Adenosine 5′-*O*-[*S*-(4-Succinimidyl-benzophenone)thiophosphate]: A New Photoaffinity Label of the Allosteric ADP Site of Bovine Liver Glutamate Dehydrogenase†

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ABSTRACT: By reaction of adenosine 5′-monothiophosphate with benzophenone-4-maleimide, we synthesized adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate] (AMPS-Succ-BP) as a photoreactive ADP analogue. Bovine liver glutamate dehydrogenase is known to be allosterically activated by ADP, but the ADP site has not been located in the crystal structure of the hexameric enzyme [Peterson, P. E., and Smith, T. J. (1999) *Structure 7*, 769−782]. In the dark, AMPS-Succ-BP reversibly activates GDH. Irradiation of the complex of glutamate dehydrogenase and AMPS-Succ-BP at $\lambda > 300$ nm causes a time-dependent, irreversible 2-fold activation of the enzyme. The k_{obs} for photoactivation shows nonlinear dependence on the concentration of AMPS-Succ-BP, with $K_R = 4.9$ μM and $k_{max} = 0.076$ min^{-1}. The k_{obs} for photoreaction by 20 μM AMPS-Succ-BP is decreased 10-fold by 200 μM ADP, but is reduced less than 2-fold by NAD, NADH, GTP, or α-ketoglutarate. Modified enzyme is no longer activated by ADP, but is still inhibited by GTP and high concentrations of NADH. These results indicate that reaction of AMPS-Succ-BP occurs within the ADP site. The enzyme incorporates up to 0.5 mol of [^{3}H]AMPS-Succ-BP/mol of enzyme subunit or 3 mol of reagent/mol of hexamer. The peptide Lys^{488}−Glu^{495} has been identified as the only reaction target, and the data suggest that Arg^{491} is the modified amino acid. Arg^{491} (in the C-terminal helix close to the GTP #2 binding domain of GDH) is thus considered to be at or near the enzyme's allosteric ADP site. On the basis of these results, the AMPS-Succ-BP was positioned within the crystal structure of glutamate dehydrogenase, where it should also mark the ADP binding site of the enzyme.

Bovine liver glutamate dehydrogenase is a homohexameric mitochondrial enzyme that reversibly catalyzes the reductive amination of α-ketoglutarate to L-glutamate using NADH as coenzyme. The mammalian form of glutamate dehydrogenase is subject to extensive allosteric regulation by nucleotides (*1*). ADP activates the enzyme, whereas GTP and high concentrations of NADH are inhibitors. The allosteric inhibitor GTP and the coenzyme NADH each bind at two sites per subunit of the enzyme, while the allosteric activator ADP occupies at least one distinctive binding site per monomer (*2, 3*). Recent reports on congenital hyperinsulinism show that the disease is associated with an altered nucleotide binding site in glutamate dehydrogenase which causes reduced inhibition by GTP (*4*−*6*).

In an important development for the field, the crystal structure of bovine liver glutamate dehydrogenase has recently been solved at 2.8 Å resolution (*7*). For each monomer, two GTP binding sites and two binding sites for NADH have been located. These sites are illustrated in Figure 1 for one subunit of the enzyme hexamer. However, the ADP binding site has not been identified.

Affinity labeling using reactive purine nucleotide analogues has provided substantial information about nucleotide binding sites (*1, 8*), much of which is summarized in Table 2 of (*7*). Nucleotide photoaffinity labels offer photocontrol of the chemical cross-linking between the label and the enzyme. Among these, benzophenone photophores (*9*−*13*), because of their higher stability at ambient temperatures, activation at 350−360 nm, and reaction with the relatively unreactive C−H bonds, hold definite advantage over azido analogues. The carbonyl oxygen also serves as a hydrogen bonding acceptor, which could contribute to the strength of protein−probe interactions.

We have now synthesized adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate] (AMPS-Succ-BP),[1] shown in Figure 2, which features an adenosine 5′-monothiophosphate moiety linked to a reactive benzophenone photophore through the succinimidyl ring to promote specific recognition and high binding affinity. In the present study, we report the photolabeling of an ADP regulatory site by

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[1] Abbreviations: GDH, bovine liver glutamate dehydrogenase; AMPS, adenosine 5′-*O*-thiomonophosphate; BP-MAL, benzophenone-4-maleimide; AMPS-Succ-BP, adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate]; AMPS-BDB, adenosine 5′-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate]; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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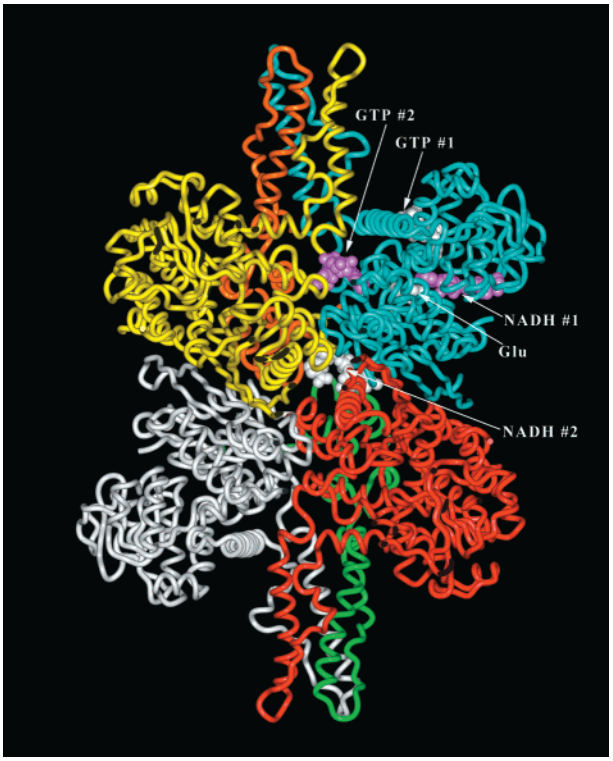

FIGURE 1: Structure of hexameric bovine glutamate dehydrogenase from (*7*). The ligands bound to the crystalline enzyme are indicated for one subunit.

AMPS-Succ-BP and locate it within the crystal structure of bovine glutamate dehydrogenase. A preliminary version of this work has been presented (*14*).

EXPERIMENTAL PROCEDURES

Materials. Bovine liver glutamate dehydrogenase was purchased from Boehringer Mannheim as a suspension in ammonium sulfate. The suspension was dialyzed against two changes of 0.1 M potassium phosphate buffer, pH 7.1 at 4 °C, for 20 h. Traces of precipitated protein were removed by centrifuging the dialyzed enzyme at 15 000 rpm for 20 min at 4 °C. The enzyme concentration was determined spectrophotometrically by using $E^{1\%}_{280nm} = 9.7$ (*15*). A molecular mass of 56 100 Da (*16*) was used for all calculations. The purified enzyme exhibited a specific activity of about 42 μmol of NADH oxidized min^{-1} (mg of enzyme)$^{-1}$, and aliquots were stored at −80 °C.

Coenzymes, nucleotides, EDTA, HEPES, Malachite green, adenosine 5′-*O*-thiomonophosphate, benzophenone-4-maleimide, PIPES, Sephadex G50-80, Tris, and *Staphylococcus aureus* V8 protease were purchased from Sigma. [2-^{3}H]-Adenosine was purchased from Amersham Life Sciences. Thiophosphoryl chloride and trimethyl phosphate were obtained from Aldrich. DE-52 cellulose was purchased from Whatman. All other chemicals were of reagent grade. Cellulose plates used for thin-layer chromatography were purchased from Kodak.

Synthesis of AMPS-Succ-BP. The adenosine 5′-*O*-[*S*-4-(succinimidyl-benzophenone)thiophosphate] (AMPS-Succ-BP) was synthesized by reacting adenosine 5′-monothiophosphate (AMPS) with benzophenone-4-maleimide, as shown in Figure 2. AMPS (3.7 mg, 10 μmol) was dissolved in 400 μL of water, and the pH of the solution was adjusted to 5.3 with acetic acid. This solution was added to a solution of BP-MAL (3 mg, 11 μmol) in 600 μL of acetonitrile, in two portions over 30 min. The mixture was stirred at 20 °C for 6 h when the reaction was found to be complete, as indicated by thin-layer chromatography on a cellulose plate with fluorescent indicator using as solvent isobutyric acid/water/ammonium hydroxide (66:33:1). Under these conditions, the product shows an R_f value of 0.7 as compared to the R_f values of 0.9 and 0.6 exhibited by BP-MAL and 5′-AMPS, respectively. The reaction mixture was lyophilized, redissolved in 1 mL of water, and centrifuged to remove any insoluble BP-MAL. The supernatant containing the crude product was subjected to purification by HPLC using a Varian model 5000 HPLC system equipped with a reverse-phase Vydac C_{18} column (0.46 × 25 cm) equilibrated with water (solvent A). After injection of the sample, the column was eluted with solvent A for 10 min to remove unreacted AMPS. A gradient was then started to 90% acetonitrile over a time period of 120 min at a flow rate of 1 mL/min. Fractions of 1 mL were collected, and the $A_{260\ nm}$ was measured. Desired fractions were pooled, lyophilized, and stored at −80 °C. A more gradual gradient was chosen for the HPLC isolation of the isomers of AMPS-Succ-BP. After the injection of the sample, the column was eluted with solvent A for 10 min to remove unreacted AMPS. A gradient was then started to 30% acetonitrile over a time period of 300 min followed by a faster gradient, reaching 100% acetonitrile in 30 min. The flow rate was 1 mL/min, and fractions of 1 mL were collected and monitored for $A_{260\ nm}$. Fractions were collected at 92 min (isomer a) and 96 min (isomer b); these were lyophilized and stored at −80 °C.

Synthesis of Radioactive AMPS-Succ-BP. [2-^{3}H]AMPS was synthesized from radioactive adenosine according to the previously reported procedure (*17*, *18*). Nonradioactive adenosine (137 mg, 0.51 mmol), mixed with [2-^{3}H]adenosine (approximately 1 mCi), was dissolved in 1 mL of dry trimethyl phosphate by warming to 60 °C in a 25 mL round-bottom flask sealed with a rubber septum. The reaction mixture was cooled to 0 °C, and freshly dried thiophosphoryl chloride (0.2 mL, 0.7 mmol) was added in two portions over 30 min. The mixture was stirred at 0 °C for 7 h to complete the reaction. After chromatographic purification of crude products on a DE-52 column (*17*), AMPS was obtained in 60% overall yield with a specific radioactivity of 1.25 × 10^{12} cpm/mol. The radioactive [2-^{3}H]AMPS-Succ-BP was synthesized by the procedures described for the synthesis of nonradioactive AMPS-Succ-BP.

Analytical Procedures. UV−Vis spectra were recorded using a Hewlett-Packard model Vectra XA spectrophotometer, and ^{1}H NMR spectra were recorded on a Bruker model DRX-400 instrument. Determination of organic phosphorus was performed after hydrolysis of the compound, according to a previously reported procedure (*19*, *20*).

The mass spectra of the samples of AMPS-Succ-BP were determined by MALDI using a Voyager DE Biospectrometry Workstation of Perspective Biosystems. The purified compound was dissolved in 0.1% trifluoroacetic acid in water. This solution (5 μL) was mixed with a saturated solution of sinapinic acid (5 μL) in a 70% water−30% acetonitrile mixture, applied to the sample plate, and air-dried. The mass of the compound was determined at an accelerating voltage

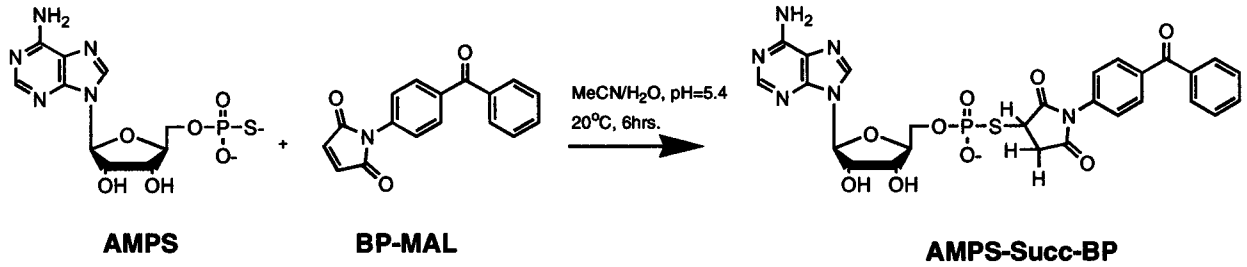

FIGURE 2: Scheme of synthesis of adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate] (AMPS-Succ-BP) from benzophenone-4-maleimide (BP-MAL) and adenosine 5′-monothiophosphate (AMPS).

of 20 kV, grid voltage 94.7%, and guide wire voltage 0.05% with a delay time of 50 ns.

Enzyme Assays. Glutamate dehydrogenase activity was measured at 25 °C and 340 nm by the oxidation of NADH ($\epsilon_{340\ nm} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) using a Gilford model 240 spectrophotometer equipped with a Linseis model L250E recorder (0.1 scale). The assays were conducted in Tris−0.01 M acetate buffer, pH 7.8, containing 10 μM EDTA. The "standard" assay solution was 50 mM in ammonium chloride, 5 mM in α-ketoglutarate, and 100 μM in NADH. The concentration of ADP in the assays "in the presence of ADP" was constant at 200 μM. Assay conditions and substrate concentrations were the same in assays used to determine the effect of varying the concentration of several regulatory nucleotides. When the assay solution contained NADH at concentrations of 0.2 mM or above, the progress of the reaction was monitored at 375 nm ($\epsilon = 1.85 \times 10^3\ M^{-1}\ cm^{-1}$). To determine the apparent K_m for NADH, the concentration of NADH was varied from 4×10^{-6} to 1×10^{-4} M while the concentrations of α-ketoglutarate and NH_4Cl were maintained at the same level used in the standard assay. Similarly, when the apparent K_m for either α-ketoglutarate or NH_4Cl was measured, the concentrations of the coenzyme and other substrate were kept constant at those of the standard assay. The concentration of α-ketoglutarate was varied from 5×10^{-5} to 5×10^{-3} M, and the concentration of NH_4Cl was varied from 2×10^{-4} to 5×10^{-2} M in determining the apparent K_m values for these two substrates. The K_m values were calculated by linear regression analysis of Lineweaver−Burk plots, and the K_ms are given along with standard errors.

Reaction of AMPS-Succ-BP with Bovine Liver Glutamate Dehydrogenase. Bovine liver glutamate dehydrogenase (0.36 mg/mL, 6.4 μM enzyme subunits) was incubated with varying concentrations of AMPS-Succ-BP in 50 mM PIPES buffer, pH 7.0. The solution (typically 250 μL) was placed in a microtiter plate kept on ice and was irradiated with long-wavelength UV light ($\lambda = 350$ nm) using a Spectroline model ENF-240C lamp. Aliquots of 10 μL were removed from the solution at various times and diluted 40 times with 50 mM potassium phosphate buffer, pH 7.1. The solution (20 μL) was assayed in the absence and presence of ADP. When the effect of substrate and regulatory nucleotides on the rate constants for irreversible activation of the native enzyme was studied, these ligands were incubated at the desired concentrations with the enzyme for 10 min prior to the addition of AMPS-Succ-BP. Rate constants are given together with their standard errors under Results.

Incorporation of [2-^{3}H]AMPS-Succ-BP into Bovine Liver Glutamate Dehydrogenase. The enzyme (0.36 mg/mL) was incubated with 20 μM [2-^{3}H]AMPS-Succ-BP and irradiated using long-wavelength UV light, as described above. Aliquots (500 μL) of the reaction mixture were withdrawn periodically, and free reagent was removed by the gel centrifugation method, as described elsewhere (*21*), using a 5 mL Sephadex G 50-80 column equilibrated with 50 mM HEPES buffer, pH 7.0. The protein concentration in the eluate was determined by Bio-Rad protein assay, which is based on the Bradford dye binding method (*22*), with native glutamate dehydrogenase as the standard protein. The absorbance was read using a Bio-Rad 2550 RIA plate reader equipped with a 600 nm filter. The amount of AMPS-Succ-BP incorporated into the enzyme was determined from the amount of radioactivity in the modified enzyme, measured using a Packard Tri-Carb model 3300 liquid scintillation counter. Typically, 50 μL of the modified enzyme was mixed with 5 mL of Liquiscint liquid scintillation cocktail, and the radioactivity was measured.

Kinetic Properties of the Modified Enzyme. Bovine liver glutamate dehydrogenase was modified by irradiating a solution of the enzyme and 20 μM AMPS-Succ-BP for 50 min in the absence of ligands, and the excess reagent was removed by gel filtration as described above. These conditions yield fully modified enzyme with about 0.5 mol of reagent incorporated/enzyme subunit. The modified enzyme was dialyzed against 0.1 M potassium phosphate buffer, pH 7.1, for 12 h at 4 °C. The dialyzed enzyme was centrifuged to remove any precipitated protein, and the protein concentration in the solution was determined using the Bio-Rad protein asssay method. The specific activity of the modified glutamate dehydrogenase was 84 μmol of NADH oxidized min^{-1} (mg of enzyme)$^{-1}$ as compared with 42 enzyme units for the native enzyme. An aliquot of 20 μL was withdrawn, diluted 20 times with 50 mM potassium phosphate buffer, pH 7.1, and assayed at 25 °C in the presence of varying concentrations of ligands.

Proteolytic Digestion of the Modified Enzyme and Isolation of Labeled Peptide. Bovine liver glutamate dehydrogenase (0.36 mg/mL) was incubated with 20 μM [2-^{3}H]AMPS-Succ-BP in 50 mM PIPES buffer, pH 7.0, and irradiated using long-wavelength UV light (350 nm region) for 45 min on ice, as described above. Typically 2 mg of enzyme was modified at a time. Excess reagent was removed using the gel centrifugation method (*21*) by applying each 500 μL aliquot to one 5 mL Sephadex G50-80 column equilibrated with HEPES buffer, pH 7.0. The eluate was dialyzed against 6 L of deionized water overnight at 4 °C followed by lyophilization. This sample was redissolved in 250 μL of 50 mM sodium acetate buffer, pH 4.0, containing 8 M urea and allowed to stand at 0 °C for 20 min. The solution was

diluted with 750 μL of 50 mM sodium acetate buffer, pH 4.0, and digested with *Staphylococcus aureus* V8 protease (2.5% w/w) at 25 °C. After 2 h, an additional 2.5% w/w of V8 protease was added, and the digestion was continued for a further 4 h. The solution was centrifuged and filtered through a 0.45 μm Millipore filter without loss of radioactivity and was immediately subjected to HPLC.

Isolation of Modified Peptide by HPLC. The proteolytic digest was injected onto a reverse phase Vydac C_{18} column (0.46 × 25 cm) which was equilibrated with the starting buffer (1 mM potassium phosphate buffer, pH 6.0, solvent A). Separation of the proteolytic fragments was carried out at a flow rate of 1.0 mL/min, and the eluant was monitored by $A_{220\ nm}$. The column was eluted for 10 min with solvent A followed by a linear gradient between solvent A and 10% solvent B (a mixture of equal volumes of 2 mM potassium phosphate and acetonitrile, with the pH adjusted to 6.0) over a time period of 40 min. A slower gradient was then started between 10% and 30% solvent B over a time period of 200 min, followed by a faster gradient from 30% to 100% solvent B over a time period of 60 min. Fractions (1 mL) were collected, and 200 μL was used to measure radioactivity.

Determination of the Amino Acid Sequence of the Labeled Peptide. The amino acid sequence of the labeled peptide was determined on an Applied Biosystems gas phase protein (peptide) sequencer, model 470, equipped with an HPLC model 120 and a model 900A computer. Typically 50−200 pmol samples were subjected to sequence determination. Fractions corresponding to individual amino acid cycles were collected and analyzed for radioactivity.

MALDI Mass Spectrometry of Labeled Peptide. The mass spectra of peptide samples were recorded on a Bruker model Biflex III instrument. The labeled peptide, purified using HPLC, was lyophilized and redissolved in 0.1% trifluoroacetic acid/water. This solution (5 μL, about 30 pmol) was mixed with 5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid, applied to the sample plate, and air-dried. The mass of the peptide was determined using an accelerating voltage of 19 kV with a delay time of 50 ns. The instrument was standardized with bombesin (MW = 1619.82) and ACTH (MW = 2465.2) as external standards.

Molecular Modeling. Molecular modeling was performed on an Iris Workstation from Silicon Graphics using the Insight II package from Biosym Technologies. The crystal structure of bovine liver glutamate dehydrogenase (1CH6) was used as deposited in the Brookhaven Protein Databank (*7*). The structure of AMPS-Succ-BP was constructed using the Builder module, and its energy was minimized using the Discover module at 5000 iterations; the molecule was soaked in buffer at pH 7.8 before performing the energy minimization. The AMP-Succ-BP was docked manually into glutamate dehydrogenase using the Docking module.

RESULTS

Characterization of AMPS-Succ-BP. The UV−Vis spectrum of pure AMPS-Succ-BP, recorded in potassium phosphate buffer, at pH 6.0, exhibits a single peak with a λ_{max} of 259 nm and a shoulder centered at 310 nm. The amount of organic phosphorus present in a given sample in solution was used to determine the concentration of AMPS-Succ-BP. From this result, an extinction coefficient of 30 700 M^{-1} cm^{-1} at 260 nm was determined for AMPS-Succ-BP. MALDI mass spectrometry of AMPS-Succ-BP showed a single signal with a *m*/*z* ratio of 641.1 for the MH^+ ion (the theoretical value for AMPS-Succ-BP is 641 mass units).

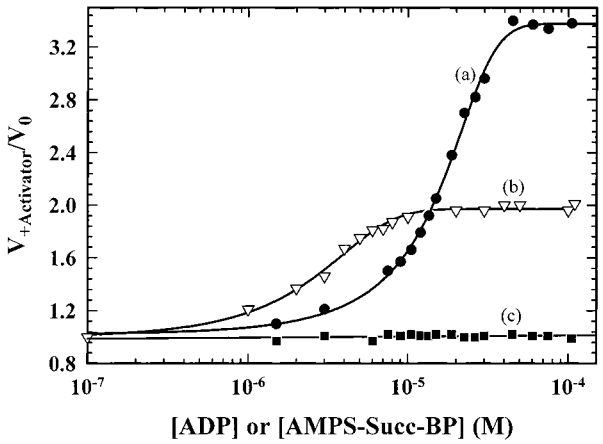

FIGURE 3: Reversible activation of native bovine liver glutamate dehydrogenase by (a) ADP (●) or (b) AMPS-Succ-BP (▽). (c) Effect of ADP on the maximum activity of enzyme modified with AMPS-Succ-BP (■). The enzymes were assayed in the presence of various concentrations of ADP or AMPS-Succ-BP at pH 7.8 and 25 °C, using 100 μM NADH as the coenzyme.

The addition of adenosine 5′-monothiophosphate to BP-MAL leads to the formation of AMPS-Succ-BP with a chiral center at the 3 position of the succinimide ring (Figure 2). The resulting isomers a and b (possibly *R* and *S*) were separated using HPLC as described under Experimental Procedures and were characterized using ^1H NMR with D_2O as solvent. For isomer a, the nine aromatic ring protons of the benzophenone system appear between 7.10 and 7.60 ppm. The 2 and 8 protons of the adenine ring exhibited chemical shift values of 7.90 (s, 1H) and 8.11 (s, 1H), respectively. The single proton at the 1′ position of the ribose appeared as a doublet between 5.84 and 5.85 ppm. The 5′, 4′, 3′, and 2′ protons of the ribose show signals centered at 3.80 (m, 2H), 3.99 (d, 1H), 4.02 (m, 1H), and 4.55 ppm (m, 1H), respectively. The single proton at the 3 position of the succinimidyl ring appears as a triplet centered at 4.32 ppm whereas the two protons at the 4 position of this ring show a chemical shift value of 3.32 ppm (m, 2H). The benzophenone ring protons of isomer b appear in the same region as that of isomer a. The 2 and 8 protons of the adenine ring are shifted upfield compared to that of isomer a and appear as singlets with chemical shift values of 7.78 and 8.04 ppm, respectively. The single proton at the 1′ position of ribose also shows an upfield shift compared to that of isomer a, appearing as a doublet between 5.77 and 5.79 ppm. For isomer b, the ribose protons 5′, 4′, 3′, and 2′ appear at 3.79 (m, 2H), 3.99 (d, 1H), 4.02 (m, 1H), and 4.54 ppm (m, 1H), respectively, while chemical shift values of 4.28−4.30 (t, 1H) and 3.28−3.30 ppm (d, 1H) are shown by the succinimidyl ring protons. The ^{31}P NMR spectrum of AMPS-Succ-BP containing the mixture of isomers shows two closely spaced signals at 22.56 and 22.63 ppm, respectively.

Reversible Effect of AMPS-Succ-BP on Assays of Bovine Liver Glutamate Dehydrogenase. Bovine liver glutamate dehydrogenase, when assayed in the presence of increasing concentrations of ADP, shows enhanced activity (Figure 3a). A maximum activation of about 3.4-fold is observed at

saturating concentrations of ADP. Under the same experimental conditions, when AMPS-Succ-BP is added to the assay solution, it activates the enzyme maximally up to 2-fold at saturating concentrations of the reagent (Figure 3b). This effect is designated as reversible, since no radioactive AMPS-Succ-BP is incorporated into the enzyme under these conditions. The dissociation constant for this reversible activation, K_a (calculated from Figure 3), equals the concentration of ADP or AMPS-Succ-BP at which $V_{max_{obs}} = (V_{+activator} + V_{-activator})/2$, as described by Frieden (*23*). For ADP, K_a is about 13 μM, while for AMPS-Succ-BP, K_a = 4.3 μM.

Irreversible Activation of Bovine Liver Glutamate Dehydrogenase by AMPS-Succ BP. Irradiation of glutamate dehydrogenase in the presence of 20 μM AMPS-Succ-BP results in a time-dependent irreversible activation of the enzyme when measured in the standard assay in the absence of ADP. (As shown in Figure 6, the photoactivation is accompanied by incorporation of radioactive AMPS-Succ-BP and therefore represents an irreversible reaction.) Since the concentration of AMPS-Succ-BP present in the assay after dilution of the enzyme is only 0.2 μM, this activation cannot be due to the reversible effect of AMPS-Succ-BP on the assay, as shown in Figure 3b. Furthermore, no such time-dependent change in activity is observed (and no incorporation of radioactive reagent is found) when the enzyme is incubated with AMPS-Succ-BP in the dark or when the enzyme alone is irradiated. Figure 4 shows typical changes in the activity (measured in the absence or presence of ADP) of bovine liver glutamate dehydrogenase upon photolysis in the presence of 20 μM AMPS-Succ-BP. The velocity assayed in the absence of ADP (Figure 4A, ▼) exhibits an increase with time of photolysis. In contrast, when assayed in the presence of 200 μM ADP, the activity decreases as a function of time of photolysis (Figure 4A, ●). Prolonged irradiation results in $V_{+ADP} = V_{-ADP}$, indicating that externally added ADP does not activate the modified enzyme. Thus, it appears that photolysis with AMPS-Succ-BP causes an irreversible activation of the enzyme, simultaneously preventing the activation by ADP. (This postulate of irreversible reaction is supported by the incorporation of radioactive AMPS-Succ-BP into the enzyme as shown in Figure 6.) The observed rate constant for the modification upon photolysis can be measured from the gain in activity assayed in the *absence* of ADP; k_{obs} can be calculated from the slope of a plot of $\ln[(V_\infty - V_t)/(V_\infty - V_0)]$ versus t, where V_∞ is the velocity at maximum activation upon photolysis and V_0 and V_t are, respectively, the velocities before photolysis and at any given time, t, of photolysis. The observed rate constant for the modification upon photolysis can also be measured from the decrease in activity assayed in the *presence* of ADP; in this case, k_{obs} can be calculated from the slope of a plot of $\ln[(V_t - V_\infty)/(V_0 - V_\infty)]$ versus t. The constants when calculated from the two sets of velocities (i.e., the velocities "in the presence of ADP" and those "in the absence of ADP") were the same. If these changes in V_{max} values measured in the presence or absence of ADP upon photomodification represent the same process, the rate of change may more accurately be represented as a ratio of velocities in the presence and absence of ADP. When the modification produced by photolysis is complete, the ratio of V_{+ADP} to V_{-ADP} equals 1. The rate constant for the reaction, k_{obs}, can therefore be calculated from a plot of $\ln[(V_{+ADP}/V_{-ADP})_t - 1]/[(V_{+ADP}/V_{-ADP})_0 - 1]$ versus time (Figure 4B).

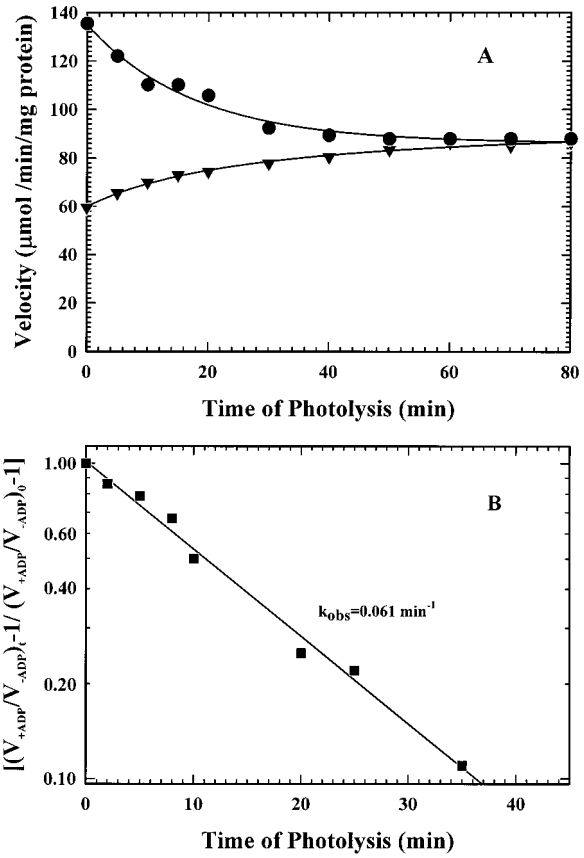

FIGURE 4: Irreversible activation of GDH by AMPS-Succ-BP upon incubation of the enzyme (0.36 mg/mL, 6.4 μM subunit) with 20 μM AMPS-Succ-BP in 0.05 M PIPES buffer at pH 7.0 and 0 °C and photolyzed, as described under Experimental Procedures. (A) Representative changes in the enzyme activity when assayed in the presence of ADP (●) and in the absence of ADP (▼). (B) Representative graph used to calculate the observed rate constant for irreversible photoactivation: a plot of $\ln[(V_{+ADP}/V_{-ADP})_t - 1]/[(V_{+ADP}/V_{-ADP})_0 - 1]$ vs t (■) using the velocities in the presence and absence of ADP, where V_0, V_t, and V_∞ are, respectively, the velocities at zero time (i.e., before photolysis), at time t of photolysis, and at very long times of photolysis. From these data, k_{obs} = 0.061 min^{-1}, with a standard error of ±0.002 min^{-1}.

The rate constants for irreversible photomodification were also measured by separately irradiating 20 μM samples of the separated isomers of AMPS-Succ-BP together with bovine liver glutamate dehydrogenase. While k_{obs} = 0.061 ± 0.002 min^{-1} using the mixture of isomers, rate constants of 0.055 and 0.064 min^{-1} were determined using the separated isomer a and isomer b, respectively. Since these k_{obs} values were close, the mixture of isomers was used in subsequent experiments.

The observed rate constants show a nonlinear dependence on the concentration of AMPS-Succ-BP in the incubation mixture (Figure 5). From the plot of $1/k_{obs}$ vs 1/[AMPS-Succ-BP] (Figure 5, inset), the apparent enzyme−reagent dissociation constant (K_R) and the value of k_{max} for irreversible photomodification are estimated to be 4.9 ± 0.2 μM and 0.076 ± 0.003 min^{-1}, respectively.

Effect of Ligands on k_{obs} for Irreversible Activation. Table 1 shows the effect of ligands on the rate constants for photomodification of glutamate dehydrogenase by 20 μM AMPS-Succ-BP. ADP strikingly decreases the rate constant,

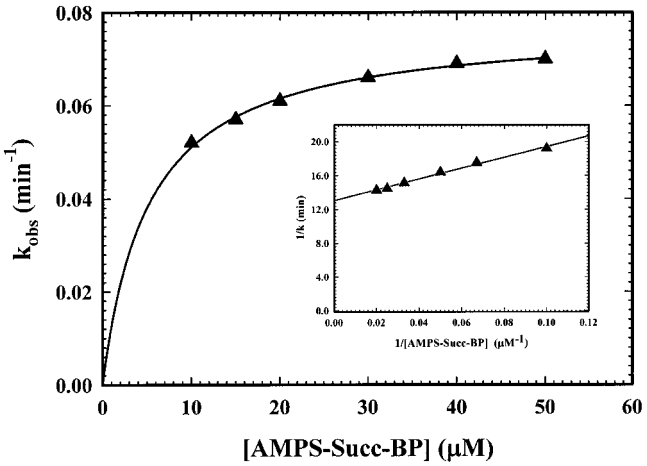

FIGURE 5: Dependence of k_{obs} on [AMPS-Succ-BP]. The enzyme was incubated with various concentrations of AMPS-Succ-BP in 0.05 M PIPES buffer at pH 7.00 at 0 °C and irradiated using long-wavelength UV light. The activity of the enzyme was measured periodically as described under Experimental Procedures. The k_{obs} was calculated at each [AMPS-Succ-BP] as illustrated in Figure 4B. Inset: The k_{max} and K_R were determined from a plot of $1/k_{obs}$ vs 1/[AMPS-Succ-BP].

Table 1: Effect of Substrate and Allosteric Ligands on the Rate Constant for Irreversible Photoactivation of Glutamate Dehydrogenase by 20 μM AMPS-Succ-BP[a]

ligand	k_{obs} (min^{-1})
none	0.061 ± 0.002
ADP (200 μM)	0.009 ± 0.001
NADH (100 μM)	0.045 ± 0.003
GTP (100 μM)	0.055 ± 0.001
GTP (200 μM)	0.044 ± 0.002
NAD (200 μM)	0.065 ± 0.003
NAD (1 mM)	0.046 ± 0.001
GTP (100 μM) + NADH (100 μM)	0.045 ± 0.002
α-ketoglutarate (1 mM)	0.036 ± 0.001
α-ketoglutarate (5 mM)	0.037 ± 0.001

[a] The enzyme was photolyzed with 20 μM AMPS-Succ-BP in PIPES buffer at pH 7.0 at 0 °C in the presence of the indicated ligand, and the rate constants for irreversible activation (along with their standard errors) were determined as illustrated in Figure 4B.

suggesting that the reaction occurs within the ADP activation site. In contrast, the allosteric inhibitor GTP, the reduced coenzyme NADH (either alone or when present together with GTP), the oxidized coenzyme NAD, and the substrate α-ketoglutarate, when present at concentrations sufficient to saturate the active site and the high-affinity inhibitory GTP site, cause less than a 2-fold decrease in the k_{obs}. These results indicate that AMPS-Succ-BP modifies covalently the allosteric ADP activation site of bovine liver glutamate dehydrogenase.

Incorporation of [2-^{3}H]AMPS-Succ-BP into Bovine Liver Glutamate Dehydrogenase. The incorporation of [2-^{3}H]-AMPS-Succ-BP into bovine liver glutamate dehydrogenase as a result of covalent modification was measured after various times of irradiation. The results, shown in Figure 6, indicate an incorporation of about 0.5 mol of AMPS-Succ-BP/ mol of enzyme subunit or 3 mol of reagent/mol of hexamer when the enzyme is maximally activated. The specific activity of this modified enzyme is 84 μmol of NADH min^{-1} (mg of enzyme)$^{-1}$, as compared with 42 μmol

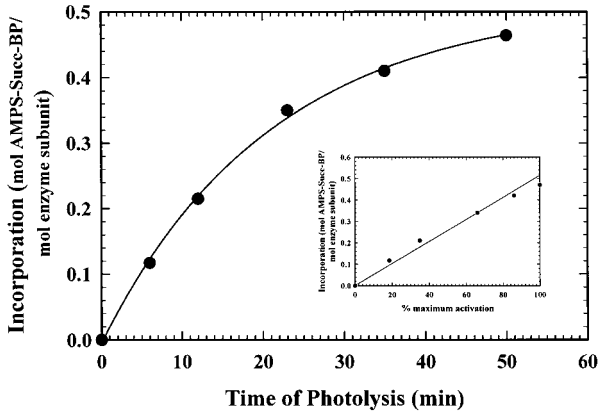

FIGURE 6: Dependence of the incorporation of [2-^{3}H]AMPS-Succ-BP into bovine liver glutamate dehydrogenase on the time of photolysis. The enzyme (0.36 mg/mL, 6.4 μM subunit) was incubated with 20 μM radioactive AMPS-Succ-BP in 0.05 M PIPES buffer at pH 7.0 at 0 °C and photolyzed using long-wavelength UV light. The activity of the enzyme and incorporation were determined at intervals of time, as described under Experimental Procedures. Inset: Relationship between reagent incorporation and percent of maximum activation [$\{(V_t - V_0)/(V_\infty - V_0)\} \times 100$].

of NADH min^{-1} (mg of enzyme)$^{-1}$ for the native enzyme. Prolonged irradiation does not lead to an increase in the amount of reagent incorporated. Addition of fresh 20 μM [2-^{3}H]AMPS-Succ-BP to the enzyme containing 0.5 mol of the reagent/mol of the enzyme subunit, followed by irradiation, also does not result in any increase in the amount of reagent incorporated.

Kinetic Properties of AMPS-Succ-BP-Modified Enzyme. To characterize the photoaffinity-labeled GDH, fully modified enzyme was isolated containing about 0.5 mol of AMPS-Succ-BP/enzyme subunit. In contrast to native enzyme (which is activated 3.5-fold), the activity of this modified enzyme remains constant as the ADP concentration is increased (Figure 3c, ■). No change in the activity of the modified enzyme is observed even at ADP concentrations as high as 1 mM. These results indicate that covalent reaction of AMPS-Succ-BP with glutamate dehydrogenase has blocked the ADP binding site to externally added ADP.

The native enzyme is inhibited by concentrations of NADH higher than 0.2 mM. The modified enzyme shows a similar dependence of velocity on [NADH] up to 0.2 mM; the K_m for NADH is 23.8 ± 3.1 and 24.7 ± 3.9 μM for native and AMPS-Succ-BP-modified enzyme, respectively. However, the modified enzyme is inhibited slightly less at high concentrations of NADH.

Figure 7 illustrates the GTP inhibition of the native and modified enzymes. While the native enzyme exhibits 90% inhibition at saturating concentrations of GTP, the enzyme modified with AMPS-Succ-BP is inhibited only to 78%. The apparent inhibition constants for the enzyme−GTP complex were 41 nM for the native enzyme and 320 nM for the modified enzyme, as calculated from Figure 7 according to the method of Frieden (*23*). This relatively small change in the affinity of modified enzyme for GTP suggests that this is an indirect effect of labeling the enzyme at a different site.

As a monitor of the integrity of the active site, we determined the substrate K_m values of the native and modified enzymes for the substrates. The Michaelis constants for α-ketoglutarate were 0.21 ± 0.016 and 0.26 ± 0.034 mM,

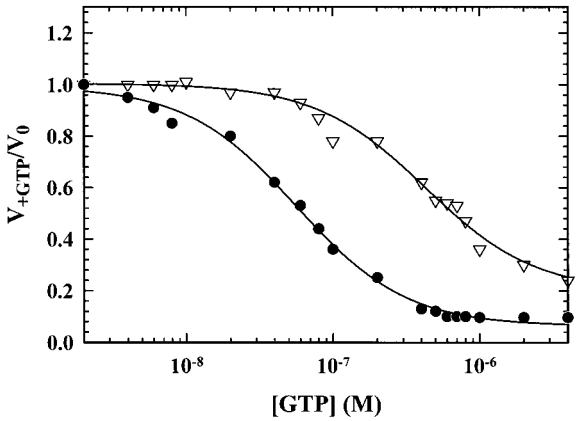

FIGURE 7: Effect of [GTP] on the maximum velocity of native bovine liver glutamate dehydrogenase (●) and enzyme modified with AMPS-Succ-BP (▽). The enzyme was assayed in the presence of various concentrations of GTP at pH 7.8 and 25 °C. The NADH concentration was 100 μM in the assay solution. The K_i value, calculated from the graph, equals the concentration of GTP yielding $(V_{-GTP} + V_{+GTP})/2$ where V_{-GTP} is the maximum velocity in the absence of GTP and V_{+GTP} is the limit at saturating GTP concentrations (*23*).

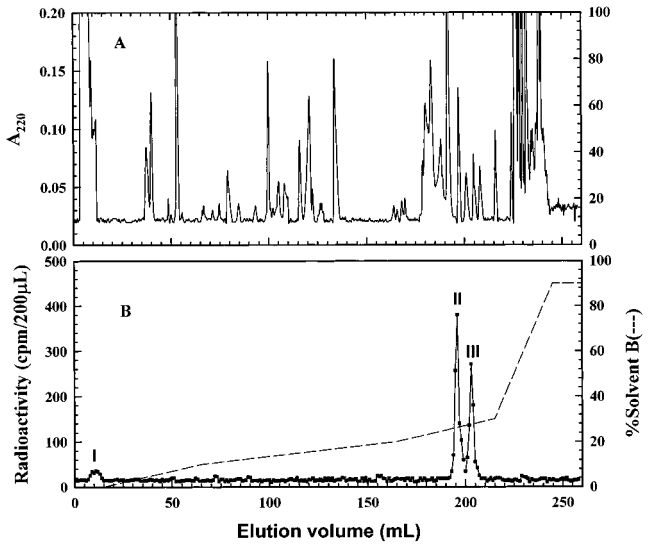

FIGURE 8: Isolation of V8 protease digested peptides by reverse phase HPLC using a Vydac C_{18} column equilibrated with 1 mM potassium phosphate, pH 6.0. (A) Absorbance of peptides at 220 nm. (B) Radioactivity of eluate (■) and elution gradient of solvent B (- - -). The Roman numerals designate the radioactive peaks.

respectively, for the native and modified enzymes, while the K_m values for NH_4Cl were 3.1 ± 0.21 and 2.0 ± 0.18 mM, respectively, for native and modified enzymes. The similarity of these values for the two types of enzyme indicates that the modification of glutamate dehydrogenase by AMPS-Succ-BP does not affect the affinity of the enzyme for these two substrates.

Purification and Characterization of Labeled Peptide from the Proteolytic Digest of AMPS-Succ-BP-Modified Enzyme. The enzyme, fully modified with AMPS-Succ-BP, was digested with *S. aureus* V8 protease, and the digest was fractionated by HPLC, using a C_{18} reverse phase column equilibrated at pH 6.0, as shown in Figure 8. Among the three radioactive peaks obtained, peaks II and III (at elution volumes of 196 and 203 mL, respectively) contained 95% of the radioactivity eluted from the column.

Table 2: Amino Acid Sequence of Modified Peptide from Peaks II and III of Figure 8

		peak II		peak III	
cycle	amino acid	amount[b] (pmol)	net radioact. (cpm)	amount[c] (pmol)	net radioact. (cpm)
1	Lys^{488}	48	5	41	3
2	Val	30	7	27	0
3	Phe	31	8	41	0
4	Arg[a]	38	22	17	9
5	Val	26	18	25	1
6	Tyr	21	19	27	1
7	Asn	26	10	26	2
8	Glu^{495}	14[d]	8	18[d]	1

[a] Modified amino acid. [b] 56 pmol of labeled peptide was subjected to analysis for 16 cycles. [c] 49 pmol of labeled peptide was subjected to sequence analysis for 12 cycles. [d] End of peptide.

The purified peptides of peaks II and III, upon gas-phase amino acid sequencing, yielded the same sequence, as shown in Table 2. The unique sequence corresponds to Lys^{488}-Val-Phe-Arg^{491}-Val-Tyr-Asn-Glu^{495} in the known amino acid sequence of bovine liver glutamate dehydrogenase (*16*, *24*). The two peptide peaks are likely the result of reaction of the two isomers of AMPS-Succ-BP with the same peptide. [It has previously been observed that peptides labeled by *N*-ethylmaleimide give two peaks on HPLC, each of which has the same amino acid sequence (*25*).] As shown in Table 2, a significant amount of radioactivity was detected in the fraction corresponding to the PTH-Arg^{491} cycle collected from the amino acid sequencer. However, the amount of phenylthiohydantoin derivative for Arg^{491} detected during the sequencing was comparable to that of the other amino acids in the same peptide. These results suggest that the derivatized amino acid is regenerated under the conditions used for peptide sequencing. During the gas-phase sequencing by Edman degradation, the peptide is exposed to pH 11.7 upon treatment with 12.5% trimethylamine. Since the modified peptide is known to be unstable above neutral pH concomitant with loss of label, this treatment is likely to cause regeneration of Arg. Similar observations have been made previously while sequencing peptides containing derivatized arginines (*18*).

Further evidence that Lys^{488}−Glu^{495} is the only labeled peptide comes from mass spectrometry. The pure peptide of peak II shows a signal with an *m*/*z* ratio of 1712.2, about 18 mass units higher than that calculated for the labeled peptide (calculated mass = 1695). When the peptide sample of peak III was subjected to mass spectrometric analysis, three peaks corresponding to *m*/*z* ratios of 641.1, 1054.5, and 1712.8 were obtained. While the first two *m*/*z* ratios correspond to the calculated masses of the reagent ($mass_{calc}$ = 641) and that of the unlabeled peptide fragment, Lys^{488}−Glu^{495} ($mass_{calc}$ = 1054), the *m*/*z* ratio of 1712.8 is about 18 units higher than that expected for the labeled peptide. The addition of 18 mass units corresponds to that of a molecule of water, which can result from the hydrolysis of the imide ring during the proteolytic digestion and peptide purification. Similar results were obtained during the mass spectroscopic analysis of the peptides labeled by glutathionyl *S*-[4-(succinimidyl)benzophenone] (*26*).

DISCUSSION

Adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate] was designed to retain most of the structural elements of ADP in order to be recognized by ADP binding sites. It not only has the normal AMP moiety, but also has a succinimide ring in which one of the carbonyls corresponds to the β-phosphoryl of ADP. In addition, AMPS-Succ-BP has features which may enhance binding to an enzyme: the phenyl rings provide the possibility of hydrophobic or aromatic stacking interaction, while the carbonyl oxygen of the benzophenone can function as a hydrogen bond acceptor. Once it binds at a specific site upon photoactivation, the benzophenone moiety reacts relatively indiscriminately with groups which are accessible from the binding site (*27*). Thus, it is not limited to reaction with nucleophilic side chains, as are many affinity labels (*28*). Benzophenone photoaffinity labels have been successfully employed in probing a variety of complex biomolecular interactions (*9*−*13*, *26*, *29*, *30*).

Our results show that AMPS-Succ-BP, when added to the assay in the dark, reversibly activates bovine liver glutamate dehydrogenase with a dissociation constant one-third that of the natural allosteric activator ADP. No incorporation of radioactive reagent is observed under these conditions. Thus, AMPS-Succ-BP mimics ADP as an allosteric activator of glutamate dehydrogenase, and strong noncovalent interactions exist between the enzyme and the reagent.

The irradiation of the enzyme−reagent complex using long-wavelength UV light results in an irreversible activation of the enzyme as observed in the assays in the absence of ADP, concomitant with loss of activation by externally added ADP. The enzyme remains activated in the absence of ADP after gel filtration and dialysis, and exhibits incorporation of radioactive reagent, consistent with covalent modification by AMPS-Succ-BP upon photoactivation. It is relatively rare for an affinity label, upon covalent modification, to mimic the activation of a regulatory enzyme by its allosteric activator (*18*). This observation suggests that AMPS-Succ-BP binds at an ADP activator site and causes the same conformational change as does the natural ADP. The nonlinear dependence on reagent concentration of the rate constant for reaction of AMPS-Succ-BP with glutamate dehydrogenase is also consistent with AMPS-Succ-BP binding at a specific site prior to the covalent modification. Furthermore, the marked effect of ADP in decreasing the rate constant for the photoaffinity labeling strongly supports the designation of the ADP site as the target site for AMPS-Succ-BP.

The kinetic characteristics of the modified enzyme provide additional information on the site of reaction of the enzyme with AMPS-Succ-BP. The isolated, covalently modified glutamate dehydrogenase is no longer activated by free ADP, as expected if the allosteric ADP site is occupied by AMPS-Succ-BP. In contrast, the modified enzyme is still inhibited by the allosteric regulator GTP (albeit at higher concentrations than for native enzyme) and is inhibited by high concentrations of NADH (although to a lesser extent than is the native enzyme). These results are best interpreted in terms of reaction at the ADP site having an indirect effect in weakening binding at other allosteric sites. Interactions among these distinguishable regulatory sites have been well documented (*1*).

When glutamate dehydrogenase is maximally activated by AMPS-Succ-BP, only 0.5 mol of reagent/mol of average subunit or 3 mol of reagent/enzyme hexamer has been incorporated. There are other examples in which covalent reaction with only some of the glutamate dehydrogenase subunits causes a functional change in all the subunits (*1*), as in the cases of modification of the NADH inhibitory site by 5′-*p*-fluorosulfonylbenzoyl adenosine (*31*, *32*) or by the 2′,3′-dialdehyde of NADPH (*33*). Peterson and Smith (*7*) have pointed out that the effects of a ligand binding to a site on one subunit might be transmitted to adjacent subunits via the C-terminal α-helix (residues 477−495) which extends through the inner core and makes contact with the opposing trimer. It is also possible that the subunit−subunit interactions are transmitted through the "antenna domain", formed by a 48 amino acid insertion (395−443) that is unique to the crystalline bovine glutamate dehydrogenase and is missing in the crystalline nonallosteric bacterial glutamate dehydrogenases (*7*). The mutual twisting of the antenna from each subunit within a trimer can be visualized in Figure 1.

A single modified peptide has been clearly identified as the target of AMPS-Succ-BP, and the evidence we have presented suggests that Arg^{491} is the only amino acid of bovine glutamate dehydrogenase labeled by our new photoaffinity label. Although we have not isolated the arginine adduct, its lability under basic conditions is characteristic of derivatized arginines, and the release of radioactivity at the Arg^{491}cycle during the sequencing of the peptide is also consistent with the modification of this amino acid. Thus, we consider that Arg^{491} is at or near the ADP binding site of this allosteric enzyme.

Previously, affinity labeling and irreversible activation of bovine glutamate dehydrogenase by adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] (AMPS-BDB) identified Arg^{459} as part of the ADP regulatory site (*18*). We further showed that the two carbonyls of the bromodioxobutyl moiety reacted with the guanido group of Arg^{459} (*18*). In the crystal structure of glutamate dehydrogenase (*7*), the closest distance between the guanido groups of Arg^{459} and Arg^{491} is about 8.6 Å. A comparison of the structure of ADP with AMPS-BDB indicates that the β-phosphate of ADP aligns with the two carbonyls of AMPS-BDB. Thus, Arg^{459} may normally facilitate the binding of ADP by electrostatic interaction with the β-phosphate of ADP. If AMPS-Succ-BP is compared with ADP, it can be seen that the succinimidyl ring of the reagent is structurally equivalent or close to the location of ADP's β-phosphate. The carbonyls of the succinimidyl moiety of AMPS-Succ-BP may thus be positioned close to Arg^{459} when the photoaffinity reagent binds to glutamate dehydrogenase. If the adenosine part of AMPS-BDB and AMPS-Succ-BP binds to the enzyme in the same region, the distance between their reactive groups must be close to the distance between the amino acids modified by these reagents. It is notable that when the adenines of the two reagents are superimposed, the distance between a carbonyl of the succinimide and the carbonyl of the benzophenone moiety of AMPS-Succ-BP is about 7 Å, which is comparable to the distance between Arg^{459} and Arg^{491} in the crystal structure of GDH. These considerations helped us to dock AMPS-Succ-BP into the crystal structure of glutamate dehydrogenase.

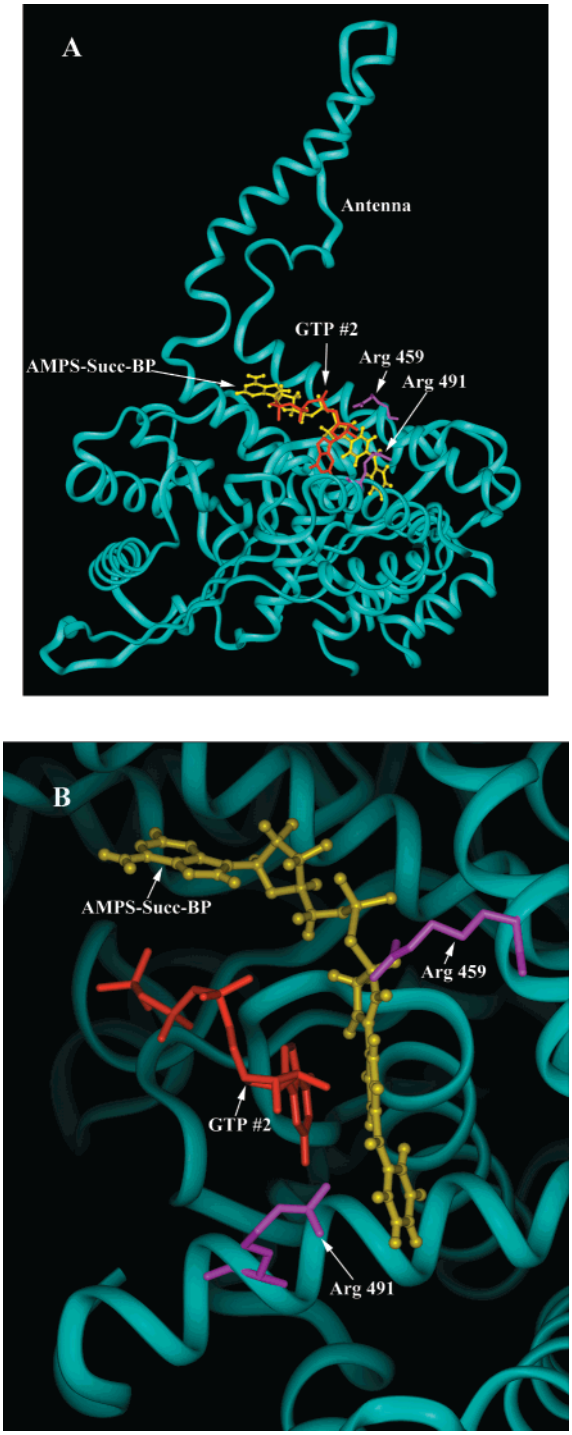

FIGURE 9: Model for the AMPS-Succ-BP binding site of GDH highlighting the interaction of the reagent with Arg^{459} (*18*) and Arg^{491}(this work). (A) The energy-minimized structure of AMPS-Succ-BP was docked into the crystal structure of GDH (*7*). The structure was visualized using INSIGHT II (Molecular Simulations Inc.). AMPS-Succ-BP is shown in yellow, GTP in red, and Arg^{459} and Arg^{491} in pink. This view shows an entire subunit, allowing comparison with the subunit with bound ligands shown in Figure 1. (B) An enlarged view of the AMPS-Succ-BP binding site in which the subunit has been rotated toward the reader to show better the separation between AMPS-Succ-BP and GTP bound to GDH.

Figure 9A shows the model of AMPS-Succ-BP bound to bovine liver glutamate dehydrogenase with the purine ring of the reagent positioned at the base of the "antenna" region, a succinimidyl carbonyl oxygen located near the guanido moiety of Arg^{459} (~2.8 Å), and the benzophenone carbonyl oxygen close to the guanido group of Arg^{491} (~2.7 Å). These interactions between the reagent and glutamate dehydrogenase may be responsible for the low dissociation constant for reversible activation of the enzyme by AMPS-Succ-BP. Arg^{459} is located at the base of the "antenna", in the middle of the penultimate or "pivot helix" connecting the "antenna" with the C-terminal helix (*7*), while Arg^{491} is in the middle of the C-terminal helix which is also close to the "antenna". It has been suggested that the "pivot helix" moves when NAD binds, and Peterson and Smith proposed that ADP in binding to the "pivot helix" causes the catalytic cleft to open, thereby releasing NAD (*7*). In Figure 9, AMPS-Succ-BP must serve as a marker of the ADP site. As shown in Figure 9B, this AMPS-Succ-BP site is clearly close to (but distinguishable from) the GTP #2 site, and it is not far from the GTP #1 and NADH #2 sites pictured in Figure 1. The proximity among these sites may account for the small decreases by NADH and by GTP in the rate constant for reaction of AMPS-Succ-BP with glutamate dehydrogenase. The proximity of AMPS-Succ-BP to these other allosteric sites may also be responsible for the weaker affinity for GTP and for the decreased inhibition by NADH exhibited by the AMPS-Succ-BP-modified enzyme. There have been several studies indicating the apparent competition between ADP and the inhibitory NADH (*34*, *35*), as well as between ADP and GTP (*1*, *36*).

In summary, we have described the synthesis of adenosine 5′-*O*-[*S*-(4-succinimidyl-benzophenone)thiophosphate], a new photoaffinity label which (in the dark) reversibly activates bovine liver glutamate dehydrogenase, as does ADP. Upon irradiation, AMPS-Succ-BP covalently modifies the enzyme at the allosteric ADP site, allowing us to locate this regulatory site in the crystal structure of bovine liver glutamate dehydrogenase. We suggest that AMPS-Succ-BP may have widespread applications as a photoaffinity label of ADP sites in other enzymes.

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