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Chemical modification of a functional arginine residue in diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) phosphorylase I from *Saccharomyces cerevisiae*

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Phenylglyoxal, a reagent with high specificity for arginine residues, inactivated Ap_4A phosphorylase I from *Saccharomyces cerevisiae* in a pseudo-first-order manner. The second-order rate constant was 11.5 ± 2.5 M^{-1} min^{-1}. The loss of activity was a linear function of the incorporation of [7-^{14}C]phenylglyoxal. The incorporation of 1.9 ± 0.4 mol of phenylglyoxal/mol of enzyme accounted for complete loss of activity. The specificity of inactivation by phenylglyoxal was tested in the presence of Ap_nA ($n = 2$–6), ADP, ATP and P_i. The substrates, Ap_4A, Ap_5A and P_i protected the enzyme against inactivation, but Ap_2A, Ap_3A and Ap_6A did not. Ap_4A, Ap_5A and P_i reduced the rate of inactivation by about 70%, 60% and 37% respectively. The Ap_4A phosphorolysis products, ADP and ATP, also partially protected the enzyme against inactivation by phenylglyoxal. Thus Ap_4A phosphorylase I probably contains an arginine residue in the binding site for Ap_4A.

INTRODUCTION

Diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) is a dinucleoside polyphosphate present in bacteria, lower eukaryotes, including *Saccharomyces cerevisiae*, and mammalian cells at basal levels of 10^{-8}–10^{-6} M (Rapaport & Zamecnik, 1976; Ogilvie, 1981; Lee *et al.*, 1983; Garrison & Barnes, 1984; McLennan & Prescott, 1984; Baker & Jacobson, 1984). Exceptions are platelets and chromaffin cells in which Ap_4A is present at 10^{-4}–10^{-3} M (Flodgaard & Klenow, 1982; Rodriguez del Castillo *et al.*, 1988).

Ap_4A is catabolized by three different types of specific enzymes. A hydrolase discovered in *Physarum polycephalum* (Barnes & Culver, 1982; Garrison *et al.*, 1982), and also present in *Escherichia coli* (Guranowski *et al.*, 1983; Plateau *et al.*, 1985), symmetrically cleaves Ap_4A to ADP. A hydrolase present mainly in higher eukaryotic species asymmetrically cleaves Ap_4A to AMP and ATP in the presence of a bivalent cation (Vallejo *et al.*, 1976; Ogilvie & Antl, 1983; Prescott *et al.*, 1989; Ogilvie *et al.*, 1989). Ap_4A phosphorylase, discovered in *Saccharomyces cerevisiae* (Guranowski & Blanquet, 1985) and also present in *Euglena* (Guranowski *et al.*, 1988*a*), catalyses the phosphorolysis of Ap_4A in the presence of P_i and a bivalent cation to yield ADP and ATP.

Ap_4A phosphorylase has been purified to homogeneity from *S. cerevisiae* (Guranowski & Blanquet, 1985), and the *DTP* (*d*iadenosine *t*etraphosphate *p*hosphorylase) gene encoding the enzyme has been cloned and sequenced (Plateau *et al.*, 1989; Kaushal *et al.*, 1990). Ap_4A phosphorylase is apparently unique in terms of catalysing the phosphorolysis of a phosphoanhydride bond between nucleoside moieties (Guranowski & Blanquet, 1985). Under certain conditions *in vitro*, the yeast enzyme also catalyses the formation of Ap_4A from ADP and ATP (Brevet *et al.*, 1987), but results from disruption of the gene indicate that the enzyme behaves catabolically *in vivo* (Plateau *et al.*, 1989). This enzyme catalyses the exchange of the β-phosphate of nucleoside diphosphates and P_i (Guranowski & Blanquet, 1986*a*), and some inorganic anions, e.g. vanadate, can substitute for P_i in the exchange reaction as well as in the cleavage of Ap_4A (Guranowski & Blanquet, 1986*b*). Yeast Ap_4A phosphorylase also catalyses the irreversible synthesis of Ap_4A from ATP and adenosine 5′-phosphosulphate (Guranowski *et al.*, 1988*b*). Recently, a second form of Ap_4A phosphorylase has been isolated from *S. cerevisiae* (Plateau *et al.*, 1990). This form of the enzyme, designated II, catalyses all of the same reactions as the first form (I), and the two forms have about 60% amino acid sequence identity. The two forms differ quantitatively in their kinetic properties (Plateau *et al.*, 1990).

Nothing is known about the amino acid residues in the active site that participate in the reactions catalysed by the yeast Ap_4A phosphorylases. Arginine residues participate in the binding and catalysis of phosphoryl groups in substrates for a number of enzymes (Riordan, 1979). We identified a potential adenine nucleotide-binding site in Ap_4A phosphorylase I based on comparison of the deduced amino acid sequence with the sequences of several enzymes known to contain this particular sequence motif (Kaushal *et al.*, 1990). One arginine residue is present in this potential nucleotide-binding site. Thus we tested the possibility that an arginine residue may by present in the Ap_4A-binding site of yeast Ap_4A phosphorylase I using phenylglyoxal, a reagent with high specificity for arginine (Takahashi, 1968, 1977).

Here we report that chemical modification of yeast Ap_4A phosphorylase I with phenylglyoxal causes loss of activity with pseudo-first-order kinetics, and that the substrates and products partially prevent this loss of activity. The loss of enzymic activity is a linear function of the stoicheiometry of incorporation of [^{14}C]phenylglyoxal.

EXPERIMENTAL

Materials

Ap_4A was purchased from Sigma Chemical Co. and was custom-labelled with tritium (Amersham Corp.). ^{3}H-labelled Ap_4A was purified by chromatography on dihydroxyboronyl-

Abbreviations used: Ap_4A, A5′pppp5′A, diadenosine 5′,5‴-P¹,P⁴-tetraphosphate; other diadenosine 5′,5‴-polyphosphates are abbreviated in a similar fashion; QAE, quaternary aminoethyl.

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Bio-Rex 70 resin (Barnes *et al.*, 1985) and analysed for purity by isocratic h.p.l.c. (Garrison & Barnes, 1984). Phenylglyoxal was purchased from Sigma Chemical Co. [7-^{14}C]Phenylglyoxal (27.8 mCi/mmol) and [^{14}C]methylated proteins used as molecular-mass standards were purchased from Amersham Corp. The QAE-Sepharose resin was manufactured by Pharmacia. NZ-amine A was from ICN Biochemicals, Inc. Kodak X-Omat AR film was from Eastman Kodak Co.

Purification of Ap_4A phosphorylase I

Ap_4A phosphorylase I was purified to homogeneity from *S. cerevisiae* CGY-339 (α his4-29 ura3-52 pep4-3) that had been transformed with the plasmid YEp352 (Hill *et al.*, 1986) with a 3.1 kb insert that contained the *DTP* gene (Kaushal *et al.*, 1990). The presence of this expression vector increased the yield of the enzyme by a factor of 15 relative to untransformed yeast (L. D. Barnes & V. Kaushal, unpublished work). Yeast was grown in 0.5% (w/v) yeast nitrogen base [without amino acids and without $(NH_4)_2SO_4$], 1% (w/v) NZ-amine A and 3% (w/v) glucose to a density of 2×10^8 cells/ml. The enzyme was purified by modification of the procedure described by Guranowski & Blanquet (1985). The order of column chromatography was changed to the sequence: DEAE-cellulose (instead of DEAE-Sephacel), hydroxyapatite and Sephadex G-75 (instead of Sephadex G-100). The enzyme was then subjected to chromatography on a QAE-Sepharose column equilibrated with 20 mM-potassium phosphate, pH 6.8, 10% (v/v) glycerol and 5 mM-2-mercaptoethanol. Purified enzyme was eluted at about 0.25 M-NaCl in a linear gradient. The enzyme was at least 95% pure based on SDS/PAGE analysis, and it exhibited the same physicochemical and kinetic properties as those described previously (Guranowski & Blanquet, 1985).

Assay of enzymic activity

Ap_4A phosphorylase I from *S. cerevisiae* was assayed by measuring formation of [^{3}H]ADP and [^{3}H]ATP from [^{3}H]Ap_4A and P_i. Activity was measured in 50 mM-Hepes/NaOH (pH 7.5)/0.5 mM-$MnCl_2$/1 mM-KH_2PO_4 at 37 °C for 10 min in a volume of 100 μl. The reaction products were separated from residual substrate by column chromatography on a boronate-derivatized resin (Barnes *et al.*, 1985). Enzymic activity was expressed as nmol of ADP + ATP formed/min per mg of protein.

Inactivation of Ap_4A phosphorylase I with phenylglyoxal

In preliminary experiments, different combinations of Hepes and phosphate buffers, glycerol, dithiothreitol or 2-mercaptoethanol, and EGTA were examined to optimize conditions for the incubation solution with phenylglyoxal and for the subsequent dilution solution. Different concentrations of the enzyme were also tested. The effects of these components on the stability of the enzyme during incubation and dilution, inactivation by phenylglyoxal and interference in the enzymic assay were examined. Glycerol stabilized the enzymic activity in general. Phosphate partially protected the enzyme against inactivation (see the Results section), so Hepes was used as the buffer in the inactivation incubation. Phosphate, glycerol and a thiol compound stabilized the enzyme after dilution for assay of residual activity. The thiol compounds and EGTA had no significant effect on the stability of the enzyme at the enzyme concentrations used in the inactivation incubations. Dithiothreitol did not significantly affect the rate of inactivation of the enzyme by phenylglyoxal.

Ap_4A phosphorylase I (0.16–0.78 mg/ml) was incubated in 50 mM-Hepes/NaOH (pH 7.5)/10% (v/v) glycerol at 25 °C with phenylglyoxal at 0–7.5 mM. Stock phenylglyoxal was prepared in the same buffer. In ligand-protection experiments, different ligands, as described in the Results section, were also present. Samples were diluted from 1:400 to 1:2000 into 20 mM-potassium phosphate (pH 6.8)/10% (v/v) glycerol/5 mM-2-mercaptoethanol at different times after addition of phenylglyoxal, and 10 μl of the dilution was assayed for enzymic activity. The phenylglyoxal carried over into the assay (maximal final concentration of 0.4 μM) did not interfere with the assay. The concentration of phenylglyoxal, diluted into water for measurement, was calculated from its absorbance at 252 nm and an ϵ value of 13200 M^{-1} cm^{-1} in water. This value of the absorption coefficient is similar to a value of 12600 M^{-1} cm^{-1} at 253 nm reported by Kohlbrenner & Cross (1978).

Incorporation of [7-^{14}C]phenylglyoxal into Ap_4A phosphorylase I

Ap_4A phosphorylase I was incubated with [7-^{14}C]phenylglyoxal under the conditions described above. A 5 μl sample was removed from the incubation mixture as a function of time and spotted on to a disc of Whatman 3MM paper soaked in cold 10% (w/v) trichloroacetic acid. Discs were then washed once with cold 10% (w/v) trichloroacetic acid (10 ml per disc), twice with 5% (w/v) trichloroacetic acid at room temperature, and twice with 95% (v/v) ethanol, and dried before measurement of the radioactivity in a liquid-scintillation counter. Control experiments demonstrated that this wash procedure removed unincorporated phenylglyoxal to less than 0.1% of the total d.p.m. in the incubation mixture. Samples also were removed from the incubation mixture at the same time points for assay of enzymic activity.

Electrophoretic analysis

Ap_4A phosphorylase I was subjected to electrophoresis on 12.5%-polyacrylamide gels containing 0.1% SDS using the discontinuous buffer system described by Laemmli (1970) and modified by Studier (1973). In experiments with [7-^{14}C]phenylglyoxal-labelled enzyme, gels were dried and subjected to autoradiography at −70 °C using Kodak X-Omat AR film. ^{14}C-methylated proteins were used as molecular-mass standards on gels analysed autoradiographically.

Data analyses

Representative data from repeated experiments are presented. The mean value ($\bar{x}$), standard deviation (S.D.), and number of experiments (n) are given for some data. Values of apparent pseudo-first-order rate constants were calculated by least-squares linear regression analysis of semi-logarithmic plots of residual enzymic activity as a function of time.

RESULTS

Inactivation of Ap_4A phosphorylase I by phenylglyoxal

Yeast Ap_4A phosphorylase I was inactivated by phenylglyoxal in a time-dependent manner. Inactivation was dependent on the concentration of phenylglyoxal and followed pseudo-first-order kinetics from 100% activity to less than 2% of the residual activity (Fig. 1). Enzyme incubated under the same conditions in the absence of phenylglyoxal exhibited negligible loss of activity (results not shown, but see Fig. 1 for the level of residual activity in the presence of 0.79 mM-phenylglyoxal). A plot of the pseudo-first-order rate constants as a function of the phenylglyoxal concentrations yielded a straight line from which the value of the second-order rate constant was calculated to be 11.5 ± 2.5 M^{-1} min^{-1} ($n = 5$). The value of the slope from a

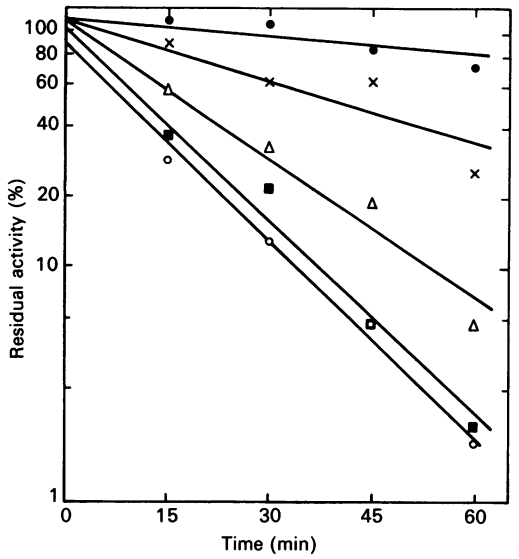

Fig. 1. Inactivation of Ap_4A phosphorylase I by phenylglyoxal as a function of time

Ap_4A phosphorylase I (0.16 mg/ml) was incubated with 0.79 (●), 1.58 (×), 2.77 (△), 3.96 (■) and 5.10 (○)-mM phenylglyoxal in 50 mM-Hepes/NaOH (pH 7.5)/10% (v/v) glycerol at 25 °C. At the indicated times, portions were removed, diluted 1:400 into 20 mM-potassium phosphate (pH 6.8)/10% (v/v) glycerol/5 mM-2-mercaptoethanol, and assayed for residual enzymic activity.

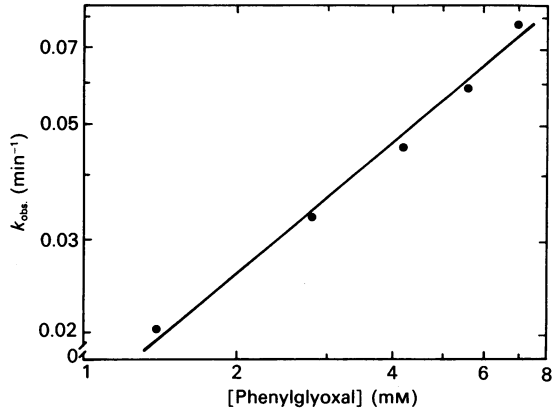

Fig. 2. Logarithmic plot of the rate constants of inactivation of Ap_4A phosphorylase I as a function of the phenylglyoxal concentration

Values of the pseudo-first-order rate constants, k_{obs}, were calculated from the slopes of a semilogarithmic plot of residual activity in the presence of different concentrations of phenylglyoxal versus time (see Fig. 1).

logarithmic plot of the pseudo-first-order rate constants as a function of the phenylglyoxal concentrations (Fig. 2) indicated that a minimum of 1.11 ± 0.20 ($n = 5$) mol of phenylglyoxal/mol of the enzyme accounted for the loss of enzymic activity.

The incorporation of [7-^{14}C]phenylglyoxal into Ap_4A phosphorylase I as a function of time was measured concomitantly with assay of residual activity and the results are shown in Fig. 3(*a*). The loss of enzymic activity was a linear function of the incorporation of phenylglyoxal (Fig. 3*b*), and the stoicheiometry of incorporation was 1.9 ± 0.4 ($n = 5$) mol of phenylglyoxal/mol of Ap_4A phosphorylase I.

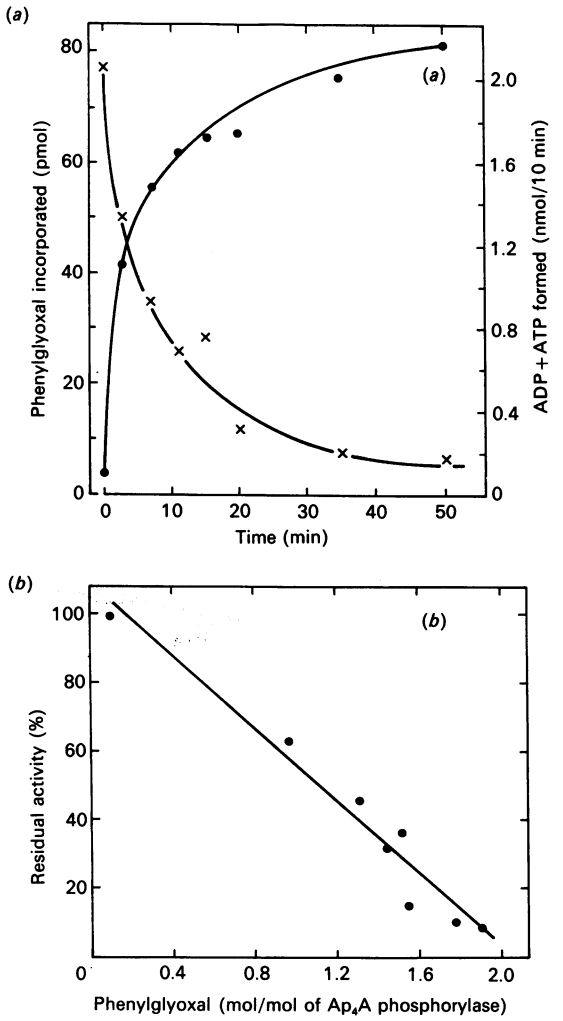

Fig. 3. Correlation of inactivation of Ap_4A phosphorylase I with incorporation of phenylglyoxal into the enzyme

(*a*) Ap_4A phosphorylase I (0.31 mg/ml) was incubated in 50 mM-Hepes/NaOH (pH 7.5)/10% (v/v) glycerol in the presence of 2.99 mM-[7-^{14}C]phenylglyoxal at 25 °C. At the times indicated, samples were removed for measurements of the incorporation of phenylglyoxal (●) and enzymic activity (×) as described in the Experimental section. (*b*) The residual activity (%) of Ap_4A phosphorylase I as a function of the stoicheiometry of phenylglyoxal incorporated into the enzyme.

Effect of ligands on inactivation of Ap_4A phosphorylase I by phenylglyoxal

The substrates Ap_4A and Ap_5A partially protected against inactivation by phenylglyoxal (Table 1). Ap_4A at a concentration of 1 mM, i.e. about 16 times the value of the K_m for Ap_4A (Guranowski & Blanquet, 1985), reduced the rate of inactivation by about 70%. Ap_5A reduced the rate of inactivation by about 60%. Autoradiographic data of the time-course of incorporation of [7-^{14}C]phenylglyoxal into the enzyme are shown in Fig. 4. This Figure also demonstrates a decrease in incorporation of [7-^{14}C]phenylglyoxal in the presence of Ap_4A. The protection provided by Ap_4A and Ap_5A was specific among the diadenosine polyphosphates tested. Ap_2A, Ap_3A or Ap_6A at a concentration of 1 mM did not protect the enzyme against inactivation by 4.2 mM-phenylglyoxal based on the values of $k_{obs.}$ (Table 1).

The substrate, P_i, at 10 mM, decreased the rate on inactivation by 4.2 mM-phenylglyoxal by about 37%. The products, ADP

Table 1. Effect of diadenosine polyphosphates on inactivation of Ap_4A phosphorylase I by phenylglyoxal

Ap_4A phosphorylase I (0.16 mg/ml) was incubated with 4.2 mM-phenylglyoxal in the absence (control) or presence of the indicated diadenosine polyphosphate, each at a concentration of 1.0–1.2 mM. Samples were removed from the incubations as a function of time (0–60 min) and assayed for activity. The rate constants were calculated from a semilogarithmic plot of residual activity versus time. n = the number of experiments with duplicate assays in each experiment. Values for $k_{obs.}$ are expressed as the mean ($\bar{x}$) and standard deviation (S.D.) for the indicated number of experiments.

		$10^3 \times k_{obs.}$ (min^{-1})		
Ap_nA added	n	$\bar{x}$	S.D.	Relative rate
Control	5	44.9	3.5	1.00
Ap_2A	3	49.4	5.3	1.09
Ap_3A	2	49.1	2.0	1.09
Ap_4A	4	13.7	2.5	0.31
Ap_5A	4	17.3	5.3	0.38
Ap_6A	2	50.0	1.0	1.11

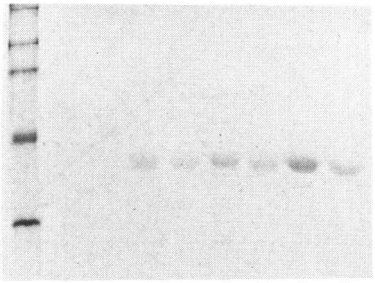

Fig. 4. Incorporation of [7-^{14}C]phenylglyoxal into Ap_4A phosphorylase I as a function of time and protection by Ap_4A

Ap_4A phosphorylase I (3.1 mg/ml) was incubated at 25 °C in 50 mM-Hepes/NaOH (pH 7.5)/10% (v/v) glycerol in the presence of 4.3 mM-[7-^{14}C]phenylglyoxal and in the absence or presence of 4.2 mM-Ap_4A. Samples (6 μg of enzyme) were removed at 0, 30, 60 and 90 min, dissociated in SDS, and subjected to electrophoresis on a polyacrylamide gel containing SDS as described in the Experimental section. The gel was dried and subjected to autoradiography. Lane 1, ^{14}C-methylated proteins as molecular-mass standards: bands in descending order from the top of the gel: myosin (200 kDa), phosphorylase *b* (92.5 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Lanes 2–9, Ap_4A phosphorylase I incubated with [7-^{14}C]phenylglyoxal for 0 (lanes 2, 3), 30 (lanes 4, 5), 60 (lanes 6, 7) and 90 (lanes 8, 9) min. Lanes 2, 4, 6 and 8, incubation in the absence of Ap_4A; lanes 3, 5, 7 and 9, incubation in the presence of Ap_4A.

and ATP, each at 1 mM, decreased the rate of inactivation by about 60% and 20% respectively.

DISCUSSION

Ap_4A phosphorylase I contains an arginine residue in the substrate-binding site for Ap_4A based on the correlation of loss of activity with the stoicheiometry of phenylglyoxal incorporation and the partial protection by specific substrates and products against inactivation by phenylglyoxal. The specificity of protection by Ap_4A and Ap_5A among Ap_nA (n = 2–6) reflects the substrate specificity of Ap_4A phosphorylase I. The rate of phosphorolysis of Ap_4A is greater than for Ap_5A, and Ap_2A and Ap_3A are not substrates for the enzyme (Guranowski & Blanquet, 1985). Ap_6A has not been tested as a substrate, but our results suggest that it is not a substrate.

The presence of an essential arginine residue in this enzyme is compatible with results indicating that arginine residues are often components of binding sites in enzymes with phosphate-containing substrates (Riordan, 1979). Riordan (1979) also observed that arginine occurred infrequently in enzymes in which arginine was present in binding sites for anionic substrates. The relative scarcity of arginine in Ap_4A phosphorylase I, only five arginines in a total of 321 amino acids (Plateau *et al.*, 1989; Kaushal *et al.*, 1990), is in agreement with the observations of Riordan (1979). In addition, Holler (1984) has demonstrated that Ap_4A and L-arginine can form a 1:1 stoicheiometric complex.

The dependence of the pseudo-first-order rate constants on the concentration of phenylglyoxal supports the conclusion that modification of one arginine residue is sufficient to account for the loss of activity. Complete inactivation of Ap_4A phosphorylase I on incorporation of 2 mol of phenylgloyoxal/mol of enzyme (Fig. 3*b*) is also compatiable with the modification of one arginine residue because Takahashi (1968) demonstrated that two phenylglyoxal moieties are incorporated per guanidino group. However, this 2:1 stoichiometry of phenylglyoxal to arginine residue does not always occur (Borders & Riordan, 1975; Koland *et al.*, 1982; Konishi & Fujioka, 1987). Thus we cannot at present dismiss the possibility that two different arginine residues in Ap_4A phosphorylase I are modified by phenylglyoxal, but with only one being essential for activity. Attempts to distinguish these possibilities, to demonstrate conclusively that an arginine residue has been modified and to identify the specific arginine that is modified have been unsuccessful. The phenylglyoxal–arginine product formed in Ap_4A phosphorylase I is unstable under the conditions required for amino acid analysis and isolation of phenylglyoxal-labelled peptides. Similar instability of phenylglyoxal–arginine products in other enzymes has been reported (Jornvall *et al.*, 1977; McKee & Nimmo, 1989). However, the stability of the inactivated enzyme to high dilution, precipitation with trichloroacetic acid and dissociation in SDS support the kinetic data that phenylglyoxal acts as an irreversible inhibitor of Ap_4A phosphorylase I.

The results support the proposal that an arginine residue is present in the potential nucleotide-binding site in Ap_4A phosphorylase I (Kaushal *et al.*, 1990). This information on the Ap_4A-binding site will be essential in elucidating the mechanism of catalysis by this enzyme.

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