INTERACTION OF THE PHOTOAFFINITY LABEL 8-AZIDO-ADP WITH GLUTAMATE DEHYDROGENASE*

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Received 10 February 1976

1. Introduction

The enzymatic activity of glutamate dehydrogenase is regulated by purine nucleotides [2,3]. Among these, ADP is of particular interest, since it may affect the enzymatic reaction as activator or inhibitor depending on the experimental conditions [4,5]. Recent binding studies have shown ADP to compete with NADH for the nonactive regulatory binding site [6]. It was, therefore, tempting to label and characterize this site with a structural analog of ADP. Compared to conventional labeling techniques, the method of photoaffinity labeling has certain advantages, as discussed by Knowles [7].

Recently, the synthesis of the 8-azidoadenine analogs of 3′,5′-cyclic AMP [8], 5′-ATP [9], NAD^+ and FAD [10] has been reported and some of these nucleotides have been used in photoaffinity labeling experiments [9,11,12]. We, therefore, synthesized 8-azido-ADP as a photoaffinity reagent for glutamate dehydrogenase. In the present communication, the properties of the nucleotide analog and its reversible interaction with glutamate dehydrogenase are described. Furthermore, covalent incorporation of the label upon irradiation is reported and preliminary localization studies are discussed.

2. Experimental

Beef liver glutamate dehydrogenase, AMP, ADP, NAD^+ and NADH were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Enzyme solutions were prepared and assayed as previously described [6]. 8-Bromo-AMP and 8-bromo-ADP were prepared from AMP and ADP by reaction with bromine in 1 M sodium acetate solution at pH 4.0. 8-Azido-ADP (yield 25%) was obtained after heating the tributylammonium salt of 8-bromo-ADP with a ten-fold molar excess of tributylammonium azide in dimethylformamide for 12 h at 75°C, the main product being 8-azido-AMP (45 %). Radioactive [β-^{32}P]8-azido-ADP was obtained by coupling 8-azido-AMP with [^{32}P]inorganic phosphate according to [13], (yield 33%). The synthesized nucleotide analogs were purified on Dowex 1 X 2 chloride using a LiCl gradient from 0–0.2 M in 10 mM HCl. The substrates and all other chemicals were of highest purity grade from commercially available sources.

Equilibrium dialysis experiments were performed overnight at 4°C in a Dianorm dialysis apparatus (Diachema AG, Zürich, Switzerland) containing 20 Teflon dialysis cells (1 ml). After equilibrium was reached, aliquots from both ligand and protein compartment were counted for radioactivity measuring the Cerenkov radiation of ^{32}P in water. Absorption and circular dichroism spectra were measured as previously described [6]. Photolabeling was performed at room temperature with a Fluotest 5241 lamp (Quarzlampen GmbH, Hanau, Germany) equipped with a glass filter to cut off radiation below 300 nm. The samples being photolyzed were place on serology plates, covered with the glass filter and located 3 cm from the light source. Labeled protein was precipitated with 5% trichloroacetic acid and collected on millipore filters which were solubilized in dioxane and counted by liquid scintillation. Labeled protein was carboxy-

*Studies on glutamate dehydrogenase, part XXV, for part XXIV see [1].

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methylated and digested with trypsin (1% by weight) for 12 h at pH 8.5 and 22°C. Electrophoresis of radioactive peptides was done as previously described [14].

3. Results and discussion

The prepared 8-azido-ADP is homogeneous as judged from thin-layer chromatography and the absorption spectrum remaining unchanged through all fractions of the 8-azido-ADP peak eluted from the ion exchange column. The absorption spectrum of 8-azido-ADP (fig.1) is different for neutral and acidic pH values due to protonation of the azidoadenine ring at N-1. For this process a pK of 3.9, identical to that of natural ADP, was determined from spectroscopic pH titrations. To get an idea about the photolability of 8-azido-ADP in aqueous solution, u.v.-irradiation was carried out followed by measurement of the absorbance. As shown in fig.2, the azidoadenine absorption at 282 nm disappears upon irradiation generating a new maximum at 274 nm with lower

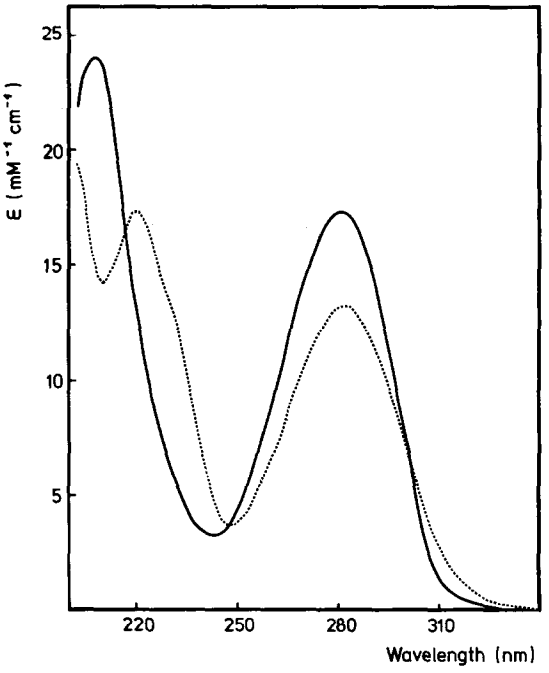

Fig.1. Absorption spectrum of 8-azido-ADP at 20°C. (———) pH 2.0, 10^{-2} M HCl, (– – – –) pH 7.6, 0.067 M phosphate buffer.

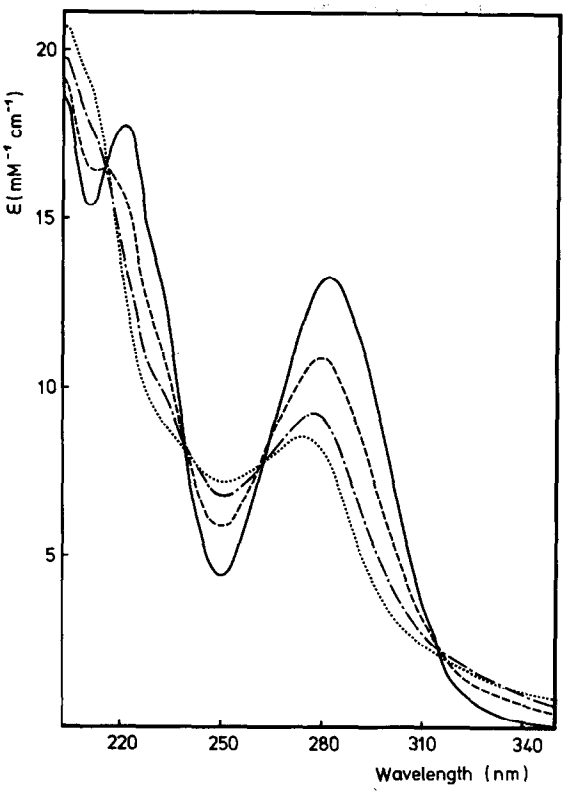

Fig.2. Change of the absorption spectra of 8-azido-ADP after irradiation. Samples in 0.067 M phosphate buffer, pH 7.6, were irradiated at 20°C for different time intervals at 300 nm and the spectra were taken. Irradiation time: (———) 0, (— — — —) 10 min, (– - – - –) 25 min, (. . . . . .) 45 min.

absorbance. The observed nearly isosbestic points indicate a fairly homogeneous reaction product. The photolysis of aromatic azides is described as a two step process [7]. In the first light dependent step, N_2 is split off leading to a highly reactive nitrene, which in the second step reacts covalently with its immediate environment. Therefore, the photolysis of 8-azido-ADP in water is expected to yield de 8-hydroxylamino derivative of ADP. Experimental support of this hypothesis comes from the reaction of 8-bromo-ADP with hydroxylamine in methanol at 60°C. Under these conditions, a purified main fraction with an absorption maximum at 274 nm is obtained.

8-Azido-ADP and 8-bromo-ADP are very poor substrates for rabbit muscle pyruvate kinase both

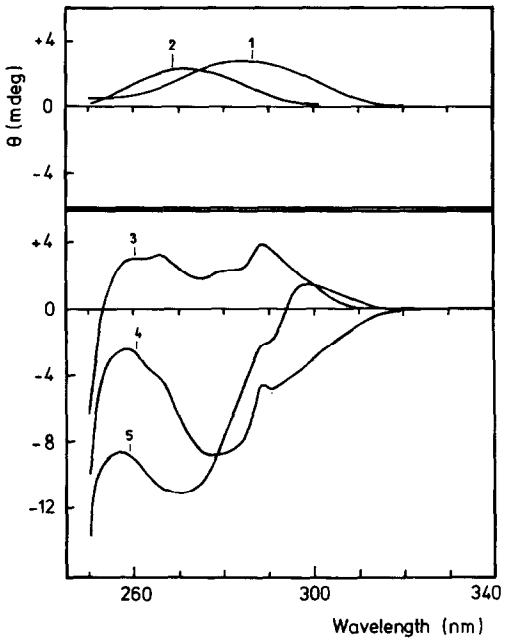

Fig.3. Circular dichroism spectra of the complexes of glutamate dehydrogenase with 8-bromo-ADP and 8-azido-ADP. Measurements in 0.067 M phosphate buffer pH 7.6 at 20°C, 1 cm-cuvette. (1) 8-bromo-ADP, 63 μM; (2) 8-azido-ADP, 86 μM; (3) glutamate dehydrogenase, 1 mg/ml; (4) complex of glutamate dehydrogenase with 8-bromo-ADP, concentrations as above; (5) complex of glutamate dehydrogenase with 8-azido-ADP, concentrations as above.

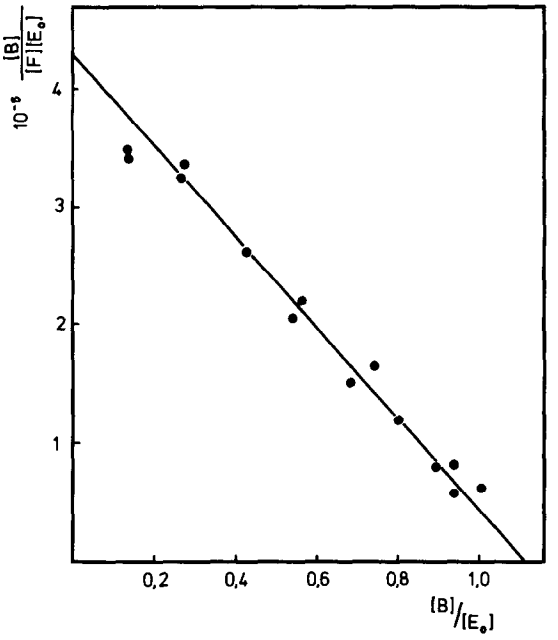

Fig.4. Scatchard plot for the binding of [^{32}P]8-azido-ADP by glutamate dehydrogenase. Equilibrium dialysis in 0.067 M phosphate buffer, pH 7.6, at 4°C; glutamate dehydrogenase, 0.5 mg/ml. B and F are the bound and free ligand, respectively, and [E_0] the enzyme concentration with respect to the polypeptide chain.

yielding less than 1% of the enzymatic activity observed with ADP as substrate under the same conditions [15]. Both 8-substituted nucleotides, however, are specifically bound by glutamate dehydrogenase. The circular dichroism spectra of the respective binary complexes (fig.3) show strong negative Cotton effects induced at the absorption maximum of the nucleotides. An analogous circular dichroism signal has been obtained for the interaction of glutamate dehydrogenase with ADP [16,17]. Quantitative determination of the binding of 8-azido-ADP to glutamate dehydrogenase was performed with equilibrium dialysis using ^{32}P-labeled 8-azido-ADP. The resulting Scatchard plot (fig.4) shows a straight line extrapolating to about one binding site for 8-azido-ADP per polypeptide chain with an apparent dissociation constant of 2.6 μM. This binding curve is consistent with six identical and independent binding sites per glutamate dehydrogenase unimer (comprising six identical polypeptide chains) and is identical within the limits of error to that observed with ADP [16].

Nevertheless, ADP and 8-azido-ADP affect the enzymatic activity of glutamate dehydrogenase to different extents. As shown in fig.5, ADP enhances the enzymatic activity up to 2.3-fold, whereas 8-azido-ADP and 8-bromo-ADP lead to only a 1.6- and 1.3-fold increase of activity, respectively. Therefore, binding alone of ADP or 8-azido-ADP to the enzyme does not appear to be a sufficient criterion for the observed activation. Moreover, a steric or allosteric interaction of the bound nucleotide with the active site has to be postulated to explain the different extents of activation. Such an interaction is obviously affected by alterations of the adenine-8 position.

The experiments discussed above well establish the specific reversible interaction between 8-azido-ADP and glutamate dehydrogenase. Therefore, covalent photoaffinity labeling of the ADP binding site of the

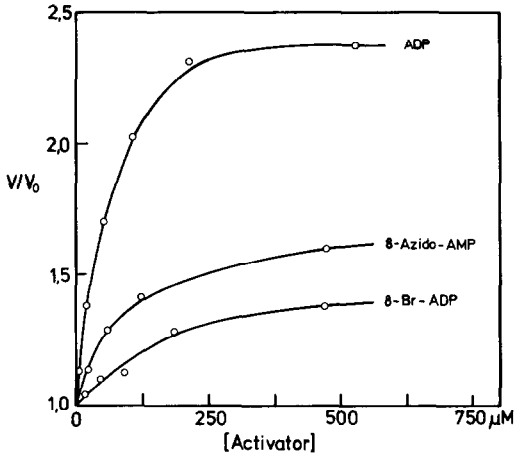

Fig.5. Activation of glutamate dehydrogenase by ADP, 8-azido-ADP and 8-bromo-ADP. Assay conditions: 1.3 mM NAD^+, 8.5 mM L-glutamate, 2 µg/ml enzyme, 0.067 M phosphate buffer, pH 8.4, 20.0°C.

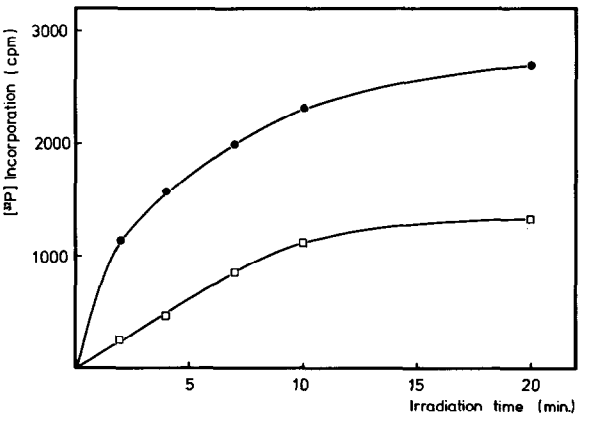

Fig.6. Photoaffinity labeling of glutamate dehydrogenase with [^{32}P]8-azido-ADP. Samples of 200 µl containing equimolar amounts of enzyme and [^{32}P]8-azido-ADP (3.2 nmol) in 0.067 M phosphate buffer pH 7.6 were irradiated at 300 nm. After precipitation, covalent incorporation of ^{32}P was determined by scintillation counting. (• – •) Experiments without ADP; (□ – □) experiments in the presence of 0.4 µmol ADP. Spec. act. of [^{32}P]8-azido-ADP, 9800 cpm/nmole.

enzyme was attempted. 8-Azido-ADP has an absorption maximum at 282 nm and thus, rapid inactivation of the enzyme due to photolysis of aromatic amino acids occurs, when this wavelength is used in photolabeling experiments. However, photolysis of the nucleotide without photoinactivation of the enzyme can be achieved by irradiating the sample at 300 nm. Under these conditions, the ^{32}P labeled nucleotide is covalently incorporated into the enzyme as shown by the content of radioactivity after denaturing precipitation. A series of control experiments showed that: (1) irradiation of the mixture of 8-azido-ADP and enzyme is necessary for covalent incorporation of the label; (2) non-covalent adsorption of the nucleotide on the enzyme or on the filter is negligible; (3) no inactivation of the enzyme occurs upon irradiation neither in presence nor in absence of 8-azido-ADP; and (4) irradiation at lower temperature (0°C instead of 20°C) does not significantly affect labeling.

The presence of ADP during irradiation reduces the incorporation of the label remarkably, indicating considerable specificity of the label with respect to the ADP binding site. Fig.6 shows the time course of incorporation of [^{32}P]8-azido-ADP in the absence and presence of 2 mM ADP. Obviously, short irradiation times favour specific labeling, however, the total amount of label incorporated in 5 min (fig.6) corresponds to the labeling of only 6% of the polypeptide chains. This value can be improved by using higher concentrations of both enzyme and label. At 2 mg/ml enzyme and a fourfold molar excess of 8-azido-ADP 22% of the polypeptide chains were labeled within 10 min of irradiation.

A sample prepared under these conditions was subjected to carboxymethylation and tryptic digest followed by gel filtration on Sephadex G-50. Subsequent high voltage electrophoresis at pH 6.5 of the lyophilized radioactive fraction yielded two radioactive ninhydrine positive spots, one remaining at the start of the pherogram and the other travelling strongly anodic. Work is in progress to purify and analyze the labeled peptides and to characterize the regulatory ADP binding site.

Acknowledgements

We thank Dr I. Rasched for his help in the separation of the labeled peptides. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138: Biologische Grenzflächen und Spezifität) and the Fonds der Chemischen Industrie.

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