDH and NAD⁺ mixture solution, we found that the reduction peak of NAD⁺–GLDH complex (at $-1.17$ V) would decrease and the reduction peak of NAD⁺ (at $-0.95$ V) would emerge again. The cyclic voltammograms were shown in Fig. 6. These indicate that the addition of either ADP or AMP would enhance the dissociation of the NAD⁺–GLDH complex and compete with NAD⁺. This was strongly

Fig. 6. Cyclic voltammograms of (a) 0.3 mmol/l NAD⁺ + 0.4 mg GLDH; (b) 0.3 mmol/l NAD⁺ + 0.4 mg GLDH + 0.2 mmol/l ADP; (c) 0.3 mmol/l NAD⁺ + 0.4 mg GLDH + 0.4 mmol/l ADP, in 50 mmol/l Tris buffer solution with final volume of 5 ml (pH = 8.0). Equilibrium time: 10 s; scan rate: 50 mV/s.

$$
\text{NAD}^+ + \text{GLDH} \rightleftharpoons \text{NAD}^+–\text{GLDH}
$$

$$
\text{NAD}^+ + \text{GLDH} \rightleftharpoons \text{NAD}^+–\text{GLDH}
$$

Scheme 1.
supported by the dissociation constants of the complexes of NAD$^+$–GLDH (≈ 0.72 mM) and ADP–GLDH (≈ 2 \mu M) [15]. The general representation of the equilibrium system of enzyme, coenzyme, and ADP (or AMP) was given as following Scheme 2.

Baker et al. [20] have reported a crystal structure of the closely homologous Clostridium symbosium GLDH–NAD$^+$ complex which showed that each sub unit consists of two domains separated by a deep cleft. The NAD$^+$ is bound in the open form with the nicotinamide ring buried deep in the (presumably) narrower interior end of the cleft, while the adenylate moiety is strung out in the direction of the sub unit surface. Simple consideration of this gross geometry makes it obvious that the only physically feasible way in which such a process could occur requires that the nicotinamide ring must bind first to the narrow end of the cleft and that the remainder of the coenzyme, which occupies the less constrained outer portion of the cleft, must bind in a subsequent step. The results of circular dichroism difference spectroscopy [15] have also showed that the two ligands in the binary complexes of GLDH–NAD$^+$ and GLDH–ADP bound at the same site. Therefore, combining our experiments, the activation mechanism of ADP or AMP will be that both ADP and AMP could interrupt the binding of NAD$^+$ to GLDH by combining preferentially at the regulatory (adenine nucleotide) binding site.

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