Volume 229, number 2, 261–264 FEB 05648 March 1988

Adenosine di-, tri- and tetraphosphopyridoxals modify the same lysyl residue at the ATP-binding site in adenylate kinase

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Received 19 December 1987

Adenosine diphosphopyridoxal modifies Lys-21 in adenylate kinase which is located in a glycine-rich loop [(1987) J. Biol. Chem. 262, 8257–8261]. We presently report that adenosine tri- and tetraphosphopyridoxals modify the same lysyl residue more rapidly than the diphospho compound does. However, susceptibilities of the Schiff bases between the labels and the lysyl residue to sodium borohydride considerably differ in the modifications with the three reagents. These observations seem to be ascribable to the mobility of the ε-amino group of Lys-21 in the active-site region of the enzyme.

Adenylate kinase; Nucleotide-binding protein; Affinity label; Adenosine polyphosphopyridoxal; Glycine-rich region; Induced fit

1. INTRODUCTION

The sequence data of nucleotide-binding proteins have recently accumulated and revealed the presence of a highly conserved region; the consensus sequence is Gly-X-X-(Gly)-X-Gly-Lys-Thr(Ser) [1–3]. Adenylate kinase shares this sequence in Gly-15 to Gly-22 except for the substitution of Gly for Thr(Ser). Among the proteins sharing this conserved sequence, this enzyme is unique in that its three-dimensional structure has been resolved by X-ray crystallography [4]. However, locations of the AMP- and ATP-binding sites have remained undefined. Different binding sites for the two nucleotides were proposed from X-ray crystallographic analysis [5] and NMR studies [6,7]. Our recent affinity labeling studies using AP_2-PL revealed that Lys-21, a lysyl residue in the conserved sequence, is located near the γ-phosphate of the bound ATP [8]. In the present study, we investigated whether AP_3-PL and AP_4-PL modify any lysyl residue in adenylate kinase or not.

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Abbreviations: AP_2-PL, adenosine diphosphopyridoxal; AP_3-PL, adenosine triphosphopyridoxal; AP_4-PL, adenosine tetraphosphopyridoxal

2. EXPERIMENTAL

All the materials used were the same as those described [8,9].

Purification of AP_3-PL- and AP_4-PL-labeled peptides was carried out as described below. Adenylate kinase from rabbit muscle was incubated with an equimolar amount of AP_3-PL or AP_4-PL for 10 min at 25°C, and then cooled to 0°C. Reductive fixation of AP_3-PL to the enzyme was performed by treatment with sodium borohydride alone or in the presence of 7.5 M urea. The amino acid composition and the sequence of the labeled peptides were determined in the former and latter cases, respectively. Fixation of AP_4-PL to the enzyme was carried out with sodium borohydride in the presence of 7.5 M urea, 1% SDS, or 3 M guanidine hydrochloride. In the first case, the sequence of the labeled peptide was determined. The amino acid compositions of the labeled peptides were determined in the latter two cases. Procedures for purification of the labeled peptides were followed essentially as described previously [8]. The protein was carboxymethylated and then cleaved by tosylphenylalanyl chloromethyl ketone-trypsin. The labeled peptides were purified by the combination of gel filtration on Bio-Gel P6 chromatography and high-performance liquid chromatography with a solvent system of (A) 0.1% trifluoroacetic acid and 5% acetonitrile and (B) 0.1% trifluoroacetic acid and 90% acetonitrile or (A) 10 mM ammonium acetate and 5% acetonitrile and (B) 10 mM am-

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00145793/88/$3.50

monium acetate and 90% acetonitrile. Gradients of the solvents were followed as described in [8].

3. RESULTS

AP_4-PL inactivated rabbit muscle adenylate kinase within 3 min (fig.1a), more rapidly than AP_2-PL did [8]. A similar time course of inactivation was observed in the modification of the enzyme by AP_3-PL. Plots of the residual enzyme activity against the ratio in the concentrations of the enzyme and AP_3-PL and AP_4-PL added were linear up to 90% inactivation. The extrapolation to complete inactivation yielded the same value for the two reagents, 0.87 mol of reagent per mol of enzyme (not shown), suggesting that the two reagents bind to the enzyme with an equimolar amount as does AP_2-PL [8]. ADP, ATP and MgATP considerably reduced the rate of inactivation by AP_4-PL, whereas AMP afforded no protection against inactivation (fig.1b). Essentially the same results were obtained in terms of inactivation by AP_3-PL. These results are similar to those of the inactivation experiment with AP_2-PL. In a quantitative comparison, however, protection by ADP, ATP and MgATP was less effective in the inactivation by AP_3-PL and AP_4-PL than by AP_2-PL. The enzyme was inactivated by only 20% when incubated with AP_2-PL for 10 min in the presence of ADP, ATP and MgATP [8]. In contrast, AP_3-PL and AP_4-PL inactivated the enzyme by 60–70% (not shown) and 70–75% (fig.1b), respectively, under similar conditions. These results suggest that AP_3-PL and AP_4-PL are more accessible to the ATP-binding site than AP_2-PL.

Inhibition by 400 μM of the reduced compounds of AP_2-PL, AP_3-PL and AP_4-PL was investigated under the standard assay conditions except that the concentration of ATP was 50 μM. Reduced AP_2-PL, AP_3-PL and AP_4-PL inhibited the enzyme by 0%, 17% and 50%, respectively. Therefore, inhibition becomes stronger as the number of the phosphate increases. The binding of AP_4-PL to the ATP-binding site of the enzyme was confirmed by the competitive inhibition of adenosine tetraphosphopyridoxine with respect to ATP. The apparent inhibition constant was 210 μM.

Treatment of the AP_2-PL-modified enzyme with sodium borohydride caused irreversible inactivation of the enzyme [8]. However, when the AP_3-PL- and AP_4-PL-modified enzymes were treated with sodium borohydride and then diluted with a buffer containing ATP, their enzyme activities were gradually recovered (fig.2). After 240 min, 93% and 53% of the original activity were recovered in the AP_4-PL- and AP_3-PL-treated enzymes, respectively. The Schiff base between a lysyl residue and pyridoxal phosphate in phosphorylase is resistant to reductive fixation by sodium borohydride [10]. This resistancy was explained by the hydrophobic nature around the Schiff base [11]. The reductive fixation of the coenzyme by sodium borohydride was carried out in the presence of deforming reagents such as SDS [12] and urea [13]. Therefore, we tried to fix AP_3-PL and AP_4-PL to adenylate kinase by sodium borohydride treatment in the presence of 6.6 M urea, and found that the labels are irreversibly bound to the enzymes under these conditions. We repeated the reactivation experiments and found that reactivation is due to the release of the labels during incubation.

The AP_3-PL- and AP_4-PL-labeled peptides were purified and their structures were determined. The sequences of AP_3-PL- and AP_4-PL-labeled peptides which had been prepared by treatment of the modified enzymes with sodium borohydride in the

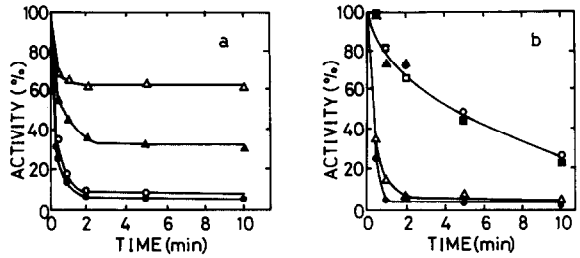

Fig.1. Inactivation of adenylate kinase by AP_4-PL. (a) Time course. The reaction mixture contained 12.8 μM adenylate kinase, 50 mM Hepes buffer (pH 7.8), 1 mM EDTA, 25% glycerol and AP_4-PL at 4 (△), 7 (▲), 10 (○) or 50 μM (●). At various times during incubation at 25°C aliquots were withdrawn, mixed with $NaBH_4$ and diluted with 50 mM triethanolamine buffer (pH 7.5) containing 1 mM EDTA and 1 mg/ml bovine serum albumin, and residual activity was measured. (b) Effect of substrates on inactivation. The reaction mixture contained 12.8 μM enzyme, 50 mM Hepes buffer (pH 7.8), 1 mM EDTA, 25% glycerol, and 20 μM AP_4-PL (●), in the presence of 0.8 mM AMP (△), 0.8 mM ADP (▲), 0.8 mM ATP (○) or 0.8 mM ATP and 8.0 mM $MgCl_2$ (□).

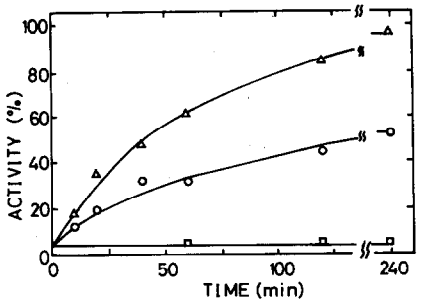

Fig.2. Reactivation of adenosine polyphosphopyridoxal-modified adenylate kinase. The inactivation mixture contained 12.8 μM enzyme, 50 mM Hepes buffer (pH 7.8), 1 mM EDTA, 25% glycerol and 50 μM AP_2-PL (□), 50 μM AP_3-PL (○) or 50 μM AP_4-PL (△). After incubation at 25°C for 20 min, the enzyme was treated with $NaBH_4$. Aliquots were withdrawn and diluted with a solution containing triethanolamine buffer (pH 7.5), 1 mM EDTA, 1 mg/ml bovine serum albumin and 1 mM ATP, and the activity recovered was measured.

presence of urea were the same, Ile-Ile-Phe-Val-Val-Gly-Gly-Pro-Gly-Ser-Gly-X-Gly-Thr-Gln-carboxymethyl Cys-Glu-Lys, where X is the residue unidentified. Since the labeled lysine cannot be identified on sequence analysis [8], X should be a labeled lysine. This sequence corresponds to Ile-10 to Lys-27 of the rabbit muscle enzyme [14]. Thus,

Table 1

Amino acid compositions of labeled peptides[a]

	AP_3-PL	AP_4-PL + Gu-HCl	AP_4-PL + SDS	No. of residue[b]
Cmcys[c]	0.57	0.14	0.50	1
Thr	1.01	0.94	1.03	1
Ser	1.06	1.00	1.14	1
Glu	2.21	2.10	2.42	2
Pro	1.25	0.99	1.05	1
Gly	5.44	5.01	5.49	5
Val	1.27	1.15	1.26	2
Ile	0.73	0.74	0.88	2
Phe	1.01	0.94	1.07	1
Lys	1.11	1.01	1.11	2
Lys(Pxy)[d]	0.69	0.64	0.72	

[a] Labeled peptides were purified by combination of gel filtration and high-performance liquid chromatography with ammonium acetate buffer
[b] Sequence from Ile-10 to Lys-27
[c] Carboxymethyl cysteine
[d] The color value of pyridoxyl lysine (Lys(Pxy)) is assumed to be the same as that of lysine

AP_3-PL and AP_4-PL modified the same lysine, Lys-21, which is also modified by AP_2-PL [8]. In order to check the possibility that the labels are transferred to Lys-21 during denaturation of the modified enzymes, we prepared three kinds of treated enzymes: the AP_3-PL-labeled enzymes treated with sodium borohydride alone, and AP_4-PL-labeled enzymes treated with sodium borohydride in the presence of SDS and guanidine hydrochloride. Throughout the purification of the labeled peptides a single fluorescent peak derived from the label was detected in every case. Table 1 shows the amino acid compositions of the labeled peptides. The labeled residue must be Lys-21 in every case, suggesting that the labels are all bound originally to Lys-21.

4. DISCUSSION

The combined results of the present and previous [8] studies showed that AP_2-PL, AP_3-PL and AP_4-PL all modify the same lysyl residue, Lys-21, at the ATP-binding site in adenylate kinase. There are two possible explanations for this unexpected finding, namely, (i) the flexibility in structures of the labels and (ii) the mobility of the ε-amino group of Lys-21. However, different susceptibilities of the Schiff bases between Lys-21 and each label against sodium borohydride cannot be explained by applying the former possibility. If the polyphosphate moieties of the labels are bent and the Schiff bases formed occupy the same position in the enzyme, it would be reasonable to expect that susceptibilities of the Schiff bases against sodium borohydride are the same. Since adenylate kinase has a high affinity pocket for diadenosine pentaphosphate [15], the polyphosphate moieties of the labels we used should occupy this site. Therefore, we prefer the latter possibility.

The movement of the ε-amino group of Lys-21 might be endurable by the rotation of the two glycyl residues which sandwich this lysyl residue. Although the free rotation around the N-C and C-C′ bond of amino acid residues in protein is restricted by steric hindrance, dihedral angles of the glycyl residue (ψ,ϕ) are exceptionally widely allowed [16]. The changes in angles of ϕ of Gly-20 and ψ of Gly-22 may cause a large movement of the ε-amino group of Lys-21. Alternatively, such a large movement might also be possible by the

movement of a peptide segment itself containing Lys-21. X-ray crystallographic analysis of the porcine enzyme showed that Gly-15 to Gly-22 construct a loop structure with a turn at Pro-17 and a large movement of this loop occurs between the two different crystal forms [5]. The consensus sequence, Gly-X-X-(Gly)-X-Gly-Lys-Thr(Ser) in other nucleotide-binding proteins might similarly move when the proteins bind nucleotides.

Acknowledgements: This investigation was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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