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ENERGY TRANSFER IN THE GLUTAMATE DEHYDROGENASE–NADH BINARY COMPLEX AND ITS RELATION TO THE BINDING OF COFACTOR*

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SUMMARY

Fluorescence spectroscopy was used to examine the interaction between glutamate dehydrogenase (EC 1.4.1.3) and NADH. The binary complex of enzyme and coenzyme showed enhanced emission at 465 nm with excitation at 295 nm, as compared to the free components. This behavior was concluded to arise from a non-radiative transfer of excitation energy between glutamate dehydrogenase and NADH. The intensity of the transferwas used to quantitate the binding of NADH to glutamate dehydrogenase. It was found that a K_{diss} from $2 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ could be measured, depending on the experimental conditions. Those conditions closest to the composition of kinetic assays gave a K_{diss} for the glutamate dehydrogenase–NADH binary complex of $1 \cdot 10^{-4}$. The fact that glutamate dehydrogenase may contain as many as four tryptophanyl residues per mole makes distance calculations based on the efficiency of the energy transfer quite hazardous. If only one of the tryptophanyl residues were shown to be the energy donor, then this transfer could prove a useful probe of the NADH binding site on glutamate dehydrogenase.

INTRODUCTION

Glutamate dehydrogenase (EC 1.4.1.3) has been used frequently as a model protein for the study of the regulation of enzymatic activity by non-covalently bound small molecules. As a result, a great deal is known about the relation between changes in this enzyme's tertiary structure and its catalytic activity. Although the identification of the primary structural features essential to its catalytic activity has also been attempted, this aspect of glutamate dehydrogenase is not so well understood.

Several reports have appeared which suggest that a tryptophanyl residue may be of some importance to the enzymatic activity of glutamate dehydrogenase. CHEN[1] described a simultaneous decay in both fluorescent quantum yield and enzymatic activity when glutamate dehydrogenase was continuously irradiated with 290 nm

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light, presumably due to the photooxidation of tryptophan. FISHER AND CROSS[2], showed that the formation of the glutamate dehydrogenase–NADH–L-glutamate ternary complex generated a difference spectrum which was qualitatively similar to that observed when *N*-acetyl tryptophanamide was perturbed with ^2H_2O. They postulated that this difference spectrum reflected the change in environment of a tryptophanyl residue located in the glutamate binding site of glutamate dehydrogenase. BROCKLEHURST *et al.*[33] noted a decrease in the intrinsic fluorescence of glutamate dehydrogenase, modified with 4-iodoacetamido salicylic acid. They concluded that this arose from quenching of an active site tryptophanyl residue.

In light of these findings, and of the proposals of CHAN AND SCHELLENBERG[3] and SCHELLENBERG[4,31,32] regarding the function of tryptophanyl residues in various other dehydrogenases, the properties of tryptophan in native glutamate dehydrogenase were examined in relation to the binding of cofactor.

MATERIALS AND METHODS

The chemicals used in these studies were of the highest purity commercially available, and underwent no further purification. Glutamate dehydrogenase was purchased as an ammonium sulfate suspension from Boehringer Mannheim. The enzyme crystals were pelleted by centrifugation, and resuspended three times in one-half the original volume of 0.05 M phosphate buffer (pH 7.39) containing 0.05 M NaCl and $1 \cdot 10^{-3}$ M EDTA. Finally, the enzyme pellet was dissolved in about 1/3 the original volume of phosphate buffer. Approximately half of the enzyme was lost due to its solubility in the buffer used to resuspend the pellet, nevertheless, this procedure gave a stock glutamate dehydrogenase preparation with a concentration of about 20–30 mg/ml, as determined by a molar absorbance at 280 nm of $5.24 \cdot 10^4$, based on a chain molecular weight of 53500 (ref. 5).

Stock solutions of NADH (Sigma) were prepared fresh daily by dissolving an appropriate amount in 0.001 M NaOH and chilling in an ice bath. A molar absorbance of $6.22 \cdot 10^3$ at 340 nm was used to determine concentrations of NADH, as recommended by P and L Laboratories*.

An Aminco Bowman Spectrophotofluorometer, equipped with an RCA 1P28 phototube and a linear X–Y recorder, was used throughout these studies. All spectra are reported without correction for phototube response and with scatter peaks omitted. The cuvette block on this instrument was maintained at 21° by means of a Lo-Temptrol 154 circulating water bath from Precision Scientific Company.

For those experiments in which the concentrations of NADH were varied, μl aliquots of a stock NADH solution were added directly to the cuvette by means of a syringe microburette and an appropriate length of small diameter polyethylene tubing. The contents of the cuvette were continuously mixed with a micro stirring bar, actuated by a stir plate placed directly under the cuvette housing. In all cases, the exciting beam of light was attenuated until spectra were actually being taken, to eliminate any photooxidation of the sample. Final concentrations were not corrected for dilution due to titrant addition since this would only involve a maximum correction of $< 3\%$. The Δ excitation at 295 nm was calculated by subtracting the

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intensity at that excitation wavelength for NADH in buffer with all components but glutamate dehydrogenase, from the intensity obtained by adding an equal amount of NADH to an identical cuvet containing glutamate dehydrogenase. This treatment presumably eliminated the contribution of any free NADH. These data were then treated similarly to those of TOMKINS *et al.*[6] and ADELSTEIN[7].

The fluorescence lifetime measurements were done using the TRW Instrument described by CHEN *et al.*[8] who generously provided his facilities for these experiments.

Ultraviolet spectra were recorded with a Cary 15 spectrophotometer. Both the 0–0.1 and 0–1.0 A slidewire were used in conjunction with these studies.

In spite of several attempts to standardize both the excitation and emission monochromators on the Aminco instrument used in these studies, the measured λ_{max} for protein absorption and emission, as well as NADH absorption and emission were consistently 10–15 nm higher than expected. Since the wavelengths of maximum absorption or emission were always higher than the reported values for glutamate dehydrogenase and NADH, it was felt that the possibility of exciting tyrosyl residues in glutamate dehydrogenase by setting the excitation monochromator on 295 nm was quite small. For simplicity, the actual uncorrected readings from the two monochromators will be used in this paper.

RESULTS

Spectral properties of glutamate dehydrogenase, NADH and their complex

The effect of binary complex formation on glutamate dehydrogenase fluorescence is presented in Fig. 1. As the intrinsic protein fluorescence at 350 nm was quenched by the presence of the NADH, there was a simultaneous emergence of NADH fluorescence at 465 nm.

This suggested that the interaction between glutamate dehydrogenase and NADH led to a non-radiative transfer of the protein excitation energy, causing NADH emission at 465 nm. An alternate, trivial, explanation is that NADH simply absorbed the protein emission at 350 nm and re-emitted at 465 nm; however, the low chromophore concentration, as well as the poor quantum yield of NADH[13] make this most unlikely. Several experiments were done on NADH alone, as described in the following sections, to be certain of the cause of the enhanced emission at 465 nm seen in the presence of enzyme.

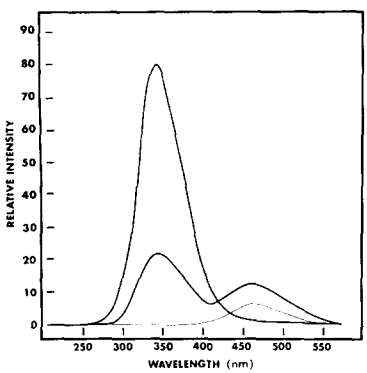
Effect of solvents on NADH emission

It is possible that the NADH which was bound to glutamate dehydrogenase might show increased absorption of 295 nm exciting light, due to its environment, resulting in a higher quantum yield for these bound NADH molecules. To test this, the effects of increasing concentrations of methanol (dielectric constant = 33) and ethylene glycol (dielectric constant = 38) on the emission of NADH dissolved in water (dielectric constant = 78) were examined. The use of these two solvents would show the effects of both polarity and viscosity on NADH fluorescence, in a manner analogous to the effects of a protein binding site. These results, shown in Fig. 2, confirmed that the 350 nm excited NADH fluorescence was dependent on solvent character while that excited at 295 nm was not.

Effect of binding of NADH absorption

Increased emission of bound NADH at 465 nm might also result from a red shift in the 260 nm absorption peak of the coenzyme; the absorption spectra of several different NADH–glutamate dehydrogenase mixtures were measured and show no indication of such a shift. In fact, extensive binding of NADH to glutamate dehydrogenase causes a slight decrease in the λ_{max} for bound NADH.

These results are emphasized by comparing the molar absorbance of NADH in various mixtures of enzyme as shown in Table I. Extensive binding of NADH to glutamate dehydrogenase caused ε (260 nm) to increase from $11 \cdot 10^3$ to $16 \cdot 10^3$. The



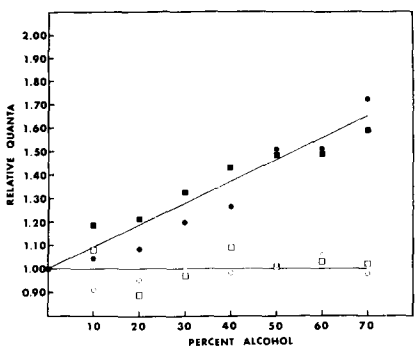


Fig. 1. Effect of complex formation with NADH on glutamate dehydrogenase emission spectrum. NADH was added to a cuvette containing 2.0 ml of 0.1 mg/ml glutamate dehydrogenase in 0.05 M Tris–HCl buffer (pH 8.2) with 0.05 M NaCl and $1 \cdot 10^{-3}$ M EDTA, so that the final NADH concentration was $6.2 \cdot 10^{-5}$ M. Excitation was constant at 295 nm. The thin line shows the emission spectrum of an equal amount of NADH, in the absence of glutamate dehydrogenase, excited at 295 nm. The most intense spectrum is that for glutamate dehydrogenase only, while the partially quenched curve with two maxima is the mixture of glutamate dehydrogenase with NADH.

Fig. 2. Effect of solvent polarity on NADH emission. Serial dilutions of either methanol or ethylene glycol were prepared in water. To 2.0 ml of each solution was added an aliquot of NADH to give a final concentration of $2.8 \cdot 10^{-5}$ M and each solution thus prepared was irradiated at either 350 nm or at 295 nm, as noted below. The area under each emission peak was measured with a planimeter and relative quanta were calculated by dividing the area under each curve for the different solvent concentrations, by the area under the curve for pure water. ●, methanol, excited at 350 nm; ○, methanol, excited at 295 nm; □, ethylene glycol, excited at 350 nm; ■, ethylene glycol, excited at 295 nm.

TABLE I

MOLAR ABSORBANCE OF GLUTAMATE DEHYDROGENASE–NADH MIXTURES AT 295 AND 260 nm

The values for the molar absorbance in each experiment were calculated on the basis of the NADH concentration. The numbers listed under NADH/glutamate dehydrogenase are molar ratios, calculated from the dilution of stock solutions. For the last two values (NADH/glutamate dehydrogenase = 0.30 and 0.07), an equal amount of glutamate dehydrogenase was present in the reference cuvette, to blank out the very strong protein absorption.

NADH/enzyme	$\varepsilon \times 10^{-3}$ *at 260 nm*	$\varepsilon \times 10^{-3}$ *at 295 nm*
5.3	11	1.2
2.5	11	1.6
0.30	15	1.1
0.07	16	1.1

molar absorbance of the mixture at 295 nm remained essentially unchanged at about $1.1 \cdot 10^3$.

The data therefore, were indicative of energy transfer between glutamate dehydrogenase and bound NADH. To support this argument, the fluorescence lifetimes of the complex and the NADH alone were examined, since the observed lifetime of a given fluorescent species is often a characteristic of that species. If the excitation of bound NADH does, in fact, come from excited protein chromophore residues the lifetime measured by exciting the complex at 295 nm might be different from that obtained by exciting the complex at 350 nm.

For determining the lifetime of the excited state of the isolated coenzyme, the activating beam was passed through a grating blazed for light at 350 nm. These results were compared with experiments using a filter transmitting the band between 280 nm and 360 nm to excite the glutamate dehydrogenase–NADH complex. In each instance, emission was measured at 465 nm.

The results of these experiments, shown in Table II, indicated that the lifetime of the NADH excited by 350 nm irradiation was increased approx. 3-fold on binding to glutamate dehydrogenase whereas that excited by 280–360 nm irradiation was increased 5–6 fold. These results provided strong evidence for the energy transfer mechanism.

TABLE II

LIFETIMES OF THE COMPONENTS OF THE GLUTAMATE DEHYDROGENASE–NADH COMPLEX

These experiments were done as described in the text.

System	λ_{exc} (*nm*)	λ_{emit} (*nm*)	τ (*nsec*)*
Enzyme	295	350	4.4
NADH	340	465	0.4**
NADH + enzyme	340	465	1.0
NADH + enzyme	280–360	465	2.2

* ± 0.5 nsec (ref. 8).

** This value is considerably smaller than that originally reported by CHEN *et al.*[8], however, its agreement with that of SPENCER AND WEBER[24], who used a much more sensitive instrument is compelling.

NADH binding constants

The quantitative relationship between the binding of NADH to glutamate dehydrogenase and the intensity of the energy transfer emission was examined in the following set of experiments. The peak height at 295 nm was corrected for free NADH and then plotted against the NADH final concentration, both as reciprocals, shown in Fig. 3. The control titration gave K_{diss} of $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$. L-Glutamate ($2.5 \cdot 10^{-2}$ M) which is known to increase the strength of NADH binding[6,11,12,14], causes the constant to decrease to a value of about $3 \cdot 10^{-6}$–$5 \cdot 10^{-6}$. An increase in ionic strength alone, in contrast, causes the K_{diss} to increase slightly, an effect also noted by others[15,16]. The interesting effect of $ZnCl_2$ on the NADH binding is not understood, but must relate to the extensive conformational change that Zn^{2+} is known to cause in glutamate dehydrogenase[17].

A similar experiment, with NADH being added to varying amounts of glutamate dehydrogenase is shown in Fig. 4. In these experiments, the buffer system was changed from Tris–HCl to phosphate, and in addition, $(NH_4)_2SO_4$ was added to the cuvette. As one would expect, the maximum amount of energy transfer is dependent on the amount of energy donor present, over the range of glutamate dehydrogenase concentrations examined. In spite of a 10-fold increase in the glutamate dehydrogenase concentration, however, the extrapolated K_{diss} for the binary complex remains the

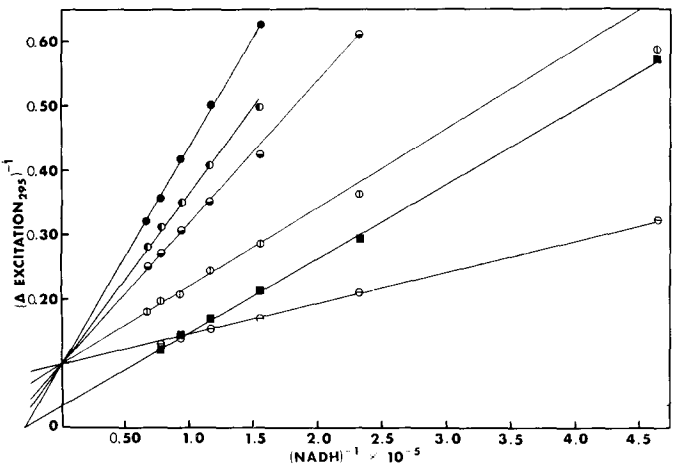


Fig. 3. Effect of Zn^{2+}, L-glutamate and ionic strength on NADH binding. Sequential addition of NADH were made to a cuvet with 0.26 mg/ml glutamate dehydrogenase dissolved in 0.05 M Tris (pH 8.2), 0.05 M NaCl, and containing the following additional solutes: ●, 0.10 M NaCl; ◐, none; ◒, $6.2 \cdot 10^{-3}$ M L-glutamate; ○, $1.2 \cdot 10^{-2}$ M L-glutamate; ⊖, $2.5 \cdot 10^{-2}$ M L-glutamate; ■, $5 \cdot 10^{-5}$ M $ZnCl_2$. The Δ excitation at 295 nm was calculated from an excitation spectrum of the mixtures described above, with emission wavelength constant at 465 nm.

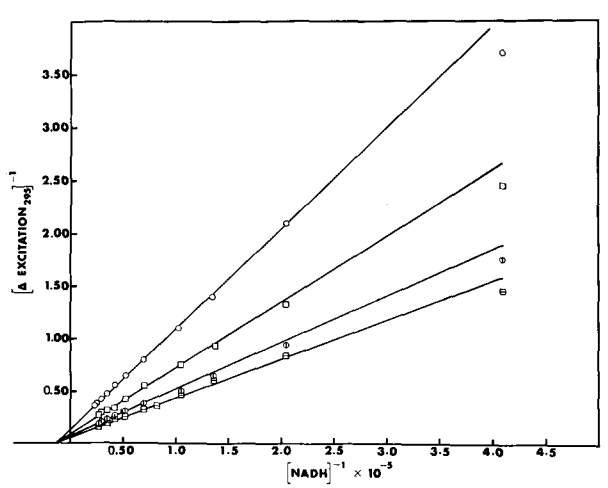


Fig. 4. Effect of glutamate dehydrogenase on NADH binding. To 2.00 ml of 0.05 M phosphate buffer (pH 7.43) containing 0.05 M NaCl, $1 \cdot 10^{-3}$ M EDTA, $5 \cdot 10^{-2}$ M $(NH_4)_2SO_4$ and glutamate dehydrogenase at a concentration of: ○, $1.6 \cdot 10^{-6}$ M; □, $3.5 \cdot 10^{-6}$ M; ◯, $6.2 \cdot 10^{-6}$ M, or ▯, $1.3 \cdot 10^{-5}$ M, were added sequential μl increments of $9.8 \cdot 10^{-4}$ M NADH. Emission wavelength was constant at 465 nm.

same, at about $1 \cdot 10^{-4}$. It is noteworthy that the curves for both Figs. 3 and 4 appear to be linear throughout the entire titration range and give no indication of two competing sites for NADH, as proposed previously[9,34].

DISCUSSION

The fluorescence studies reported here have shown that excitation of the binary NADH–glutamate dehydrogenase mixture at a wavelength where only tryptophan absorbs appreciably, results in fluorescence at a wavelength characteristic of NADH. Observations of this type have been interpreted to mean that the energy of excitation is transferred from the excited state of the donor molecule to the unexcited acceptor molecule[18,19]. This interpretation is of particular interest, since it implies a special relationship between NADH and tryptophan in the binary complex. Because of this important implication, alternate explanations must be examined carefully.

It has been demonstrated that in solution, NADH exists in two general conformations[18,20–22]. These two orientations, referred to as closed and extended, result in the planes of the nicotinamide and adenine rings being parallel and perpendicular, respectively. It has also been shown that a rather efficient intramolecular energy transfer occurs in the isolated NADH molecule[20,21], causing the 275-nm maximum in its excitation spectrum. This process has been investigated by FREED *et al.*[23], among others, who found that the intramolecular transfer was considerably affected by the chemical character of the solvent, presumably by altering the distribution between closed and extended structures.

Although VELICK[18] showed that lactate dehydrogenase binds "extended" NADH, it is conceivable that when NADH is bound to glutamate dehydrogenase, it is bound only in the closed form, causing an increase in the observed emission at 465 nm. However, the data of Table II show that there is not a remarkable alteration in either the position or the intensity of the NADH–adenine absorption band on binding to glutamate dehydrogenase. Thus, an alteration in the NADH structure on binding resulting in a more efficient adenine–nicotinamide intramolecular interaction, is not sufficient to explain the observations.

The rate of transfer of energy from tryptophan to NADH is not known, but must be no slower than the lifetime of the excited tryptophan residue in glutamate dehydrogenase, or about 4.4 nsec. Since the lifetime of excited NADH is only 0.4 nsec, as measured by SPENCER AND WEBER[24] and confirmed by the data shown here, if the rate of transfer is not appreciably different from the rate of tryptophan emission, the energy transfer step should be rate limiting to the overall process of tryptophan absorption → NADH emission. This can be tested by measuring the lifetime of the 465-nm emitting species, in the presence of glutamate dehydrogenase, first by exciting the nicotinamide and then by exciting at the protein. When the NADH–glutamate dehydrogenase mixture was excited at 340 nm, the fluorescent lifetime was 1.0 nsec. This lifetime of bound NADH is exactly what one would predict from the data of WINER AND SCHWERT[11], who found that the intrinsic fluorescence of NADH was enhanced by a factor of about 2 upon binding to glutamate dehydrogenase. If excitation of bound NADH by 295 nm light were occurring by the same mechanism as excitation by 340 nm light, one would expect to measure an identical lifetime by exciting at 295 nm. This is clearly not the case. The lifetime obtained by exciting the glutamate

dehydrogenase–NADH mixture at 295 nm is over twice as long as that from 340 nm excitation. This is the most compelling evidence to favor energy transfer.

The utility of this energy transfer in studying the binding of NADH to glutamate dehydrogenase is shown by the experiments in Figs. 3 and 4. It is clear that the measured value for the dissociation of NADH from the glutamate dehydrogenase–NADH binary complex is determined by the ionic milieu of the experiment, a fact previously noted[16,25,26]. Note that a change in the experimental conditions to approximate more closely the ionic environment of the enzyme during kinetic assays, as shown in Fig. 4, generates K_{diss} values completely compatible with those obtained from a Lineweaver–Burke plot of the initial velocity data. This is the first demonstration of a fluorimetrically determined NADH K_{diss} which is essentially identical with the Michaelis constant for NADH of about $1 \cdot 10^{-4}$.

The tryptophan content of glutamate dehydrogenase has been reported as being 2.4 (ref. 27), 4 (ref. 28), and 4.7 (ref. 29) moles per mole of glutamate dehydrogenase (mol. wt. 53500). Since there is obviously more than one tryptophanyl residue in glutamate dehydrogenase, it becomes important to consider which of these might be responsible for the donation of its 295-nm excitation energy to bound NADH. The fact that the NADH binding curves shown in Figs. 3 and 4 are linear may give an important clue to this question.

There seem to be three appropriate explanations for this failure to observe a second NADH binding site: (1) The energy transfer method is not a valid NADH binding monitor. (2) The tryptophanyl residue(s) involved in the transfer are so specifically located that they transfer preferentially to NADH bound at the catalytic site, and do not interact at all with NADH bound to the second site. (3) There is no second NADH binding site.

The first of these tentative explanations can be effectively ruled out by the good agreement between previously observed effects on the NADH binding behavior[6,11,12,14–16,25,26] and those reported here.

There is no good theoretical or experimental basis for selecting between the two remaining alternatives, although there has been substantial difficulty in showing conclusive evidence of a second NADH binding site. Based on the extreme dependence of the measured K_{diss} values for the NADH–glutamate dehydrogenase binary complex on the experimental conditions, as shown here and elsewhere[25,26], one might speculate that the variation in these values could represent the weighted average of identical NADH binding sites on a multitude of conformations of glutamate dehydrogenase rather than two separate sites on the same molecule. In either event, one is still faced with the problem of deciding which, and how many tryptophanyl residues in glutamate dehydrogenase might be involved in transferring energy to bound NADH. If it could be established that a single tryptophanyl residue is involved in the energy transfer, then the use of the relationship of FÖRSTER[30] to calculate distances and orientations for the two chromophores might give some indication of the dimensions of the catalytic site on glutamate dehydrogenase.

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