NICOTINAMIDE 1, N^6-ETHENOADENINE DINUCLEOTIDE, A COENZYME FOR GLUTAMATE DEHYDROGENASE*

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Received 9 August 1974

1. Introduction

The 1,N^6-ethenoadenine analogue (ϵ-NAD^+) described by Leonard and coworkers [2,3] allows fluorescence studies in the oxidized state of the coenzyme. Provided the analogue is active as a coenzyme in the dehydrogenase reaction, these studies should give useful information about coenzyme binding and the influence of effectors or substrates on enzyme–coenzyme complexes. In the case of glutamate dehydrogenase an additional aspect can be considered. Since this enzyme binds the reduced coenzyme at six active and six non-active sites [4,5], the latter being also accessible for ADP, the introduction of coenzyme analogues may serve to differentiate between these binding sites, as has previously been shown with α-NADH, which is competing only for the one set of NADH binding sites which also binds ADP [6]. Thus, the interaction of ϵ-NAD^+, ϵ-NADH, and ϵ-ADP with glutamate dehydrogenase was examined.

The experiments presented here show that ϵ-NAD^+ and ϵ-NADH are fully active as coenzymes in the glutamate dehydrogenase reaction, and that both coenzyme analogues exhibit strong fluorescence enhancement upon binding to the enzyme. Furthermore, ϵ-ADP and ϵ-NADH appear to be unable to bind to the six nonactive coenzyme binding sites.

* Studies on glutamate dehydrogenase, part XXI; for part XX see [1].

2. Experimental

Beef liver glutamate dehydrogenase, ADP, GTP, NAD^+ and NADH were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Solutions of these compounds were prepared and assayed as previously described [4]. ϵ-ADP and ϵ-NAD^+ were prepared according to [2,3] and purified to homogeneity by chromatography on Dowex X 1. Their concentrations were measured photometrically using the absorption coefficients $\epsilon_{265} = 5.7\ cm^{-1}\ mM^{-1}$ for ϵ-ADP and $\epsilon_{265} = 10.0\ cm^{-1}\ mM^{-1}$ for ϵ-NAD^+ [2,3]. ϵ-NADH was prepared by enzymatic reduction of ϵ-NAD^+ with liver alcohol dehydrogenase and 3% cyclohexanol at pH 9.4 as described for NADH [7]. It was purified by adsorption on DEAE-cellulose and eluted with 0.05 M ammonium carbonate pH 8.5. With this procedure ϵ-NADH could be well separated from unreacted ϵ-NAD^+ and a small amount of a decomposition product, probably ϵ-ADPR which eluted at the beginning of the ϵ-NADH peak. Only ϵ-NADH fractions with a constant ratio $\epsilon_{334}/\epsilon_{275} = 1.07$ were collected and used in the experiments. The absorption coefficient of ϵ-NADH at 334 nm of $6.9 \pm 0.1\ cm^{-1}\ mM^{-1}$ was determined on the basis of the phosphate content of the molecule according to [8]. ϵ-NADH is stable in 0.05 M carbonate or phosphate buffer at pH 8.5. After three days in these solutions no decomposition could be detected. All other materials were of highest purity grade from E. Merck (Darmstadt, Germany). Absorption, circular dichroism and fluorescence measurements as well as the determination of sedimentation coefficients were performed as previously described [4,6]. Fluorescence enhancement was determined according to [5].

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3. Results and discussion

In order to characterize the reduced coenzyme analogue the absorption, fluorescence and circular dichroism spectra of purified ε-NADH are shown in fig. 1. The absorption spectrum contains the typical bands of the ε-adenosine chromophore with maxima at 265 and 275 nm and shoulders at 260 and 294 nm, the latter overlapping with the broad 340 nm band of the dihydronicotinamide absorption. The determined absorption coefficient of 6.9 cm^{-1} mM^{-1} at 334 nm is close to the value of 6.7 which is calculated from the absorption of NADH and the trailing end of the 294 nm band of ε-NAD^+.

The CD curve of ε-NADH (fig. 1) exhibits several cotton effects corresponding to the absorption bands of the dihydronicotinamide and ε-adenosine

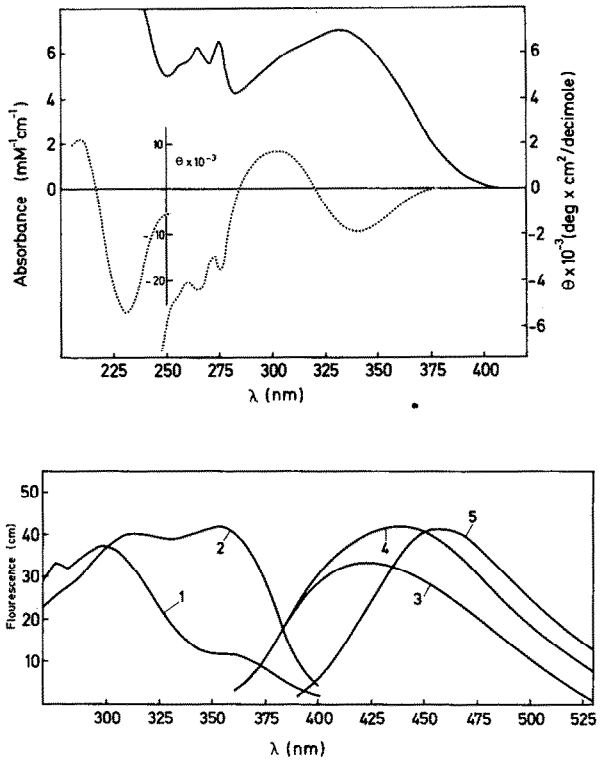

Fig. 1. Spectral properties of ε-NADH in 0.05 M phosphate buffer, pH 8.5, at 20°C. A) Absorption ——; circular dichroism. . . . . B) Fluorescence of 150 μM ε-NADH (uncorrected); slitwidths 5 nm; excitation spectra recorded at the emission wavelength of 410 (1) and 460 (2) nm; emission spectra excited at 275 (3), 305 (4) and 350 (5) nm.

chromophores. Compared to the natural coenzyme β-NADH [10] a stronger cotton effect at 340 nm is observed which might indicate reduced mobility of the dihydronicotinamide chromophore of ε-NADH, as is also suggested from NMR experiments [9]. A marked difference between β-NADH and ε-NADH is also seen in the 200 to 240 nm region where the natural coenzyme shows only a small deflection [10] whereas ε-NADH has a strong and complex cotton effect which is sensitive to dioxane (unpublished results). This suggests stronger chromophore–chromophore interaction of ε-NADH in the folded state as compared to β-NADH.

The fluorescence emission spectrum strongly depends on the excitation wavelength (fig. 1B). Excitation at 275 nm yields primarily the ε-adenine fluorescence at 420 nm whereas excitation at 350 nm favors the dihydronicotinamide emission at 460 nm. With an excitation at 305 nm, however, nearly equal fluorescence intensities of both chromophores are observed, suggesting radiationless energy transfer from the ε-adenine to the dihydronicotinamide chromophore. This is supported by the excitation spectra showing excitation of the 460 nm emission at both, 315 and 355 nm. The energy transfer from the dihydronicotinamide to the ε-adenine chromophore is small as indicated by the shoulder at 355 nm in the excitation spectrum recorded at the emission wavelength of 410 nm. The quantum yield of the ε-NADH fluorescence is very low. Compared to the emission of the ε-NADH 'half molecules' ε-AMP and NMNH under the same experimental conditions, the ε-NADH fluorescence is about 38-fold reduced in the ε-adenine emission and 4-fold reduced in the dihydronicotinamide emission.

Some properties of the investigated ε-nucleotides with respect to the glutamate dehydrogenase reaction are summarized in table 1. It is evident that both, oxidized and reduced analogue, are fully active as coenzymes under the conditions of the standard assay, indicating low specificity of the active coenzyme binding site for the adenine moiety. GTP inhibits the enzymatic reaction with ε-NADH to a similar extent as observed with NADH, the reaction with ε-NAD^+, however, is much stronger inhibited than that with NAD^+. ADP has only a small effect, yielding 14% activation of the ε-NADH and 12% inhibition of the ε-NAD^+ reaction under standard assay conditions in contrast to the reaction with NADH of NAD^+ which is strongly

Table 1
Enzymatic activity and fluorescence enhancement of ϵ-NAD^+ and ϵ-NADH with glutamate dehydrogenase as compared to NAD^+ and NADH

	NAD^+	ϵ-NAD^+	NADH	ϵ-NADH
Specific activity without effectors* (IU/mg)	5.3	5.3	37.8	38.0
Relative activity* in the presence of 15 μM GTP (%)	87	42	32	44
Relative activity* in the presence of 100 μM ADP (%)	166	83	171	114
Relative activity* in the presence of 100 μM ϵ-ADP (%)	98		100	
Fluorescence enhancement factor Q	–	21**	4.3***	10,5**

* Standard assay in 0.067 M phosphate buffer, pH 7.6, at 20°C with 1.4 mM NAD^+ or ϵ-NAD^+, 8.5 mM L-glutamate in the forward reaction and 100 μM NADH or ϵ-NADH, 2 mM 2-oxoglutarate, 50 mM NH_4Cl in the reverse reaction.
** Excitation at 320 nm, emission at 400 nm; fluorescence of ϵ-NAD^+ measured in the presence of 100 mM glutarate.
*** From ref. [5].

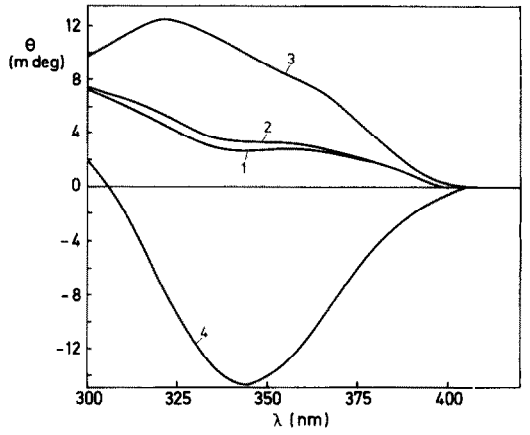

Fig. 2. Circular dichroism spectra of glutamate dehydrogenase complexes with ϵ-NADH. 3.1 mg/ml enzyme; 173 μM ϵ-NADH; 0.067 M phosphate, pH 7.6, 20°C. 1) Binary enzyme-ϵ-NADH complex (ER); 2) ER plus 475 μM GTP; 3) ER plus 10 mM 2-oxoglutarate; 4) ER plus 10 mM L-glutamate, with or without 475 μM GTP.

activated. The altered effects of ADP and GTP in the presence of the coenzyme analogue seem at least to be partially due to the unability of ϵ-NADH to bind to the nonactive coenzyme binding site (see below).

ϵ-ADP does not influence the enzymatic activity of glutamate dehydrogenase (table 1) nor does it show any interaction signal in the circular dichroism of fluorescence spectra. Therefore, since ϵ-ADP cannot substitute for ADP, the nonactive NADH binding site for which ADP competes with NADH [4] appears to be highly specific with respect to the intact adenine part. This suggests that ϵ-NADH, like ϵ-ADP does not bind to the nonactive NADH binding site. This is further supported by the lack of the ϵ-NADH self-inhibition and by the circular dichroism spectra of ϵ-NADH complexes with glutamate dehydrogenase in the presence of GTP (fig. 2). The Cotton effects of the dihydronicotinamide chromophore observed with the binary enzyme-ϵ-NADH complex and the ternary complexes with L-glutamate or 2-oxoglutarate closely resemble those obtained with the natural coenzyme [11]. In the presence of GTP (fig. 2, curves 2 and 4), however, no change of the 340 nm Cotton effects to more negative ellipticity occurs, which would have been expected from the results of NADH binding studies to the nonactive binding site [11,12].

The fluorescence of both coenzyme analogues is strongly enhanced upon binding to glutamate dehydrogenase (table 1). From the comparison of the quantum yields of ϵ-AMP and ϵ-NAD^+ [2] an about 12-fold increase of the fluorescence is expected for the open conformation of ϵ-NAD^+. However, the observed 21-fold enhancement most likely in addition reflects interactions of the ϵ-adenine chromophore with the enzyme environment. Contrary to these results, in the case of lactate dehydrogenase no significant fluorescence enhancement of ϵ-NAD^+ was observed [9], indicating a different environment of the bound ϵ-adenine chromophore in both enzymes. The fluorescence of bound ϵ-NADH contains the emission of both, the ϵ-adenine and the dihydronicotinamide chromophores. Since the emission bands overlap, only an estimate of the enhancement of either chromophore can be given.

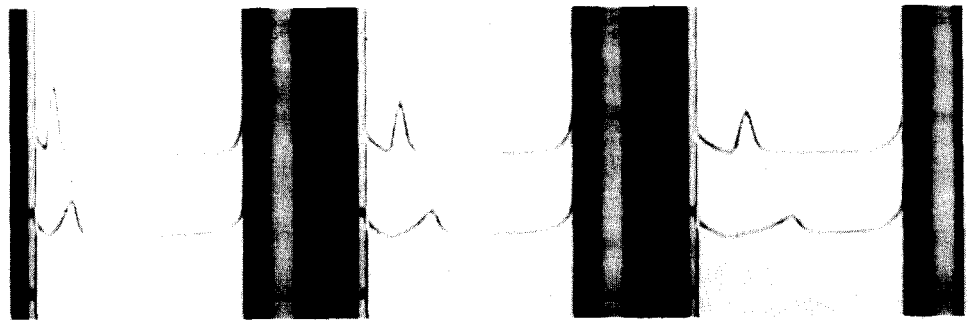

Fig. 3. Sedimentation pattern of glutamate dehydrogenase in the presence of ε-NADH. 2.5 mg/ml enzyme; 2 mM ε-NADH; measurements in 0.067 M phosphate buffer, pH 7.6, at 20°C in 12 mm normal and wedge-window cells. Lower curve: enzyme-ε-NADH (s_{20} = 23.3 S); Upper curve: enzyme-ε-NADH-2 mM GTP (s_{20} = 12.8 S); pictures were taken 4, 8, and 12 min after the rotor had reached full speed (68 000 rpm).

The value of Q = 10.5 (table 1) is obtained at wavelengths where the ε-adenine fluorescence predominates, whereas excitation at 340 nm (which favors the dihydronicotinamide chromophore) yields Q = 8.5 at the emission wavelength of 450 nm.

The plot program used for the determination of the enhancement factor is very accurate with respect to Q, but allows only a rough estimate of the dissociation constants [5]. On the assumed basis of one binding site per polypeptide chain $K_{E,\ \epsilon-NADH}$ = 60 μM and $K_{E,\ glutarate,\ \epsilon-NAD^+}$ = 45 μM can be calculated, indicating a reduced affinity of the coenzyme analogues to glutamate dehydrogenase as compared to the natural coenzyme [4,13].

Finally, the influence of ε-NADH on the association equilibrium of glutamate dehydrogenase was studied. Fig. 3 shows that ε-NADH in the presence of GTP provokes dissociation into the 13 S component, the unimer [1], whereas ε-NADH alone leaves the association equilibrium unchanged as is shown by the persistence of the 23 S peak. Thus, ε-NADH can substitute for NADH also with respect to the dissociation of glutamate dehydrogenase, indicating again that the 'dissociating' NADH binding site is the 'active' binding site.

Acknowledgements

We thank Mr L. Cobianchi for his excellent experimental help. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138: Biologische Grenzflächen und Spezifität) and the Fonds der Chemischen Industrie.

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