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Pig Liver 6-Phosphogluconate Dehydrogenase

I. FLUORESCENCE STUDY OF COENZYME BINDING

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INTRODUCTION

The fluorescence characteristics of the reduced pyridine nucreotides are altered by binding to specific apodehydrogenase (1). Usually the fluorescence emission maximum is shifted to a shorter wave length and, in most cases, the fluorescence intensity is increased (except for glyceraldehydephosphate dehydrogenase, where a decrease was observed (2)). The changes in fluorescence yields are significant and have been used by many workers to study the stoichiometry of nucleotide binding, site dissociation constant, and other properties of the enzyme-nucleotide complex. They have yielded useful information toward the understanding of the mechanism of action of many nicotinamide adenine dinucleotide-reduced (NADH)-linked dehydrogenases (2—7). However, information on nicotinamide adenine dinucleotide phosphate-reduced (NADPH)-linked dehydrogenases is limited to two enzymes, NADPH-linked isocitrate dehydrogenase (8) and malic enzyme (9).

The availability of large amounts of purified 6-phosphogluconate dehydrogenase (11) permitted a study of the NADPH binding of this enzyme by fluorimetry.

This paper presents the results of studies on the fluorescence spectra of the enzyme-NADPH complex, the stoichiometry of binding, the affinity between enzyme and NADPH, and the specificity of binding.

The number of possible binding sites was determined. Some aspects of the interaction between 6-phosphogluconate and enzyme-NADPH complex are presented and discussed.

MATERIALS AND METHODS

Purified pig liver 6-phosphogluconate dehydrogenase was obtained by the method of reference 11. This enzyme does not contain bond $NADP^+$ as is evidenced by its $A_{280} : A_{260}$ ratio of 1.75. Purity and homogeneity of the enzyme were demonstrated (11). The enzyme was dialyzed against 0.1 M potassium phosphate buffer at pH 7.0 before use.

NADH, NADPH, 6-phosphogluconate (Boehringer Mannheim Yamanouchi) were purchased from the specified sources. Deionized, distilled water was used throughout these experiments. Nucleotide solutions were made up fresh daily, and the concentrations of $NADP^+$ and NADPH were determined spectrometrically with the use of molar extinction coefficients of 14.9×10^3 and 6.2×10^3 at 260 nm and 340 nm, re-

spectively. Protein concentration was determined by the biuret method (10).

Fluorescence studies were carried out with the recording Hitachi MPF-2A spectrofluorometer equipped with a thermojacketed cuvette holder. Temperature was controlled with a constant temperature circulator to give 25 ℃ in the cuvvete. The sensitivity of the instrument was changed between experiments to meet the requirements of each experiment ; the fluorescence intensity values from different experiments can not be directly compared.

LESULTS

Fluorescence Spectra of Free NADPH, Free Enzyme-NADPH Complex

The excitation and emission spectra of NADPH and enzyme-NADPH complex are shown in Fig. 1 and Fig. 2, respectively.

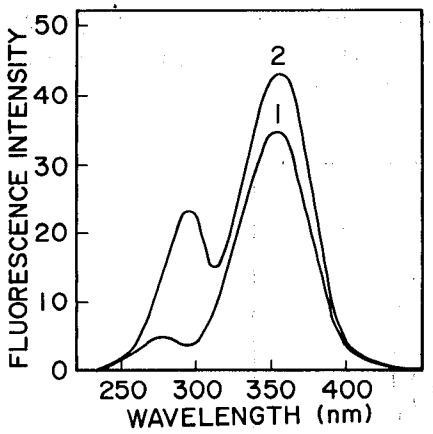

Fig. 1.

Nucleotide excitation spectra of NADPH and of 6-phosphogluconate dehydrogenase with bound NADPH. excitation spectra ; 1, cuvette contained 100 μ moles potassium phosphate buffer at pH 7.0 and 2.16 mμmoles of NADPH in a total volume of 1.0 ml, 2, 20 μl of purified 6-phosphogluconate dehydrogenase (0.25 mg)was added to cuvette 1. Emission was measured at 460 nm.

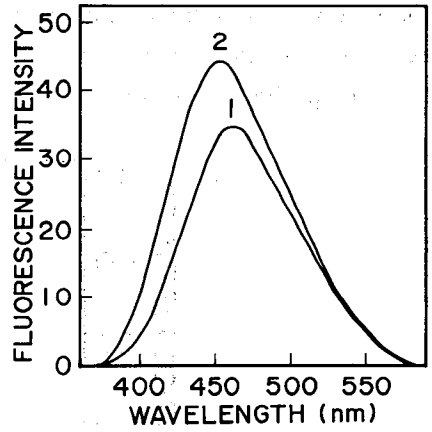

Fig. 2.

Nucleotide emission spectra of NADPH and of NADPH bound to 6-phosphogluconate dehydrogenase. emission spectra ; cuvettes 1 and 2 were the same as in Fig. 1. Excitation was at 355 nm.

The spectra were monitored at emission and excitation wave lengths of 460 nm and 355 nm, respectively, where free nuleotide fluorescence occurs. Curves 1 and 2 represent the spectra for free NADPH and enzyme-NADPH complex, respectively. The spectra for the complex were determined with an excess of enzyme so that free NADPH concentration was minimal. Two excitation maxima were observed for free NADPH, a week peak at 275 nm, and a strong peak at 355 nm. Upon the addition of 6-phosphogluconate dehydrogenase, the 275 nm peak disappeared, and a new peak at 293 nm was formed. The 355 nm excitation maximum was not shifted; however, the emission intensity was enchanced. The emissin maximum for free NADPH was at 462 nm. Enzyme binding was accompanied by enhancement of fluorescence and a blue shift to a new emission maximum at 455 nm.

The nucleotide effect was specific for NADPH, and no fluorescence enhancement was observed when NADH was used instead of NADPH. The excitation and emission spectra of 6-phosphogluconate dehydrogenase and enzyme-NADPH complex are presented in Fig. 3 and Fig. 4, respectively.

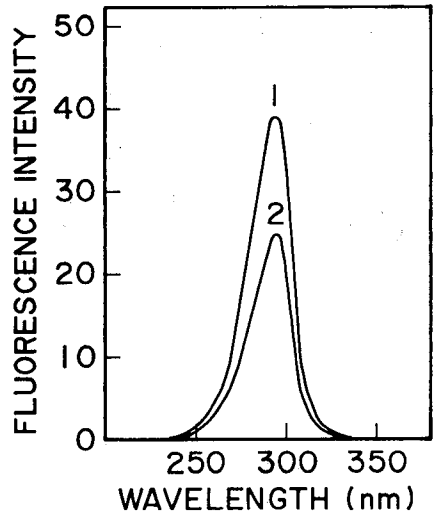

Fig. 3.

Protein excitation spectra of 6-phosphogluconate dehydrogenase-NADPH complex. excitation spectra; 1, cuvette contained 100 mμmoles of potassium phosphate buffer, pH 7.0 and 0.25 mg of purified 6-phosphogluconate dehydrogenase in a total volume of 1.0 ml; 2, 20 μl of NADPH (10.2 mμmoles) were added to cuvette 1. Emission was measured at 335 nm.

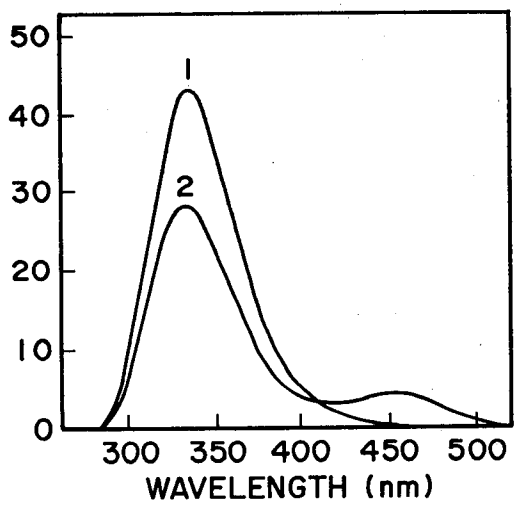

Fig. 4.

Protein emission spectra of 6-phosphogluconate dehydrogenase and of 6-phosphogluconate dehydrogenase-NADPH complex.

emission spectra; cuvettes 1 and 2 were the same as in Fig. 3. Excitation was at 293 nm.

Protein fluorescence was measured. Cuvette 1 contained free enzyme and cuvette 2 contained enzyme—NADPH complex. The spectra for the complex were determined with an excess of NADPH so that free enzyme concentration was minimal. The free protein had an excitation maximum at 293 nm, and an emission maximum at 335 nm. After the addition of NADPH, protein fluorescence intensity was diminished, but the excitation and emission maxima remained the same. A small emission maximum was also observed near 455nm.

The addition of 6-phosphogluconate had no effect on either the protein fluorescence of 6-phosphogluconate dehydrogenase or the nucleotide fluorescence of free NADPH.

However, as shown in Fig. 5, the fluorescence intensity of enzyme-NADPH complex was enhanced.

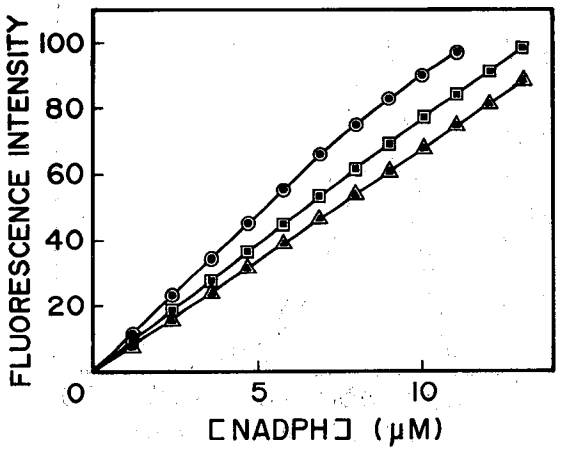

Fig. 5.

Effect of 6-phosphogluconate and $MnCl_2$ or $MgCl_2$ on nucleotide fluorescence of 6-phosphogluconate dehydrogenase-NADPH complex.

Each cuvette cotained 100 μmoles of potassium phosphate buffer at pH 7.0 and the following reagents in a total volume of 1.0 ml : ▲—▲, water ; △—△ 0.47 μmoles of 6-phosphogluconate and 2.9 μmoles of $MnCl_2$ or $MgCl_2$; ■—■, 3.25 mμmoles (0.34 mg) of purified 6-phosphogluconate dehydrogenase ; □—□, 1.96 mμmoles of enzyme and 2.9 μmoles of $MnCl_2$ or $MgCl_2$; ●—●, 1.96 mμ moles of enzyme and 0.47 μmoles of 6-phosphogluconate ; ○—○, 1.96 mμmoles of enzyme, 0.47 μmoles of 6-phosphogluconate, and 1.2 μmoles of $MnCl_2$ or $MgCl_2$.

Titration was made with 122 μM NADPH in 10 μl aliquots. Fluorescence was measured at excitation and emission wave lengths of 355 and 460 nm, respectively.

The metal requirment is unessential from the results of Fig. 5.

The titration curve of 6-phosphogluconate with enzyme-NADPH complex is shown in Fig. 6.

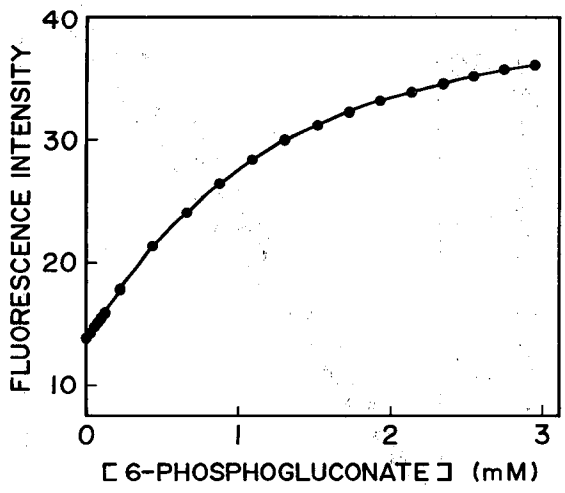

Fig. 6.

Effect of 6-phosphogluconate concentration on fluorescence intensity of 6-phosphogluconate dehydrogenase-NADPH complex. Cuvette contained 100 μmoles of potassium phosphate buffer at pH 7.0, 29 mμmoles of NADPH, 6.8 mμmoles (0.72 mg) of enzyme, and water to make 1.0 ml. 6-Phosphogluconate was used as the titrating agent. Excitation was at 355 nm; emission at 460 nm.

At low 6-phosphogluconate concentration a linear increase in fluorescence value was obtained.

The fluorescence value approached a maximum after the addition of 3.0 μmoles of 6-phosphogluconate to 6.8 mμmoles of enzyme.

Stoichiometric Titration of Enzyme with NADPH

The fluorescence enhancement of NADPH resulting from the formation of the binary enzyme-NADPH complex was large and was used to determine the stoichiometry of binding. Titration were made by the addition of small aliquots of NADPH to cuvettes containing either buffer or the enzyme solution. Fig. 7 shows a typical titration experiment with use of two levels enzyme.

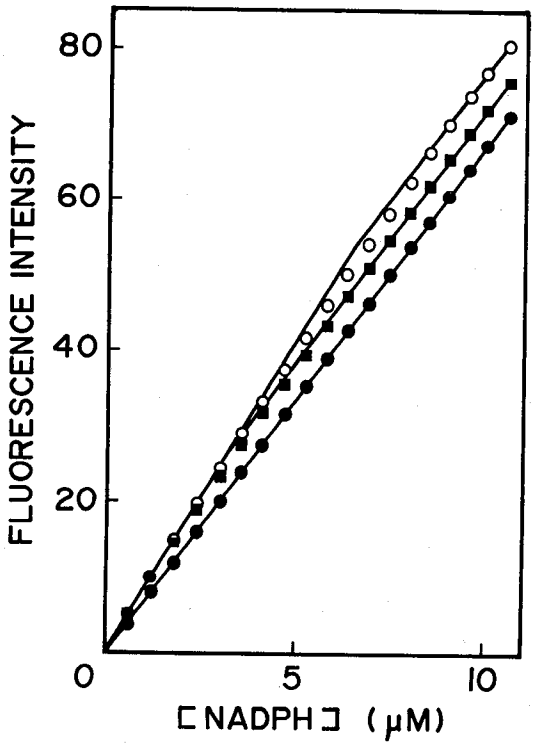

Fig. 7.

Titration curves of 6-phosphogluconate dehydrogenase with NADPH. All cuvettes contained 100 μmoles of potassium phosphate buffer at pH 7.0, water and the following: ●—●, no enzyme; ■—■, 1.63 mμmoles of enzyme; and ○—○, 3.25 mμmoles of enzyme. Total volume, 1.0 ml. Titration was carried out with the addition of 0.122 mM NADPH in 5 μl aliquots. Excitation was at 355 nm; emission at 460 nm.

Addition of NADPH to buffer alone (lower line) yielded linear increases in fluorescence intensity. In the presence of enzyme, the addition of aliquots of NADPH initially produced large increases in fluorescence value, owing to the formation of enzyme-NADPH complex. Fluorescence intensity was also proportional to NADPH concentration. When the titration was complete, further increments of NADPH were not bound, and the slope became parallel to the free NADPH line. As shown in Fig. 7, higher protein concentration did not produce an increased initial slope,

thus indicating that added NADPH was totally bound and that free NADPH concentration was negligible in this region. The intercept points were measured.

The NADPH equivalents estimated from the extrapolated intercepts were proportional to the amount of protein used in each cuvette.

Binding weights (grams of protein per mole of NADPH) were calculated to be 52,000 and 52,000 for 1.63 and 3.25 mμmoles of enzyme, respectively. Site dissociation constant (K) was obtained graphically by the method of Adelstein (7) from those titration values on Fig. 7 near the equivalent point where both free NADPH concentration were significant. The reciprocal plot of 1/F against 1/c (Fig. 8) gave straight lines for the two enzyme concentrations and intersect at the abscissa, indicating independent coenzyme site.

The site dissociation constant was calculated to be 6.7×10^{-7} M.

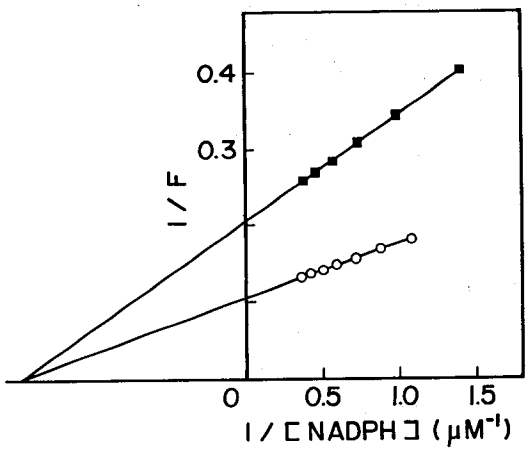

Fig. 8.

Titration plot of 1/F against 1/c of 6-phosphogluconate dehydrogenase with NADPH. All cuvettes contained 100 μmoles of potassium phosphate buffer at pH 7.0, water and the following: ■—■, 1.63 mμmoles of enzyme; and ○—○, 3.25 mμmoles of enzyme.

Total volume, 1.0 ml. Titration was carried out with the addition of 0.122 mM NADPH in 5 μl aliquots. Excitation was at 355 nm; emission at 460 nm.

Effect of 6-*Phosphogluconate and* $MnCl_2$ *or* $MgCl_2$ *on NADPH Binding*

The enhancement of enzyme-NADPH complex fluorescence intensity by 6-phosphogluconate indicated the formation of a tertiary complex.

The effect of 6-phosphogluconate could be on the fluorescence yield alone, on the number of binding sites for NADPH, or both.

Therefore, binding weight and site dissociation constant for NADPH in the presence of 6-phosphogluconate were determined by the titration method. Fig. 9 shows titration curves for two protein concentrations.

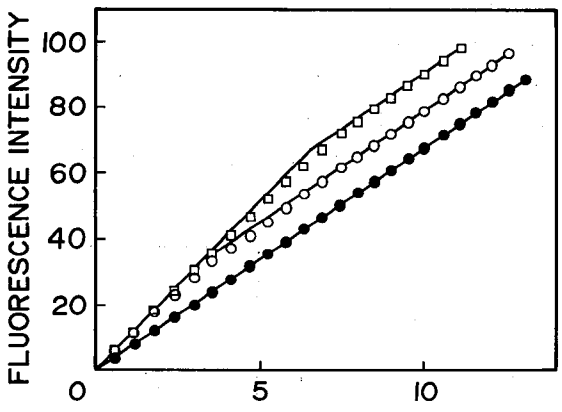

Fig. 9.

Titration of 6-phosphogluconate dehydrogenase with NADPH in the presence of 6-phosphogluconate. All cuvettes contained 100 μmoles of potassium phosphate buffer at pH 7.0, 0.468 μmoles of 6-phosphogluconate, water and the following: ●—● , no enzyme; ○—○ , 1.63 mμmoles of enzyme (1.71 mg); and □—□ , 3.25 mμmoles (3.41 mg) of enzyme; in a total volume of 1.0 ml. Titration was carried out with 5 μl aliquots of NADPH (0.122 mM). Excitation was at 355 nm; emission at 460 nm.

Except for the intensitified fluorescence, the binding patterns are similar to Fig. 7. The calculated binding weight were 52,000 and 52,000 for 1.63 and 3.25 mμmoles of enzyme, respectively. Site dissociation constant (K) was calculated to be 6.7×10^{-7} M.

Competition between $NADP^+$ and NADPH for Enzyme Binding

When enzyme-NADPH complex was titrated with $NADP^+$, fluorescence intensity

decreased, apparently owing to competition for the coenzyme-binding site. This effect was specific and not produced by NAD^+.

The ratios of site dissociation constants between NADPH and $NADP^+$ (K_{NADP^+} / K_{NADPH}). determined by titration of the complex with $NADP^+$ according to the method of Velick (2), are shown in Table I.

Table I

Competition of $NADP^+$ and NADPH for enzyme binding ※

The reaction mixtures consisted of 100 μmoles of potassium phosphate buffer pH 7.0, 9.6 mμmoles of NADPH, 0.24 mg of purified 6-phosphogluconate dehydrogenase, and water in a total volume of 1.0 ml.

Excitation was at 355 nm; emisson at 460 nm.

$NADP^+$ added	Fluorescence	Fraction increment	NADPH		$NADP^+$		K_{NADP^+} / K_{NADPH}
			Bound	Free	Bound	Free	
mμmoles			mμmoles	mμmoles	mμmoles	mμmoles	
0	80.4						
20.0	77.9	0.25	3.45	6.15	1.10	18.90	9.20
40.0	76.6	0.38	2.85	6.75	1.75	38.25	9.25
60.0	75.7	0.47	2.44	7.16	2.16	57.84	9.11
80.0	74.9	0.54	2.12	7.48	2.48	77.52	8.82
100.0	74.5	0.58	1.93	7.67	2.67	97.33	9.19
120.0	74.1	0.62	1.75	7.85	2.85	117.15	9.14
140.0	73.8	0.65	1.61	7.99	2.99	137.01	9.23
160.0	73.5	0.68	1.47	8.13	3.13	156.87	9.08
180.0	73.3	0.70	1.38	8.22	3.22	176.78	9.22
200.0	73.0	0.73	1.24	8.36	3.36	196.64	8.70

※ End point of titration was estimated to be 70.3 in fluorescence intensity by a cuvette containing buffer and the same amount of NADPH in a total volume of 1.0 ml with no enzyme present. Calculations were based on the assumption that $NADP^+$ bound equals NADPH released and on a binding weight of 52,000 g per mole.

The end point of titration (*i.e.* all bound NADPH replaced by $NADP^+$) was estimated by the fluorescence value of the same amount of NADPH in the absence of enzyme. The (K_{NADP^+} / K_{NADPH}) values obtained for different $NADP^+$ concentrations were in good agreement and gave an average of 9.09 ± 0.19. In a separate experiment, with the use of double amounts of NADPH and enzyme, the

average (K_{NADP^+} / K_{NADPH}) value was found to be 8.79 ± 0.15. The over-all average is thus 8.95 ± 0.23.

Subunit of Purified 6-Phcsphogluconate Dehydrogenase

Dissociation of native 6-phosphogluconate dehydrogenase was brought about by the addition of sodium laurylsulfate (11). The molecular weight of the subunit was determined by the method of Weber and Osborn (12). The molecular weight of the native enzyme and the subunits were 105,000 and 52,000 respectively (11). The dissociated protein of this size is not catalytically active nor was it reactivated by dialysis against pH 7.0, 0.1 M potassium phosphate buffer.

DISCUSSION

Free NADPH exhibits activation peaks at 275 and 355 nm, and an emission peak at 462 nm. The excitation peaks correspond to the 260 and 340 nm absorption maxima, respectively (2). Previously, extinction and emission maxima of NADPH were reported to be 352 and 478 nm, respectively, by Langan (8). The apprecible discrepancy of the observed emission maxima may be due to instrumentation. The 275 nm excitation peak indicates the weak, although significant, transfer of energy from the adenine to the reduced pyridine moiety (13).

The disappearance of the 275 nm maximum when coenzyme is bound to 6-phosphogluconate dehydrogenase is analogous to the case of lactate dehydrogenase which was interpreted to indicate that during binding the coenzyme may change from a compact form to an extended form, thus decreasing the probability of energy transfer from the adenine group (13). The 293 nm extinction peak was contributed by protein absorption, and could be due either to intramolecular energy transfer from the protein to the reduced nucleotide or to an intermolecular process, since the protein emitted strongly at the excitation wave length of NADPH. The fluorescence enhancement and blue shift of the emission maximum indicated higher fluorescence efficiency for the enzyme-bound nucleotide. 6-Phosphogluconate dehydrogenase did not change the fluorescence characteristics of NADH, suggesting that this nucleotide is not bound. This is in good agreement with the absolute NADPH specificity for the catalytic activity (14).

The 293 nm excitation maximum for protein differs appreciably from its 281 nm absorption maximum. Binding with NADPH did not cause a shift in either the excitation or the 335 nm emission maxima.

The decrease in 335 nm fluorescence is , at least in part, the result of energy transfer to the reduced nucleotide which fluoresces maxmally near 450 nm (Fig. 4). It could, in part, also result from a change in protein conformation uodn coenzyme binding as suggested by Shifrin, Kaplan, and Ciotti (15) for lactate dehydrogenase from bovine heart.

6 - Phosphogluconate dehydrogenase gives strong protein fluorescence, which could be used to develop a method estimation with a sensitivity many times that of ultraviolet absorption measurements.

Formation of enzyme, nucleotide, and substrate or substrate analogue complexes have been shown for several dehydrogenase by the fluorescence technique(3, 4, 8, 16-18). Usually fluorescence intensity is enhanced. In the case of 6- phosphogluconate dehydrogenase, enzyme-NADPH complex apparently binds 6-phosphogluconate. Enhancement of fluorescence of enzyme-NADPH complex must be due to the formation of an 6-phosphogluconate-enzyme-NADPH tertiary complex. The metal effect is different from that observed for isocitrate dehydrogenase (8) and malic enzyme (9) where Mn^{++} is involved in the binding of substrate molecules. Enhancement of enzyme -NADPH complex fluorescence by 6-phosphogluconate was observed over a wide range of 6-phosphogluconate concentrations, and therefore these data could not be applied to a study of its stoichiometry. The above phenomenon a large dissociation constant for 6-phosphogluconate-binding capacity by enzyme-NADPH complex, or both. It is possible that the oxidation state of the nucleotide determines the substrate-binding properties of the enzyme.

The same binding weight and dissociation constant were obtained by stoichiometric titration of NADPH. Save for an increase in fluorescence yield, 6-phosphogluconate binding did not change either the NADPH binding weight or the site dissociation constant.

The over-all average values of binding weight and site dissociation constants determined from several separate experiments are 52,000 g per mole of NADPH and 6.7×10^{-7} M, respectively. From a molecular weight of 1.05×10^{5} (10), the number of NADPH-binding sites was estimated to be 2. Sodium laulylsulfate polyacrylamide gel electrophoresis studies also implied 2 subunits in the native enzyme (11).

When enzyme-NADPH complex was titrated with $NADP^{+}$ the fluorescence intensity decreased with increasing concentration of $NADP^{+}$ owing to competition of free $NADP^{+}$ for the coenzyme-binding site and the simultaneous release of free NADPH.

From the average ratio of site dissociation constants (K_{NADP^+} / K_{NADPH}) and the site dissociation constant calculated for NADPH (6.7×10^{-7} M), the site dissociation constant for $NADP^+$ is estimated to be 6.0×10^{-6} M. Thus 6-phosphogluconate dehydrogenase has the different affinity for the oxidized and the reduced coenzyme.

SUMMARY

Purified pig liver 6-phosphogluconate dehydrogenase (phosphogluconate dehydrogenase (decarboxylating), EC 1.1.1.44) fluoresces strongly with excitation and emission maxima at 293 nm and 335 nm, respectively. Binding of nicotinamide adenine dinucleotide phophate-reduced (NADPH) by 6-phosphogluconate dehydrogenase was studied by the fluorescence technique. The purified enzyme binds NADPH strongly and causes a blue shift in the emission maximum of NADPH from 462 to 455 nm, and an enhancement of fluorescence intensity. Titration of 6-phosphogluconate dehydrogenase with NADPH yields a binding weight of 52,000 g of protein per mole of NADPH and a site dissociation constant of 6.7×10^{-7} M. 6-Phosphogluconate binds to the enzyme-NADPH complex in the abscence of Mn^{++} or Mg^{++}, but at a site other than the NADPH-binding site. The binding of enzyme with NADPH is specific, and is not observed with NADH.

$NADP^+$ binds to 6-phosphogluconate dehydrogenase in competition with NADPH. The ratio of site dissociation constants was determined, and from this, the dissociation constant for $NADP^+$ is calculated to be 6.0×10^{-6} M.

REFERENCES

1, Udenfriend, S., Fluorescence assay in biology and medicine, Academic press, New York, 1962.

2, Velick, S, F., J. Biol. Chem., **233**, 1455 (1958).

3, Theorell, H., and Langan, T. A., Acta Chem. Scand., **14**, 993 (1960).

4, Pfleiderer, G., and Hohnholz, E., Biochem. Z., **331**, 245 (1959).

5, Theorell, H., and Bonnichsen, F. K., Acta Chem. Scand., **5**, 1105 (1951).

6, Winer, A. D., Schwert, G. W., and Miller, D. B. S., J. Biol. Chem., **234**, 1149 (1959).

7, Adelstein, S. J., Biochemistry, **4**, 891 (1965).

8, Langan, T. A., Acta Chem. Scand., **14**, 936 (1960).

9, Hsu, R. Y., and Lardy, H. A., J. Biol. Chem., **242**, 527 (1967).
10, Gornall, A. G., Bardawill, C. J., and David, M. M., J. Biol. Chem., **177**, 751 (1949).
11, Yasue, S., Aizutankidaigaku Gakuhô (natural science) **37**, 21 (1980).
12, Weber, K., and Osborn, M., J. Biol. Chem., **244**, 4409 (1969).
13, Shifrin, S., and Kaplan, N. O., Advan. Enzymol., **22**, 337 (1960).
14, Rutter, W. J., and Lardy, H. A., J. Biol. Chem., **233**, 374 (1958).
15, Shifrin, S., Kaplan, N. O., and Citti, M. M., J. Biol. Chem., **234**, 1555 (1959).
16, Winer, A. D., and Theorell, H., Acta Chem. Scand., **13**, 1038 (1959).
17, Winer, A. D., and Schwert, G. W., Biochim. Biophys. Acta, **29**, 424 (1958).
18, Winer, A. D., Nova, W. B., and Schwert, G. W., J. Am. Chem. Soc., **79**, 6571 (1957).