Vol. 86, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1979 Pages 285-292

THE INTERACTION OF ADENINE WITH ITS BINDING SITE IN RABBIT MUSCLE GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE STUDIED BY FLUORESCENCE DECAY

Ari Gafni

Department of Chemical Physics
The Weizmann Institute of Science
Rehovot, Israel

Received November 21,1978

SUMMARY

The fluorescence decay mechanism of $1,N^6$-ethenoadenosine diphosphoribose bound to rabbit muscle glyceraldehyde 3-phosphate dehydrogenase markedly differs from that of the intact coenzyme analog (εNAD^+) bound to the same enzyme. In the latter case the fluorescence is partially quenched by interactions between the ethenoadenine ring and amino acid residues in its binding site. Binding of the nicotinamide moiety of the coenzyme thus affects the relative orientation of the adenine ring within its binding site leading to the quenching interactions. The interactions of the adenine group with its binding site induce conformational changes in the enzyme which affect the binding of additional coenzyme molecules. The nicotinamide base thus determines, indirectly, the negative cooperativity found in NAD^+ binding.

INTRODUCTION

The interactions of NAD^+ and NADH with dehydrogenases have been the subject of numerous studies. The conformation of the bound coenzyme molecule was found to be fully unfolded in most cases (1-3). Similar results were obtained for the fluorescent coenzyme analog, εNAD^+[1] bound to several dehydrogenases, both by steady state and nanosecond fluorescence decay measurements (4,5). Some ambiguity existed regarding the conformation assumed by the coenzyme when bound to GPDH. The fluorescence energy transfer study of Velick (3) indicated that the reduced coenzyme was in the folded

[1] Abbreviations: εNAD^+, nicotinamide, $1,N^6$-ethenoadenine dinucleotide; εADP and $\varepsilon ADPR$, $1,N^6$-ethenoadenosine diphosphate and diphosphoribose, respectively; GPDH, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase; LDH, beef heart lactate dehydrogenase; LADH, horse liver alcohol dehydrogenase.

0006-291X/79/020285-08$01.00/0

conformation in this case and the NMR results of Lee *et al.* (6) led these authors to the conclusion that the conformation of NAD^+ when bound to GPDH, differed from that of the coenzyme bound to other dehydrogenases. However more recent X-ray crystallographic studies have shown the coenzyme to be bound to GPDH in the unfolded conformation (7).

GPDH differs from other dehydrogenases in the mode of interaction with NAD^+, and exhibits strong negative cooperativity in its binding (8,9). A similar effect was reported to accompany the binding of εNAD^+ to GPDH (10). It was suggested that the conformation of the nicotinamide binding subsite in one subunit was unaltered upon NAD^+ binding to another subunit and that the sequential conformational changes were limited to the adenine binding subsite (10). The possibility that the conformational changes in the adenine binding site are affected by the nicotinamide moiety has been recently suggested by Henis and Levitzki (11).

In a previous study (5) a dramatic difference was found between the fluorescence decay mechanisms of εNAD^+ bound to GPDH and when bound to LADH or LDH. It was concluded that the fluorescence of εNAD^+ bound to GPDH was partially quenched by (dynamic) interactions with amino acid residues in its binding site. In the present study we examined the fluorescence decay of εADPR in solution and when bound to GPDH. A comparison of the results with those of the GPDH:εNAD^+ complex provides direct evidence for the marked effect of the nicotinamide moiety on the interactions between the adenine ring and its binding site.

MATERIALS AND METHODS

εADP and NADase (N. Crassa) were both purchased from Sigma and used without further purification. GPDH and εNAD^+ were kindly donated by A. Levitzki and Y. Henis (The Hebrew University, Jerusalem) who had prepared the enzyme from rabbit muscle according to Ferdinand (12) and further purified it to yield homogeneous enzyme of high specific activity (11).

Apo GPDH was prepared from GPDH following the procedure of Henis and Levitzki (11). The apo enzyme was substantially free from NAD^+ as evidenced by the A_{280}/A_{260} ratio of 1.8-1.9 . Enzyme concentration was

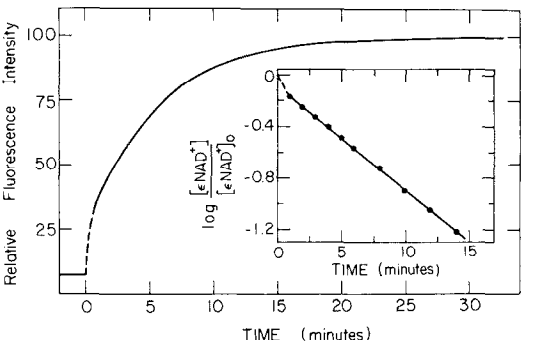

Figure 1. Time dependence of the fluorescence intensity of εNAD^+ following the addition of NADase. The excitation wavelength was 315nm and the fluorescence was observed at 410nm. Initial εNAD^+ concentration ($[\varepsilon NAD^+]_o$) was 7×10^{-5}M. More details are given in the experimental section. Insert, relative concentration of εNAD^+ (log. scale) calculated from the fluorescence enhancement curve, as a function of time following NADase addition.

determined spectrophotometrically from the optical density at 280nm (13). Concentrations of εNAD^+ and εADP were determined spectrophotometrically according to Luisi *et al.* (4).

εADPR was prepared from εNAD^+ by cleavage with NADase. The contents of one vial of NADase (∿0.8 units) were dissolved in ∿0.5ml of 50mM HEPES buffer solution (pH=7.4) which contained 10mM EDTA. This solution was added to 1ml of εNAD^+ solution in the same buffer and the mixture was kept at room temperature (∿23°C). The reaction was followed fluorometrically using the enhancement of the ethenoadenine fluorescence upon cleavage.

Complexes with GPDH were prepared by adding the appropriate volume of the εNAD^+ or εADPR solution to a 8×10^{-5}M GPDH solution in 50mM HEPES 10mM EDTA buffer (pH=7.4).

Nanosecond fluorescence decay measurements were done with an instrument built in this laboratory and described elsewhere (14). The excitation wavelength was 315nm and the fluorescence was observed through a Schott KV394 filter transmitting light above ∿400nm. The fluorescence decay curves were analyzed for one or two exponential components using the method of nonlinear least squares (15). The quality of the fit between the experimental and calculated curves was judged from the root mean square of the deviations (RMS) and the autocorrelation function of the deviations (15).

RESULTS AND DISCUSSION

The cleavage of εNAD^+ by NADase, to form εADPR, is accompanied by a marked increase in the fluorescence intensity as the possibility for stacking of the ethenoadenine and nicotinamide bases is removed. This fluorescence enhancement was used to follow the cleavage and a typical experiment is shown in Figure 1. The concentration of εNAD^+

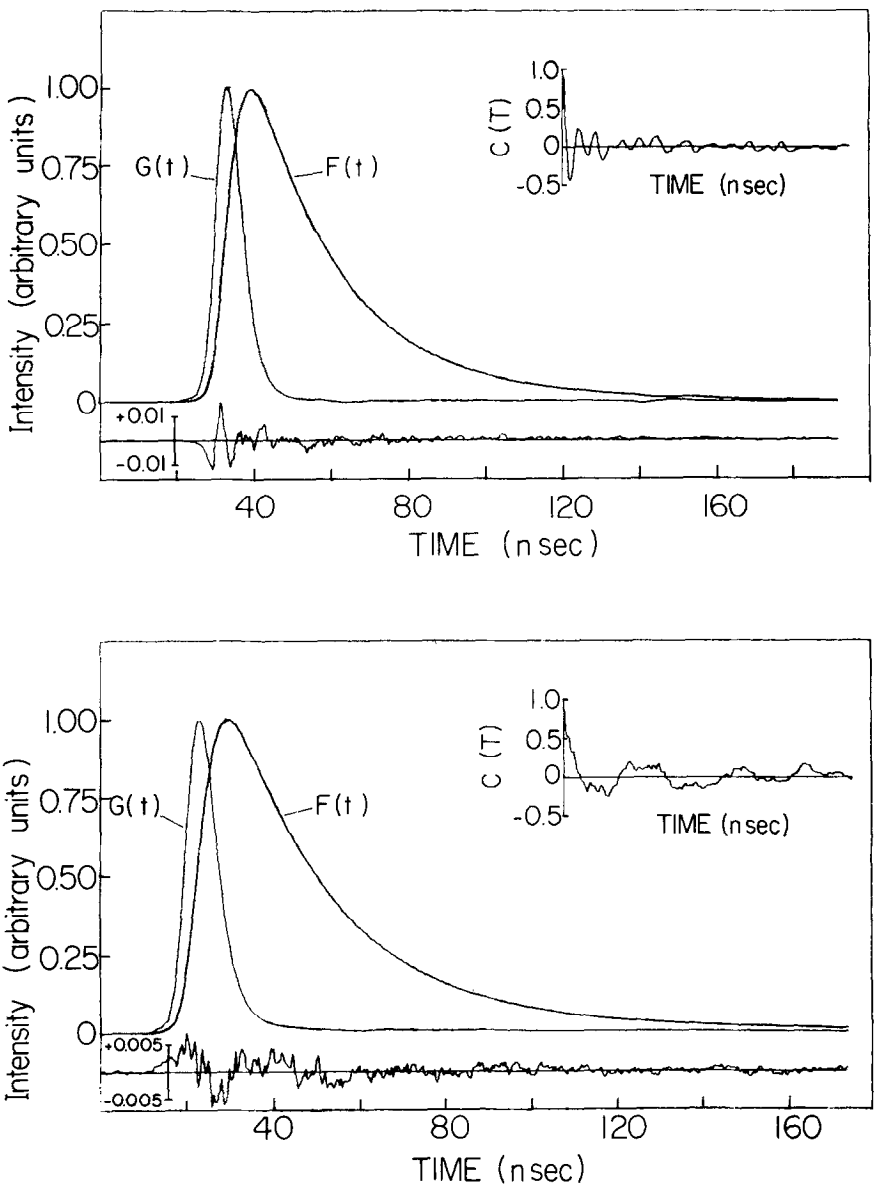

Figure 2. Analysis for two exponential components of the fluorescence decay curves of 6×10^{-5}M εADPR in neutral aqueous buffer solution (upper part) and of the GPDH:εADPR complex (lower part). The sample contained 6×10^{-5}M GPDH and 12×10^{-5}M εADPR. More experimental details are given in the text. G(t), excitation light profile. F(t), experimental and calculated decay curves. Noisy curves at the bottom of the two plots are the traces of the deviations between the experimental and calculated curves. The inserts describe the autocorrelation function of the deviations (15). The decay times and the relative amplitudes obtained in the analyses are summarized in Table I.

was found to decrease according to a first order reaction mechanism. No change in the absorption at 315nm (the excitation wavelength) was detected during the cleavage.

The fluorescence decay curve of εADPR in HEPES:EDTA buffer (pH=7.4) is shown in the upper part of Figure 2. A good fit to the experimental data could not be obtained using a single exponential component. Analysis using two components resulted in a very good fit. This finding is in contrast to the fluorescence of εADP in phosphate buffer which follows a monoexponential decay mechanism (see Table I). The origin of the short decay component in the εADPR sample may be the presence of a fluorescent impurity (possibly originating in the NADase extract) in this sample (note that the short decay component constitutes only about 2% of the steady state fluorescence intensity). The lower part of Figure 2 presents the fluorescence decay curve of a GPDH:εADPR complex prepared by mixing the enzyme and nucleotide in a molar ratio of 1:2. Similar results were obtained when a molar ratio of 1:1 was used as shown in Table I. The binding constant of the GPDH:εADPR complex has been recently found to be $4.2 \times 10^{4} M^{-1}$ (16). Hence at the concentrations used more than 60% of the εADPR molecules were bound to the enzyme. The similarity between the decay constants of free and GPDH bound εADPR cannot, therefore, be attributed to a large excess of free nucleotide. This similarity is striking especially when one compares it with the decay of εNAD^{+} bound to GPDH. While the fluorescence of the dinucleotide bound to LDH or LADH was found to decay monoexponentially with a lifetime similar to that of (free) εADP or εAMP, the decay of εNAD^{+} when bound to GPDH is biexponential and the major decay component has a lifetime of 7.3nsec (see Table I). This shortening of the fluorescence decay time of the ε-adenine ring (compared to the values obtained for εNAD^{+} bound to LDH or LADH) cannot be due to interactions with the nicotinamide moiety (which are responsible for the short lifetime of free εNAD^{+} fluo-

Table I. Fluorescence Decay Data for Ethenoadenine Derivatives Under Various Conditions

Sample[a]	One component		Two components				
	τ nsec	RMSx10^4	τ_1 nsec	τ_2 nsec	α_1	α_2	RMSx10^4
εADP (30μM) in 0.1M phosphate buffer (pH=7.4)	24.3	25	single exponential				
εADPR (60μM)	21.6	97	1.3	22.4	0.19	0.81	31
GPDH (60μM):εADPR (60μM)	22.3	106	0.6	22.6	0.22	0.78	16
GPDH (60μM):εADPR (120μM)	22.4	93	1.3	22.9	0.09	0.91	17
GPDH (80μM):εNAD^+ (80μM)	8.6	91	7.3	22.5	0.91	0.09	18

[a] Unless otherwise stated, measurements were carried out in 50mM HEPES buffer (pH=7.4) containing 10mM EDTA.

rescence (5)) since X-ray crystallographic studies clearly prove the coenzyme to be fully unfolded when bound to GPDH. We conclude that the quenching is due to an interaction between the ethenoadenine and an amino acid residue in its binding site. Tryptophan and methionine have indeed been shown to quench ε-adenine fluorescence (16,17). The lack of fluorescence quenching of εADPR bound to GPDH proves that in this case the quenching residue is not in contact with the adenine, hence the interaction of the adenine with its binding site is different when the nicotinamide is absent. Binding of the pyridine moiety of the coenzyme thus affects the relative orientation of the adenine moiety with respect to its binding site. The mode of interaction of the adenine group with its binding site determines the conformational changes in the enzyme which affect the binding of additional coenzyme molecules to the enzyme (10,11). The negative cooperativity found in NAD^+ binding to GPDH may thus be (indirectly) determined by the binding of the nicotinamide base.

REFERENCES

1. Rossman, M.G., Liljas, A., Bränden, C.I., and Banaszak, L.J. in Boyer, P.D. (ed.) The Enzymes, Vol. 11, part A, 61-102, Academic Press, New York (1975).
2. Bränden, C.I., Jörnvall, H., Eklund, H., and Furugren, B. in Boyer, P.D. (ed.) The Enzymes, Vol. 11, part A, 103-190, Academic Press, New York (1975).
3. Velick, S.F. in McElory, W.D., and Glass, B. (eds.) Light and Life, 108-143, Johns Hopkins Press, Baltimore (1961).
4. Luisi, P.L., Baici, A., Bonner, F.J., and Aboderin, A.A., Biochemistry (1975) 14:362-368.
5. Gafni, A., in Sund, H. (ed.) Pyridine Nucleotide Dependent Dehydrogenases, 237-248, Walter de Gruyter, Berlin (1977).
6. Lee, C.Y., Eichner, R.D., and Kaplan, N.O., Proc.Nat.Acad.Sci., USA (1973) 70:1593-1597.
7. Harris, J.I., and Waters, M., in Boyer, P.D. (ed.) The Enzymes, Vol. 13, 1-50, Academic Press, New York (1976).
8. Conway, A., and Koshland, D.E., Jr., Biochemistry (1968) 7:4011-4022.
9. Devijlder, J.J.M., and Slater, E.C., Biochim.Biophys.Acta (1968) 167:23-34.
10. Schlessinger, J., and Levitzki, A., J.Mol.Biol. (1974) 82:547-561.
11. Henis, Y.I., and Levitzki, A., J.Mol.Biol. (1978) in press.
12. Ferdinand, W., Biochem.J. (1964) 92:578-585.

13. Fox, J.B., Jr., and Dandliker, W.B., J.Biol.Chem. (1956) 218:53-57.
14. Hazan, G., Grinvald, A., Maytal, M., and Steinberg, I.Z., Rev.Sci. Inst. (1974) 45:1602-1604.
15. Grinvald, A., and Steinberg, I.Z., Anal.Biochem. (1974) 59:583-598.
16. Gafni, A., Biochemistry (1979) in press.
17. Harvey, S.C., and Cheung, H.C., Biochem.Biophys.Res.Commun. (1976) 73:865-868.