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Effects of mutation at arginine-218 residue on the reaction of synthetic thymidylate synthase and 5-fluorouracil

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Abbreviations: TS, thymidylate synthase; FdUMP, 5-fluoro-2'-deoxyuridylate; CH_2H_4folate, 5,10-methylenetetrahydrofolate; H_2folate, 7,8-dyhydrofolate

Abstract

The importance of Arg-218 residue for the catalytic activity of thymidylate synthase chemically synthesized based on the amino acid sequence of thymidylate synthase from *Lactobacillus casei* has been studied by cassette mutagenesis and chemical modification with the arginine-specific dicarbonyl reagent phenylglyoxal. A series of mutations were constructed and functionally acceptable substitutions were confirmed by genetic complementation of thymidylate synthase deficient cells. The mutants were further characterized by determination of kinetic parameters using 5-fluoro-2'-deoxyuridylate as an active site titrant. Analysis of the mutants by genetic complementation and kinetic studies showed that Arg-218 could be essential for enzyme activity. The k_{cat}/K_m values of the inactive mutants were undetectable or much lower than those of wild type. SDS-PAGE analysis and activity measurements of crude extracts prepared from each of the mutants showed that the absence of catalytic activity is not due to a lack of production or stability of the different mutants. The results indicate that Arg-218 is critical for substrate binding or catalytic activity of thymidylate synthase.

Keywords: thymidylate synthase mutants, site-directed mutagenesis, genetic complementation

Introduction

Thymidylate synthase (EC 2.1.1.45) catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate (CH_2H_4folate) to dTMP and 7,8-dihydrofolate (H_2folate). The catalytic mechanism of thymidylate synthase (TS) has been extensively studied (Santi and Danenberg, 1984; Santi *et al.*, 1987). The amino acid sequences of the enzyme from 17 sources are known, and the three-dimensional structures of TS from several species have been solved (Hardy *et al.*, 1987). The primary sequences of TS have revealed that it is one of the most conserved proteins known; the similarity of TS sequences among species implies functional importance of this molecule in biological system.

The X-ray structures of the *Lactobacillus casei* TS-P_i complex and the *Escherichia coli* TS·dUMP·10-propargyl-5,8-dideazafolate ternary complex have provided insight about the possible role of specific residues of the protein, especially conserved 4 arginine residues (Arg-23, 178, 179, and 218 in *L. casei*) which are located within bonding distance of the liganded phosphate. Chemical modification and ^{13}C NMR studies also have suggested that one or more arginine residues are involved in binding of dUMP (Cipollo and Dunlap, 1979; Cipollo *et al.*, 1982; Belfort *et al.*, 1980). Hence, it is reasonable to predict that arginine residue, especially located at position 218 may play a role in the binding of phosphate moiety of dUMP. The putative importance of Arg-218 to the structure-function of TS is supported by the fact that this residue is completely conserved in all TSs sequenced to date (Bzik *et al.*, 1987). From these observations, we adopted a plan to produce mutants at a residue of Arg-218.

In this report, we describe the strategy and preliminary results of an approach to understanding the structure of TS by saturation site-directed mutagenesis. The mutagenic DNA cassettes contained mixtures of 32 codons that encode 20 amino acids and the amber (TAG) stop codon at the target site (Reidhaar-Olson and Sauer, 1988). "Replacements sets" were constructed in which Arg-218 residue was replaced by a large number of substitutions. The approach we have used has several practical advantages. First of all, a synthetic gene with conveniently placed, unique restriction sites permits mutagenesis at high mutagenesis efficiency rate. The advantages of a synthetic TS gene as a mutagenesis vehicle (Climie and Santi, 1990) were demonstrated by the construction of a series of 12 site-directed mutations and 1 amber codon at Arg-218 in a single experiment. Second, a mutagenesis vector which also serves as a high expression vector avoids further subcloning. Since the recombinant enzyme is highly overexpressed, large quantity of the pure enzyme has been obtained with no difficulties in purification. Catalytically active recombinant TS is indistinguishable from that isolated from *L. casei.* Third, functionally acceptable substi-tutions were defined by genetic complementation of TS deficient cells. This combination of genetic and biochemical techniques could be used to address a broad range of questions relating to the structure and function of the enzyme.

Materials and Methods

Materials

Restriction endonucleases, polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, and DNA polymerase I were purchased from New England Biolabs. [α-^{35}S]dATP (>600 Ci/mmol) was purchased from Amersham. Sequenase DNA sequencing kit was from Amersham. Oligonucleotides were synthesized at the University of California, San Francisco, Biomolecular Resource Center. Phenylglyoxal monohydrate and dUMP were obtained from Sigma. Other materials have been reported (Bruice and Santi, 1982; Pinter *et al.*, 1988) or were the purest commercial grade available.

Bacterial strains and plasmids

Strain DH5α [*ø80lacZΔM15; ara, Δ (lac-proAB*), *rpsL, hsdR*] was used as the host strain for plasmid-mediated transformations for the initial isolation of mutants. A synthetic DNA for TS (pSCTS9) and a Thy$^-$ tetracyclin-resistant *E. coli* strain x2913recA (*ΔthyA572, recA56*) were gifts from Dr. Daniel V. Santi, University of California, San Francisco, U.S.A., and used to test plasmids for TS activity by genetic complementation and for the production of recombinant TS.

Mutagenesis of Arg-218

A series of single amino acid substitutions of Arg-218 was constructed by cassette mutagenesis of plasmid pSCTS9 (see Figure 1). General methods for DNA manipulation were carried out as described by Maniatis *et al.* (1982). Plasmid DNA was digested with *Esp*I and *Avr*II to remove a 39-bases fragment, and vector DNA was purified by electrophoresis using 1% low melting point agarose, and the excised bands were used directly in the ligation reaction (Crouse *et al.*, 1983). The *Esp*I/*Avr*II fragment was replaced with a 39-bases synthetic DNA duplex containing multiple substitutions on both DNA strands at position encoding Arg-218. The substitutions included equal mixtures of all four bases at the first and second positions of the codon and an

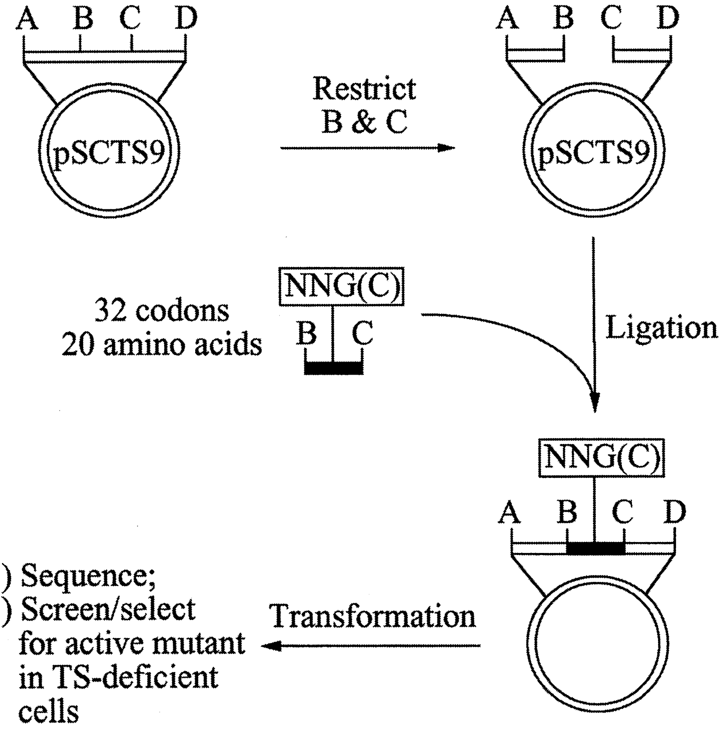

Figure 1. Strategy for saturation site-directed mutagenesis of TS. *In vitro* cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9. A fragment of the TS gene between two restriction sites (*Esp*I and *Avr*II) was removed and replaced by a mixture of oligonucleotides containing NNG/C at the Arg-218 codon. The resultant plasmids were sequenced and screened in *E. coli* Thy$^-$ cell (x2913recA) for catalytic activity.

equal mixture of G and C at the third position. Mutagenic oligonucleotides were annealed in 10 μl reaction mixtures that contained 1, 5, 10, 50, and 100 pmol of each unphosphorylated oligonucleotide. The annealed DNA fragments were ligated with 0.5 μg of gel-purified vector DNA as described above. The ligation mixtures were digested with *Not*I (10 units) for 2 h at 37°C and 50 ng of the ligated DNA were used to transform strain DH5α to ampicillin resistance. The resulting colonies were pooled by flooding the plates with 3 ml of L broth and collecting the cell suspension with a sterile pipette. Plasmid DNA was prepared from the pooled cells and used to transform *E. coli* strain χ2913recA (Thy$^-$), which was then plated on LB agar containing 100 μg/ml of ampicillin and 50 μg/ml of thymidine. Plasmid DNA was prepared from individual χ2913recA transformants and Arg-218 mutants were identified by the dideoxynucleotide sequencing method or by the automated DNA sequence analysis. Arg-218 mutants were further characterized by their ability to grow on minimal agar in the absence of thymidine.

Protein preparations and enzyme assay

For small scale preparations, the transformed χ2913recA (Thy$^-$) was grown by inoculation of 50 ml of LB containing 100 μg/ml ampicillin and 50 μg/ml thymidine with 0.25 ml of overnight cultures and incubation for 16-20 h at 37°C. Cells were harvested by centrifugation, washed with cold 150 mM NaCl, then resuspended in 5 ml of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, and disrupted by sonication on ice. The cell debris was removed by centrifugation at 10,000 *g* for 15 min. Protein concentration was determined by the method of Read and Northcote (1981) using bovine serum albumin as a standard. TS activity was assayed spectrophotometrically at 25°C using the methods of Pogolotti *et al.* (1986). One unit of activity is the amount of TS that catalyzes the formation of 1 mmol of product per min. TS synthesis was examined by SDS-PAGE of crude cell extracts. SDS-PAGE was performed by the method of Laemmli (1970) and gels were stained in Coomassie brilliant blue R-250.

Filter binding assay

The formation of the enzyme-[^{3}H]5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) complex was performed in a standard assay mixture containing 50 mM Tes (pH 7.4), 25 mM $MgCl_2$, 6.5 mM HCHO, 1 mM 2-mercaptoethanol, 0.15 mM FAH_4, [6-^{3}H]FdUMP, and 10 μg of cell extract. The reaction was initiated by addition of the cell extract in a total assay volume of 100 μl. After 45 min incubation, 40 μl aliquots were assayed in duplicate for complex formation and 10 μl was removed for determination of [^{3}H]FdUMP concentration in the assay mixture. Nitrocellulose membranes were soaked before use in 75 mM potassium phosphate (pH 7.4). Filters which were not wetted within 2 min were discarded. The filter discs were placed on a filter manifold (Hoefer-Scientific) and a gentle vacuum was applied to remove excess moisture. Without removing the vacuum, filters were washed with 2 ml of the same buffer and then 40 μl aliquots of the reaction mixture were applied to the discs and allowed to permeate the membrane. After washing the filters with 6 ml of the same buffer, the damped filters were placed in scintillation vials and dissolved in 10 ml of Aquasol. Optimal solubilization of filters required approximately 24 h. Counting efficiencies were determined using external standards.

Modification of arginine residues with phenylglyoxal

Solutions of phenylglyoxal were prepared by dissolving the solid in a minimal volume of methanol and then adding water to give a 20 mM solution. The concentration of phenylglyoxal was determined by measuring the absorbance in methanol ($\varepsilon_{247\ nm} = 11300\ M^{-1}cm^{-1}$) (Kohlbrenner and Cross, 1978). The extent of arginine modification by phenylglyoxal in phosphate buffer was determined by measuring the difference absorbance at 250 nm using the estimated molar extinction coefficient for the diphenylglyoxal adduct of 11000 $M^{-1}cm^{-1}$ (Takahashi, 1968). Two mg/ml of TS in 50 mM sodium phosphate, pH 7.5 containing 1 mM EDTA and 1 mM dithiothreitol was incubated for 1 h at 30°C with 0.1-2 mM phenylglyoxal. Reaction mixtures were dialyzed for 24 h against the same buffer used above. Solutions were assayed for enzyme activity and protein, and measured for absorbance change at 250 nm.

Results and Discussion

Our current understanding of the structure and catalytic activity of TS has been the result of many years of biochemical study. In an effort to extend these studies, we genetically approached for the production of a large number of site-directed mutations within the coding region of the TS gene. As a step toward that goal, we have used a synthetic gene constructed based on the amino acid sequence of TS from *L. casei* (Climie and Santi, 1990). The resulting gene product has been characterized and the synthetic gene has been used to construct a series of mutations at Arg-218 by cassette mutagenesis. Identification of catalytically active mutants by genetic complementation of χ2913recA, which is deficient in TS is an important step in our strategy. Individual mutant isolates were grown under nonselective conditions and then patched onto minimal agar lacking thymidine. Cell growth in the absence of exogenous thymidine identifies mutants that express catalytically active TS.

Arg-218 targeted for mutagenesis was chosen because it shows highly conserved sequence and has putative roles in phosphate binding as determined by solution or structural studies (Bzik *et al.*, 1987; Hardy *et al.*, 1987). The strategy used in the construction and characterization of the replacement set is outlined in Figure 1. Following ligation of oligonucleotide mixtures and initial transformation, colonies were pooled, and the mutagenized plasmid DNA pool was recovered and used to transform χ2913recA (Thy$^-$) resistant to ampicillin. Individual mutants were identified by DNA sequencing, and the resulting plasmids were used to re-transform χ2913recA. Plasmid DNA from the secondary χ2913recA transformants was sequenced again and tested for TS activity. Passage of the mutagenized DNA mixtures through several rounds of transformation ensured segregation and/or repair of the heteroduplex DNA molecules that were created during the construction of the mutants. It was not practical to identify all possible mutants in a replacement set by this strategy, which would require sequencing of over 160 clones to obtain a 95% confidence level of obtaining a complete set. Generally, 20~30 isolates of a replacement set were sequenced to give 10~15 different mutants of a given residue.

The results obtained with regard to complementation of χ2913recA for the different mutants of Arg-218 are shown in Table 1. After sequencing 35 clones, we have isolated 12 of the 20 possible amino acid substitutions, 1 amber codon, and 2 synonymous Arg codons in the case of Arg-218 mutagenesis. The 12 mutants arose at a frequency close to that expected on the basis of the codon distribution in the mutagenic DNA cassette (data not shown). None of the 12 mutants (Ala, Asp, Gln, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, and Val) were able to complement the *thyA* deletion in χ2913recA on minimal

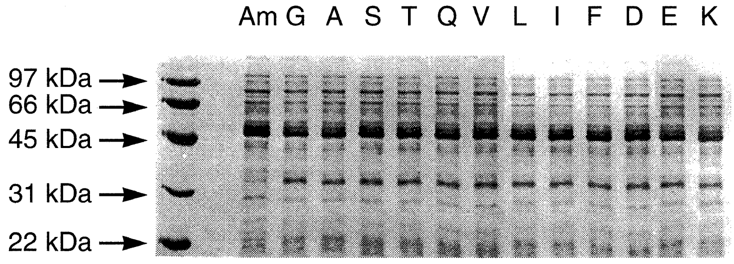

Figure 2. SDS-PAGE analysis of TS synthesis from x2913recA. SDS-PAGE was performed on 12% separating gel and 4% stacking gel and stained with Coomassie blue. Crude extracts from various Arg-218 mutants were expressed as one-letter codes for amino acids except Amber (Am). Lane 1, molecular weight marker proteins.

Table 1. Genetic complementation screen and kinetic properties of Arg-218 mutants of TS. Steady-state kinetic constants for Arg-218 of TS were determined as described in Materials and Methods. Initial rates of TS activity were monitored spectrophotometrically. When dUMP was varied, CH$_2$H$_4$folate was kept at 380-480 mM; when CH$_2$H$_4$folate was varied, dUMP was kept at 750 mM. Reactions were initiated with dUMP.

Amino acid substitution	Genetic complementation	k_{cat} (sec^{-1})	K_m (μM)		k_{cat}/K_m (μM^{-1} sec^{-1})	
Wild type	Active	4.20	3	18	1.400	0.2333
Ala 218	Inactive	n.d.	n.d.	n.d.	n.d.	n.d.
Asp218	Inactive	0.13	31	211	0.004	0.0006
Gln218	Inactive	0.19	41	245	0.005	0.0008
Glu218	Inactive	n.d.	n.d.	n.d.	n.d.	n.d.
Gly218	Inactive	n.d.	n.d.	n.d.	n.d.	n.d.
Ile218	Inactive	0.08	35	362	0.002	0.0002
Leu218	Inactive	0.08	43	327	0.002	0.0002
Lys218	Inactive	n.d.	n.d.	n.d.	n.d.	n.d.
Phe218	Inactive	0.07	38	264	0.002	0.0002
Ser218	Inactive	0.07	46	489	0.002	0.0001
Thr218	Inactive	0.12	34	377	0.003	0.0003
Val218	Inactive	0.12	29	413	0.004	0.0003

n.d.; not detectable

agar in the absence of thymidine, an indication that catalytically inactive TS was being synthesized. A comparison of the specific activity (data not shown) of TS in crude extracts from a variety of mutants indicated that a specific activity of ~0.002 μmol/min/mg is necessary for complementation of χ2913recA, and the TS synthesized in these cells was incapable of providing sufficient thymidylate to sustain growth. From this result, it is suggested that Arg-218 is strictly essential for TS activity. Analysis of crude cell extracts by SDS-PAGE showed that all 12 mutant plasmids encoding an amino acid substitution at position 218 directed the synthesis of a 37-kDa protein that comigrated with wild type TS (Figure 2). SDS-PAGE and activity measurements of crude extracts showed that TS was expressed to a level of ~8% of the total soluble protein except amber substitution. Thus, the absence of catalytic activity is not due to a lack of production or stability of the different mutants.

Further characterization of the Arg-218 mutants was assessed by kinetic studies. Determination of k_{cat} was performed by measuring the formation of FdUMP-enzyme complex using the filter binding assay. Calculations of the pmol of dimer/mg protein were performed assuming that the stoichiometry is 1.7 mol bound per mol of enzyme in the ternary complex. The k_{cat} values for the both native and mutant enzymes were calculated (Table 1). The k_{cat} values of the inactive mutants were only 2~5 % of wild type. Similar observations were made in the k_{cat}/K_m values. For dUMP, the k_{cat}/K_m values at saturating CH_2H_4folate of the inactive mutants were dramatically decreased. For CH_2H_4folate, the k_{cat}/K_m values at saturating dUMP for the mutants were also undetectable or much lower than those of wild type. Since k_{cat} values of the mutants are much lower than wild type, we conclude that once substrates are bound within the ternary complex the catalytic steps of the reaction are profoundly affected by the nature of the residue at 218. As shown in Table 1, the k_{cat}/K_m values of CH_2H_4folate at saturating dUMP are also very low in the inactive mutant. We suggest that the mutants significantly differ to the wild type in any of the steps after formation of the binary TS·dUMP complex. Since k_{cat} values of the mutants are dramatically decreased compared to wild type, and

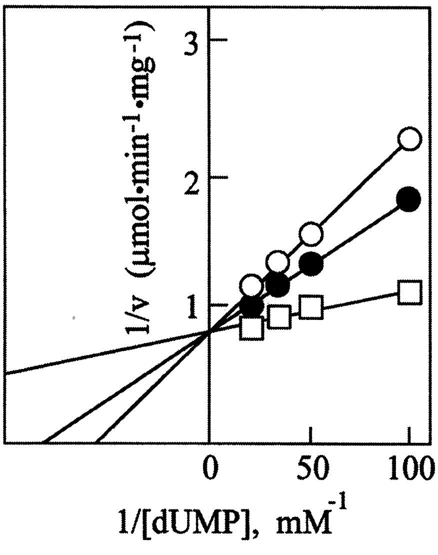

Figure 3. Inhibition of TS activity by phenylglyoxal. The concentration of CH_2H_4folate was kept constant (450 μM) and dUMP was varied. Results were obtained at concentrations of phenylglyoxal of 0 μM (open square), 100 μM (closed circle), and 200 μM (open circle).

Table 2. Stoichiometry of incorporation of phenylglyoxal and FdUMP. The values for incorporation of reagents were measured using spectral titration at 250 nm and filter binding assay as described under Materials and Methods.

Enzyme	Incorporation (mol/mol of enzyme)	
	Phenylglyoxal	FdUMP
Wild type	4.0	1.7
R218A mutant	2.8	0.01
R218G mutant	3.1	0.02
R218F mutant	3.2	0.06
R218V mutant	2.9	0.10

Residue	G	A	S	T	C	M	P	N	Q	V	L	I	F	Y	W	D	E	H	R	K	Reference
Arg 218	○	○	○	○					○	○	○	○	○			○	○		●	○	This work
Arg 179		●		●													●		●	●	Santi *et al.*, 1990
Arg 178			○	●			○			○	○	○	○	●		○	○		●	●	Cho & Choi, 1992
Arg 23		○	○		○	○	○	○	○	○	○	○	○	○		○	○	○	●	○	Cho *et al.*, 1995

Figure 4. Results of complementation screen of Arg mutants in TS-deficient cells. Growth of colonies harboring plasmids (closed circle); no growth (open circle).

the apparent K_m of CH_2H_4folate is increased, the effects can be attributed to binding of the cofactor. From the X-ray structure of the TS-P_i complex and molecular modeling (Hardy *et al.*, 1987), it was deduced that a conformational change of the protein occurs to accommodate binding of dUMP. It is not apparent why the inactive mutants would significantly affect both the rate of association of dUMP and binding of CH_2H_4folate.

Chemical modification studies with the arginine-specific dicarbonyl reagent phenylglyoxal confirmed the importance of the Arg-218 residue for substrate binding. When dUMP is preincubated with the enzyme, it strongly protects the enzyme activity from inactivation caused by phenylglyoxal, whereas CH_2H_4folate does not protect TS (data not shown). The results suggest that the arginine residues are involved in dUMP binding site and phenylglyoxal and dUMP are competitively binds to the same site. Competition between phenylglyoxal and dUMP was confirmed by kinetic studies as shown in Figure 3. Phenylglyoxal is a competitive inhibitor with respective to dUMP (K_i = 180 μM) and a noncompetitive inhibitor with respect to CH_2H_4folate (data not shown). Thus phenylglyoxal and dUMP compete for the same binding site. Inhibition of TS by phenylglyoxal results from reversible binding rather than from irreversible inactivation since linear initial rates were observed. The extents of incorporation of phenylglyoxal and FdUMP of the wild type and some of the mutant proteins were determined from the spectral titration at 250 nm and filter binding assay, respectively. As shown in Table 2, the replacement of Arg-218 with other amino acids such as Ala, Gly, Phe, or Val lowered the incorporation of phenylglyoxal by about 1 mol/mol of enzyme compared to that of wild type. The extents of incorporation of FdUMP of the mutants were dramatically decreased compared to wild type. These results indicate that Arg-218 is essential for substrate binding.

Arg-218 is completely conserved in all TSs sequenced to date, indicating an important role in structure-function of the enzyme (Bzik *et al.*, 1987). This feature has been observed with other conserved residues which are also apparently important to structure-function (Bzik *et al.*, 1987; Dev *et al.*, 1989; Maley *et al.*, 1990). The Arg residues conserved at positions 23, 178, 179 and 218 can form a positively charged binding surface for the 5'-phosphate of dUMP. Five of the guanidine NH groups are within appropriate distance (2.5~3.2 Å) to provide hydrogen bonding or electrostatic stabilization of the phosphate anion of dUMP (Hardy *et al.*, 1987). It was reported that Arg-179 could be replaced by amino acids as diverse as Ala, Thr, Lys and Glu without dramatic differences on binding or catalysis (Santi *et al.*, 1990). Recently we reported that 11 mutants, amber codon and 3 synonymous Arg codons at position 178 after sequencing 30 colonies were isolated (Cho and Choi, 1992). Three mutants were fully active in the complementation assay. Very recently, it has been reported that none of the mutants at position of Arg-23 are active in the complementation assay (Cho *et al.*, 1995). The amino acid replacement sets obtained from mutagenesis of the TS arginine residues reported are summarized in Figure 4. Of the 4 arginine residues involved in phosphate binding, only Arg-23 and Arg-218 have thus far been shown to be essential.

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