On the binding of ATP to the autophosphorylating protein, Ptk, of the bacterium *Acinetobacter johnsonii*

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Abstract The autophosphorylating protein, Ptk, of the bacterium *Acinetobacter johnsonii* was overproduced, purified to homogeneity and assayed for ATP binding by using the nucleotide analog 5′-*p*-fluorosulfonylbenzoyl adenosine. The ATP binding site of this bacterial autophosphorylating protein was found to be different from that generally used by eukaryotic protein kinases. It consists of two amino acid sequences that closely resemble the Walker motifs A and B. This observation was confirmed by site-directed mutagenesis experiments which showed, in addition, that the ATP molecule bound to these motifs is effectively employed by the bacterial protein to autophosphorylate on tyrosine. It is concluded that even though the overall autophosphorylation reaction is similar in eukaryotic and prokaryotic proteins, the mechanism involved is likely different.

Key words: Bacterial protein phosphorylation; ATP binding site; Autophosphorylation mechanism

1. Introduction

Phosphorylation of proteins on tyrosine has long been considered to occur exclusively in eukaryotes. In these organisms, it has been shown to play a key role in a series of fundamental biological functions including cell proliferation, cell differentiation and signalling processes [1,2]. In prokaryotes, the presence of a similar phosphorylating activity was suggested, much later than in eukaryotes, by the finding of phosphotyrosine first in the proteins of *Escherichia coli* [3], then in the proteins of a variety of bacterial species (reviewed in [4,5]). But the significance of this finding was found questionable for several years due to the objection that the phosphotyrosine residues might be generated by nucleotidylation rather than phosphorylation of proteins [6,7]. However this uncertainty has recently been cleared up by demonstrating, for the first time, the presence of a tyrosine phosphorylating activity in the bacterium *Acinetobacter johnsonii* [8,9]. This enzyme, termed Ptk, is a 82-kDa protein located in the inner-membrane fraction of cells, which has been purified to homogeneity by anti-phosphotyrosine immunochromatography [8]. It is able to autophosphorylate at multiple tyrosine residues by using ATP as phosphoryl donor [8]. In this work, we have undertaken experiments to characterize the mechanism of phosphorylation of Ptk by analyzing in particular the first step of this reaction, i.e. the binding of the precursor nucleotide ATP to the protein. The data presented hereafter have been compared to those previously reported for eukaryotic tyrosine kinases.

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2. Materials and methods

2.1. Bacterial strains and plasmids

A. johnsonii was obtained from A.M. Gounot (University of Lyon). *E. coli* XL1-Blue strain was used to propagate plasmids in cloning experiments. *E. coli* BL21 (pREP4-*gro*ESL), used for pQE30 and pGEX-KT expression experiments, was previously described [10]. It was a gift from I. Martin-Verstraete (Pasteur Institute, Paris). Plasmid vectors pQE30 and pGEX-KT were purchased from Qiagen and Pharmacia, respectively.

2.2. Culture media and growth conditions

A. johnsonii and *E. coli* strains were grown in 2YT medium at 30°C and 37°C, respectively. In the case of strains carrying drug resistance genes, the antibiotics kanamycin, ampicillin or tetracyclin, were added to the medium at a concentration of 25 μg/ml, 50 μg/ml, and 15 μg/ml, respectively. Growth was monitored by measuring the absorbance at 600 nm.

2.3. DNA manipulations

Small- and large-scale plasmid isolations were carried out by the alkaline lysis method, and plasmids were purified by using caesium chloride-ethidium bromide gradients [11]. All restriction enzymes, calf intestine phosphatase, T4 DNA ligase and T4 polynucleotide kinase, were used as recommended by the manufacturer (Eurogentec). Transformation of *E. coli* cells was performed by the method previously described [12].

2.4. Construction of the ptk expression plasmids

Construction of the pQE30-*ptk* plasmid: A pUC19 vector carrying a 5-kb chromosomal fragment from *A. johnsonii* containing the *ptk* gene was used [9]. This plasmid served as template in PCR amplification for preparing the *ptk* gene with appropriate restriction sites at both ends. The sequences of the two primers were 5′-TAT*GGATCC*-**GAT GACGATGACAAA**TACGTTATGAGCCAAACTACAAAT-3′ at the N-terminus (the *Bam*HI site is italicized; the enterokinase site is bolded; the second codon of *ptk* is underlined) and 5′-TAT*AAGCTT*TTAGTCTGATTCTTTATTGGC-3′ at the C-terminus (the *Hin*dIII site is italicized; the stop codon of *ptk* is underlined). The amplified fragment was digested with *Bam*HI and *Hin*dIII restriction enzymes, then ligated into pQE30 vector, previously opened with the same enzymes, to yield plasmid pQE30-*ptk*. The nucleotide sequence of the synthesized *ptk* gene was checked according to [13].

Construction of the pGEX-*ptk* plasmid: The pQE30-*ptk* plasmid was digested with the *Hin*dIII restriction enzyme and the resulting recessed end was filled with the Klenow fragment of DNA polymerase I. After linearization, pQE30-*ptk* was restricted by *Bam*HI and the DNA fragment carrying the *ptk* gene was purified and inserted in the pGEX-KT vector, opened with *Bam*HI and *Sma*I enzymes. The resulting plasmid was termed pGEX-*ptk*.

2.5. Site-directed mutagenesis

Site-directed mutagenesis was carried out by using the Transformer Site-Directed Mutagenesis Kit from Clontech, based on the method developed by [14]. The main characteristic of this strategy lies in the fact that, in addition to the mutagenic oligonucleotide, a selection oligonucleotide containing a mutation in a unique restriction site within the target plasmid is used. This procedure was applied directly to the pGEX-*ptk* construct. The oligonucleotide primers used for the desired substitutions were as follows: K436M, 5′-CCAATTGCACC-AATGAAACTACAGATTTTAATTC-3′; K549M, 5′-GCTCCAGA-AGTTGGTATGTCCTTTATCTCAACC-3′; S550C, 5′-CCAGAAG-

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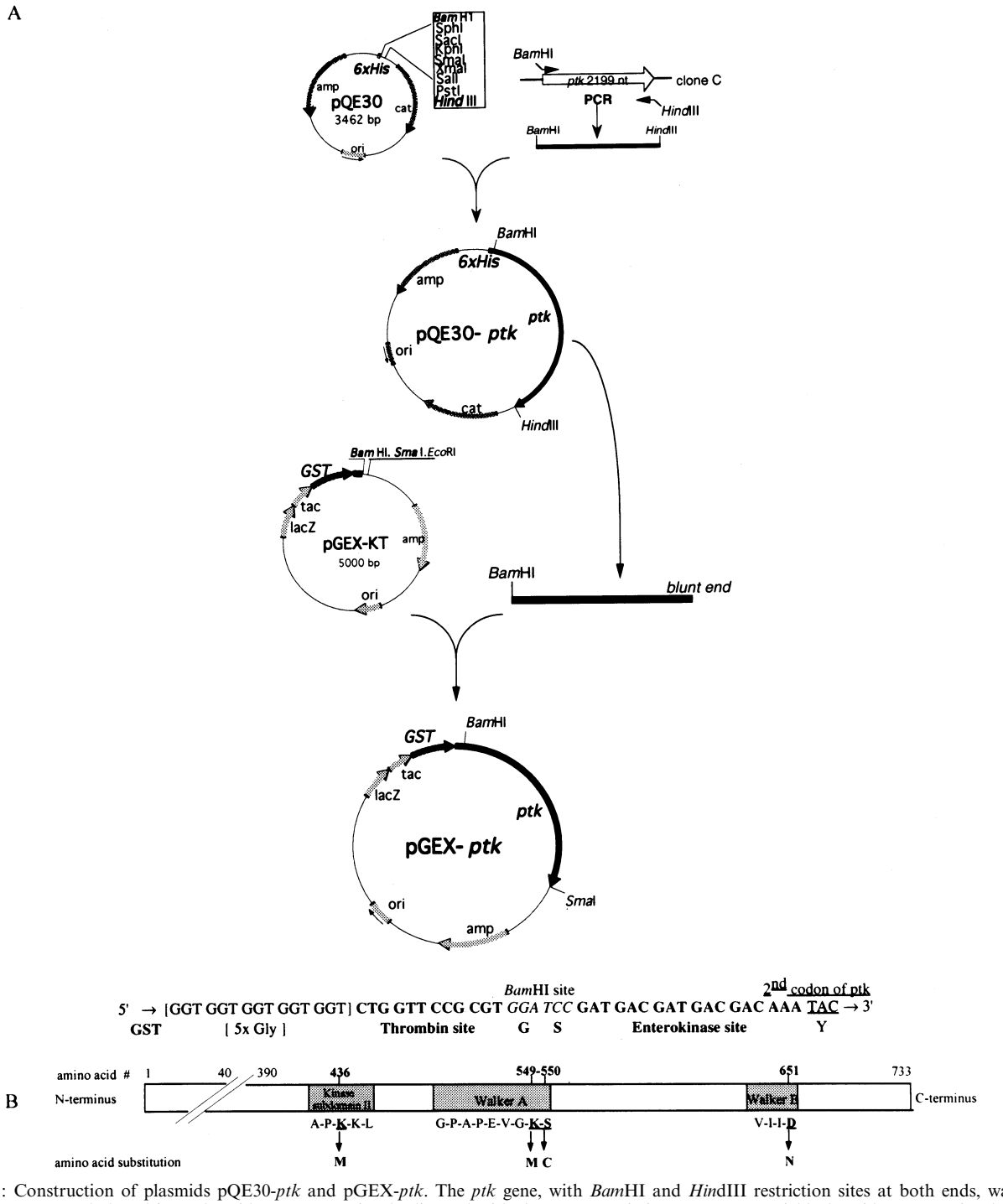

Fig. 1. A: Construction of plasmids pQE30-*ptk* and pGEX-*ptk*. The *ptk* gene, with *Bam*HI and *Hin*dIII restriction sites at both ends, was synthesized by PCR and cloned into plasmid pQE30, previously digested with the same restriction enzymes, to yield plasmid pQE30-*ptk*. The *Bam*HI-*Hin*dIII DNA fragment, containing the *ptk* gene with the enterokinase site, was excised from pQE30-*ptk* and inserted into pGEX-KT which was opened with *Bam*HI and *Sma*I restriction enzymes. The N-terminal end of the recombinant protein is shown at the bottom. B: Schematic presentation of the primary structure of Ptk. Numbering of amino acids is indicated from N-terminus to C-terminus. Shaded areas represent nucleotide binding regions. The locations of amino acid substitutions in mutant proteins are shown by vertical arrows.

TTGGTAAATGCTTTATCTCAACCAAC-3′; D651N, 5′-GATCATGTGATTATCAATACTCCACCAGTC-3′; and a *Mlu*I restriction site-eliminating oligonucleotide, 5′-CTTCTCGCGCAGCGCGTCAGTGG-3′ (the underlined bases indicate the changes from naturally occurring nucleotides). All mutations were checked by DNA sequencing [13].

2.6. Purification of Ptk

E. coli BL21 (pREP4-*gro*ESL) cells were transformed with plasmid pGEX-*ptk*. These cells were used to inoculate 1 l of 2YT medium supplemented with ampicillin and kanamycin, and were incubated at 37°C under shaking until A_{600} reached 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.1 mM, and growth was continued for 2 h at 30°C under shaking. Cells were harvested by centrifugation at 3000×*g* for 10 min and suspended in 12 ml of buffer A (10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1 mM EDTA, 10% glycerol) containing 1 mM PMSF, and deoxyribonuclease I and ribonuclease A at a final concentration of 100 μg/ml

each. Cells were disrupted in a French pressure cell at 16 000 psi. The resulting suspension was supplemented with Triton X-100 at a final concentration of 1% and centrifuged at 4°C for 30 min at 30 000×*g*. The supernatant was incubated with glutathione-Sepharose 4B matrix (Pharmacia Biotech), suitable for purification of glutathione-*S*-transferase (GST) fusion proteins, for 30 min at 4°C. The protein-resin complex was packed into a column for the washing and elution steps. The column was washed with 50 ml of buffer A containing 1% Triton X-100. Protein elution was carried out with buffer B (50 mM Tris-HCl (pH 8.0), 5 mM MgCl, 10% glycerol) containing 0.1% Triton X-100 and 10 mM gluthatione. Eluted fractions were analyzed by gel electrophoresis [15]. Fractions containing Ptk were pooled and dialyzed against buffer C (20 mM Tris-HCl (pH 8.8), 1 mM EDTA, 10% glycerol) supplemented with 20 mM NaCl. This protein solution was then loaded onto a column of Q-Sepharose High Performance matrix (Pharmacia Biotech). Proteins were eluted with buffer C containing 0.1% Triton X-100 and NaCl varying from 150 to 500 mM. The GST-Ptk fusion protein was eluted at a concentration of 300 mM. Fractions containing the purified GST-Ptk protein were dialyzed against buffer A and stored at −20°C.

2.7. Phosphorylation assays

In vitro phosphorylation of about 3 μg of purified wild or mutant Ptk protein was performed at 30°C in 10 μl of a buffer containing 25 mM Tris-HCl (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, and 10 μM ATP with 200 μCi/ml [γ-^{32}P]ATP. After 1, 2, 3, 5, or 10 min of incubation, the reaction was stopped by addition of an equal volume of 2×sample buffer and the mixture was heated at 100°C for 5 min. One-dimensional gel electrophoresis was performed as described [15]. After electrophoresis, gels were soaked in 16% TCA for 10 min at 90°C. They were stained with Coomassie Blue, and radioactive proteins were visualized by autoradiography.

2.8. ATP binding assays

Wild or mutant Ptk proteins were tested for their ability to bind ATP by using a covalent ATP analog, the 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA), and an anti-FSBA antibody for specific detection. Three μg of wild or mutant GST-Ptk were first labelled with 1 mM FSBA for 20 min at 30°C. The reaction was stopped by boiling in an equal volume of 2×sample buffer for 5 min. Proteins were run on SDS polyacrylamide gel [15]. FSBA-derivitized proteins were transferred to a nitrocellulose membrane [16], which was then treated with 2% gelatin in buffer D (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) for 1 h. The membrane was incubated with anti-FSBA antibody (Boehringer Mannheim) diluted 1:500 in buffer D containing 0.1% Tween-20. Alkaline phosphatase-conjugated secondary antibody (Boehringer Mannheim) served as the second ligand. The blot was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt as substrates (Boehringer Mannheim).

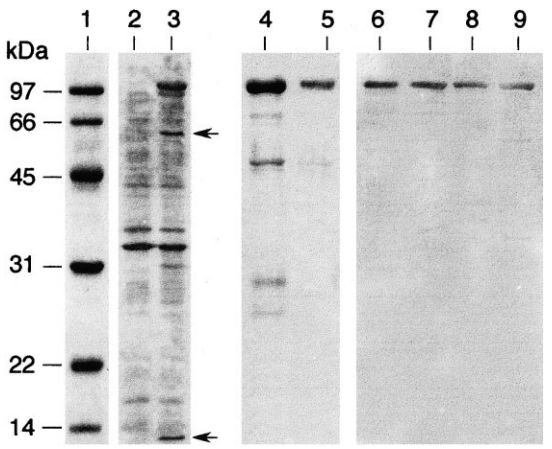

Fig. 2. Overproduction and purification of Ptk. Total soluble proteins from BL21 (pREP4-groESL)(pGEX-*ptk*) cells and purified GST-Ptk were analyzed by SDS-PAGE and stained with Coomassie Blue. Lane 1, molecular mass standards; lane 2, soluble fraction from BL21 (pREP4-groESL)(pGEX-*ptk*) cells grown in the absence of IPTG; lane 3, soluble fraction from BL21 (pREP4-groESL)-(pGEX-*ptk*) cells grown in the presence of 0.1 mM IPTG (the location of GroEL and GroES chaperone proteins is indicated by arrows); lane 4, purification of GST-Ptk by affinity chromatography on glutathione-Sepharose 4B; lane 5, purification to homogeneity of GST-Ptk by additional anion exchange chromatography on Q-Sepharose; lane 6, purification of mutant protein GST-Ptk K436M by double chromatography; lane 7, purification of mutant protein GST-Ptk K549M by double chromatography; lane 8, purification of mutant protein GST-Ptk S550C by double chromatography; lane 9, purification of mutant protein GST-Ptk D651N by double chromatography.

3. Results

3.1. Overproduction and purification of GST-Ptk

Since a relatively large quantity of protein Ptk was required for performing the different assays planned in this study, a series of experiments was first carried out to overproduce this protein by using the expression vector pQE30 from *E. coli*. The *ptk* gene lacking the start codon and containing a sequence encoding the enterokinase site was synthesized by PCR, and cloned in pQE30 previously digested with *Bam*HI and *Hin*dIII restriction enzymes. The resulting plasmid, termed pQE30-*ptk*, was used to encode a fusion protein consisting of the entire Ptk protein and 16 extra amino acids, including six histidine residues and the enterokinase site at its N-terminal end (Fig. 1). This construct was used to transform competent cells from the BL21 (pREP4-*groESL*) strain of *E. coli*; this strain can overproduce the two chaperone proteins GroES and GroEL and is suitable for the overproduction of proteins that exhibit a high degree of hydrophobicity, such as Ptk. Upon induction by IPTG, an effective overexpression of a 85-kDa protein, consistent with the calculated molecular mass of the fusion protein, was obtained. However, the attempt made to purify this 6His-Ptk protein by using a Zn^{2+} matrix, suitable for the purification of fusion proteins carrying a polyhistidine tag, failed because the fusion protein could not bind to the resin.

Therefore, to overcome this problem, another approach using the expression vector pGEX-KT was used. The *Bam*HI-*Hin*dIII DNA fragment, containing the *ptk* gene with the enterokinase site, was excised from pQE30-*ptk* and inserted

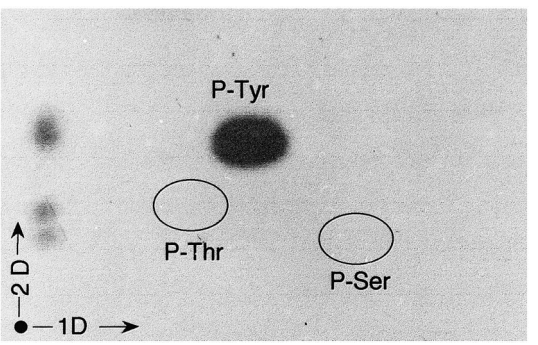

Fig. 3. Two-dimensional analysis of the phosphoamino acids of protein GST-Ptk. The fusion protein GST-Ptk was purified by double chromatography and subjected to acid hydrolysis. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1st) and ascending chromatography in the second dimension (2nd). After migration radioactive molecules were revealed by autoradiography. Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) were run in parallel and visualized by staining with ninhydrin.

A

Kinase subdomain I

c-Src	273/	EVKL**G**Q**G**CF**G**E**V**WMGTWNG
EGFR	715/	IKVL**G**S**G**AF**G**T**V**YKGLWIP
PDGFR-β	602/	GRTL**G**S**G**AF**G**Q**V**VEATAHG
INS.R	1023/	LREL**G**Q**G**SF**G**M**V**YEGNARD
PTK	408/	IAKA--**G**EI**G**N**V**RIVDTAV

Kinase subdomain II

c-Src	292/	TRV**A**I**K**T**L**KPG
EGFR	740/	IPV**A**I**K**E**L**REA
PDGFR-β	629/	MKV**A**V**K**M**L**KST
INS.R	1052/	TRV**A**V**K**T**V**NES
PTK	431/	EPI**A**P**K**K**L**QIL

B

Walker A motif

GKY	6/	S**G**PS-GT**GKS**TL
Ras	9/	V**G**AG-GV**GKS**AL
ADK	13/	V**G**GP-GS**GK**GTQ
ETU	18/	I**G**HV-DH**GKT**TL
PTK	541/	S**G**PAPEV**GKS**FI

Fig. 4. A: Comparison of the amino acid sequence of Ptk with the conserved subdomains I and II of eukaryotic protein tyrosine kinases. The alignment of the amino acid residues from 408 to 424 and from 431 to 441 of the Ptk protein with respectively subdomains I and II of various protein kinases is presented: c-Src, human P60-SRC proto-oncogene protein tyrosine kinase (P12931); EFGR, human epidermal growth factor receptor (P00533); PDGFR, human platelet-derived growth factor receptor (P09619); INS.R, human insulin receptor (P06213). B: Comparison of the amino acid sequence of Ptk with the Walker A motif of nucleotide binding proteins. The alignment of the amino acid residues from 541 to 552 of the Ptk protein with the Walker A motif of various proteins is presented: GKY, phosphoglycerate kinase (P15454); Ras, Ras GTP binding protein (P01112); ADK, adenylate kinase (P00571); ETU, elongation factor EF-Tu (P02990).

in the pGEX-KT vector opened with *Bam*HI and *Sma*I restriction enzymes. The resulting plasmid, termed pGEX-*ptk*, was able to encode a fusion protein consisting of Ptk with the glutathione-*S*-transferase at its N-terminal end (Fig. 1). This plasmid was used to transform the BL21 (pREP4-groESL) strain of *E. coli*. Upon induction by IPTG, the GST-Ptk was effectively overproduced, and further purified to homogeneity in a two-step chromatographic procedure, including an affinity chromatography on glutathione-Sepharose 4B matrix followed by an anion exchange chromatography on a Q-Sepharose column (Fig. 2, lane 5). In these conditions, about 1 mg of pure protein was obtained from 1 l of bacterial culture.

Different attempts were then made to obtain the Ptk protein in a native state, i.e. without the GST at its N-terminal end, by using either enterokinase or thrombin proteases. The fusion protein was actively hydrolyzed but the native Ptk protein thus obtained had no more autophosphorylating activity. This loss of activity might be related to the aggregation of the Ptk protein due to its high degree of hydrophobicity. The fusion protein GST-Ptk was therefore used in all following experiments. However, before analysing the ATP binding site of GST-Ptk, a control experiment was performed to check whether the behavior of this fusion protein in terms of autophosphorylating activity at tyrosine sites was identical to that of the free Ptk protein, i.e. unbound to GST. For this, pure GST-Ptk was incubated in the presence of radioactive ATP, subjected to acid hydrolysis, and the phosphoamino acids thus released were separated in two successive dimensions and revealed by autoradiography. The pattern presented in Fig. 3 clearly shows that GST-Ptk behaved like the unbound protein Ptk [8] in the sense that it also was phosphorylated exclusively at tyrosine.

3.2. Localization of the ATP binding site

The first step of the chemical mechanism of autophosphorylation of the Ptk protein, like that of any phosphorylating enzyme of this type, consists in the binding of ATP to the protein. Previous analysis of the amino acid sequence of Ptk, deduced from the nucleotide sequence of the *ptk* gene, had revealed two putative ATP binding sites [9]. This protein indeed contains amino acid sequences or individual amino acids at invariant positions that are homologous to certain sequences specific to the protein kinase family [17,18]. These conserved sequences are, first, the glycine-rich loop G^{415}-E-I-G^{418} followed by the invariant V^{420} which is similar to the subdomain I of eukaryotic protein kinases and, second, the invariant K^{436} which is homologous to the subdomain II of these same kinases (Fig. 4A). In the eukaryotic protein kinase family, these two conserved features, especially the invariant K, have been shown to be involved in the binding of the ATP phosphoryl donor. On the other hand, the Ptk protein contains two conserved sequences at the C-terminal end, G^{542}PAPEVGKS^{550} and V^{648}IID^{651}, which are similar respectively to the Walker A motif [AG]X_4GK[ST] and to the Walker B motif [hhhD] (X refers to any amino acid; h represents a hydrophobic amino acid; alternative residues at a given position are bracketed) (Fig. 4B). These conserved motifs are commonly found in adenine and guanine nucleotide binding proteins such as guanylate kinase, protein Ras, ad-

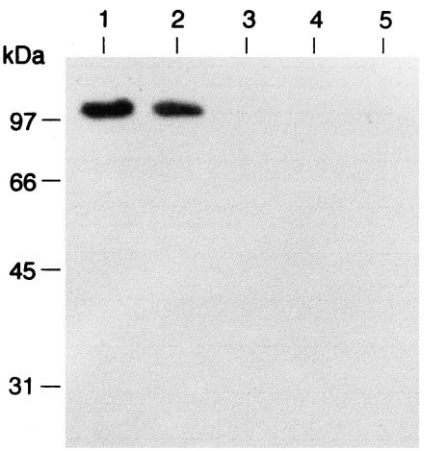

Fig. 5. FSBA binding assays. About 3 μg of purified wild or mutant Ptk protein were labelled with FSBA, run on polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. FSBA-labelled proteins were revealed with an anti-FSBA antibody, which was itself detected with alkaline phosphatase-conjugated secondary antibody. The blot was treated with nitroblue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt as substrates. Lane 1, wild GST-Ptk; lane 2, mutant GST-Ptk K436M; lane 3, mutant GST-Ptk K549M; lane 4, mutant GST-Ptk S550M; lane 5, mutant GST-Ptk D651N.

enylate kinase and elongation factor EF-Tu. But, no protein kinase has been reported so far to use this sequence for binding the nucleotide phosphoryl donor [19–21].

The involvement of these different conserved features in the binding of ATP by Ptk was assessed. For this, various conservative mutant proteins were generated by site-directed mutagenesis of the *ptk* gene, overproduced from pGEX plasmid, and purified by affinity chromatography followed by anion exchange chromatography (Fig. 2, lanes 6 to 9), as in the case of wild-type Ptk (Fig. 2, lanes 4 and 5). The levels of expression of the different mutants were similar to that of the wild-type *ptk* gene since, in each case, about 1 mg of pure mutant protein was obtained from 1 l of bacterial culture. After purification, each mutant protein was assayed for its capacity to bind to the covalent ATP analog 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) [22]. The first mutant protein analyzed was the GST-Ptk K436M, modified in the eukaryotic kinase-like subdomain II which is crucial, even more than subdomain I, for ATP binding in eukaryotes. It was observed that this mutant protein could still be labelled with FSBA, which indicated that the concerned amino acid, K^{436}, is not essential for ATP binding to Ptk (Fig. 5, lane 2). Since, by contrast, the substitution of this conserved amino acid in eukaryotic protein kinases results in the complete loss of affinity of the enzymes for ATP [23], it was therefore suggested that Ptk does not utilize the same process as eukaryotic enzymes

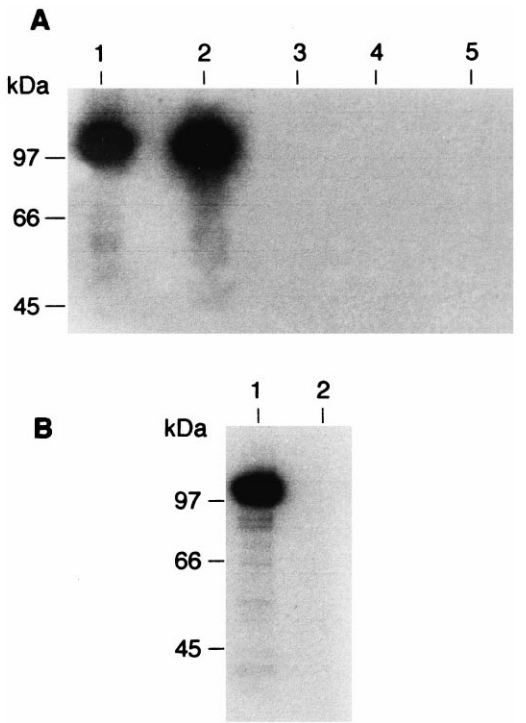

Fig. 6. Ptk autophosphorylation assay. A: About 3 μg of purified wild or mutant GST-Ptk protein were incubated with [γ-^{32}P]ATP. Proteins were then separated by polyacrylamide gel electrophoresis, gels were soaked in 16% TCA and radioactive bands were revealed by autoradiography. Lane 1, wild GST-Ptk; lane 2, mutant GST-Ptk K436M; lane 3, mutant GST-Ptk K549M; lane 4, mutant GST-Ptk S550M; lane 5, mutant GST-Ptk D651N. B: About 3 μg of purified wild GST-Ptk protein were incubated in the absence (lane 1) or in the presence (lane 2) of FSBA for 20 min at 30°C. The protein was then assayed for autophosphorylation with [γ-^{32}P]-ATP, as described above. Phosphoproteins were revealed by autoradiography.

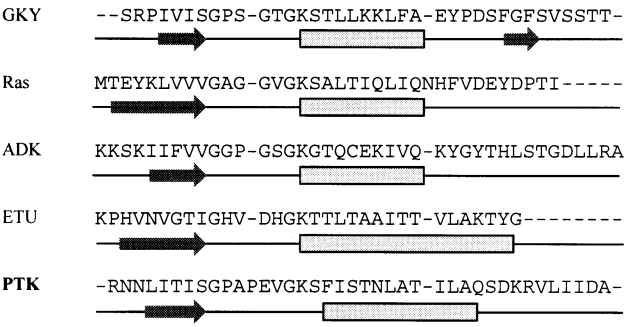

Fig. 7. Comparison of the predicted secondary structure of Ptk with that of various nucleotide binding proteins. The structures of the amino acid sequences surrounding, upstream and downstream, the Walker A-like motif of Ptk were predicted by using the SOPMA method. They were compared to those surrounding the Walker A motif of different nucleotide binding proteins whose crystal structure had been previously established, as indicated in the legend of Fig. 3. The black arrows represent β-sheets and the shaded boxes refer to α-helices.

for binding ATP. Other mutants of Ptk were prepared by changing either the Walker A or the Walker B motif. Two mutant proteins consisted in conservative replacements within motif A, K549M and S550C, and in a third protein the aspartate at position 651 was mutated to an asparagine within motif B. Such mutations were chosen on the basis that in the eukaryotic ATP binding protein family, except protein kinases, the conserved amino acids lysine and aspartate have been previously shown to be essential for Mg^{2+}-nucleotide binding and the serine residue has been reported to be important for both ATP binding and catalysis [21,24–26]. Our results surprisingly showed that the three mutant proteins GST-Ptk K549M, S550C, and D651N, were all unable to bind FSBA (Fig. 5, lanes 3 to 5). This finding therefore suggested that, contrary to eukaryotic protein kinases, both Walker motifs A and B present in the prokaryotic phosphorylating enzyme Ptk are required for the binding of ATP to the enzyme.

3.3. Utilization of ATP as phosphoryl donor

Further experiments were performed to determine whether the binding of ATP to the Walker motifs of Ptk actually represented the first step of the overall phosphorylation mechanism of this bacterial enzyme and, namely, was followed by the utilization of the nucleotide as phosphoryl donor. To check this point, the different mutant Ptk proteins described above were tested individually for their ability to autophosphorylate. After purification, each protein was incubated in vitro with [γ-^{32}P]ATP for 10 min, a time length which allows optimal autophosphorylation of the wild Ptk protein, and was separated by gel electrophoresis. After autoradiography, it was observed that only the GST-Ptk K436M was labelled to the same extent as the wild protein, which indicated that lysine 436 is not essential for the autophosphorylation of Ptk (Fig. 6A, lane 2). In contrast, the three proteins mutated in the Walker motifs could no more autophosphorylate, even after longer incubation with [γ-^{32}P]ATP (Fig. 6A, lanes 3 to 5), thus showing that not only these motifs are required for ATP binding but, furthermore, they are needed for the autophosphorylation reaction. Finally, to confirm the importance of the Walker motifs in both the binding of ATP and its utilization as phosphoryl donor, the wild GST-Ptk protein

was first incubated with FSBA then assayed for autophosphorylation in the presence of radioactive ATP. In these conditions, no labelling of the protein was detected, which brought evidence that the target site of the ATP analog is the same binding site as that used by the ATP molecule involved in the autophosphorylation process (Fig. 6B, lane 2).

4. Discussion

The main result of this study is that, in the bacterial phosphorylating enzyme Ptk of *A. johnsonii*, the molecule of ATP which serves as phosphoryl donor for the autophosphorylation reaction binds to a protein site different from that generally used by eukaryotic protein kinases. This site includes two amino acid sequences that resemble respectively the two Walker motifs: first, the G^{542}PAPEVGKS^{550} sequence which is similar to the consensus motif A, also called P-loop [20,21], consisting of [AG]XXXXGK[ST] and, second, the V^{648}IID^{651} sequence which is similar to the consensus sequence hhhD of motif B [19]. The similarity of the G^{542}PAPEVGKS^{550} sequence of Ptk with a Walker A motif is further supported by the theoretical analysis of the amino acid sequences surrounding this sequence within the protein. Indeed, by using the SOPMA (self optimized prediction method from alignment) method described in [32], we observed that the G^{542}PAPEVGKS^{550} sequence is located between a β-sheet structure and a short α-helix (Fig. 7). The same structural organization has been found to occur in a number of nucleotide binding proteins harboring a genuine Walker A motif, namely guanylate kinase [33], Ras protein [34], adenylate kinase [35] and elongation factor EF-Tu [36]. In these different proteins, whose crystal structure has been determined, such structural organization has been demonstrated to be required for ATP binding.

The occurrence of Walker motifs A and B has been described in a wide variety of prokaryotic and eukaryotic ATP or GTP binding proteins [27]. However, searches that we made in the Swissprot 34 database as well as observations made by other authors [28] indicated that no known protein kinase had so far been shown to contain such motifs. Only, in a few exceptions, a motif similar, but not identical, to the Walker A motif was detected in eukaryotic protein kinases. These concern, for instance, the DNA damage response protein kinase Dun1 [29] which harbors the GDRIVFGKS motif, the tyrosine protein kinase receptor MDK-5 [30] with the sequence AVNGVSGKS, or the proto-oncogene tyrosine protein kinase Yes [31] with the sequence ARSIATGKS. In these cases, the consensus motif [AG]XXXXGK[ST] is replaced with a motif including five, instead of four, varying amino acid residues X in the central part of the sequence. And, moreover, when checked by site-directed mutagenesis, the phosphorylating activity of such kinases has been demonstrated to be linked not to this Walker-like motif but to other amino acids within the protein molecule, namely in subdomain II [29].

Together, our data provide evidence, for the first time, that (1) a bacterial phosphorylating enzyme that closely resembles a protein tyrosine kinase, contains two amino acid sequences similar to the Walker motifs A and B, (2) these two sequences are essential for the binding of ATP, (3) the ATP molecule bound to these sequences is the nucleotide precursor used by the enzyme to autophosphorylate. However, these two amino acid sequences do not contain any tyrosine residue liable to undergo phosphorylation. This means that the phosphorylation reaction that follows the binding of ATP to the kinase occurs at site(s) different from the nucleotide binding site. Further studies are therefore needed to elucidate the catalytic mechanism which directs the transfer of the γ-phosphate of ATP to its peptide substrate within the protein molecule. In any case, in terms of evolution, it now seems clear that even though the overall reaction of autophosphorylation on tyrosine is similar in eukaryotic and prokaryotic enzymes, the corresponding mechanism is likely different.

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