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SPECTROPHOTOMETRIC OBSERVATION OF A GLUTAMATE DEHYDROGENASE–L-GLUTAMATE COMPLEX

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SUMMARY

Ultraviolet differential spectroscopic measurements show the existence of a glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating) EC 1.4.1.3)–L-glutamate complex. The spectral features resemble perturbation difference spectra of enzyme aromatic amino acid chromophores and allow the determination of the L-glutamate concentration dependence. The dissociation constant for this enzyme–L-glutamate complex was approximately 48 mM and was independent of enzyme concentration. The lack of interaction between the binding of L-glutamate and the activating monocarboxylic amino acids indicates that they bind at totally separate sites.

INTRODUCTION

Recently the existence of several L-α-amino acid binary complexes of glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating) EC 1.4.1.3) has been established for a number of monocarboxylic amino acids[1]. An enzyme–L-glutamate complex has been indirectly demonstrated by glutamate protection from urea denaturation[2] and the existence of a reactive enzyme–L-glutamate complex has been postulated in transient-state kinetic studies[2]. This paper will present spectrophotometric evidence for the existence of an enzyme–L-glutamate complex and will define the interaction of this glutamate binary complex with other binary complexes of glutamate dehydrogenase.

MATERIALS AND METHODS

The glutamate dehydrogenase was purchased from the Sigma Chemical Company as the Type I crystalline ammonium sulfate suspension. The suspension was dialyzed, treated with Norit A, filtered, and assayed as described previously[1]. Enzyme concentrations were calculated from 280 nm absorbance readings using a value of 0.97 as the extinction coefficient of a 1 mg/ml solution of glutamate dehydroge-

nase[3]. The enzyme used had an A_{280}/A_{260} ratio of 1.95–1.98 and a specific activity of 3.5 ± 0.5 units per mg protein[1]. The pH values of all solutions were determined using a Radiometer PHM26 pH meter. Unless stated, the buffer employed for all spectroscopy experiments was 0.1 M potassium phosphate, pH 7.6 and the temperature was 20 °C. The amino acids and α-ketoacids used were purchased from the Sigma Chemical Company or the Mann Research Laboratories.

The binding difference spectra were obtained using a Cary Model 14 double-beamed spectrophotometer with the photomultiplier interfaced directly to a Varian Model 620i computer[1]. Each spectrum presented in this study represents an average of 3 to 4 spectra collected from consecutive runs. The spectrum and glutamate concentration dependence were determined in 1.000 cm quartz cells and the enzyme concentration dependent experiments employed either 1.000, 2.000, or 5.000 cm cells depending on enzyme concentration. For convenience, the final spectra were obtained as standard binding difference spectra by subtracting an averaged base line spectrum of equal amounts of glutamate dehydrogenase in the sample and reference cells from the spectrum of a solution of enzyme mixed with ligand in the sample cell and solutions of enzyme and ligand separated in the reference cell.

RESULTS AND DISCUSSION

An ultraviolet difference spectrum obtained by subtracting the absorbance of solutions of glutamate dehydrogenase and L-glutamate measured separately from that of a solution of the same concentrations of enzyme mixed with L-glutamate can be seen in Curve A of Fig. 1. At lower L-glutamate concentrations, the differential absorbance at the shorter wavelengths appeared to increase with time. A plot of log ΔA *vs* log wavelength (excluding the major signal from 278 to 295 nm) yielded a straight line with a slope of −4.2 indicating that this increasing differential absorption at the shorter wavelengths is probably due to light scattering.

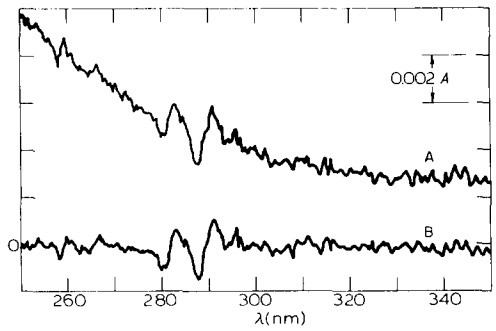

Fig. 1. The ultraviolet difference spectrum of the glutamate dehydrogenase–L-glutamate complex. The glutamate dehydrogenase concentration was 1.04 mg/ml and the L-glutamate concentration was 65 mM. Curve A is the spectrum of the complex and Curve B is the spectrum obtained by subtracting a curve with a reciprocal fourth power dependence on wavelength.

Curve B of Fig. 1 shows the spectrum of the L-glutamate dehydrogenase-L-glutamate complex with the light scatter effect subtracted. Both difference spectra

of Fig. 1 have maxima at 291 and 283 nm and minima at 287 and 280 nm. The general shape of both difference spectra suggest the involvement of enzyme aromatic amino acid chromophores and the 291 nm spectral feature could be characterized as a red-shifted tryptophan perturbation difference spectrum. While the signals are too small to resolve, the minima at 287 and 280 nm suggest that a blue-shifted tyrosine perturbation difference spectrum may contribute to the overall signal. Since this binding signal does not resemble the large tryptophan and tyrosine solvent perturbation spectra of glutamate dehydrogenase caused by general perturbers which are the same size as L-glutamate, the signal is probably due to a specific perturbation upon L-glutamate binding.

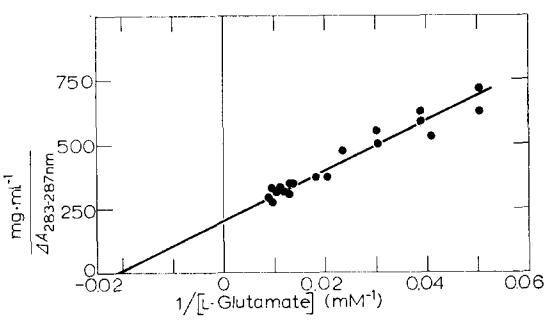

Fig. 2. The concentration dependence of L-glutamate binding.

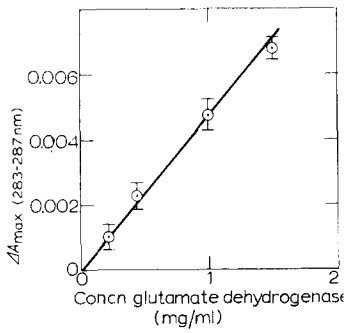

Fig. 3. The dependence of ΔA_{max} from L-glutamate binding plots on glutamate dehydrogenase concentration.

TABLE I

THE DISSOCIATION CONSTANTS FOR THE GLUTAMATE DEHYDROGENASE-L-GLUTAMATE COMPLEX AT VARIOUS CONDITIONS

The dissociation constants were determined from double reciprocal plots similar to Fig. 2.

Temp. °C	K_d *(mM)*	
	pH 6.5	*pH 7.6*
20	—	47 ± 5
25	52 ± 10*	38 ± 8

* This experiment was performed using 0.2 M potassium phosphate buffer.

The L-glutamate binding dependence of this enzyme-L-glutamate complex is shown in the double reciprocal plot in Fig. 2. The ΔA values were measured from maxima to minima of the 283–287 nm spectral feature and the ΔA_{max} was found to be 0.0049 A units/mg per ml. A plot of ΔA_{max} *vs* glutamate dehydrogenase concentration was linear from below 0.2 to 1.6 mg/ml (Fig. 3). The dissociation constants at two pH values and two temperatures are shown in Table I.

Recently we have shown that many monocarboxylic amino acids form complexes with glutamate dehydrogenase and have postulated that these complexes are

the activating amino acid complexes observed by several other workers[4,5,6]. High concentrations of L-leucine (2 mM) and L-isoleucine (2.8 mM) did not affect the spectroscopic signal or dissociation constant of the enzyme–L-glutamate complex nor did 50–100 mM L-glutamate affect the signal or dissociation constant of the L-leucine and L-isoleucine complexes. Therefore, the activating monocarboxylic amino acids and L-glutamate have separate binding sites.

The relationship of the enzyme-L-glutamate complex to the transient state kinetics will be described elsewhere.

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