stabilized when enzyme bound, which would facilitate the reductive carboxylation reaction by the mechanism proposed by Rose (1960).

The ^{13}C NMR studies have shown that isocitrate binds to isocitrate dehydrogenase with all carboxyls ionized, as predicted from kinetic and binding studies (Colman, 1983). They have indicated that metal significantly changes the electronic environment of the β-carboxyl. The alteration of the chemical shift of the β-carboxyl in the enzyme–metal–isocitrate complex and the failure to observe distinct shifts in either carboxyl in the enzyme–α-ketoglutarate complex suggest that the polarization of the β-carboxyl by the enzyme contributes to the tighter binding of isocitrate and is significant for the oxidative decarboxylation function of isocitrate dehydrogenase.

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Photoaffinity Labeling of Rabbit Muscle Fructose-1,6-bisphosphate Aldolase with 8-Azido-1,N^6-ethenoadenosine 5′-Triphosphate†

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ABSTRACT: Steady-state kinetic measurements have shown that 8-azido-1,N^6-ethenoadenosine 5′-triphosphate (8-N_3-ϵATP) can be noncovalently bound to rabbit muscle fructose 1,6-bisphosphate aldolase with K_i = 0.075 mM at pH 8.5. This binding is purely competitive with substrate and occurs at the strong binding site for mononucleotides. Photoaffinity labeling of aldolase in the presence of 8-azido-1,N^6-ethenoadenosine 5′-triphosphate results in inactivation of the enzyme. Aldolase is protected against modification in the presence of the inhibitors hexitol 1,6-bisphosphate or ATP. The labeling is saturable, and a good correlation is observed between the loss of enzymatic activity and the incorporation of 8-N_3-ϵATP into aldolase. In addition, aldolase loses its ability to bind to phosphocellulose following modification. Digestion of labeled protein with trypsin, chymotrypsin, and cyanogen bromide revealed substantial modification of peptide 259–269. Thr-265 was identified as the residue that was covalently modified by 8-N_3-ϵATP. On the basis of these results and other data we propose a model for the mononucleotide binding site.

The inhibitory effect of adenine nucleotides on aldolases A and B was discovered by Spolter and co-workers over twenty years ago (Spolter et al., 1965). Recently our knowledge concerning interaction of these physiologically important molecules with aldolase has been extended. It has been shown that each subunit of the aldolase tetramer has one strong and one weak binding site for mononucleotides (Kasprzak & Kochman, 1980a). The electronegative phosphate group of the nucleotide plays a predominant role in binding to the muscle enzyme. A secondary role has been ascribed to the adenosine moiety (Kasprzak & Kochman, 1980a). It has been found that binding to the weak site can be markedly reduced

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at pH 8.5, indicating that a basic group with pK_a below 8 is presumably associated with the binding (Kasprzak & Kochman, 1980a). Several lines of evidence indicate that the strong binding site overlaps partially with the active site of enzyme, whereas the weak binding site is outside of this region (Palczewski et al., 1985; Kasprzak & Kochman, 1980a). There are large differences among the nucleotides in their affinity for aldolase. ATP was found to be the most effective inhibitor of muscle and brain aldolase, whereas AMP was the least effective one (Kasprzak & Kochman, 1980a; Buczylko et al., 1983). The reverse order of inhibition was observed for liver aldolase (Kasprzak & Kochman, 1981). Ultraviolet difference spectroscopy and NMR analysis have indicated that at least the γ-phosphate residue of ATP and N-1 or N-7 atom of the purine residue are engaged in the interaction with the protein matrix (Kochman & Mas, 1981; Palczewski, 1986). It has been demonstrated also that 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine can be covalently linked to aldolase residues Lys-107 and Tyr-363 (Palczewski et al., 1985). A role for Lys-107 in binding the C-6 phosphate group of fructose-1,6-P_2[1] has been previously established (Shapiro et al., 1968). Tyr-363 plays an as yet undefined role in enzyme catalysis, but aldolase activity toward fructose-1,6-P_2 decreases by 96% in its absence (Drechsler et al., 1959). It has been postulated that Tyr-363 is in the vicinity of Lys-107 and both residues are engaged in binding the mononucleotide phosphate group (Palczewski et al., 1985).

In light of above findings the question is raised as to which residue(s) in the aldolase molecule is (are) involved in binding the purine moiety of ATP. The answer to this question may help in understanding the topography of the mononucleotide binding site in aldolase when the three-dimensional structure of this enzyme becomes available. It is important to note that a dinucleotide fold has been postulated in aldolase on the basis of both theoretical and experimental considerations (Kasprzak & Kochman, 1980b; Stellwagen, 1976; Matteuzzi et al., 1985).

In this paper 8-N_3-1,N^6-ethenoadenosine 5′-triphosphate, an analogue of ATP, has been used for photolabeling the enzyme. We demonstrate that this reagent is specifically bound only to the strong nucleotide binding site and that the purine moiety is mainly covalently linked to Thr-265.

Materials and Methods

8-N_3-εATP was synthesized from ATP according to the method of Schäfer et al. (1978). Its chromatographic and spectroscopic properties were identical with those previously reported (Schäfer et al., 1978).

Hexitol-1,6-P_2 was synthesized by reduction of fructose-1,6-P_2 with sodium borohydride as described by Ginsburg and Mehler (1966). The preparation was free from fructose-1,6-P_2 as shown by enzymatic analysis using aldolase (Michal & Beutler, 1974).

All other chemicals used in this work were commercially available and of analytical grade.

Enzymes. Fructose-1,6-P_2 aldolase from rabbit muscle was prepared by phosphocellulose chromatography (Penhoet et al., 1969). Its specific activity was 15.5–17.0 μmol of fructose-1,6-P_2 cleavage min^{-1} (mg of protein)$^{-1}$. The activity of aldolase was assayed spectrophotometrically at 25 °C (Blostein & Rutter, 1963). The molar concentration of aldolase was calculated by assuming a molecular weight of 160 000 (Kawahara & Tanford, 1966) and extinction coefficient $\epsilon = 0.91$ cm^{-1} mL mg^{-1} at 280 nm (Baranowski & Niederland, 1949).

The protein concentration of modified aldolase was determined by using the Bio-Rad protein assay according to the procedure of Bradford (1976). Native aldolase was used as the standard.

Trypsin and chymotrypsin were obtained from Sigma Chemical Co. Carboxypeptidase Y was purchased from Pierce Chemical Co.

Spectroscopic Data. All fluorescence measurements were performed with a Perkin-Elmer MPF-44 spectrofluorometer. Measurements were performed at 25 ± 0.5 °C with a 8-nm excitation and emission slit width. Absorbance measurements were performed with an Acta MVI spectrophotometer (Beckman).

Kinetic Analysis. Steady-state kinetic measurements were obtained from initial velocities by spectrophotometrically recording the disappearance of NADH. Triosephosphate isomerase and glycerol-1-phosphate dehydrogenase were employed in the linked enzyme assay. The reaction was initiated by adding 0.4 μg of aldolase diluted in 0.2% bovine serum albumin. These experiments were made in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA. The concentrations of ATP or 8-N_3-εATP were 0.05 and 0.1 mM, whereas the concentrations of frustose-1,6-P_2 were 2.4, 5.2, 9.6, and 24 μM. From a Hanes–Woolf plot of the data, we calculate K_m (fructose-1,6-P_2) = 4 μM, K_i(ATP) = 0.11 mM, and K_i(8-N_3-εATP) = 0.075 mM.

Photochemical Modification of Aldolase by 8-N_3-εATP. Photolysis of 8-N_3-εATP in the presence of aldolase was done in a 3-mL cuvette by using a mercury quartz lamp (180 W) at a distance of 12 cm. The cuvette was cooled in a thermostatic holder. A Pyrex glass plate was placed between the light source and the cuvette to eliminate UV light below 300 nm.

Aldolase (2.5 mL, 10 μM) was irradiated in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 0.45 mM 8-N_3-εATP (= 12.9 mM cm^{-1} at 288 nm; Schafer et al., 1978). After a 5-min irradiation, a 50-μL aliquot was withdrawn and added to 2 mL of 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, for determination of enzymatic activity.

CNBr Cleavage of Aldolase Modified by 8-N_3-εATP. S-Carboxymethylation of the modified aldolase was performed as described by Crestfield et al. (1963). CNBr cleavage and separation of CNBr peptides was performed as described by Lai (1968) and Schettino et al. (1981), respectively.

Tryptic and Chymotryptic Digestion. Tryptic digestion of the C-terminal CNBr peptide was performed according to the method of Lai (1975). Separation of tryptic peptides was performed on Sephadex G-25F. The column (145 × 2 cm) was equilibrated with 50 mM ammonium acetate, pH 4. The 2.1-mL fractions were collected at a flow rate of 21 mL/h. Chymotryptic digestion of tryptic peptide T-1 (residues 259–288 in the aldolase sequence) was performed as described by Lai (1975).

Separation of chymotryptic peptides was performed on SP-Sephadex C-25. The column (13 × 1.5 cm) was equilibrated with 10 mM phosphate buffer, pH 3.0. Elution was carried out with 150 mL of a linear NaCl gradient (0–0.3 M NaCl) in the same buffer, at a flow rate of 30 mL/h.

The fluorescent fraction fCh-A (see Results), derived from the SP-Sephadex column after the desalting step on the Sephadex G-25 column, was purified on QAE-Sephadex A-25. The column (13 × 1.5 cm) was equilibrated with 0.04 M

[1] Abbreviations: 8-N_3-εATP, 8-azido-1,N^6-ethenoadenosine 5′-triphosphate; fructose-1,6-P_2, fructose 1,6-bisphosphate; hexitol-1,6-P_2, hexitol 1,6-bisphosphate; EDTA, ethylenediaminetetraacetic acid; P-cellulose, phosphocellulose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Incorporation of 8-N_3-ϵATP into Aldolase: Effect of ATP on Extent of Modification and Activity Loss[a]

sample	ATP (8 mM)	8-N_3-ϵATP (0.45 mM)	sp act.	% act.	phosphate found (mol/mol of protein subunit)	calcd[b] 8-N_3-ϵATP incorpd/aldolase subunit
1	−	−	16.4	100	0.36	0
2	+	−	16	98	0.36	0
3	+	+	15.5	94	0.66	0.1
4	−	+	7.9	48	1.68	0.44

[a] Aldolase (2.5 mL, 10 μM) was irradiated for 5 min in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA at 25 °C in the presence of the following: sample 1, no addition; sample 2, with 8 mM ATP; sample 3, with 8 mM ATP and 0.45 mM 8-N_3-ϵATP; and sample 4, with 0.45 mM 8-N_3-ϵATP. After illumination of each sample, 4 mL of saturated ammonium sulfate was added and each sample was gently mixed at 5 °C for 30 min. The precipitated protein was centrifuged and washed 3 times with 2 mL of 60% saturated ammonium sulfate. Then the protein was dissolved in 2 mL of deionized water, transferred to a dialysis bag, and dialyzed again vs. five changes of 250 mL of 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.5. Phosphate analysis was performed according to the procedure of Chen et al. (1956). [b] These values were corrected for phosphate content in the blank sample 1.

NH_4HCO_3, pH 8.0. Elution was carried out with 150 mL of a linear NH_4HCO_3 gradient (0.04–0.4 M NH_4HCO_3), at a flow rate of 30 mL/h; 1.5-mL fractions were collected.

The fluorescent fCh-B fraction, obtained from the QAE-Sephadex A-25 column, was purified by high-voltage electrophoresis at 10 °C by using a Savant Instruments electrophorator with Whatman 3MM paper (96 × 23 cm) in 1.5 M formic acid for 3 h. The current was 125 mA at 3.5 kV. The paper was sprayed with 1% triethylamine in acetone. Any peptide modified by 8-N_3-ϵATP was visualized by ultraviolet light (blue spot), and then all peptides were detected by spraying the paper with 0.01% fluorescamine in acetone. The fluorescent fCh-2, which had migrated about 5 cm, was eluted from the paper with 20% formic acid and was then lyophilized. The N-terminal residue of the peptide was determined by the dansyl chloride method (Gray, 1972).

Amino Acid Analysis. Hydrolysis of peptide was performed in vacuo at 107 °C in 6 M HCl containing 0.5% 2-mercaptoethanol for 24, 48, and 72 h. Peptides were analyzed for amino acid composition by using the AAA 881 (Prague) amino acid analyzer.

Digestion with Carboxypeptidase Y. The peptide (50 nmol) in 0.2 mL of 100 mM pyridine/acetate buffer, pH 5.5, was digested at a carboxypeptidase Y to peptide molar ratio of 1:300. After 10 and 60 min these samples were acidified to pH 2.2 with 1 M HCl, diluted with an equal volume of 0.2 M citrate buffer (pH 2.2), and applied directly to the amino acid analyzer.

Results

Kinetic Analysis. All experiments described below were performed at pH 8.5, i.e., under conditions in which interaction between nucleotides and the weak nucleotide binding site is decreased. Two sets of experiments were designed to study the binding of the azide analogue of ATP, 8-N_3-ϵATP, with rabbit muscle aldolase. The *noncovalent* interaction was investigated in the dark by analysis of inhibition of aldolase activity. *Covalent* bond formation was investigated upon irradiation of the aldolase sample in the presence of 8-N_3-ϵATP.

Steady-state kinetics measurements performed in the dark revealed pure competitive inhibition of aldolase by 8-N_3-ϵATP with a K_i of 0.075 mM. Similar results were found for ATP (0.11 mM) (data not shown).

In the preliminary photolabeling experiments it was found that in the absence of protein most of the 8-N_3-ϵATP is decomposed after 100 s of illumination (Figure 1A). The reaction obeys first-order kinetics (Figure 1B). Aldolase activity is only slightly affected by light. No incorporation of the ATP derivative or its photolysis product into protein was observed in the dark (data not shown). The time course of irradiation of aldolase with 8-N_3-ϵATP shows that under these conditions

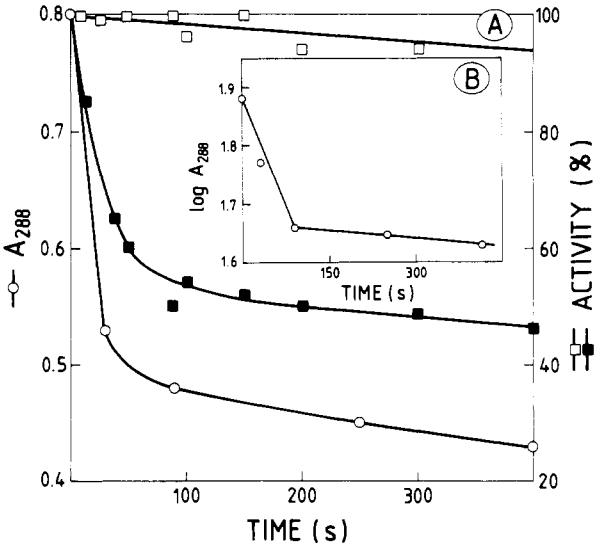

FIGURE 1: Kinetics of aldolase photoinactivation by 8-N_3-ϵATP and photodecomposition of 8-N_3-ϵATP. (A) The absorption of 8-N_3-ϵATP at 288 nm is plotted for various times of irradiation (○). Three milliliters of 8-N_3-ϵATP (60 μM) in citrate buffer, pH 2.6, was irradiated at 25 °C. At the indicated time, the cuvette with nucleotide was taken out and absorption at 288 nm was measured. The time course of inhibition of aldolase activity by 8-N_3-ϵATP (■) is shown. A volume of 2.5 mL of aldolase (10 μM) in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 0.45 mM 8-N_3-ϵATP was irradiated at 25 °C as described under Materials and Methods. Aliquots (50 μL each) were withdrawn at the indicated time and diluted in 2 mL of 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. This solution was used for measurements of enzymatic activity. As a control (□), 2.5 mL of 10 μM aldolase in 50 mM Tris-HCl, 1 mM EDTA, pH 8.5, was mixed with 0.45 mM ATP and irradiated as indicated above. (B) First-order plot of 8-N_3-ϵATP decomposition by irradiation (○). Data from (A).

45% of enzyme activity is lost after 100 s of photolabeling (Figure 1). Further illumination of the reaction mixture, for 300 s, causes only a slight additional decrease of the enzyme activity (by ca. 5%), indicating that most of the photolabeling reaction occurred during the first 100 s. In the next photolabeling experiments a 5-min irradiation time was used. The effect of 8-N_3-ϵATP concentration on enzyme activity loss caused by photolabeling is shown in Figure 2. Nearly 50% of aldolase activity is lost at a 0.2 mM concentration of reagent. Aldolase activity loss due to this modification corresponds to the number of ATP derivative molecules incorporated into the aldolase tetramer (Table I). Substrate or inhibitor (hexitol-1,6-P_2) at 0.2 mM protects aldolase against modification (Table II).

Preparative photolabeling was performed with 0.45 mM 8-N_3-ϵATP and 10 μM aldolase. The resulting preparation exhibited about 50% of initial activity. As shown in Figure

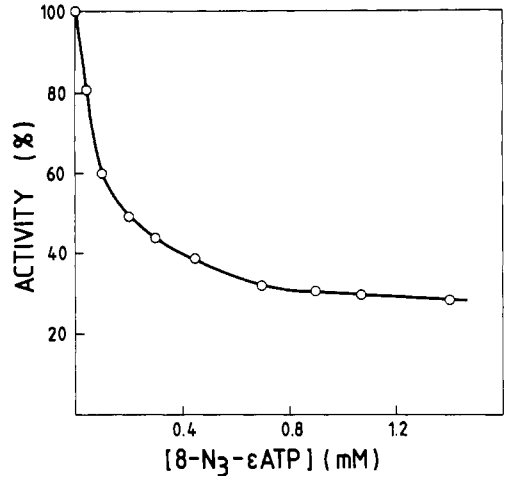

FIGURE 2: Percentage of aldolase activity remaining after 5-min illumination in various concentrations of 8-N_3-ϵATP. Ten micromolar aldolase was irradiated for 5 min with various concentrations of 8-N_3-ϵATP in 50 mM Tris-HCl buffer, pH 8.5, and the activity was measured as described in the legend of Figure 1.

Table II: Effect of Protecting Agents on Photoinactivation of Aldolase by 8-N_3-ϵATP[a]

agents	E_t/E_0 × 100	agents	E_t/E_0 × 100
no protective agent	48	ATP (0.2 mM)	55
fructose-1,6-P_2 (0.2 mM)	93	ATP (0.5 mM)	60
hexitol-1,6-P_2 (0.2 mM)	93	ATP (1 mM)	73

[a] Aldolase (10 μM) was irradiated with 8-N_3-ϵATP (0.45 mM) in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and various protecting agents. Activity was measured as described in the legend to Figure 2. E_t and E_0 represent the activity after 5 and 0 min of irradiation, respectively.

3, this preparation contained a mixture of modified and unmodified aldolase molecules; when this preparation was chromatographed on P-cellulose, only part of the applied material was retained, whereas the remaining material passed through the column with washing buffer. Retained molecules could be eluted with the substrate, exhibited almost 100% of activity of the native aldolase and exhibited the same UV spectrum as the native enzyme (data not shown).

Identification of an Amino Acid Residue Involved in the Reaction with 8-Azido-1,N^6-ethenoadenosine 5'-Triphosphate. Aldolase (80 mg) was covalently labeled with 8-N_3-ϵATP and separated from the reaction mixture by ammonium sulfate precipitation (as described in Table I). The modified protein, after S-carboxymethylation, was subjected to CNBr cleavage, and the resulting peptides were separated by chromatography on Sephadex G-75. The highest incorporation of fluorescent compound was found in the C-terminal peptide (CN-2) containing residues 250–363 (Table III). Twenty-seven percent of labeling was detected in the N-terminal peptide containing residues 1–164, and the remaining 12% of fluorescence was distributed between peptide CN-3 (containing functional Lys-229) and peptide CN-4.

In order to further characterize the major site of incorporation of 8-N_3-ϵATP into aldolase, the C-terminal cyanogen bromide peptide, CN-2, was subjected to trypsin digestion. From this digestion, peptide T-1, comprising residues 259–288, was found to contain 86% of the total fluorescence (Table III). The amino acid analysis of this peptide (T-1) shows that it contains one less Thr residue than the analogous peptide obtained from nonphotolabeled aldolase (data not shown). Peptide T-1 was subjected to chymotryptic digestion, and the resulting mixture was separated by chromatography on SP-

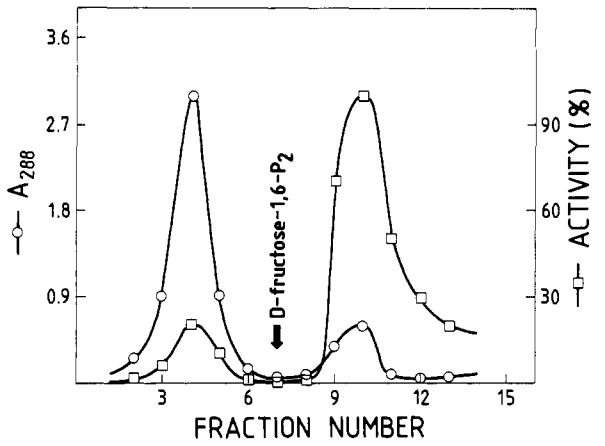

FIGURE 3: P-cellulose chromatography of aldolase modified by 8-N_3-ϵATP. Aldolase (2.5 mL, 10 μM) was irradiated in the presence of 8-N_3-ϵATP (0.45 mM) in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, for 5 min. After irradiation, the sample was dialyzed vs. 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. The sample, which exhibited 50% of initial enzyme activity, was applied to a P-cellulose column (0.6 × 10 cm) equilibrated with 10 mM Tris-HCl buffer, Ph 7.5, containing 1 mM EDTA. The column was washed with equilibration buffer, and then 2.5 mM fructose-1,6-P_2 was applied in the equilibration buffer. Fractions (1.2 mL) were collected at a flow rate of 10 mL/h.

Table III: Relative Fluorescence of Cyanogen Bromide Peptides and Tryptic Peptides Obtained from Aldolase Modified with 8-N_3-ϵATP[a]

peptide[b]	relative fluorescence (%)	distribution of fluorophore (%)
CN-1 (N-terminal) (1–164 aa)	44	27
CN-2 (C-terminal) (251–363 aa)	100	61
CN-3 (containing Lys-229) (165–232 aa)	13	8
CN-4 (233–250 aa)	7	4
T-1	100	86
T-2	16	14
T-3	0	0
T-4	0	0

(T-1 to T-4: obtained from CN-2)

[a] Covalently labeled aldolase was obtained as described in Table I. The modified protein was cleaved by CNBr and separated into peptides CN-1–CN-4 as described under Materials and Methods. Cyanogen bromide peptide CN-2 was as and subjected to tryptic digestion and Sephadex G-25 column chromatography as described under Materials and Methods. The resulting peptides are designated as T-1–T-4. Fractions containing peptides were pooled and lyophilized. Then, each peptide was dissolved in 3 mL of 0.1 M $(NH_4)_2CO_3$, pH 9, and fluorescence was measured at λ_{ex} = 328 nm and λ_{em} = 410 nm. Identification of peptides was performed on the basis of their amino acid composition. [b] Using the designation and the position in aldolase sequence according to Lai (1968, 1975) and Tolan et al. (1984), respectively; aa, amino acids.

Sephadex C-25F (Figure 4A). The fluorescent peptide (peptide fCH-A) was isolated and purified by using QAE-Sephadex A-25 column chromatography (Figure 4B; peptide fCh-B) and high-voltage electrophoresis (peptide fCh-2; data not shown). The amino acid analysis of purified peptide fCH-2 was similar to that for a peptide comprising residues Thr^{259}-Val-Pro-Pro-Ala-Val-Thr^{265}-Gly-Val-Thr^{268}-Phe in native aldolase except that one threonine residue was missing (data not shown). Dansylation analysis showed that peptide fCh-2 contained N-terminal threonine, indicating that this residue in the photolabelled peptide was not modified. In the aldolase sequence, the two remaining threonine residues in this peptide are in positions 265 and 268. The photolabeled peptide was subjected to carboxypeptidase digestion (data not shown). It appears that, in the modified peptide, proteolysis stopped

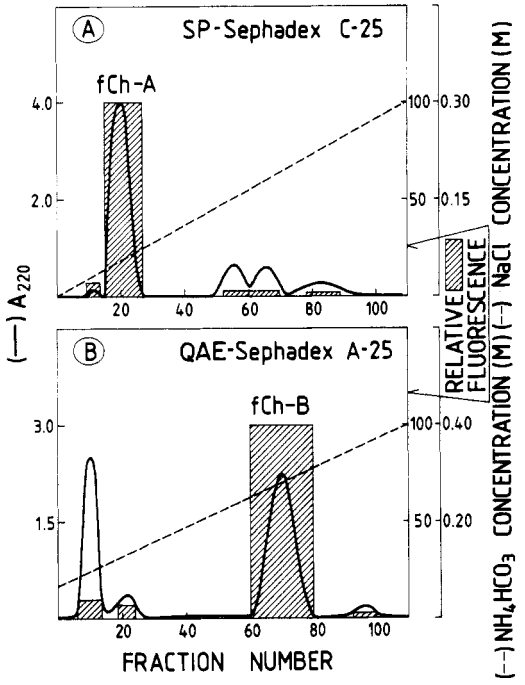

FIGURE 4: Isolation of aldolase peptide modified by 8-N_3-ϵATP. (A) Chromatography of the peptide obtained from chymotryptic digest of peptide T-1 on SP-Sephadex C-25. Peptide T-1 (3 mg, in 2 mL of NH_4HCO_3, pH 8.0), was digested with 20 μg of chymotrypsin. After 2.5 h, 200 μL of 85% formic acid was added and the sample was lyophilized and applied on the column. The column was eluted with 150 mL of a linear NaCl gradient (0–0.3 M) in 10 mM phosphate buffer, pH 3.0. Fractions (1.5 mL) were collected at a flow rate of 30 mL/h. The fractions were combined, and fluorescence at λ_{ex} = 328 nm and λ_{em} = 410 nm was measured. (B) Chromatography of peptide A obtained from SP-Sephadex, on QAE-Sephadex A-25. Peptide A was desalted on Sephadex G-25, lyophilized, and applied on a QAE-Sephadex column. The column (1.5 × 13 cm) was equilibrated with 0.04 N NH_4HCO_3, pH 8, and the column was eluted with 150 mL of a linear NH_4HCO_3 gradient (0.04–0.4 M). Fractions (1.5 mL) were collected at a flow rate of 30 mL/h. The fractions were combined, and fluorescence at λ_{ex} = 328 nm and λ_{em} = 410 nm was measured.

at residue 266, indicating that Thr-265 was chemically modified by 8-N_3-ϵATP.

Discussion

Steady-state kinetics showed that reversible interaction of 8-N_3-ϵATP with aldolase is similar to or identical with that observed previously when ATP is bound at pH 8.5, since binding occurs with the same dissociation constant (Kasprzak & Kochman, 1980a). This may indicate that in aldolase A there is no hindrance at position 8 of the purine binding subsite. This conclusion, however, should be taken cautiously because we do not know the exact contribution of free energy change due to purine residue binding.

The following lines of evidence suggest that covalent modification of aldolase under our conditions is highly specific and occurs only at the strong binding site. The modified aldolase is not bound to phosphocellulose, the binding of which Felicioli et al. (1975) have shown to occur at the active site. Substrate and competitive inhibitors protect the enzyme against the modification. The extent of modification correlates nicely with activity loss. All of these findings are consistent with the previous view that the strong nucleotide binding site must overlap partially with the active site of aldolase (Palczewski et al., 1985; Kasprzak & Kochman, 1980a).

In a preceding paper it was found that the γ-phosphate group of ATP may occupy two positions in the active site of

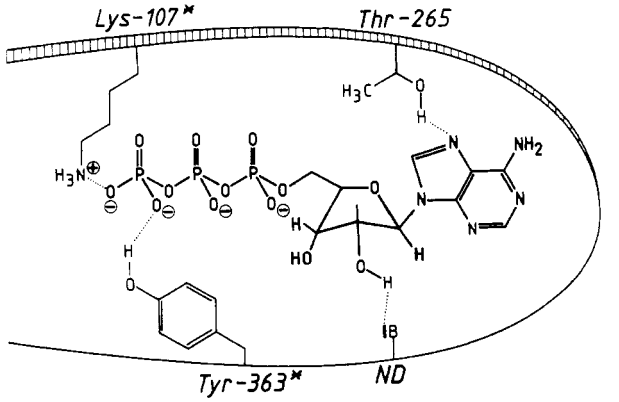

FIGURE 5: Scheme of mononucleotide binding site in aldolase A. Position of group B inferred from Kasprzak and Kochman (1981) (ND, not determined). Positions of Tyr-363 and Lys-107 inferred from Palczewski et al. (1985). Position Thr-265 inferred from this paper. *, residues present in aldolase active-site region.

aldolase (Lys-107 was modified to the extent of 80% and Tyr-363 ca. 20% by 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; Palczewski et al., 1985). Results presented in this paper suggest that in aldolase there are also two main points of attachment of the purine residue, comprising 88% of total labeling. The position of 61% of total labeling was identified as Thr-265. The second position, representing 27% of the labeling, is yet to be defined. Taking into consideration that the azide group represents an extremely reactive species during irradiation, our finding indicates that the purine moiety must not rotate freely in its aldolase binding site. This result correlates with our previous data which have shown that in rabbit liver aldolase the purine moiety is only partially exposed to solvent and rotates with a rotational correlation time similar to the whole protein molecule (Kasprzak & Kochman, 1981), indicating its immobilization in the protein matrix.

The results in this paper, in conjunction with previous data, are consistent with the positioning of the ATP molecule in the aldolase subunit as shown in Figure 5. Apparently, the general mode of binding of adenine nucleotide to aldolase parallels that for binding the mononucleotide moiety in NAD dehydrogenases or in kinases in the sense that the phosphate residue, ribose, and purine ring occupy fixed positions, involved in direct contacts with the protein matrix. In dehydrogenases, the pyrophosphate linkage of NAD is usually buried and bound by arginine or lysine side chains, the 2-OH of ribose is bound by a carboxyl residue, and the adenine moiety is usually located in the hydrophobic cleft between leucine and valine near the surface of the protein (Rossmann et al., 1975; Holbrook et al., 1975; Blake & Race, 1981; Vans et al., 1981). In dehydrogenases, threonine has been found to be located close to the pyrimidine ring of NAD rather than the purine ring (Eklund et al., 1984). A detailed X-ray analysis is obviously required to rigorously define the three-dimensional pattern of ATP binding to rabbit muscle aldolase and the overall folding of the protein in this region in comparison to the mononucleotide binding domain previously found in dehydrogenases and kinases.

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Amino Acid Sequence of Guinea Pig Prostate Kallikrein†

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ABSTRACT: The primary structure of the major arginine esteropeptidase from guinea pig prostate has been deduced from automated Edman degradation of peptides generated by clostripain, cyanogen bromide, endoproteinase Lys-C, and *Staphylococcus aureus* V8 protease digestion of the protein. The esteropeptidase is a single polypeptide chain comprised of 239 amino acids and contains 2 apparent sites of carbohydrate attachment, Asn-78 and Asn-169. Both occur in consensus sequences for N-linked glycosylation sites. The esteropeptidase exhibits approximately 35% homology with trypsin including conservation of the catalytic residues and the aspartic acid which confers specificity toward basic amino acids. The sequence identity, however, extends to greater than 60% with the kallikrein family of serine proteases. In addition to the overall homology, the guinea pig enzyme displays a number of features characteristic of kallikreins including 10 conserved half-cystine residues, a C-terminal proline, and the "kallikrein loop". On the basis of this structural relatedness, the enzyme has been designated as guinea pig prostate kallikrein. In contrast to many of the kallikreins of other species and tissues, this enzyme does not contain any sites within the kallikrein loop sensitive to proteases that result in internal breaks in the polypeptide chain.

Glandular kallikreins are a distinct subset of the serine protease family of esteropeptidases (Schachter, 1980). They preferentially hydrolyze synthetic substrates composed of esters and amides of arginine and, to a lesser extent, lysine (Fielder, 1979). In contrast to trypsin, kallikreins have very little general protease activity but rather display a high degree of substrate selectivity (Schachter, 1969). These enzymes were originally defined as kininogenases with the capacity, in vitro, to release kinin from kininogen. However, the kininogenase activity varies considerably among members of the kallikrein family, and many esteropeptidases have now been identified as kal-

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