Biochimica et Biophysica Acta, 994 (1989) 187–190
Elsevier

BBA 30240

BBA Report

Time-resolved fluorescence studies on NADH bound to mitochondrial malate dehydrogenase

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(Received 18 May 1988)

Key words: Malate dehydrogenase; NADH; Time resolved fluorescence; Fluorescence Polarization

Time-resolved fluorescence studies on the emission of NADH bound to porcine heart mitochondrial malate dehydrogenase ((*S*)-malate:NAD^+ oxidoreductase, EC 1.1.1.37), in the presence and absence of saturating levels of hydroxymalonate, were carried out. The lifetime of NADH bound in the ternary complex was determined to be 9.5 ns compared to 1.74 ns as reported in the literature. Steady-state and dynamic polarization data indicated a Debye rotational relaxation time in the range of 106–109 ns for the dimeric enzyme. This value is significantly larger than that calculated for a spherical protein and is consistent with the asymmetric dimer found by crystallographic studies.

The photophysical properties of reduced nicotinamide adenine dinucleotide (NADH) and related compounds have been the subject of numerous investigations (see, for example Refs. 1–5). The alterations in the absorption and fluorescence properties of NADH upon binding to certain proteins, specifically dehydrogenases, have been known for many years. In most cases a significant increase in the quantum yield and a blue-shift in the emission maximum accompanies binding of NADH to the coenzyme site. The extent of these changes is different for binary (enzyme plus coenzyme) and ternary (enzyme, coenzyme plus substrate/analog) complexes. These spectroscopic changes have primarily been used to obtain binding isotherms and kinetic parameters; less attention has been given to the utilization of the intrinsic NADH fluorescence to monitor hydrodynamic aspects of the protein. Two recent reports [2,3] on NADH bound to porcine heart mitochondrial malate dehydrogenase (mMDH) quoted lifetime and dynamic polarization data on the ternary complex (NADH/mMDH/hydroxymalonate). Hönes et al. [2,3] used the multifrequency phase fluorometry method for their time-resolved studies and reported a lifetime of 1.74 ns for the NADH emission from this ternary complex and a rotational correlation time (at 25°C) of 27 ns for the enzyme. Furthermore, these researchers reported a fast, local motion for NADH bound to mMDH in the ternary complex. The results of our time-resolved studies on this system are, however, quite different as described below.

Porcine heart mitochondrial malate dehydrogenase ((*S*)-malate:NAD^+ oxidoreductase, EC 1.1.1.37) was purchased from U.S. Biochemicals, Inc. (Cleveland, OH) and was further purified on a Matrix Gel Red A column (Amicon Corp., Danvers, MA) following the protocol of Smith and Sundaram [6]. The specific activity of these preparations were typically in the 1000–1300 units/mg range. NADH was purchased from Sigma and used without further purification. Hydroxymalonate (tartronic acid) was from Aldrich Chemical Co. and was recrystallized from ethanol/water.

Steady-state polarization measurements were carried out on a homebuilt, photon-counting polarization photometer equipped with calcite prism polarizers. The sample temperature was maintained at 25°C with a circulating bath. Time-resolved data were obtained using a homebuilt multifrequency phase and modulation fluorometer based on the Gratton design [7]. Excitation for both steady-state and time-resolved measurements was accomplished using the 364 nm line of a Spectra-Physics Model 2025 Argon-Ion laser. For lifetime measurements the exciting light was polarized parallel to the vertical laboratory axis while the emission was viewed through a polarizer oriented at 55°. Emission was observed through a Schott KV399 cuton filter which passes wavelengths longer than 390 nm.

Abbreviation: mMDH, mitochondrial malate dehydrogenase, EC 1.1.1.37.

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In the multifrequency phase and modulation technique the intensity of the exciting light is modulated (in our instrument a Pockels Cell is utilized) and the phase shift and relative modulation of the emitted light, with respect to the excitation, are determined. Phase and modulation lifetimes (τ^P and τ^M) are then calculated according to:

$$\tan[\theta] = \omega\tau^P$$

$$M = [1+(\omega\tau^M)^2]^{-1/2}$$

where θ is the phase shift in degrees, M the relative modulation (AC/DC ratio) and ω the angular modulation frequency [8]. An emitting system characterized by a single exponential decay will give identical phase and modulation lifetimes irrespective of the light modulation frequency whereas, in the case of heterogeneous emission (multiple non-interacting fluorescent species) the phase lifetime will be less than the modulation lifetime and both values will decrease with increasing light modulation frequency [8]. The measured phase and modulation values may be analyzed assuming either a sum of exponentials [9] or with other models such as a continuous distribution of lifetimes [10].

In the dynamic polarization measurements the sample is illuminated by light polarized parallel to the vertical laboratory axis with intensity modulated at variable frequencies. The phase delay ($\Delta\phi$) between the perpendicular and parallel polarization components of the emission can then be directly determined as well as the ratio (Y) of their AC components. For an isotropic rotation one obtains the expressions [11].

$$\Delta\phi = \tan^{-1}\{(3\omega rR)/[(k^2+\omega^2)(1+r-2r^2)/6+R(6R+2k+kr)]\}$$

$$Y^2 = \{[k+6R/(1-r)]^2+\omega^2\}/\{[k+6R/(1+2r)]^2+\omega^2\}$$

where r is the limiting anisotropy, R the rotational diffusion coefficient and k the radiative rate constant ($1/\tau$).

The phase and modulation data obtained on NADH alone, NADH bound to mMDH, and NADH bound to mMDH in the presence of saturating hydroxymalonate are shown in Fig. 1. The solid lines correspond to the best fits calculated with the non-linear least-squares method [9]. For the case of free NADH, the best single exponential value obtained was 0.46 ns. The value is in reasonably good agreement with previously reported lifetimes of NADH which range from 0.40 to 0.44 ns for single exponential fits [4,5]. We note that biexponential decay for NADH has been reported [5]; since the present work is not intended to focus on free NADH we did not attempt more refined analysis on this system. For the NADH/mMDH binary sample the data were best fit by two exponential decays of 0.46 and 1.1 ns

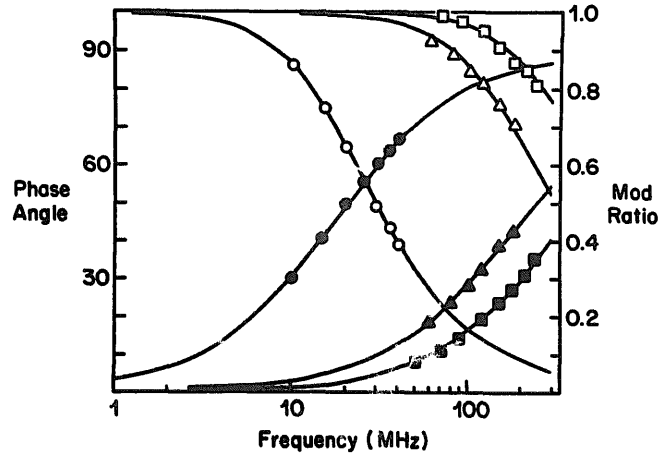

Fig. 1. Multifrequency phase (closed symbols) and modulation (open symbols) data for 10^{-5} M NADH in buffer (squares), 10^{-5} M NADH plus 10^{-5} M mMDH dimer (triangles) and 10^{-5} M NADH/10^{-5} M mMDH plus 10^{-2} M hydroxymalonate circles). The buffer was 50 mM Tris-HCl (pH 7.4) (25 °C). Solid lines correspond to calculated curves with component lifetimes and fractional intensities as indicated in the text.

with fractional intensities of 0.30 and 0.70, respectively. The 0.46 ns component is attributable to free NADH whereas the 1.1 ns component corresponds to protein bound NADH. For the ternary complex the best fit was obtained using discrete exponentials of 9.5 and 1.1 ns with relative fractional intensities of 0.995 and 0.005, respectively. We note that at the protein and coenzyme concentrations utilized a significant fraction of the NADH remains unbound in the absence of hydroxymalonate whereas addition of hydroxymalonate decreases the dissociation constant for the enzyme/coenzyme interaction and leads to almost complete binding of the NADH. A steady-state polarization value of 0.374 ± 0.004 (with excitation at 364 nm) was obtained for the ternary complex at 25 °C. The dynamic polarization results, shown in Fig. 2, were best fit with a

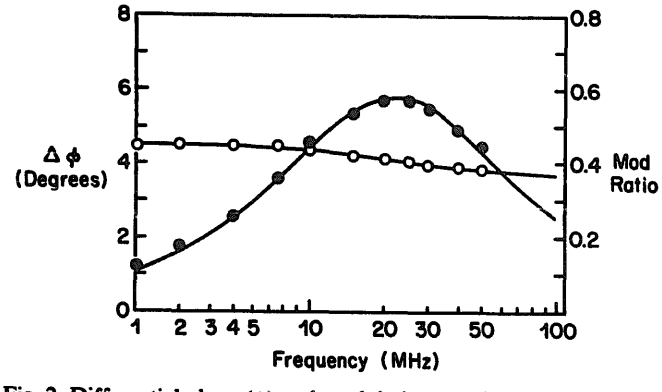

Fig. 2. Differential phase (●) and modulation (○) data for the NADH emission from the ternary complex, NADH/mMDH/hydroxymalonate. Solid lines are the calculated curves corresponding to a Debye rotational relaxation time of 109 ns and a limiting polarization of 0.46.

single Debye rotational relaxation time of 109 ± 3 ns and a limiting polarization value of 0.460 (which corresponds to a limiting anisotropy value of 0.362). The fit did not improve by incorporation of a second, fast rotation which would correspond to local motion of the fluorophore.

Our results clearly indicate that the lifetime of NADH bound to mMDH increases substantially (1.1 to 9.5 ns) upon formation of the ternary complex with hydroxymalonate. This result is consistent with the increase in the quantum yield of NADH upon formation of the ternary complex [3] and suggests that hydroxymalonate may effectively exclude water molecules from the coenzyme's environment as Baumgarten and Hönes suggest. The difference between our lifetime results for the ternary complex and those of Hönes et al. may be only apparent since they did not specify if their value of 1.74 ns represented the best fit for the entire frequency range utilized or if it actually was the phase lifetime recorded at a single frequency. In the latter case, a high modulation frequency would weight a short component, which could correspond to NADH in the binary complex or free NADH. Since our present purpose was to demonstrate the significant increase in the lifetime of NADH in the ternary complex compared to the binary complex and free NADH we did not carry out a more refined analysis of the decay mode. Biexponential decays for NADH bound in ternary complexes to liver alcohol dehydrogenase have been reported [4] and a more complex decay pattern for NADH in the mMDH ternary complex has not been ruled out. We observed that our lifetime data for the ternary complex could also be well fit to a continuous distribution model [8] with a center lifetime value near 9.5 ns and a width of several tenths of a nanosecond; more detailed work is required, however, to ascertain if the distribution approach to these results are more appropriate than the discrete component approach and, if so, the molecular origins of the distribution.

Using the 9.5 ns lifetime value, in conjunction with the steady-state polarization value (P) and the limiting polarization (P_0) of 0.460 (obtained independently from the dynamic polarization results and from the excitation polarization spectrum of NADH in glycerol at $-55\,^\circ$C (Jameson and Wong, unpublished data)) we find a Debye rotational relaxation time of 106 ± 8 ns according to the Perrin equation [12]:

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + 3\tau/\rho)$$

where τ is the fluorescence lifetime and ρ the Debye rotational relaxation time. The dynamic polarization results on the ternary complex indicate a Debye rotational relaxation time of 109 ± 6 ns, in good agreement with the steady-state results.

The Debye rotational relaxation time of a rigid, spherical protein, ρ_0, may be approximated by [13]:

$$\rho_0 = \frac{3\eta V}{RT} = \frac{3\eta M(\bar{v} + h)}{RT}$$

where V is the protein's molar volume, η the solvent viscosity, R the gas constant, T the absolute temperature, M the molecular weight, $\bar{v}$ the partial specific volume and h the degree of hydration. For mMDH with a molecular weight of 66600 in aqueous buffer ($\eta = 0.89$ cP), assuming a partial specific volume of 0.75 ml/g (calculated from the amino acid sequence [14] using the method of Cohn and Edsall [15]) and a hydration of 0.22 ml/g, one calculates a rotational relaxation time of 71 ns.

Hönes et al. [2,3] reported a rotational correlation time of 27 ns for mMDH at 25 °C which corresponds to a Debye rotational relaxation time of 81 ns. They have interpreted their results as evidence for a spherical mMDH molecule. The 106–109 ns range we found is, however, significantly longer than the value calculated for a spherical protein, and suggests a nonspherical mMDH dimer. Sedimentation and diffusion results, from analytical ultracentrifugation, have also suggested that pig heart mMDH is an asymmetrical dimer [17]. In fact, X-ray crystallographic analysis [18] has indicated that the mMDH dimer is an ellipsoid with a 2 : 1 axial ratio. One should note, however, that the actual rotational relaxation values obtained depend upon detailed aspects of the fluorophore/macromolecular complex, such as axial ratios and relative orientation of the absorption and emission oscillators of the fluorophore to the principal rotational axes of the protein [16]. Hence, attempts to assign observed rotational relaxation rates to specific macromolecular axes can be equivocal.

Hönes et al. [2,3] also reported observing a fast local rotation with a correlation time of 60 ps for NADH in the ternary complex while no such local motion was detectable for NADH in the binary complex. We were not able to sensibly improve on differential phase fits for the ternary complex by incorporation of a second rotation component. However, since the associated amplitude of the fast component was not indicated by Hönes et al. a quantitative comparison of the two results is not possible.

In summary our results indicate that the lifetime of NADH bound to mMDH increases quite substantially upon binding of hydroxymalonate and that the mMDH dimer exhibits a hydrodynamic character consistent with a nonspherical molecule.

This work was supported in part by Grant DMB-8706440 from the National Science Foundation. D.M.J. is an Established Investigator of the American Heart Association.

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