Modification of Lactate Dehydrogenase by Pyridoxal Phosphate and Adenosine Polyphosphopyridoxal†

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ABSTRACT: Pyridoxal phosphate reacts with not only the lysyl residue(s) essential for enzymatic activity but also other reactive lysyl residues in rabbit muscle lactate dehydrogenase (EC 1.1.1.27). To raise the specificity of pyridoxal phosphate, adenosine diphospho-, triphospho-, and tetraphosphopyridoxals have been newly synthesized and used for modification of the enzyme. Incubation of the enzyme for 30 min with the diphospho, triphospho, and tetraphospho compounds all at 1 mM followed by reduction by sodium borohydride resulted in the loss of enzymatic activity by 64, 51, and 34%, respectively. NADH almost completely protected the enzyme from inactivation, whereas pyruvate showed no protection. Binding of the reagents to the enzyme subunit in an equimolar amount corresponds to the complete inactivation. The adenosine diphosphopyridoxal modified enzymes with different residual activities were chromatographed on a Blue Toyopearl affinity column. The results showed the presence of at least four enzyme species besides the intact enzyme that are significantly different from one another in the amount of the reagent bound, the affinity for NADH, and the specific activity. The decrease in the affinity of the enzyme for NADH and the loss of enzymatic activity paralleled in the modification by adenosine diphosphopyridoxal, whereas, in the modification by pyridoxal phosphate, the decrease in the affinity for NADH preceded the inactivation. It is concluded that modifications by adenosine polyphosphopyridoxal compounds are specific for the active site lysyl residue(s) in lactate dehydrogenase.

Pyridoxal phosphate modifies primary amino groups in or near the phosphate-binding sites of many proteins including dehydrogenases, glycolytic enzymes, and enzymes involved in the nucleotide metabolism [for a reveiw, see Feeney et al. (1975)]. With a few exceptions, however, the modification of lysyl residues occurred not only in the active site but also in other regions of enzymes. The nonspecific modification by this reagent might be due to the electrostatic interaction between its phosphate group and cationic groups in enzymes. The addition of affinity moiety to pyridoxal phosphate might make the resultant reagent a specific modification reagent. We previously synthesized uridine diphosphopyridoxal, a conjugate of uridine monophosphate and pyridoxal phosphate through a pyrophosphate linkage, as an affinity label for glycogen synthase (EC 2.4.1.11). This reagent rapidly and almost completely inactivates the enzyme. The results of the kinetic analyses of modification combined with those of the sequence study of the modified enzyme indicate that the reagent is exclusively bound to the active site lysyl residue of the enzyme (Tagaya et al., 1985).

In view of the multifunctional roles of adenine nucleotides in cellular metabolism, it would be of great value to synthesize and use such a type of affinity labeling reagents as adenosine–pyridoxal conjugates. Through comparison of the modification by this type of reagents with that by pyridoxal phosphate, the effectiveness of the new reagents may be clarified. For this purpose, lactate dehydrogenase (EC 1.1.1.27) is suitable, because rather nonspecific inactivation of the enzyme with pyridoxal phosphate was observed (Chen & Engel, 1975; Gould & Engel, 1980, 1982). Even if any lysyl residue exists in the nucleotide-binding site of lactate dehydrogenase, the relative location of that residue to the bound base is not known. Taking this fact into account, we have synthesized adenosine diphosphopyridoxal (AP_2-PL),[1] AP_3-PL, and AP_4-PL to find the most effective and specific reagent. In this paper, we describe the results of modification of rabbit muscle lactate dehydrogenase by AP_n-PL as well as pyridoxal phosphate. The equimolar binding of the reagent to the enzyme subunit and the protection of the enzyme from inactivation indicate the specific modification of the active site lysyl residue of the enzyme by AP_n-PL.

Experimental Procedures

Lactate Dehydrogenase. Rabbit muscle lactate dehydrogenase (lot no. 32522402) was obtained from Oriental Yeast (Osaka, Japan). This preparation was apparently homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 5% gels (Weber & Osborn, 1969). A suspension of the crystalline enzyme in an ammonium sulfate solution was dialyzed against 250 volumes of 50 mM HEPES (pH 7.8) containing 1 mM EDTA for about 20 h with one change of the dialysis buffer. The enzyme solution (about 1 mg/mL) was stored at 4 °C and used within a week. The protein concentration of the enzyme was measured spectrophotometrically by using an absorbance at 280 nm of 14.9%$^{-1}$ cm^{-1} (Pesce et al., 1964) and a subunit M_r of 36000 (Taylor, 1977). The enzyme had a specific activity of 730–829 μmol of NAD^+ formed min^{-1} (mg of protein)$^{-1}$.

Assay for Enzymatic Activity. Lactate dehydrogenase activity was measured spectrophotometrically at 340 nm in a UVIDEC 505 spectrophotometer. The assay mixture, in a total volume of 3.17 mL, contained 95 mM potassium phos-

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[1] Abbreviations: AP_2-PL, adenosine diphosphopyridoxal; AP_3-PL, adenosine triphosphopyridoxal; AP_4-PL, adenosine tetraphosphopyridoxal; AP_n-PL, adenosine polyphosphopyridoxals in which the numbers of phosphate groups are 2, 3, and 4; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid trisodium salt.

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phate (pH 7.0), 0.26 mM NADH, and 0.8 mM pyruvate. To the assay mixture was added an adequate amount of enzyme solution (5–60 μL), and the mixture was incubated at 30 °C.

Synthesis of AP_2-PL. Pyridoxal compounds were all protected as well as possible from irradiation with fluorescent light to minimize the degradation of aldehyde groups. AP_2-PL was synthesized from pyridoxal phosphate and AMP by the anion-exchange method of Michelson (1964). For the synthesis of P^1-5′-adenosyl P^2-diphenyl diphosphate, AMP monohydrate (0.365 g) was dissolved at 40 °C in a solution of dimethylformamide (7 mL; dried over molecular sieves 3A) and tributylamine (0.24 mL). The solution was evaporated on a rotary evaporator at 37–39 °C. The residue was dissolved in 5 mL of dimethylformamide, and then 0.45 mL of tributylamine and 0.3 mL of diphenyl phosphochloridate were successively added. After the solution had been kept for 3 h at room temperature with mild stirring, the solvent was removed by evaporation. Ice-cold diethyl ether (30 mL) was added to the residue, and the resultant solution was left for 30 min at 0 °C. After the supernatant had been carefully discarded, the tributylammonium salt of pyridoxal phosphate (0.9 g) in 2 mL of pyridine (dried over KOH) was added to the precipitate. After the resultant solution had been kept overnight at room temperature, the solvent was removed by evaporation. The residue was dissolved in water (25 mL) and extracted with 30 mL of diethyl ether twice. The aqueous layer was diluted to about 300 mL with water and applied to a column (2 × 20 cm) of Dowex 1-X8 (Cl^- form; 200–400 mesh) washed with water. The column was washed with 100 mL of water followed by 930 mL of 7.5 mM HCl to remove AMP and pyridoxal phosphate. AP_2-PL was eluted with 7.5 mM HCl containing 15 mM LiCl. Elution was conducted at a flow rate of 5 mL/min by using a FMI Lab Pump Model RP-SY, and the absorbance at 290 nm of the effluent was continuously monitored on a UVILOG 5 IIIA UV detector. Appropriate fractions were pooled and adjusted to pH 6.8 with 1 N LiOH. The solution was concentrated to a small volume (about 2 mL) on a rotary evaporator below 25 °C. The concentrated solution was placed in a centrifugal tube, and 30 mL of cold acetone/methanol (4:1 v/v) was added all at once. The mixture was kept at 0 °C for a while and then centrifuged. After removal of the supernatant, the precipitate was washed successively with acetone/methanol (4:1 v/v), acetone, and diethyl ether. After the precipitate had been dried over phosphorus pentoxide in vacuo overnight, it was dissolved in 30 mL of water and applied to a column (1.2 × 12 cm) of Dowex 50-X8 (H^+ form; 200–400 mesh) washed with water. The column was developed with water, and 15-mL fractions were collected. An aliquot of each fraction was mixed with 2 mL of 0.1 N NaOH, and the absorbances at 260 and 390 nm of the mixture were measured. The fractions in which the value of A_{260nm}/A_{390nm} was 2.30 ± 0.02 were pooled and neutralized with 0.2 N NaOH. The solution was concentrated to less than 1 mL, and 40 mL of ice-cold acetone was added. The precipitate was washed successively with acetone and diethyl ether and then dried over phosphorus pentoxide in vacuo overnight. The material was ground in an agate mortor with a pestle and dried at 40 °C overnight. The yield was 75 mg. Anal. Calcd for $C_{18}H_{24}N_6O_{14}P_2Na_2$ (AP_2-PL·2Na·2H_2O): C, 32.91; H, 3.69; N, 12.80. Found: C, 33.15; H, 3.99; N, 12.06. The molar absorption coefficient at 394 nm of AP_2-PL in 0.1 N NaOH was 5060 M^{-1} cm^{-1}. The NMR spectrum of AP_2-PL (in D_2O, internal standard sodium 3-(trimethylsilyl)-1-propanesulfonate; in ppm) showed the following: 10.2 (1 H, s, 4″-CHO), 8.32 (1 H, s, 2-H), 8.11 (1 H, s, 8-H), 7.40 (1 H, s, 6″-H), 5.96 (1 H, d, 1′-H), 5.01 (1 H, m, 5″-CH_2), and 2.29 (3 H, s, 2″-CH_3).

Synthesis of AP_3-PL. AP_3-PL was synthesized from pyridoxal phosphate and ADP in essentially the same manner as described above for the synthesis of AP_2-PL. AP_3-PL was purified as follows. The crude solution of AP_3-PL was applied to a column (2 × 20 cm) of Dowex 1-X8 (Cl^- form; 200–400 mesh) at a flow rate of 8.6 mL/min. The column was washed with 80 mL of water followed by 100 mL of 10 mM HCl, and finally, the materials were eluted with a linear gradient of 10 mM HCl to 10 mM HCl containing 0.1 M LiCl (1 L/1L). The absorbance at 290 nm of the effluent was continuously monitored. Fractions corresponding to the second broad peak were pooled and neutralized with 1 N LiOH. The neutralized solution was concentrated to a small volume, and the precipitate was obtained as described above. After the precipitate had been dried in vacuo over phosphorus pentoxide, it was applied to a column (2.8 × 12 cm) of (aminoethyl)cellulose (Cl^- form). After the column had been successively washed with 45 mL of water and 45 mL of 10 mM HCl, the materials were eluted with 300 mL of 10 mM HCl containing 20 mM LiCl. Fractions containing AP_3-PL were pooled and neutralized with 1 N LiOH. The solution was concentrated to a small volume, and the precipitate was obtained as described above. After the precipitate had been dried in vacuo over phosphorus pentoxide, it was dissolved in a small volume of water and applied to a column of (aminoethyl)cellulose (Cl^- form) again. The materials were eluted as described above. Fractions in which the value of A_{260nm}/A_{390nm} in 0.1 N NaOH was 2.22 ± 0.02 were pooled and neutralized with 1 N LiOH. The neutralized solution was concentrated to a small volume, and the precipitate was obtained as described above. The trilithium salt of AP_3-PL was dried at 40 °C in vacuo over phosphorus pentoxide and stored at −20 °C. The yield was 53 mg. Anal. Calcd for $C_{18}H_{28}N_6O_{19}P_3Li_3$ (AP_3-PL·3Li·4H_2O): C, 28.97; H, 3.79; N, 11.26. Found: C, 28.87; H, 4.09; N, 10.38. The molar absorption coefficient at 393 nm in 0.1 N NaOH was 5550 M^{-1} cm^{-1}. The NMR spectrum of AP_3-PL (in D_2O; in ppm) showed the following: 10.2 (1 H, s, 4″-CHO), 8.37 (1 H, s, 2-H), 8.13 (1 H, s, 8-H), 7.51 (1 H, s, 6″-H), 5.94 (1 H, d, 1′-H), 5.06 (1 H, d, 5″-CH_2), and 2.32 (3 H, s, 2″-CH_3).

Synthesis of AP_4-PL. Since the synthesis of P^1-5′-adenosyl P^4-diphenyl tetraphosphate in the same manner as described above was unsuccessful, we synthesized AP_4-PL by the anion exchange between P^1-5′-pyridoxalyl P^2-diphenyl diphosphate and ATP. To P^1-5′-pyridoxalyl P^2-diphenyl diphosphate, which was synthesized as described above, the tributylammonium salt of ATP dissolved in 5 mL of dimethylformamide and 20 mL of pyridine were successively added. The resultant solution was kept at room temperature overnight and then evaporated. The residue was dissolved in water (30 mL) and extracted with 40 mL of diethyl ether twice. The aqueous layer was diluted to about 300 mL with water and applied to a column (2 × 20 cm) of Dowex 1-X8 (Cl^- form; 200–400 mesh). The column was successively washed with 50 mL of water, 300 mL of 10 mM HCl, and 400 mL of 10 mM HCl containing 40 mM LiCl. The materials were eluted with a linear gradient of 10 mM HCl containing 40 mM LiCl to 10 mM HCl containing 0.2 M LiCl (1 L/1 L). Effluent was monitored by the absorbance at 254 nm. Fractions containing AP_4-PL and ATP were combined and neutralized with 1 N LiOH. The solution was concentrated to a small volume, and the precipitate was obtained as described above and dried in vacuo over phosphorus pentoxide. It was dissolved in a small

volume of water and applied to a column (1.2 × 20 cm) of (aminoethyl)cellulose (Cl^- form). The column was successively washed with 40 mL of 10 mM HCl and 70 mL of 10 mM HCl containing 70 mM LiCl. The materials were eluted with 350 mL of 10 mM HCl containing 80 mM LiCl. The fractions in which the values of A_{260nm}/A_{390nm} in 0.1 N NaOH were 2.27 ± 0.06 were combined and neutralized with 1 N LiOH. The solution was concentrated to a small volume, and the precipitate was obtained as described above. The tetralithium salt of AP_4-PL was dried at 40 °C in vacuo over phosphorus pentoxide and stored at −20 °C. The yield was 70 mg. Anal. Calcd for $C_{18}H_{32}N_6O_{24}P_4Li_4$ (AP_4-PL·4Li·6H_2O): C, 24.90; H, 3.72; N, 9.68. Found: C, 24.92; H, 3.73; N, 8.59. The molar absorption coefficient at 392 nm in 0.1 N NaOH was 5800 M^{-1} cm^{-1}. The NMR spectrum of AP_4-PL (in D_2O; the ppm) showed the following: 10.3 (1 H, s, 4″-CHO), 8.40 (1 H, s, 2-H), 8.14 (s, 8-H), 7.51 (1 H, s, 6″-H), 5.98 (1 H, d, 1′-H), 5.07 (2 H, d, 5″-CH_2), 2.34 (3 H, s, 2″-CH_3).

High-Performance Liquid Chromatography of Pyridoxal Compounds. The purity of AP_n-PLs synthesized was evaluated on high-performance liquid chromatography with an anion-exchange Whatman Partisil 10-SAX column (4.6 × 300 mm) at room temperature on a Gilson liquid chromatography system at a flow rate of 1 mL/min. The solvents used were (A) 30 mM ammonium phosphate buffer (pH 4.1) and (B) 0.4 M ammonium phosphate buffer (pH 4.5) containing 0.8 M KCl. A linear gradient from 0 to 90% buffer B was applied for 30 min followed by an isocratic period of 25 min with 90% buffer B. Since AP_n-PLs were not eluted from the column under these conditions, they were reduced with $NaBH_4$. The sample at a concentration of about 1 mM (10 μL) was injected, and the absorbance at 254 or 290 nm of the effluent was monitored on a Variactor 311 UV detector. The retention times of AMP, ADP, and ATP were 9.96, 29.3, and 50.6 min, respectively. Each preparation of AP_n-PLs gave a single absorption peak with retention times of 15.8, 27.2, and 40.8 min for AP_2-PL, AP_3-PL, and AP_4-PL, respectively.

Affinity Chromatography of Lactate Dehydrogenase. Blue Toyopearl (type I) is commercially available Cibacron Blue beads of Toyo Soda Manufacture. The resin was kindly donated from the manufacturer and packed into a stainless steel column (4.6 × 250 mm) in our laboratory. Affinity chromatography of lactate dehydrogenase was carried out by using the column at room temperature on a Gilson liquid chromatography system at a flow rate of 1 mL/min. The solvents used were (A) 20 mM potassium phosphate buffer (pH 7.5) and (B) 20 mM potassium phosphate buffer (pH 7.5) containing 1 M KCl. After 10 min of an isocratic period with buffer A, a linear gradient to buffer B was applied for 50 min, and finally, an isocratic period of 10 min of buffer B. Unless otherwise stated, elution was monitored by the absorbance at 280 nm with a Variactor 311 UV detector. Fractions of 1-mL each were collected into test tubes to which 0.5 mL of a solution containing 25 mM HEPES (pH 7.5), 1 mM EDTA, and 0.25 mg/mL bovine serum albumin had been poured. When the elution program was finished, the test tubes were shaken and stored in an ice bath.

RESULTS

Modification by Pyridoxal Phosphate. Treatment of rabbit muscle lactate dehydrogenase at 30 °C with pyridoxal phosphate followed by reduction by sodium borohydride resulted in a time-dependent loss of enzymatic activity (Figure 1). The inactivation showed biphasic reaction kinetics: the initial rapid phase and the second slower phase. A similar biphasic pattern of inactivation by pyridoxal phosphate was observed for pig

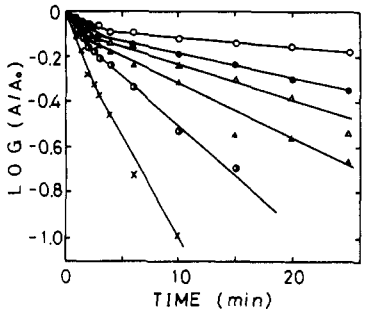

FIGURE 1: Inactivation of lactate dehydrogenase by pyridoxal phosphate. The reaction mixture contained 42 mM HEPES (pH 7.8), 0.83 mM EDTA, 86 μg of enzyme, and pyridoxal phosphate at 2 (○), 3 (●), 4 (△), 5 (▲), 8 (◑), or 12 mM (×) in a final volume of 0.24 mL. The mixture was incubated at 30 °C. At the time shown, 20 μL of the mixture was taken, and 3 μL of 0.1 M $NaBH_4$ was added. After 10 s, an ice-cold solution (0.5 mL) containing 25 mM HEPES (pH 7.8), 1 mM EDTA, and 0.25 mg/mL bovine serum albumin was added. Aliquots of the solution were assayed for the residual activity.

lactate dehydrogenase and porcine malate dehydrogenase (Wimmer et al., 1975; Gould & Engel, 1982). Since pyridoxal phosphate or lactate dehydrogenase alone is stable under these conditions, the biphasic nature observed is probably intrinsic to the inactivation reaction. A possibility of a change in quaternary structure of the enzyme during inactivation is unlikely because no difference between the native and the inactivated enzymes was observed in the behavior on TSK-GEL G3000SW gel filtration chromatography (data not shown). In the double-reciprocal plots of the apparent first-order rate constant (k_{app}) vs. the reagent concentration, a straight line for the initial rapid phase has a finite vertical intercept, suggesting that the reagent forms a dissociable complex prior to inactivation. By contrast, the plot for the second slow phase gives a hyperbolic line. Extrapolation of this line intercepts at zero on the ordinate, suggesting that the reagent binds to the enzyme in this phase through the bimolecular reaction.

The effect of substrates on the inactivation of the enzyme by pyridoxal phosphate was tested. NADH at a concentration of 25 μM afforded almost complete protection from inactivation, whereas pyruvate at a concentration of 1.1 mM provided only slight protection (data not shown). This implies that the modification occurs at a portion of the active site that is involved in the binding of the coenzyme. The spectrum of the pyridoxal phosphate–enzyme reduced by sodium borohydride followed by extensive dialysis against potassium phosphate buffer (pH 7.0) showed an absorption maximum at 325 nm in addition to the typical absorption maximum of protein at 280 nm. The absorption maximum at 325 nm is characteristic of the formation of ε-[(aminophospho)-pyridoxyl]lysine (Fischer et al., 1963). The stoichiometry of bound pyridoxal phosphate with respect to inactivation was calculated in two separate experiments with a value of 10 150 M^{-1} cm^{-1} for the absorption coefficient at 325 nm of bound pyridoxal phosphate (Fischer et al., 1963). When the residual activity is plotted against moles of pyridoxal phosphate incorporated, there was a linear relationship to 80% inactivation (not shown). Extrapolation to 100% loss of enzymatic activity gave a value of 5.1 mol/mol of enzyme subunit.

In order to further characterize the inactivation reaction, pyridoxal phosphate modified enzymes with different residual activities were subjected to affinity chromatography on a Blue Toyopearl column, which can separate proteins according to the affinity for nucleotides including NADH. Figure 2 shows the elution profiles of the modified enzymes. The intact en-

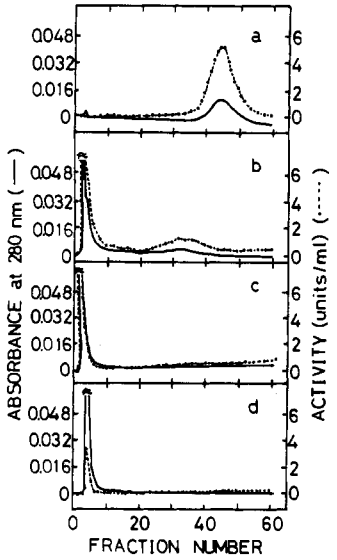

FIGURE 2: Blue Toyopearl chromatography of pyridoxal phosphate modified lactate dehydrogenase. Each reaction mixture contained 42 mM HEPES (pH 7.8), 0.83 mM EDTA, 0.59 mg of enzyme, and 10 mM pyridoxal phosphate in a final volume of 0.84 mL. After incubation at 30 °C for 1, 2, and 15 min, 120 μL of 0.1 M $NaBH_4$ was added. After the mixture was extensively dialyzed against 20 mM potassium phosphate (pH 7.0), a 200-μL portion of the mixture was applied to a Blue Toyopearl column (4.6 × 250 mm) and eluted as described in the text. Unmodified enzyme with K_m for NADH of 7.1 μM (a) and modified enzymes with residual activities of 71 (b), 52 (c), and 7.0% (d), having K_m values for NADH of 20.0, 20.8, and 22.2 μM, respectively.

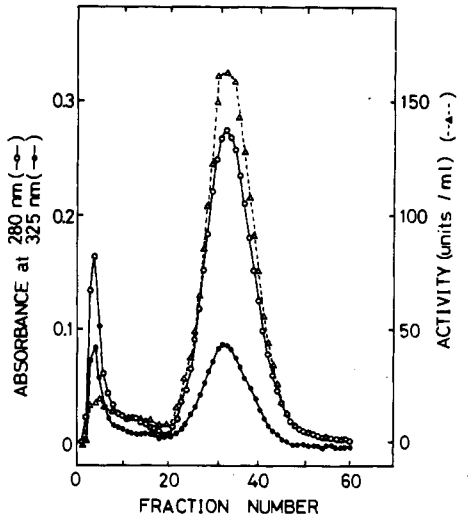

FIGURE 3: Blue Toyopearl chromatography of lactate dehydrogenase modified by pyridoxal phosphate in the presence of NADH. The enzyme (4.9 mg) was incubated at 30 °C with 10 mM pyridoxal phosphate in the presence of 60 μM NADH for 30 min and then treated with $NaBH_4$. The modified enzyme was precipitated with 80% saturation of ammonium sulfate. The precipitates were collected by centrifugation and dissolved in 1 mL of 20 mM potassium phosphate buffer (pH 7.0). After extensive dialysis, the sample was applied to a column (4.6 × 250 mm) of Blue Toyopearl and eluted as described in the text. The effluent was collected in test tubes. The absorbances at 280 and 325 nm of each fraction were measured on a UVIDEC 505 spectrophotometer with a 1-mL cuvette.

zyme showed a single peak eluted at 0.70 M potassium chloride in addition to a trace amount of unbound materials with no enzymatic activity (Figure 2a). Treatment of the enzyme with pyridoxal phosphate significantly changed its elution profile. In the elution profile of the modified enzyme with 71% residual activity, the intact enzyme species almost completely disappeared, and several new species were detectable (Figure 2b). All of the new species detected were eluted prior to the native enzyme. These observations are consistent with the results of kinetic studies that the modified enzymes had lower affinities for NADH (the legend to Figure 2). As the inactivation further proceeded, the species that eluted at 0.46 M potassium chloride disappeared, and unbound materials further increased (Figure 2c). A precise comparison of the absorbance at 280 nm with enzymatic activity revealed that the pass-through fractions contained at least two species, one with a relatively high enzymatic activity and the other with little or no enzymatic activity. As the inactivation proceeded, the amount of the former species decreased, whereas that of the latter species increased.

Since NADH almost completely protects the enzyme from inactivation by pyridoxal phosphate, it would be of interest to investigate whether the behavior on affinity chromatography of the enzyme modified in the presence of the coenzyme differs from that of the intact enzyme. Figure 3 shows the elution profile of the enzyme modified by pyridoxal phosphate in the presence of 60 μM NADH. A major peak was eluted at 0.43 M potassium chloride. The specific activity and K_m for NADH of fraction 33 were 780 units/mg of protein and 11.6 μM, respectively. Although this species had essentially the same specific activity as the intact enzyme did, the affinity for NADH slightly decreased. The analysis of the absorption spectrum of this species revealed that it bound pyridoxal phosphate as much as 1.6 mol/mol of enzyme subunit.

Modification by AP_n-PL. The above results of the modification of lactate dehydrogenase by pyridoxal phosphate suggest that this enzyme has an essential lysyl residue(s) at or near the NADH-binding site. However, the modification by pyridoxal phosphate is not limited to these lysyl residues, because more than 5 mol of the reagent was actually incorporated into the enzyme subunit concomitantly with the complete loss of enzymatic activity. These results are essentially the same as those observed in lactate dehydrogenase from other sources (Chen & Engel, 1975; Gould & Engel, 1982). To prepare the reagents with higher specificities for the active site of the enzyme, we have introduced adenosine polyphosphate moieties to pyridoxal phosphate. AP_2-PL, AP_3-PL, and AP_4-PL were actually synthesized. When lactate dehydrogenase was incubated at 30 °C with 0.5 mM AP_2-PL and then reduced by sodium borohydride, the enzyme was inactivated by 52% in 30 min (Figure 4a). Further addition of AP_2-PL did not increase the extent of inactivation. We have applied reduction by sodium borohydride to fix the aldehyde reagent to lysyl residue(s) of the enzyme. However, inactivation proceeded in essentially the same manner even without sodium borohydride treatment. Therefore, binding of AP_2-PL to the enzyme appears to be stable to hydrolysis.

Figure 4b shows the dependence of inactivation on the concentration of AP_n-PL. Since the inactivation reactions with all the AP_n-PLs were completed within 30 min, the residual activities after 30-min incubation were measured. At 1 mM each, AP_2-PL, AP_3-PL, and AP_4-PL inactivated the enzyme by 64, 51, and 34%, respectively. The effectiveness of AP_n-PLs for inactivation of the enzyme appears to be directly related to the number of the phosphate groups in the reagents. At lower concentrations of the reagents, however, the order of the effectiveness is not apparent. Incubation of the enzyme with an equimolar amount of AP_n-PL for 30 min resulted in a loss of enzymatic activity by approximately 30%, regardless of the reagents used. NADH almost completely protected the enzyme from inactivation, whereas pyruvate showed no protection (data not shown). The results of X-ray crystallographic studies on dogfish lactate dehydrogenase at 2.8-Å resolution

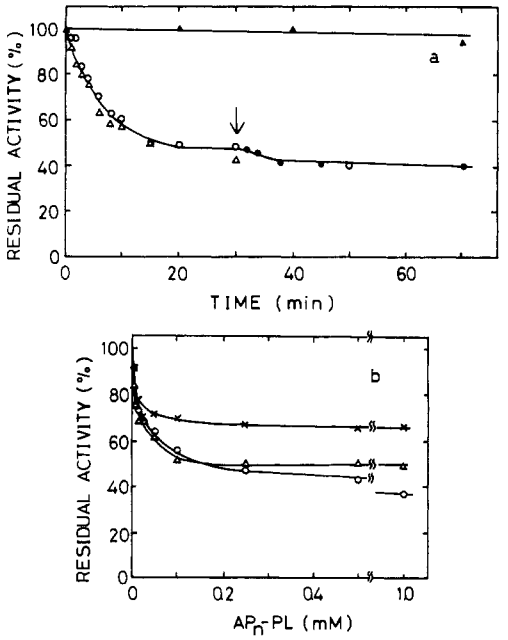

FIGURE 4: Inactivation of lactate dehydrogenase by AP_n-PL. (a) Time dependence. The reaction mixture contained 42 mM HEPES (pH 7.8), 0.83 mM EDTA, 0.33 mg of enzyme, and 0.5 mM AP_2-PL in a final volume of 0.72 mL. The mixture was incubated at 30 °C. At the time shown, 20 μL of the mixture was taken and mixed with 3 μL of 0.1 M $NaBH_4$ (○) or water (△). After 10 s, an ice-cold solution (0.5 mL) containing 25 mM HEPES (pH 7.8), 1 mM EDTA, and 0.25 mg/mL bovine serum albumin was added. Aliquots were assayed for the residual activity. After incubation for 30 min, 0.2 mL of the remaining mixture was taken, and 50 μL of 3.0 mM AP_2-PL was further added. Incubation was continued at 30 °C. At the time shown, 20 μL of the mixture was taken, and 3 μL of 0.1 M $NaBH_4$ was added. After dilution, the residual activity was measured (●). For control, the enzyme was incubated without AP_2-PL (▲). (b) Concentration dependence. The reaction mixture contained 42 mM HEPES (pH 7.8), 0.83 mM EDTA, 9.4 μg of enzyme, and the indicated concentrations of AP_n-PL in a final volume of 30 μL. After incubation at 30 °C for 30 min, 3 μL of 0.1 M $NaBH_4$ was added to the mixture. After 10 s, the mixture was diluted, and the residual activity was assayed. AP_2-PL (○), AP_3-PL (△), and AP_4-PL (×).

showed that AMP and ADP bind to the NADH-binding site and induce a conformation change of the enzyme in the same way as the coenzyme does (Chandrasekhar et al., 1973). These results suggest that AP_n-PLs, which contain an ADP moiety, bind to the NADH-binding site in the active site of the enzyme.

To determine the relationship between the moles of AP_2-PL incorporated and the loss of enzymatic activity, the enzyme was modified with AP_2-PL for different periods of time and reduced by sodium borohydride. The amount of the reagent incorporated was estimated on the basis of the assumption that its absorption coefficient at 325 nm is the same as that of bound pyridoxal phosphate, 10 150 M^{-1} cm^{-1} (Fischer et al., 1963). When the residual activity is plotted as a function of moles of the reagent incorporated, there was a linear relationship to 65% inactivation (Figure 5). Extrapolation to 100% loss of enzymatic activity gave a value of 1.25 mol of AP_2-PL/mol of enzyme subunit. Alternatively, the amount of AP_2-PL incorporated was estimated on the basis of the increase in the absorbance at 430 nm on a difference spectrum, which results from the formation of a Schiff base between the reagent and a lysyl residue in the enzyme (Metzler, 1957). The absorption coefficient at 430 nm of the Schiff base was assumed to be the same as that of a pyridoxal–*n*-hexylamine complex, 6970 M^{-1} cm^{-1} (Tagaya et al., 1985). A linear relationship between the residual activity and moles of the

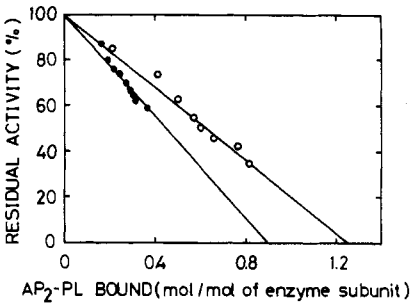

FIGURE 5: Stoichiometry of the reaction between lactate dehydrogenase and AP_2-PL. Each reaction mixture contained 42 mM HEPES (pH 7.8), 0.83 mM EDTA, 0.66 mg of enzyme, and 0.5 mM AP_2-PL. After the mixture had been incubated at 30 °C for 1.5, 3, 4.5, 6, 8, 10, 15, and 30 min, 60 μL of 0.1 M $NaBH_4$ was added to the mixture. The solution was extensively dialyzed against 20 mM potassium phosphate (pH 7.0), and then the concentration of the enzyme, the amount of bound AP_2-PL, and the residual activity were determined as described in the text (○). In another series of experiments, one compartment of a cuvette contained 0.75 mg of enzyme, 49 mM HEPES (pH 7.8), 0.99 mM EDTA, and 55 μM AP_2-PL in a final volume of 2.03 mL. In another compartment, enzyme was omitted. Incubation was carried out at 30 °C. The absorbance at 430 nm was continuously monitored. At 1, 2, 3, 4, 6, 8, 10, 15, and 30 min, 20 μL of the mixture was taken, and 3 μL of 0.1 M $NaBH_4$ was added. After 10 s, an ice-cold solution (0.5 mL) containing 25 mM HEPES (pH 7.8), 1 mM EDTA, and 0.25 mg/mL bovine serum albumin was added. Aliquots were taken and assayed for the residual activity. The amount of bound AP_2-PL was calculated from the absorbance at 430 nm as described in the text (●).

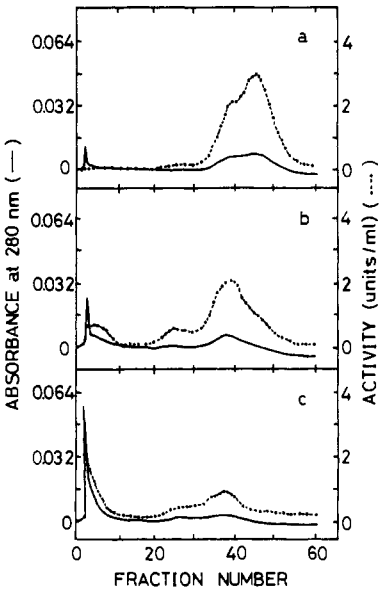

FIGURE 6: Blue Toyopearl chromatography of AP_2-PL-modified enzyme used for the experiments of Figure 5. The modified enzyme (200 μL) was applied to a Blue Toyopearl column (4.6 × 250 mm) and eluted as described in the text. Modified enzymes with residual activities of 88 (a), 71 (b), and 56% (c).

reagent incorporated was obtained up to 40% inactivation (Figure 5). Extrapolation to 100% loss of enzymatic activity gave a value of 0.90 mol/mol of enzyme subunit. Experiments using AP_3-PL and AP_4-PL gave values of 0.82 and 0.79 mol/mol of enzyme subunit, respectively. All of these results indicate that binding of AP_n-PL to enzyme subunit in an equimolar amount corresponds to the complete loss of enzymatic activity.

For further analysis of the relationship between modification and inactivation, the AP_2-PL-modified enzymes with different residual activities were subjected to affinity chromatography on a Blue Toyopearl column (Figure 6). In the elution profile for the modified enzyme with 88% residual activity, an enzyme

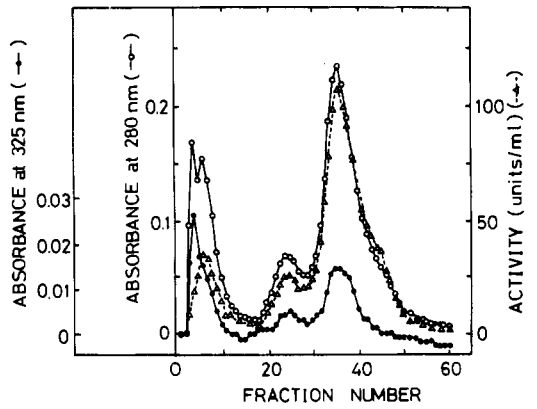

FIGURE 7: Blue Toyopearl chromatography of AP_2-PL-modified lactate dehydrogenase. The enzyme (4.2 mg) was incubated at 30 °C with 0.5 mM AP_2-PL for 6 min. After $NaBH_4$ treatment, the modified enzyme was precipitated with 80% saturation of ammonium sulfate. The precipitates were collected by centrifugation and then dissolved in 1 mL of 20 mM potassium phosphate buffer (pH 7.0). After extensive dialysis, the material was applied to a column (4.6 × 250 mm) of Blue Toyopearl and eluted as described in the text. The effluent was collected in test tubes. The absorbances at 280 and 325 nm of each fraction were measured on a UVIDEC 505 spectrophotometer with a 1-mL cuvette.

Table I: Properties of Peaks I–IV Separated on Blue Toyopearl Chromatography of AP_2-PL-Modified Lactate Dehydrogenase[a]

peak	elution position (M KCl)	sp act. (units/mg of protein)	K_m for NADH (μM)	AP_2-PL bound (mol/mol of enzyme subunit)
I	0	171	35.7	0.81
II	0	345	16.1	0.51
III	0.28	556	12.5	0.39
IV	0.53	645	9.6	0.32

[a] The experimental procedures are described in the legend to Figure 7.

species eluted at 0.56 M potassium chloride was detected besides the intact enzyme. As inactivation proceeded, the amount of unbound materials increased. However, a considerable amount of the above new species remained even after 30-min incubation, at which time nearly half of the original enzymatic activity was lost. In order to analyze more precisely the enzyme species formed during modification, a larger amount of the enzyme was modified by AP_2-PL and analyzed on an affinity column of Blue Toyopearl. Figure 7 shows the elution profile, in which the presence of at least four enzyme species besides the intact enzyme are apparent. The enzyme species are referred to as peaks I–IV, according to the order of elution. Table I summarizes the specific activity, the K_m for NADH, and the amount of bound AP_2-PL of peaks I–IV. Although peaks I and II were eluted close together, they are significantly different in their affinities for NADH. The amount of bound AP_2-PL in peak I is slightly underestimated, due to the unbound materials that contaminated in the original enzyme preparation used.

Discussion

Pyridoxal phosphate has been utilized for selective chemical modification of lysyl residues in a variety of enzymes. In most cases, however, the modification with this reagent is nonspecific for the active site lysyl residues. Our previous study has demonstrated that the introduction of a UMP moiety to pyridoxal phosphate makes the resultant reagent an effective probe for the active site of glycogen synthase (Tagaya et al., 1985). The main purpose of this study was to evaluate the effectiveness of AP_n-PLs as affinity probes for adenine nucleotide binding sites of enzymes. For this purpose, lactate dehydrogenase was chosen on the basis of the following reasons. (1) This enzyme requires NADH, which contains an ADP moiety in its structure. (2) The primary and tertiary structures of dogfish lactate dehydrogenase have been well studied by Taylor (1977) and Holbrook et al. (1975), respectively. (3) The nonspecific modifications of the M_4 and H_4 isozymes of pig lactate dehydrogenase have been reported by Gould and Engel (1982). To compare the effectiveness of AP_n-PL with that of pyridoxal phosphate, the kinetics and stoichiometry of inactivation of rabbit muscle lactate dehydrogenase by these reagents were investigated.

The present results of inactivation by pyridoxal phosphate showed that the reagent reacts with not only the lysyl residue(s) essential for enzymatic activity but also other reactive residues in the rabbit muscle enzyme. In the presence of NADH, despite the almost complete protection of the enzyme from inactivation, 1.6 mol of the reagent was incorporated to enzyme subunit (Figure 3), suggesting that at least this amount of the bound pyridoxal phosphate does not affect enzymatic activity. The inactivation reaction by pyridoxal phosphate is characterized by its biphasic nature. In the initial phase, the enzyme has a considerable affinity for pyridoxal phosphate, whereas in the second phase it has little or no affinity for the reagent. The analyses of the modified enzyme on Blue Toyopearl affinity chromatography and by the kinetic study showed that the decrease in the affinity of the enzyme for NADH preceded the loss of enzymatic activity (Figure 2). These results might be consistent with the fact that the modified enzyme in the second phase has little or no affinity for pyridoxal phosphate.

In contrast with the modification by pyridoxal phosphate, AP_n-PL was exclusively bound to the nucleotide-binding site of the rabbit muscle enzyme. The decrease in the affinity of the enzyme for NADH and the loss of enzymatic activity of the enzyme paralleled in the modification by AP_2-PL. Four enzyme species having different affinities for NADH were separated on Blue Toyopearl affinity chromatography of the enzyme modified by AP_2-PL (Figure 7). Peaks I–IV contain 0.81, 0.51, 0.39, and 0.32 mol of the bound reagent/mol of enzyme subunit, respectively (Table I). Thus, the change in the affinity of the enzyme for NADH correlates with the incorporation of AP_2-PL. Since lactate dehydrogenase is tetrameric (Holbrook et al., 1975), it is tempting to assume that they correspond to the tetrameric enzymes containing 4, 3, 2, and 1 mol of the bound reagent, respectively. We suggest that the modification of one subunit causes a conformational change of the other subunits, which results in the decrease in the affinity of the whole enzyme for NADH.

One problem for the modification of lactate dehydrogenase by AP_n-PL is that inactivation was not complete even at a high concentration of the reagent and for a prolonged time of incubation. This pattern of inactivation has also been observed for the modification of lactate dehydrogenase by pyridoxal phosphate (Chen & Engel, 1975; Gould & Engel, 1980). Chen and Engel (1975) explained that the residual activity is due to the existence of an equilibrium between the noncovalent and covalent enzyme–reagent complexes. Treatment of the modified enzyme with sodium borohydride might fix only the covalent complex, whereas the noncovalent complex might dissociate rapidly on dilution in the assay. Another explanation for the incomplete inactivation would be that the enzyme is incompletely modified by the reagent. In the analysis of the modified enzyme on the affinity column, the intact enzyme

disappeared in the early phase of modification, whereas peak III having a considerable enzymatic activity remained even after 30-min incubation (Figure 7). It appears that the modification of one subunit causes a conformational change of the other subunits, which results in not only the decrease in the affinity of the whole enzyme for NADH but also the decrease in the reactivity of the remaining subunits with AP_n-PL. A similar explanation was made for the modification of isocitrate dehydrogenase by the 2′,3′-dialdehyde derivative of ADP in which the rate of inactivation gradually decreased as modification proceeded (King & Colman, 1983).

We have initially expected that only one of the AP_n-PLs tested could specifically modify the active site of lactate dehydrogenase. In contrast to our expectation, the enzyme was considerably inactivated by all of the AP_n-PLs tested; the difference in the effectiveness is not apparent at lower concentrations of AP_n-PLs. The X-ray crystallographic analysis of dogfish lactate dehydrogenase revealed that only Lys-57 locates near the pyrophosphate moiety of the bound coenzyme (Holbrook et al., 1975; Taylor, 1977). The N atom of the ϵ-amino group of Lys-57 locates 3.7 and 6.0 Å apart from the 3′-O and 2′-O atoms, respectively, of the ribose in the adenosine moiety and 6.2 Å apart from the adenosine proximal P atom. The results of our model building study of AP_n-PLs show that the 4-formyl group of any compound can be adequately located close to the N atom of the ϵ-amino group of Lys-57, if the rotation of the polyphosphate moiety is allowed to a considerable extent. The free energy of binding of AMP and ADP to lactate dehydrogenase is nearly equal (McPherson, 1970). The X-ray crystallographic analysis of ADP-bound dogfish lactate dehydrogenase revealed that the β-phosphate of ADP occupies partially the two separate sites (Chandraseikar et al., 1973). These results suggest that the β-phosphate of ADP is insignificant for the binding of an ADP molecule. Therefore, the binding of the polyphosphate moiety of AP_n-PLs except for the adenosine proximal phosphate is not strictly restricted, and the 4-formyl group of any AP_n-PL can be located in similar positions in the enzyme.

On the other hand, it is clear that the degrees of inactivation by AP_n-PL vary according to the number of the phosphate groups in the compounds. Incubation of the enzyme with AP_2-PL, AP_3-PL, or AP_4-PL at a concentration of 1 mM each resulted in the loss of enzymatic activity by 64, 51, and 34%, respectively (Figure 4b). If we assume that the residual activity is due to the existence of an equilibrium between the noncovalent and covalent complexes, the equilibrium constants for interconversion between the two complexes decrease as the number of the phosphate groups increases. These results suggest that the reactive group of AP_2-PL is most naturally fitted to the essential lysyl residue, possibly Lys-57, without steric hindrance.

After completion of this study, we noted a preliminary report by Tamura et al. (1985). They have synthesized AP_2-PL and tested it as an affinity label for the essential lysyl residues on several nucleotide-binding enzymes. The reagent binds more tightly and exhibits greater specificity for inactivation than pyridoxal phosphate. These results, combined with the previous (Tagaya et al., 1985) as well as the present results of our studies, show that this type of reagent is useful in the modification of the lysyl residue at the nucleotide-binding sites of proteins.

Registry No. AP_2-PL, 4500-99-6; AMP, 61-19-8; AP_2-PL·2Na, 101418-65-9; AP_3-PL, 101418-63-7; ADP, 58-64-0; AP_3-PL·3Li, 101418-66-0; AP_4-PL, 101418-64-8; ATP, 56-65-5; AP_4-PL·4Li, 101418-67-1; NADH, 58-68-4; P^1-5′-adenosyl P^2-diphenyl diphosphate, 32452-84-9; P^1-5′-adenosyl P^3-diphenyl triphosphate, 101418-68-2; P^1-5′-pyridoxyl P^2-diphenyl diphosphate, 101418-69-3; L-lysine, 56-87-1; lactate dehydrogenase, 9001-60-9; pyridoxal phosphate, 54-47-7.

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