Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*. Conformational Transitions Induced by Nicotinamide Adenine Dinucleotide, Nicotinamide Adenine Dinucleotide Phosphate, and Glucose 6-Phosphate Monitored by Fluorescent Probes†

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ABSTRACT: The interactions between the dual nucleotide-specific glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and NAD^+, $NADP^+$, and glucose 6-phosphate were monitored by using three fluorescent probes. The enzyme's intrinsic protein fluorescence is quenched upon binding of NAD^+, $NADP^+$, or the thionicotinamide analogue of NADPH (S-NADPH). Binding of S-NADPH to the enzyme leads to a large enhancement of the fluorescence of this coenzyme analogue; this fluorescence is markedly influenced by the presence of other ligands on the enzyme. The fluorescence of the phosphopyridoxyl group, bound covalently to a unique lysine residue in the enzyme [Haghighi, B., Flynn, T. G., & Levy, H. R. (1982) *Biochemistry* (preceding paper in this issue)], is also perturbed by the binding of other ligands. Equilibrium dialysis measurements with $NADP^+$ established that the dimeric enzyme contains two, independent coenzyme binding sites. Dissociation constants were determined for binding of $NADP^+$ (by equilibrium dialysis), NAD^+, and S-NADPH (by quenching of protein fluorescence) to the native enzyme and for binding of $NADP^+$ and NAD^+ (by quenching of phosphopyridoxyl fluorescence) to the covalently modified enzyme. The binding of $NADP^+$, NAD^+, and glucose 6-phosphate to the native and phosphopyridoxyl enzyme was also monitored by their effects on the fluorescence (quenching or enhancement) of bound S-NADPH and on the wavelength of its maximum fluorescence emission. Simultaneous measurements of protein fluorescence in these experiments demonstrated that the ligands did not displace the bound S-NADPH. Binding of NAD^+ to native enzyme (K_D = 7.2 mM) leads to a large quenching of protein fluorescence; $NADP^+$ binding (K_D = 6.5 μM) causes only a small fluorescence quenching. NAD^+ binding to covalently modified enzyme (K_D = 0.79 mM) causes less quenching of the phosphopyridoxyl group than does $NADP^+$ binding (K_D = 26.7 μM). The fluorescence of S-NADPH bound to native enzyme is quenched, and its emission maximum blue shifted, by NAD^+ binding; but it is enhanced, without any change in emission maximum, when $NADP^+$ binds. Glucose 6-phosphate binding to native enzyme enhances the fluorescence of bound S-NADPH and, at high concentration, blue shifts its emission maximum; it has no effect on S-NADPH fluorescence of covalently modified enzyme. The emission maximum of S-NADPH bound to covalently modified enzyme is also blue shifted. These results indicate that binding of NAD^+ and $NADP^+$ to the enzyme induces different conformational changes in the enzyme. The effects of glucose 6-phosphate and of the covalently bound phosphopyridoxyl group are similar and result in enhanced NAD^+ binding. The results are consistent with previous studies on differences in kinetic mechanisms of the NAD- and NADP-linked reactions and the postulated regulatory role of glucose 6-phosphate.

The coenzymes NAD and NADP serve distinctly different metabolic roles in all forms of life. Most dehydrogenases, therefore, are either NAD or NADP linked. Glucose-6-phosphate dehydrogenases in eukaryotes and many prokaryotes are NADP specific or NADP preferring (Levy, 1979). An important function of these enzymes is to generate NADPH, used for various biosynthetic reactions. In some prokaryotes glucose-6-phosphate dehydrogenases participate in two or more metabolic pathways. Such organisms possess either two distinct glucose-6-phosphate dehydrogenases, NAD and NADP preferring, respectively, or a single enzyme that can catalyze both reactions (Levy, 1979). Among the latter, the best studied example is glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (DeMoss et al., 1953; Olive & Levy, 1967). This is a particularly interesting enzyme because its reactions with NAD^+ and $NADP^+$ serve catabolic and anabolic roles, respectively (Kemp & Rose, 1964). Steady-state kinetic analysis of the NAD- and NADP-linked reactions revealed differences in their kinetic mechanisms (Olive et al., 1971). Recent studies, employing a dual wavelength assay that permits analyzing both reactions simultaneously, described means by which the enzyme may be regulated that exploit these kinetic differences (Levy & Daouk, 1979; Levy et al., 1979).

There are other dehydrogenases that display dual nucleotide specificity such as animal mitochondrial glutamate dehydrogenase (Smith et al., 1975) and homoserine dehydrogenase in plants (Bryan, 1980). Little is known about the mechanisms which control the utilization of NAD and NADP in such enzymes. In this connection it is pertinent to ask what protein structural features underlie such dual nucleotide specificity and how enzymes that can utilize both coenzymes differ from those which are NAD or NADP specific. The steady-state kinetic analysis (Olive et al., 1971), differential protection of NAD^+ and $NADP^+$ against arginine modification (Levy et al., 1977), and previous fluorescence studies (Grove et al., 1976) all suggested that, in the case of *L. mesenteroides* glucose-6-phosphate dehydrogenase, the dual nucleotide specificity is associated with different conformational isomers of the enzyme. Fluorescent probes have been widely used to measure protein conformational changes (Brand

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& Gohlke, 1972). Therefore, we have exploited three fluorescent probes to monitor such transitions in the interaction of *L. mesenteroides* glucose-6-phosphate dehydrogenase with NAD^+, $NADP^+$, and glucose 6-phosphate.

First, the intrinsic protein fluorescence was quenched by binding various ligands to the enzyme. These measurements permitted assessing the overall conformational changes of the enzyme upon ligand binding and calculations of binding constants. Second, the covalently bound phosphopyridoxyl group in the enzyme modified with pyridoxal 5'-phosphate (PLP)[1] and sodium borohydride (Haghighi et al., 1982) served as a fluorescence reporter group to monitor the conformational changes produced by NAD^+ or $NADP^+$ and to calculate their binding constants. Third, the fluorescence of S-NADPH was used as an extrinsic probe of the coenzyme binding region of the native and pyridoxylated enzyme. The results of these experiments indicate that NAD^+, $NADP^+$, and pyridoxylation produce different conformational changes in the enzyme. The significance of these conformational transitions for ligand binding is described and consistent with previous kinetic studies as well as with experiments on the reassociation of inactive enzyme subunits into active enzyme (Haghighi & Levy, 1982).

Experimental Procedures

Materials. Glucose-6-phosphate dehydrogenase was isolated from *L. mesenteroides* (ATCC 12291, American Type Culture Collection) as described previously (Haghighi et al., 1982). NAD^+, $NADP^+$, S-$NADP^+$, and glucose 6-phosphate were purchased from Sigma Chemical Co.; PLP was obtained from ICN Pharmaceuticals Inc.; DE-52 anion exchanger was from Whatman Co.; all other chemicals were of reagent grade. All solutions were made up with double glass-distilled water and, except for the enzyme solutions, were filtered through a 0.45-μm membrane filter (Millipore) 3 times before use.

Preparation of S-NADPH. S-NADPH was prepared in the dark by reducing S-$NADP^+$ with glucose-6-phosphate dehydrogenase, which can utilize thionicotinamide analogues of both NAD^+ and $NADP^+$ (Levy & Daouk, 1979). S-$NADP^+$ (2.5 mM) was incubated with glucose 6-phosphate (30 mM) and glucose-6-phosphate dehydrogenase (20 μg/mL) in 33 mM Tris-HCl, pH 7.8, for 2 min at 25 °C, and the absorbance at 400 nm was monitored. The reduction was completed rapidly, and no more increase in the absorbance was observed. S-NADPH was then purified on a DE-52 column by the procedure originally described by Winer (1964) for purifying NADH. Fractions that displayed the ratio of absorbances at 260 and 399 nm characteristic for S-NADPH (Stein et al., 1963) were pooled. The concentration of S-NADPH was determined by using an extinction coefficient of 15 800 and 11 700 M^{-1} cm^{-1} at 260 and 399 nm, respectively (Stein et al., 1963). S-NADPH concentration was also measured enzymatically with glutamate dehydrogenase (Alex & Bell, 1980), but in the presence of 5 mM α-ketoglutarate and 100 mM NH_4Cl in 50 mM Tris-HCl, pH 7.8. There was good agreement between these two methods.

The photosensitivity of S-NADH has been noted (Shifrin, 1963). Joppich-Kuhn & Luisi (1978b) and Baici et al. (1978) observed different photochemical processes in free S-NADH and in S-NADH bound to alcohol dehydrogenase and octopine dehydrogenase. Because of these findings, we conducted all experiments with S-NADPH rapidly with a minimum exposure to light and observed no such effects in our experiments.

PLP Modification. Glucose-6-phosphate dehydrogenase was inactivated with PLP and subsequently reduced by $NaBH_4$ as described previously (Haghighi et al., 1982). Samples containing 0.6–1.4 mol of pyridoxyl groups/mol of enzyme were prepared.

Equilibrium Dialysis. Binding of $NADP^+$ to glucose-6-phosphate dehydrogenase was measured in 0.03 M potassium phosphate buffer, pH 7.6, at room temperature (~20 °C) by equilibrium dialysis. Dialysis tubing was boiled in 16 mM $NaHCO_3$ containing 5 mM EDTA and washed thoroughly with water before use. $NADP^+$ was present both inside and outside the dialysis bag at the beginning of dialysis. For each sample, 0.5 mL of enzyme solution was dialyzed against 7.0 mL of buffer containing $NADP^+$. At low $NADP^+$ concentration both volumes were increased. The protein concentration always was 1 mg/mL, and the range of $NADP^+$ concentrations used is given in the figure legend. A control experiment indicated that equilibration was achieved in less than 8 h; however, the experimental samples were allowed to dialyze for about 16 h to ensure complete equilibration. The dialyses were performed in appropriate size test tubes that were slowly rotated. The concentration of $NADP^+$ was measured enzymatically with either glucose-6-phosphate dehydrogenase (Haghighi et al., 1982) or glutamate dehydrogenase (Dalziel & Egan, 1972).

Fluorescence Studies. All fluorescence studies were carried out with a Perkin-Elmer MPF-3L fluorescence spectrophotometer with an attached Leeds & Northrup Speedomax XL 620 series flatbed recorder. The temperature of the cell compartment was controlled with a Forma constant temperature bath and circulator and remained at 25 °C. A microcell assembly with 4 × 4 mm cells was used in order to attenuate internal absorption, especially at high coenzyme concentrations. All experiments were done in 0.03 M potassium phosphate buffer, pH 7.6. The concentrations of NAD^+, $NADP^+$, and glucose 6-phosphate were measured enzymatically with glucose-6-phosphate dehydrogenase (Haghighi et al., 1982), and calculated by using an extinction coefficient of 6220 M^{-1} cm^{-1} for reduced coenzyme at 340 nm.

It is important to note that the fluorescence spectra presented in the figures are uncorrected. In those experiments where corrections for dilution and internal absorption were necessary (see below), these were applied, and the corrected values are recorded in the tables.

In the titrations of native glucose-6-phosphate dehydrogenase with NAD^+ and S-NADPH, protein fluorescence was monitored as described by Grove et al. (1976). The protein was excited at 290 nm (bandwidth = 2 nm) and the fluorescence measured at 330 nm (bandwidth = 16 or 22 nm).[2] The protein concentration was maintained between 1.7 and 2.8 μM. A correction factor for internal absorption was determined experimentally by titration of glycyl-L-tryptophan with the absorbing ligand (Price & Radda, 1971).

When the fluorescence of the pyridoxyl group in pyridoxyl-enzyme was monitored, the protein was excited at 325 nm (bandwidth = 2 nm) an the fluorescence measured at 392 nm (bandwidth = 16 nm) (Forrey et al., 1971). Corrections for the inner-filter effect were made by titrating *N*-5'-phospho-

[1] Abbreviations: PLP, pyridoxal 5'-phosphate; S-$NADP^+$ and S-NADPH, thionicotinamide analogues of $NADP^+$ and NADPH, respectively; Tris, tris(hydroxymethyl)aminomethane. Enzyme modified with PLP and sodium borohydride is referred to as pyridoxyl-enzyme.

[2] The fluorescence excitation and emission wavelengths are designated by subscripts preceding and following the letter *F*. Thus, $_{290}F$ signifies the fluorescence when an excitation wavelength of 290 nm is used; F_{350} means the fluorescence measured at 350 nm; $_{290}F_{350}$ means, specifically, the fluorescence measured at 350 nm when the sample is excited at 290 nm.

pyridoxyllysine, prepared from poly(L-lysine hydrobromide) by the method of Schnackerz & Noltmann (1971), with the ligand used. The correction factor was negligible for $NADP^+$ and 1–5% for NAD^+ over the concentration range used.

In the experiments where the fluorescence of S-NADPH was measured, the excitation wavelength was 399 nm (bandwidth = 2 nm) and the emission wavelength 490 nm (bandwidth = 30 nm). Glucose-6-phosphate dehydrogenase, as well as the ligands used, did not show any absorbance at 399 nm in the concentration range employed; thus no correction was made for internal absorption. In most of the experiments the ligands used were included in the total volume of the sample; therefore no correction for dilution was necessary.

The quantum yield of S-NADPH was measured by using quinine sulfate in 0.1 N H_2SO_4 as a reference and assuming the quantum yield of this compound to be 0.55 (Melhuish, 1961). A solution of quinine sulfate in 0.1 N H_2SO_4 was prepared so that its absorbance at the excitation wavelength was identical with that of the sample. The efficiency of energy transfer (T) from tryptophan residues in the enzyme to the pyridoxyl group was calculated from

$$T = 1 - Q/Q_0$$

were Q and Q_0 are the quantum yields of enzyme and pyridoxyl-enzyme, respectively.

Other Methods. Assays were performed as described by Haghighi et al. (1982). Protein concentration was determined by a modification of the biuret method (Zamenhof, 1957).

Data Analysis. The binding parameters for NAD^+, $NADP^+$, and S-NADPH for both native enzyme and pyridoxyl-enzyme were calculated according to Scatchard (1949), using the data obtained from either fluorescence quenching experiments or equilibrium dialysis. The fluorescence data were analyzed according to Grove et al. (1976). The maximum fluorescence quenching, F_{max}, for each titration was determined from a plot of the reciprocal of the change in fluorescence, corrected for dilution and inner-filter effects, vs. the reciprocal of the total ligand concentration (Luisi et al., 1973). The lines for double-reciprocal plots were drawn with a computer program based on the direct linear plot (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1978). The accuracy of F_{max}, which is critical for the correct evaluation of binding data, is reflected in the degree to which the abscissa intercept in the linear Scatchard plots yields a molar fraction of ligand bound per enzyme subunit of 1.0 (Luisi et al., 1973). In all experiments reported here this value was 1.0 ± 0.15.

According to Holbrook et al. (1972), for a number of dehydrogenases fluorescence quenching is not proportional to the concentration of bound ligand. This nonproportionality was a problem with glucose-6-phosphate dehydrogenase, especially for $NADP^+$ which has a low dissociation constant. Thus, in calculating F_{max}, K_D, and ligand binding sites per enzyme molecule, only the data representing the higher ligand concentrations were used, as specified by Luisi et al. (1973). The lines for all Scatchard plots were drawn by linear regression analysis.

Where fluorescence quenching or enhancement or blue shifts in the maximum emission wavelength of S-NADPH are reported, the data quoted are for a particular experiment performed under the given conditions. All such experiments were repeated at least twice, and usually several times, to ensure that the results were reproducible. In titrations of native or pyridoxylated enzyme with coenzymes, the results of two to four experiments were averaged before calculating the F_{max} and K values, except for the titration with $NADP^+$, where the values are averages of duplicate titrations.

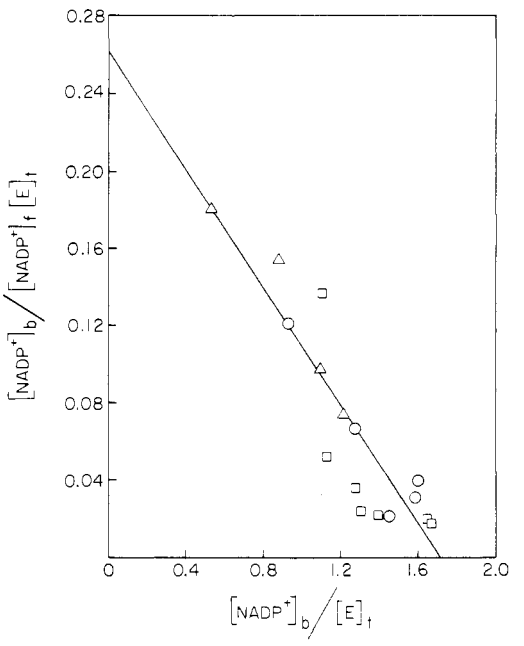

FIGURE 1: Scatchard plot of equilibrium binding of $NADP^+$ to glucose-6-phosphate dehydrogenase. $NADP^+$ binding was measured by the equilibrium dialysis technique as described under Experimental Procedures. The results of three separate experiments are presented. The $NADP^+$ concentration was measured enzymatically with glutamate dehydrogenase for the first (○) and glucose-6-phosphate dehydrogenase for the second (□) and third (△) experiments. Initial $NADP^+$ concentrations were 10–90 μM in the first, 10–100 μM in the second, and 3–18 μM in the third experiment. Each point represents the average of duplicate or triplicate assays. The line was computed by least-squares linear regression. Subscripts b, f, and t represent bound, free, and total concentrations, respectively.

Results

Equilibrium Dialysis. The dissociation constant for $NADP^+$ was determined previously by fluorescence quenching measurements (Grove et al., 1976). For confirmation of this result and determination of the number of $NADP^+$ binding sites on *L. mesenteroides* glucose-6-phosphate dehydrogenase, equilibrium dialysis experiments were performed. The data, displayed in the form of a Scatchard plot (Figure 1), show a stoichiometry of 1.7 binding sites per enzyme dimer with a K_D of 6.5 μM. The results indicate that there are two, independent $NADP^+$ binding sites, one per subunit.

Binding of NAD^+ and S-NADPH to Native Enzyme Monitored by Quenching of Protein Fluorescence. Binding of S-NADPH and NAD^+ to the enzyme was measured by monitoring the intrinsic protein fluorescence. Titration experiments were conducted and the data depicted as double-reciprocal and Scatchard plots, illustrated in Figure 2 for NAD^+. From such plots maximum fluorescence quenching of 16.5 and 77.8% was obtained for S-NADPH and NAD^+, respectively. The dissociation constants calculated from the Scatchard plots were 16.9 μM for S-NADPH and 7.2 mM for NAD^+. Table I contains a summary of all K_D values determined for the native enzyme and pyridoxyl-enzyme.

Binding of NAD^+ and $NADP^+$ to Pyridoxyl-Enzyme Monitored by Quenching of Pyridoxyl Fluorescence. Quenching of protein fluorescence could not be used to monitor coenzyme binding to pyridoxyl-enzyme because energy transfer occurs from tryptophan residues to the pyridoxyl group (see below). Addition of NAD^+ or $NADP^+$ to pyridoxyl-enzyme quenched the fluorescence of the pyridoxyl group. This quenching was used to determine the dissociation constants.

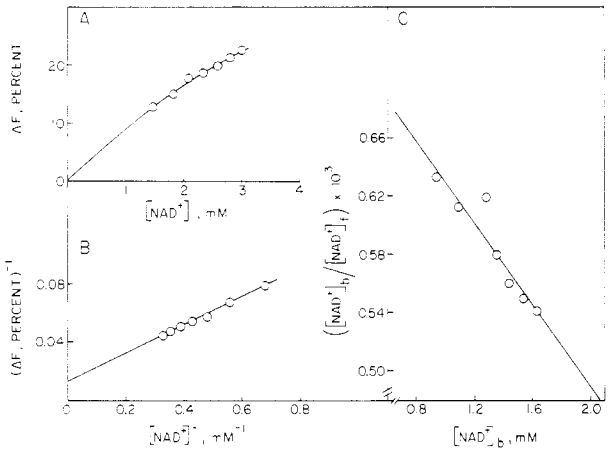

FIGURE 2: Fluorescence titration of glucose-6-phosphate dehydrogenase with NAD^+. Binding of NAD^+ to the enzyme was measured by monitoring the protein fluorescence ($_{290}F_{330}$) as described under Experimental Procedures. The protein concentration was 2.83 μM. (A) Dependence of the percentage of fluorescence quenching on coenzyme concentration; (B) double-reciprocal plot; (C) Scatchard plot. ΔF = change in fluorescence; subscripts b and f, bound and free, respectively.

Table I: Coenzyme Binding to Native and Pyridoxylated Glucose-6-phosphate Dehydrogenase[a]

	native enzyme		pyridoxyl-enzyme	
ligand	K_D (mM)	F_{max}[b] (%)	K_D (mM)	F_{max}[b] (%)
NAD^+	7.2 (2)	77.8 (2)	0.79 (4)	13.1 (4)
	2.5[c]	50[c]	–	–
$NADP^+$	0.0065[d] (3)	–	0.0267 (2)	23.4 (2)
	0.003[c]	5[c]	–	–
S-NADPH	0.0164 (3)	16.5 (3)	–	–

[a] Numbers in parentheses indicate number of experiments; data are averages that have been corrected (see Experimental Procedures); (–) indicates not measured. [b] Maximum fluorescence quenching. [c] From Grove et al. (1976). Measured in 33 mM Tris-acetate, pH 7.8. [d] Measured by equilibrium dialysis. All other values are from fluorescence quenching measurements.

The fluorescence titrations of pyridoxyl-enzyme with NAD^+, monitoring the fluorescence of the pyridoxyl group, are shown in Figure 3. The maximum quenching caused by the coenzymes, evaluated from double-reciprocal plots, corresponds to 23.4 and 13.1% for $NADP^+$ and NAD^+, respectively. Neither the pyridoxyl nor the protein fluorescence maxima were shifted upon binding of either coenzyme. The dissociation constants calculated for $NADP^+$ and NAD^+ are 26.7 μM and 0.79 mM, respectively. The addition of glucose 6-phosphate (up to 0.92 mM) had no effect on the fluorescence of the pyridoxyl group.

Binding of NAD^+, $NADP^+$, and Glucose 6-Phosphate to Native Enzyme Monitored by S-NADPH Fluorescence. The corrected emission spectrum of S-NADPH was reported by Wright & Takahashi (1977). Uncorrected spectra show excitation and emission maxima at 399 and 490 nm, respectively (Figure 4). S-NADPH exhibited a very low quantum yield of $(3.2 \pm 0.2) \times 10^{-4}$ at 24 °C upon excitation at 399 nm with quinine sulfate as a reference fluorescence compound. This value is very similar to the quantum yield of S-NADH reported by Baici et al. (1978). Upon binding to glucose-6-phosphate dehydrogenase, the fluorescence of S-NADPH is enhanced, and its emission maximum is blue shifted 2–4 nm (Figure 4). From the K_D for S-NADPH it could be calculated that the fluorescence of bound S-NADPH is enhanced approximately 6-fold.

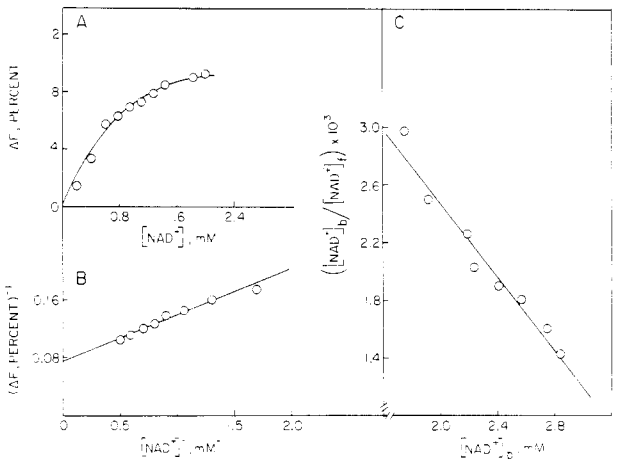

FIGURE 3: Fluorescence titration of pyridoxylated glucose-6-phosphate dehydrogenase, containing 0.90 pyridoxyl group per enzyme dimer, with NAD^+. Binding of NAD^+ to pyridoxylated enzyme was measured by monitoring the fluorescence of the pyridoxyl group ($_{325}F_{392}$) as described under Experimental Procedures. The protein concentration was 2 μM. (A), (B), and (C) as in Figure 2.

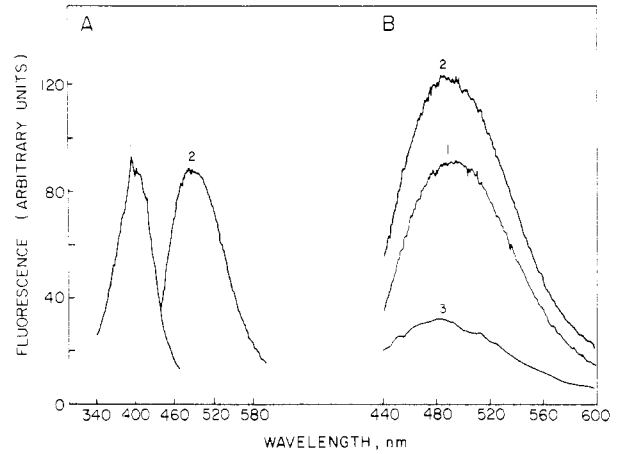

FIGURE 4: Excitation and emission spectra of S-NADPH. (A) Excitation spectrum (F_{490}, curve 1) and emission spectrum ($_{399}F$, curve 2) of 33 μM S-NADPH. (B) Emission spectra ($_{399}F$) of free (1) and enzyme-bound (2) S-NADPH. The concentrations were 33 μM for S-NADPH and 1.72 μM for glucose-6-phosphate dehydrogenase. Curve 3 represents the calculated spectrum of bound S-NADPH (2.25 μM).

The enhanced fluorescence of enzyme-bound S-NADPH ($_{399}F$)[2] was used to monitor ligand binding to glucose-6-phosphate dehydrogenase. Concentrations of S-NADPH and ligands were chosen, based on the K_D values (Table I), so that ligand binding could occur without displacing bound S-NADPH. When the protein fluorescence ($_{290}F_{330}$) was monitored simultaneously, it was possible to ascertain whether or not S-NADPH was displaced, based on the differences in the extent of quenching of protein fluorescence produced by the different ligands (see Table II). Control experiments showed that NAD^+, $NADP^+$, or glucose 6-phosphate did not affect S-NADPH fluorescence in the absence of enzyme.

Addition of 1.05 mM NAD^+ to the enzyme·S-NADPH complex quenched the fluorescence of S-NADPH with a blue shift of approximately 15 nm in its maximum emission wavelength (Figure 5A).[3] Quenching of protein fluorescence by S-NADPH, NAD^+, and S-NADPH plus NAD^+ was 6.5%, 7.2%, and 13.7% (Table II), respectively, indicating that both

[3] All wavelength shifts are given with respect to that of free S-NADPH.

Table II: Fluorescence Changes on Ligand Binding to Native Glucose-6-phosphate Dehydrogenase

expt	ligand added (concn)	effect on fluorescence max of S-NADPH		effect on protein fluorescence,
		$_{399}F$ (%)	blue shift (nm)	$_{290}F_{330}$ (% quenching)
1	S-NADPH (29.3 μM)	(100)		6.5
	NAD^+ (1.05 mM)			7.2
	S-NADPH (29.3 μM) + NAD^+ (1.05 mM)	64	15	13.7
2	S-NADPH (29.3 μM)	(100)		11.3
	$NADP^+$ (2 μM)			1.7
	S-NADPH (29.3 μM) + $NADP^+$ (2 μM)	114	0	14.7
3	S-NADPH (29.3 μM)	(100)		10.5
	$NADP^+$ (100 μM)			3.5
	S-NADPH (29.3 μM) + $NADP^+$ (100 μM)	106	0	4.9
4	S-NADPH (29.3 μM)	(100)		10.8
	G6P (0.34 mM)			0
	S-NADPH (29.3 μM) + G6P (0.34 mM)	119	0	15.0
5	S-NADPH (29.3 μM)	(100)		10.8
	G6P (0.9 mM)			0
	S-NADPH (29.3 μM) + G6P (0.9 mM)	110	10	15.0

[a] The fluorescence of the enzyme·S-NADPH complex in each experiment was normalized to 100%.

S-NADPH and NAD^+ were bound to the enzyme simultaneously and S-NADPH was not displaced. From the dissociation constants (Table I) and assuming independent binding, it can be calculated that the concentrations of bound S-NADPH and NAD^+ in this experiment were 3.8 μM (61% saturated) and 0.79 μM (13% saturated), respectively.

The effect of $NADP^+$ on the fluorescence of the enzyme·S-NADPH complex is shown in Figure 5B. In contrast to the NAD^+ effect, the fluorescence of S-NADPH in the binary complex was enhanced about 14% without shifting its emission maximum wavelength when 2 μM $NADP^+$ was added. This enhancement was only 5.6% at 100 μM $NADP^+$, suggesting that S-NADPH was partially displaced by $NADP^+$. The binding of $NADP^+$ to the binary complex was also characterized by monitoring the protein fluorescence. At an $NADP^+$ concentration of 2 μM, the percentages of protein fluorescence quenching were 1.7%, 11.3%, and 14.7% for the $NADP^+$·enzyme, enzyme·S-NADPH, and $NADP^+$·enzyme·S-NADPH complexes, respectively; at 100 μM $NADP^+$ the quenching in $NADP^+$·enzyme·S-NADPH dropped to 4.9% (Table II). These observations suggest that $NADP^+$ at low concentration ($0.3K_D$) was bound to the enzyme·S-NADPH complex, presumably at the free subunit, and changed the environment of S-NADPH in the second subunit. At a higher concentration of $NADP^+$, partial displacement of S-NADPH was observed. Calculations (see above) show that in the experiment depicted in Figure 5B the concentrations of bound S-NADPH and $NADP^+$ were 3.8 μM (61% saturated) and 0.90 μM (15% saturated), respectively. When 100 μM $NADP^+$ was added to enzyme·S-NADPH the expected concentrations of bound S-NADPH and $NADP^+$ would be 1.45 μM and 4.75 μM, respectively. The decreased enhancement of S-NADPH fluorescence and decreased quenching of protein fluorescence observed when 100 μM $NADP^+$ is added to enzyme·S-NADPH are consistent with the calculated reduced S-NADPH binding under these conditions.

The fluorescence of S-NADPH in the enzyme·S-NADPH complex was enhanced (19%) when glucose 6-phosphate was added at a concentration of 0.34 mM ($0.5K_D$; Milhausen & Levy, 1975); no change in the emission maximum wavelength was observed (Figure 5C). Glucose 6-phosphate at 0.9 mM shifted the emission spectrum 10 nm toward the blue, and the fluorescence enhancement dropped to 10% (Figure 5D). Glucose 6-phosphate also enhanced the quenching of protein fluorescence caused by S-NADPH (Table II).

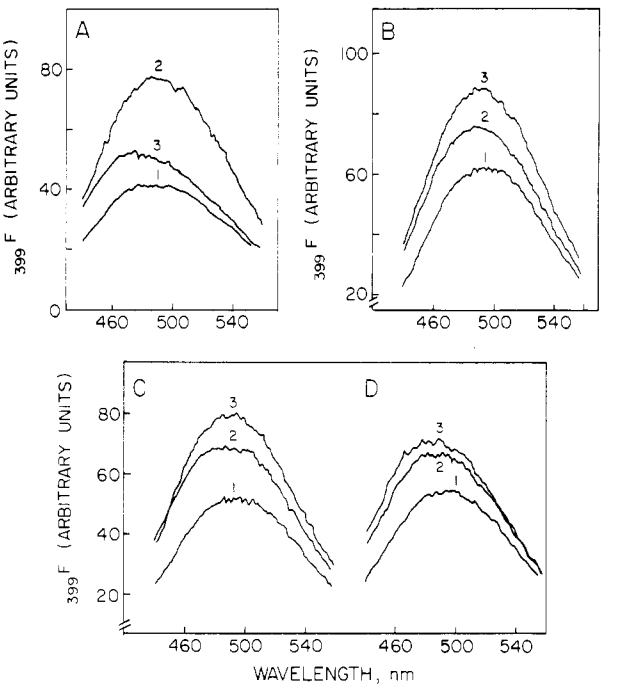

FIGURE 5: Effects of ligands on the enzyme·S-NADPH complex. (For explanation of symbols used to designate fluorescence in Figures 5 and 6, see footnote 2.) (A) Emission spectra of S-NADPH plus NAD^+ (1) and S-NADPH in the enzyme·S-NADPH (2) and NAD^+·enzyme·S-NADPH (3) complexes. (B) Emission spectra of S-NADPH in the presence of $NADP^+$ (1) and in the enzyme·S-NADPH (2) and $NADP^+$·enzyme·S-NADPH (3) complexes. Parts C and D represent the emission spectra of S-NADPH in the presence of glucose 6-phosphate (1) and in the enzyme·S-NADPH (2) and glucose 6-phosphate·enzyme·S-NADPH (3) complexes. The spectrum of S-NADPH alone was indistinguishable from those shown in spectra 1. The concentrations of enzyme (3.1 μM) and S-NADPH (29.3 μM) were identical for all experiments. The NAD^+ concentration in (A) was 1.05 mM, the $NADP^+$ concentration in (B) was 2.0 μM, and the glucose 6-phosphate concentrations were 0.34 mM in (C) and 0.9 mM in (D).

Binding of NAD^+ and $NADP^+$ to Pyridoxyl-Enzyme Monitored by S-NADPH Fluorescence. Experiments similar to those described in the previous section were conducted with pyridoxyl-enzyme containing 0.6 mol of pyridoxyl group/mol of dimer. Preliminary experiments showed that enzyme containing 0.6–1.4 mol of pyridoxyl group/mol of dimer responded similarly to ligand binding.

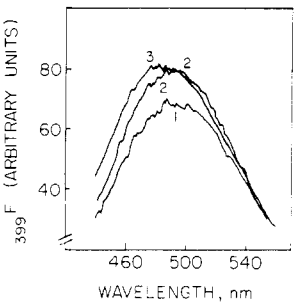

FIGURE 6: Effect of NAD^+ on the pyridoxyl-enzyme·S-NADPH complex. The enzyme contained 0.6 pyridoxyl group per enzyme dimer. S-NADPH emission spectra of S-NADPH + NAD^+ (1), pyridoxyl-enzyme·S-NADPH (2), and NAD^+·pyridoxyl-enzyme·S-NADPH (3) complexes. The spectrum of S-NADPH alone was indistinguishable from that shown in curve 1. The concentrations were 2.57 μM, 26.5 μM, and 0.76 mM for pyridoxyl-enzyme, S-NADPH, and NAD^+, respectively.

The binding of S-NADPH to pyridoxyl-enzyme was accompanied by an increase of S-NADPH fluorescence and a 6-nm blue shift in the emission spectrum of S-NADPH (Figure 6). Figure 6 also shows the effect of NAD^+ on the fluorescence of S-NADPH bound to the modified enzyme. Unlike the results with native enzyme, NAD^+ binding to the modified enzyme·S-NADPH complex caused an increase in the fluorescence of S-NADPH with a concomitant 10-nm blue shift in the emission spectrum. This suggests that the conformational form of the pyridoxyl-enzyme is different from that of native enzyme.

Binding of $NADP^+$ at 2.3 μM to the modified enzyme did not produce any shift in the emission maximum of bound S-NADPH but slightly enhanced its fluorescence (less than 5%). This fluorescence decreased when 69 μM $NADP^+$ was present, indicating some displacement of S-NADPH. displacement of S-NADPH by this concentration of $NADP^+$ was also demonstrated by a 63% decrease in the fluorescence quenching by S-NADPH of the pyridoxyl group ($_{325}F_{392}$). Similar observations were obtained when the protein fluorescence was monitored, but no quantitative analysis was made due to the complexity of this system, resulting from energy transfer from tryptophan residues to the pyridoxyl group and subsequent transfer from the latter group to S-NADPH (see below).

The addition of glucose 6-phosphate up to 0.92 mM to the modified enzyme·S-NADPH complex produced no significant change in the fluorescence of S-NADPH.

Energy Transfer. The fluorescence of pyridoxyl-enzyme activated at 290 nm increased at 390 nm and decreased at 330 nm with increase in the extent of PLP modification. This phenomenon is a result of the overlap of the absorption spectrum of the pyridoxyl group (Forrey et al., 1971) and the emission spectrum of tryptophan residues. The ratio of the fluorescence at 390 nm to the fluorescence at 330 nm increased with the number of pyridoxyl groups bound per enzyme dimer in a linear fashion. No shift in the emission spectra of either the protein or the pyridoxyl group was observed. These observations led to the conclusion that the appearance of a new fluorescence peak around 390 nm arises by energy transfer from excited aromatic amino acid residues (mainly tryptophan) of the protein to the bound pyridoxyl group. The efficiency of energy transfer was 0–0.47 in pyridoxyl enzyme samples containing 0–1.4 mol of PLP/mol of enzyme.

Titration of pyridoxyl-enzyme with S-NADPH, monitoring the fluorescence of the pyridoxyl group, excited at 325 nm, showed a maximum fluorescence quenching of 72%, calculated from the double-reciprocal plot. Since the emission spectrum of the pyridoxyl group completely overlaps with the absorption spectrum of S-NADPH, this quenching probably results from the transfer of energy from the pyridoxyl to the S-NADPH group. Titration of phosphopyridoxyllysine with S-NADPH did not generate such an effect, indicating that the quenching is not due to complex formation between the pyridoxyl group and S-NADPH.

Discussion

Binding Constants. Equilibrium dialysis was used to monitor the binding of $NADP^+$ to glucose-6-phosphate dehydrogenase. The dissociation constant calculated from these data is somewhat higher than the value previously determined from fluorescence quenching measurements (Table I). The dissociation constant for NAD^+, determined from protein fluorescence quenching measurements, is also higher than the value reported previously (Table I). Presumably these differences reflect differences in the buffers used in the two sets of experiments. The sensitivity of pyridine nucleotide coenzyme binding constants to the kind of buffer has been noted for various dehydrogenases (Winer & Schwert, 1958; Holbrook & Wolfe, 1972; Fisher, 1973; Venard et al., 1975).

The Scatchard plot (Figure 1) indicates that there are 1.7 $NADP^+$ binding sites per enzyme dimer, i.e., approximately one, independent binding site per subunit. Evidence for the identity of the subunits has been presented (Haghighi et al., 1982). The high dissociation constants for glucose 6-phosphate (Milhausen & Levy, 1975) and NAD^+ precluded performing equilibrium binding measurements to determine the number of binding sites for these ligands. The fact that NADPH inhibits competitively with respect to $NADP^+$ (Olive et al., 1971) and that the binding of NADPH and NAD^+ is competitive (Grove et al., 1976) makes it reasonable to assume that there are two, independent binding sites for NAD^+ that are largely coincident with the $NADP^+$ binding sites.

The K_D for S-NADPH is less than half that for NADPH, determined kinetically and in a different buffer (Olive et al., 1971). Lower K_m values were found for S-NAD^+ and S-$NADP^+$ than for NAD^+ and $NADP^+$, respectively (Levy & Daouk, 1979), suggesting that the thio analogues of both oxidized and reduced nicotinamide coenzymes bind more tightly to *L. mesenteroides* glucose-6-phosphate dehydrogenase than the natural coenzymes. S-$NADP^+$ is a highly reactive $NADP^+$ analogue for *L. mesenteroides* glucose-6-phosphate dehydrogenase, with k_{cat}/K_m almost identical with that for the natural coenzyme; NADPH inhibits competitively with respect to S-$NADP^+$ (Levy & Daouk, 1979) as well as $NADP^+$ (Olive et al., 1971). We have assumed, therefore, that S-NADPH binds to the same sites as $NADP^+$ and to no additional binding sites.

S-NADPH as a Fluorescent Probe. Pyridine nucleotide coenzymes have been used extensively as fluorescent probes to monitor conformational changes in glyceraldehyde-3-phosphate dehydrogenase (Bell & Dalziel, 1975; Henis & Levitzki, 1977), glutamate dehydrogenase (Bell & Dalziel, 1973; Venard et al., 1975), and other enzymes (Bell, 1981). The thionicotinamide analogues of NADH and NADPH have been used previously as fluorescent probes of enzyme structures. Wright & Takahashi (1977) showed that the weak fluorescence of S-NADPH is enhanced on binding to *Escherichia coli* aspartokinase–homoserine dehydrogenase. They were able to measure the distance between the S-NADPH binding site and the site of binding of a fluorescent ATP analogue on this enzyme by fluorescence energy transfer (Wright & Takahashi, 1977). Extensive studies from Luisi's

laboratory showed that the weakly fluorescent S-NADH binds to horse liver alcohol dehydrogenase and to the octopine·octopine dehydrogenase complex from *Pecten maximus* L. with large fluorescence enhancement (Joppich-Kuhn & Luisi, 1978a,b; Baici et al., 1978). This fluorescence enhancement with both enzymes is accompanied by a blue shift in the emission maximum.

Coenzyme Binding to Native Enzyme. The binding of NAD^+ to *L. mesenteroides* glucose-6-phosphate dehydrogenase causes a large quenching of protein fluorescence. Grove et al. (1976) found a maximum fluorescence quenching of 50%; in our studies, using a different buffer, we found maximum quenching of 77.8% (Figure 2). The binding of $NADP^+$ produces a maximum quenching of only 5% (Grove et al., 1976).

When S-NADPH fluorescence was used to monitor binding of NAD^+ and $NADP^+$, marked differences were seen also. The addition of 1.05 mM NAD^+ ($0.15K_D$) to the enzyme·S-NADPH complex resulted in 36% quenching of S-NADPH fluorescence with a 15-nm blue shift in its emission maximum (Figure 5A, Table II). When 2.0 μM $NADP^+$ ($0.31K_D$) was added to the same concentration of the enzyme·S-NADPH complex, the fluorescence of S-NADPH was enhanced 14%, and there was no detectable shift in the emission maximum (Figure 5B, Table II). Measurements of protein fluorescence ($_{290}F_{330}$) carried out simultaneously on these samples showed that little, if any, S-NADPH was displaced by these concentrations of NAD^+ or $NADP^+$. Such displacement would have been signaled by an extent of quenching, in the presence of S-NADPH plus NAD^+ or S-NADPH plus $NADP^+$, intermediate between the values found when each ligand was bound alone. In these experiments S-NADPH acts as a probe to monitor the conformation at the coenzyme binding site of one subunit when either NAD^+ or $NADP^+$ binds to the other subunit. When 100 μM $NADP^+$ was added to the enzyme·S-NADPH complex (Table II), there was little fluorescence enhancement at 399 nm and less quenching of protein fluorescence than in the presence of 2 μM $NADP^+$, indicating that most of the S-NADPH had been displaced by $NADP^+$.

Glucose 6-Phosphate Binding. Addition of 0.34 mM glucose 6-phosphate enhances the fluorescence of enzyme-bound S-NADPH without a shift in its absorbance maximum. Protein fluorescence measurements suggest that this may result from enhanced S-NADPH binding. Increasing the glucose 6-phosphate concentration to 0.9 mM lowers the fluorescence of enzyme-bound S-NADPH and blue shifts its fluorescence maximum. Protein fluorescence measurements show this is not due to S-NADPH release. Thus, high glucose 6-phosphate concentration has a similar effect on bound S-NADPH as does NAD^+, suggesting that both promote similar conformational changes in the enzyme.

Coenzyme Binding to Pyridoxyl-Enzyme. The maximum degree of fluorescence quenching of the covalently bound pyridoxyl group of the pyridoxyl enzyme was almost twice as great with $NADP^+$ as it was with NAD^+. The order is the reverse of what was observed for protein fluorescence quenching of native enzyme. This may be attributed to the fact that the pyridoxyl group promotes a conformational change in the enzyme similar to that produced by NAD^+ (see below). Binding of NAD^+ and $NADP^+$ to pyridoxyl-enzyme was also monitored by their effect on the fluorescence of bound S-NADPH. The binding of S-NADPH itself to pyridoxyl-enzyme is accompanied by enhancement of S-NADPH fluorescence and a blue shift of 5–6 nm in its emission maximum. Addition of NAD^+ to pyridoxyl-enzyme·S-NADPH produces a further blue shift, i.e., a total of 10 nm in the experiment shown in Figure 6. The addition of $NADP^+$ has little or no effect on the fluorescence of S-NADPH in the pyridoxyl-enzyme·S-NADPH complex.

Pyridoxylation of *L. mesenteroides* glucose-6-phosphate dehydrogenase occurs at a single lysine residue per subunit (Haghighi et al., 1982) believed to function in glucose 6-phosphate binding (Milhausen & Levy, 1975). Previous studies, indicating that glucose 6-phosphate binds to the same lysine residue which interacts with PLP, include glucose 6-phosphate competitive inhibition by PLP (Olive et al., 1971) and kinetic analysis of the inactivation of the enzyme by PLP in the presence of glucose 6-phosphate, showing that PLP cannot bind to the glucose 6-phosphate·enzyme complex (Milhausen & Levy, 1975). Further support is provided in the studies reported here by the fact that glucose 6-phosphate has no effect on the fluorescence of either the pyridoxyl group or bound S-NADPH when added to pyridoxyl-enzyme, although it does affect the fluorescence of S-NADPH bound to native enzyme. Thus, glucose 6-phosphate binding cannot be demonstrated with pyridoxyl-enzyme. The enzyme apparently cannot bind glucose 6-phosphate and the pyridoxyl group simultaneously.

We interpret these results to indicate that pyridoxylation of the enzyme locks the enzyme into a conformation that binds NAD^+ better, and $NADP^+$ less well, than native enzyme (Table I). A similar conformational change apparently is produced in the native enzyme by glucose 6-phosphate that is reflected in a lowering of the K_m for NAD^+ (but not for $NADP^+$) as the substrate concentration is increased; similarly, the K_m for glucose 6-phosphate is lowered as the concentration of NAD^+ (but not $NADP^+$) is increased (Olive et al., 1971; Levy & Daouk, 1979). This mutual enhancement of each other's binding by NAD^+ and glucose 6-phosphate suggests that they have similar effects on the enzyme's conformation. Some support for this idea comes from the fact that the addition of either NAD^+ or high glucose 6-phosphate concentration causes quenching of, and a blue shift in, the fluorescence of enzyme-bound S-NADPH (Table II).

Implications for Mechanism and Regulation. Two principal conclusions emerge from our fluorescence studies. First, binding of NAD^+ and $NADP^+$ probably produces different conformational changes in *L. mesenteroides* glucose-6-phosphate dehydrogenase, and second, pyridoxylation of the enzyme at a specific lysine residue and glucose 6-phosphate binding apparently promote similar conformational changes in the enzyme that enhance NAD^+ binding. Also, pyridoxylation hinders $NADP^+$ binding (fluorescence studies), and high glucose 6-phosphate concentrations inhibits the NADP-linked reaction in dual wavelength assays (Levy & Daouk, 1979). Together with previous findings, these effects bear on the mechanism of the enzyme and the postulated regulatory role of glucose 6-phosphate.

Although both NAD^+ and $NADP^+$ are reduced by *L. mesenteroides* glucose-6-phosphate dehydrogenase, the enzyme appears to assume different conformations to catalyze the two reactions. This was surmised from kinetic studies (Olive et al., 1971), from differential protection by NAD^+ and $NADP^+$ of arginine residues against chemical modification (Levy et al., 1977), and from earlier fluorescence experiments (Grove et al., 1976). More extensive fluorescence studies reported here strengthen this conclusion. The effects of NAD^+ and $NADP^+$ were monitored by three independent fluorophores: tryptophan, S-NADPH, and the pyridoxyl group. Intrinsic protein fluorescence of glucose-6-phosphate dehydrogenase

results principally from tryptophan residues; changes in this fluorescence ($_{290}F_{330}$) reflect the average of changes in the local environments of some or all of the enzyme's 16 (Ishaque et al., 1974) tryptophans. Changes in the fluorescence of enzyme-bound S-NADPH signal changes in the local environment of the coenzyme binding site. Changes in the fluorescence of the covalently bound pyridoxyl group, introduced at a specific lysine residue that is thought to function in glucose 6-phosphate binding (Haghighi et al., 1982), reflect changes in the environment of this particular residue. With each of these three fluorescent probes, the effects of the addition of NAD^+ and $NADP^+$ to glucose-6-phosphate dehydrogenase are profoundly different.

Earlier studies showed that increased glucose 6-phosphate concentration leads to enhanced NAD-linked and decreased NADP-linked activity as measured in dual wavelength assays (Levy & Daouk, 1979). Thus, glucose 6-phosphate concentration helps to regulate coenzyme utilization in this dual nucleotide-specific enzyme (Levy & Daouk, 1979). Recent studies indicate that the kinetic mechanism of the NAD-linked reaction catalyzed by *L. mesenteroides* glucose-6-phosphate dehydrogenase is random[4,5] whereas that of the NADP-linked reaction is ordered,[5] providing a kinetic basis for the regulatory effect of glucose 6-phosphate.

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