

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/21007899>

# Introduction by site-directed mutagenesis of a tryptophan residue as a fluorescent probe for the folding of E. coli phosphofructokinase

ARTICLE *in* BIOCHIMIE · JUNE 1990

Impact Factor: 2.96 · DOI: 10.1016/0300-9084(90)90064-N · Source: PubMed

---

CITATION

1

READS

29

## 3 AUTHORS, INCLUDING:



Marie-Claude Serre

French National Centre for Scientific Resea...

21 PUBLICATIONS 448 CITATIONS

[SEE PROFILE](#)



Jean-Renaud Garel

Pierre and Marie Curie University - Paris 6

84 PUBLICATIONS 1,871 CITATIONS

[SEE PROFILE](#)

# Introduction by site-directed mutagenesis of a tryptophan residue as a fluorescent probe for the folding of *Escherichia coli* phosphofructokinase

W Teschner<sup>1</sup>, MC Serre<sup>2</sup>, JR Garel<sup>3</sup>\*

<sup>1</sup>Immuno AG, Industriestrasse 67, A-1220 Vienna, Austria;

<sup>2</sup>Department of Microbiology, University of Texas, Austin, TX 78712, USA;

<sup>3</sup>Laboratoire d'Enzymologie du CNRS, 91198 Gif-sur-Yvette, France

(Received 23 April 1990; accepted 28 June 1990)

**Summary** — The leucine residue at position 178 in the major allosteric phosphofructokinase from *Escherichia coli* has been replaced by a tryptophan using site-directed mutagenesis. Transformation by the mutated gene of *pfk*-bacteria results into the expression of a *pfk*+ phenotype and the production of an active enzyme. The modified protein has been purified and its fluorescence properties show that it contains 2 tryptophan residues, the original Trp 311 and the new Trp 178. During unfolding of the protein by guanidine hydrochloride, the changes in the fluorescence of these 2 residues take place at different steps: Trp 311 becomes exposed to solvent when the dimeric form dissociates into monomers, while Trp 178 is exposed only when a folded chain loses its tertiary structure. The mutant enzyme is stabilized by its substrate fructose-6-phosphate against denaturation induced by heat or guanidine hydrochloride.

**phosphofructokinase / tryptophan fluorescence / site-directed mutagenesis / protein folding**

## Introduction

Phosphofructokinase (PFK) catalyses the reaction:

ATP + fructose-6-phosphate → ADP + fructose-1,6-bisphosphate. In *Escherichia coli*, the enzyme present as the major species, PFK, is a tetramer of 4 identical subunits of 35 000 Da each [1, 2]. The sequence of the 320 amino acids of the PFK chain has been deduced from the nucleotide sequence of the corresponding gene, *pfkA* [2]. The three-dimensional structure of PFK has been solved by X-ray crystallography at a resolution of 0.24 nm [3]. The tetramer is arranged as a dimer of dimers with a D<sub>2</sub> symmetry. Each subunit is composed of 2 domains and is in contact with only 2 other subunits along 2 different interfaces: the active interface (A) contains the Fru-6P binding site, and the regulatory interface (R) contains the effector binding site. The tetrameric species is the only one which can bind both substrates and effectors, and thus possess both catalytic and regulatory properties.

*E. coli* PFK has only 1 tryptophan residue at position 311 [2, 3]. Trp 311 is located in the R interface, close to the effector binding site [3]. The fluorescence emitted by Trp 311 changes upon formation or dissociation of the R interface, and these changes show that

association (respectively dissociation) of PFK is an ordered process in which the A interface is formed after (respectively broken before) the R interface [4–6]. The lower stability of the A interface is in agreement with an area of contact between subunits which is one-third smaller ≈ 12 nm<sup>2</sup> vs 18 nm<sup>2</sup>, than that of the R interface [3]. The fluorescence of Trp 311 was, however, almost insensitive to the first step of PFK self-assembly, the folding of a single polypeptide chain. We decided to incorporate a second tryptophan group into PFK by site-directed mutagenesis to create a fluorescent probe which would report on the first step in the renaturation, the folding of a single chain. An attempt was already made to introduce a tryptophan residue in PFK, at position 246 instead of a valine. This replacement was not well tolerated by the conformation of the protein and led to an inactive enzyme [7].

We selected position 178 as the site of mutation, where the residue is a leucine, taking the following restrictions into consideration: (i) the modified residue must not be in a functional site, active or regulatory, not to interfere with the binding of ligands to PFK; (ii) the site of mutation must correspond to a position which is exposed in the unfolded state and buried in the folded monomer; (iii) the replacement by a tryptophan residue requires that the original residue be hydro-

\*Correspondence and reprints

Abbreviations: Fru-6P: D-fructose-6-phosphate; PFK: phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC2.7.1.11); Gdn-HCl: guanidine hydrochloride; DTT: dithiothreitol; PEP: phospho-enol-pyruvate

phobic and large; (iv) the site must not be part of the hydrophobic core of a subunit not to interfere with the folding. The modified protein, PFK-LW178 is enzymatically active, and its fluorescence properties show that the 2 tryptophan residues become exposed to solvent at different steps in unfolding.

## Materials and Methods

### Mutagenesis and purification of the modified enzyme

The synthetic primer 5'CGCAGCC~~C~~ACGTCAGATC3' containing a single mismatch with the wild-type gene was designed to introduce a Trp codon at position 178. Mutagenesis and subcloning of the mutated *pfkA* gene into the pEMBL8(+) vector [8] were as previously described [9]. The complete nucleotide sequence of the mutated gene was determined in order to verify the lack of any other alteration than the single base change. Wild-type and mutant phosphofructokinases were prepared from an *E coli* strain, DF1008 [10], deleted for both *pfkA* and *pfkB* genes, transformed with pH11 (11) or the pEMBL8(+) recombinant plasmid, after a 20 h culture in 2 × TY broth containing 100 µg/ml ampicillin. Wild-type phosphofructokinase was purified as previously described [5]. For purification of the mutant enzyme, the same affinity chromatography was utilized with the following modifications: the Blue-Dextran column was washed with 20 mM sodium phosphate, pH 7.6, 1 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 M NaCl prior to elution of phosphofructokinase by an ATP gradient (0–1.2 mM). The enzyme was stored at 4°C in 100 mM Tris–HCl buffer, 1 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 2 mM Fru-6P, at pH 8.2.

### Phosphofructokinase assay

Enzymatic activity was measured at 340 nm by using the coupled enzyme assay as previously described [12]. Unless otherwise stated, conditions for assays were 1 mM ATP and 1 mM Fru-6P, in 100 mM Tris–HCl, pH 8.2 buffer containing 10 mM MgCl<sub>2</sub> and 0.2 mM NADH, at 27°C. In these conditions, the modified enzyme has a specific activity of 90 ± 10 units (*i.e.* 90 µmol of fructose-1,6-bisphosphate produced by mg and by min).

### Fluorescence measurements

Protein fluorescence was followed with a Spex Fluorolog 2 fluorometer equipped with a Spex DM1B spectroscopy laboratory co-ordinator. Fluorescence measurements of PFK-LW178 were carried out in a buffer composed of 0.1 M sodium phosphate, pH 7.6, 1 mM MgCl<sub>2</sub>, 2 mM DTT. Complete denaturation of PFK-LW178 was achieved by adding Gdn–HCl to a final concentration of 5 M in the preceding buffer.

### Denaturation of PFK-LW178

For thermal denaturation, PFK-LW178 was heated at a given temperature in a buffer composed of 0.1 M Tris–HCl, pH 8.2, 10 mM MgCl<sub>2</sub>, 2 mM DTT, in the presence or absence of different ligands (Fru-6P 2 mM, PEP 20 mM, or GDP 2 mM). After a given time, the residual enzymatic activity was measured in standard conditions. For denaturation by Gdn–HCl, PFK-LW178 was incubated for a given time in a buffer containing 0.1 M sodium phosphate, pH 7.6, 1 mM MgCl<sub>2</sub>, 2 mM DTT and a given concentration of Gdn–HCl, with or without Fru-6P 2 mM. Alternatively, the mutated enzyme was incubated under the same conditions, for 24 h in the presence or absence of 2 mM Fru-6P. The residual enzymatic activity was measured in standard conditions after diluting Gdn–HCl at least 50-fold.

## Results and Discussion

### Expression of an active enzyme by the plasmid carrying the gene with the mutation

The *Escherichia coli* strain DF 1008 is deleted for both the *pfkA* and *pfkB* genes and is unable to grow on mannitol as a unique carbon-energy source [13]. When this *pfk*<sup>−</sup> strain is transformed with the pEMBL8(+) plasmid carrying either a truncated *pfkA* gene [9] or no inserted DNA, the *pfk*<sup>−</sup> phenotype is conserved and no transformant is found to grow on a minimal medium supplemented with mannitol. However, transformation with the recombinant pEMBL8(+) plasmid carrying the mutated *pfkA* gene leads to many transformants possessing the *pfk*<sup>+</sup> phenotype which are able to grow on mannitol. These transformed cells have a normal level of mannitol-1-phosphate dehydrogenase activity showing that the *pfk*<sup>+</sup> phenotype does not result from a higher amount of the enzymes coded by the *mtl* operon [13]. Also, when these transformed cells are allowed to grow in a complete medium, a significant PFK activity can be detected in the crude extracts. It is therefore probable that it is the modified PFK-LW178 which provides the cell with enough PFK activity to support growth on mannitol.

### Purification of PFK-LW178

PFK-LW178, the protein possessing the PFK activity expressed in the *pfk*<sup>+</sup> transformed cells, was purified using slight modifications of the procedure described earlier for wild type PFK [5]. Cells were broken in the presence of Fru-6P in order to stabilize PFK-LW178. A better yield of the affinity chromatography step was obtained by washing the Dextran Blue–Sepharose column with 1 M NaCl instead of 1.5 M before applying the ATP gradient to elute PFK-LW178. After concentration and dialysis, the mutant protein is stored at 4°C in the presence of 2 mM Fru-6P, in 100 mM Tris buffer, 1 mM MgCl<sub>2</sub>, 2 mM DTT, at pH 8.2, and it remains stable for a few weeks. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate shows that PFK-LW178 is homogeneous.

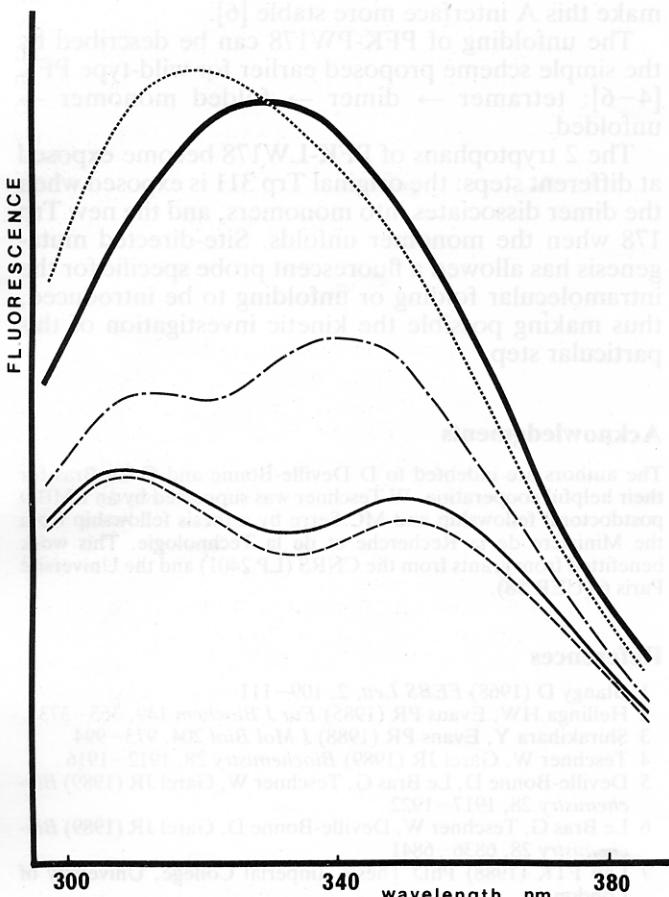
### Functional properties of PFK-LW178

Preliminary results show that PFK-LW178 is slightly less active than wild-type PFK, with a specific activity of 90–100 U per mg (as compared to 130 to 150 U), and that saturation by ATP or Fru-6P takes place in the same concentration range as for wild-type PFK. The saturation by ATP is hyperbolic, with a Michaelis constant *K<sub>M</sub>* for ATP of 7 10<sup>−5</sup> M. The saturation by Fru-6P is cooperative, with a Hill coefficient *n<sub>H</sub>* around 3 and a half-saturating concentration of Fru-6P of 4.5 10<sup>−4</sup> M. A more detailed study of the enzymatic

properties of PFK-LW178 has shown that the homotropic cooperative interactions are the same as in wild-type PFK, and that only the heterotropic interactions are modified by the mutation [14].

#### Fluorescence properties of PFK-LW178

The fluorescence spectra emitted by the PFK-LW178 mutant upon excitation at 280 nm are shown in figure 1 for the protein in the presence of different concentrations of Gdn-HCl. The spectrum of the protein completely denatured by 5 M Gdn-HCl resembles that of a mixture of tryptophan and tyrosine, in a 2:11 molar ratio, with 2 maxima at 304 nm and 350 nm [15]. The amplitude emitted at 350 nm is much larger for PFK-



**Fig 1.** The fluorescence emission spectrum of PFK-LW178 upon excitation at 280 nm. The buffer is 0.1 M sodium phosphate, 1 mM MgCl<sub>2</sub>, 2 mM DTT, pH 7.6, and Gdn-HCl at the concentrations of: 0 M (—), 1 M (.....), 2.3 M (·—·—), 5 M (—). Temperature is 23°C. The spectrum of the unfolded protein is compared to the emission spectrum of a mixture of tyrosine and tryptophan in an 11/2 molar ratio (---).

LW178 than for the wild-type, as expected from a twice as large tryptophan content. The native protein shows a broad maximum in the emission wavelength around 330 nm, indicating that the tryptophan residues are shielded from the aqueous solvent [15].

#### Stability of PFK-LW178 towards inactivation induced by heat or Gdn-HCl

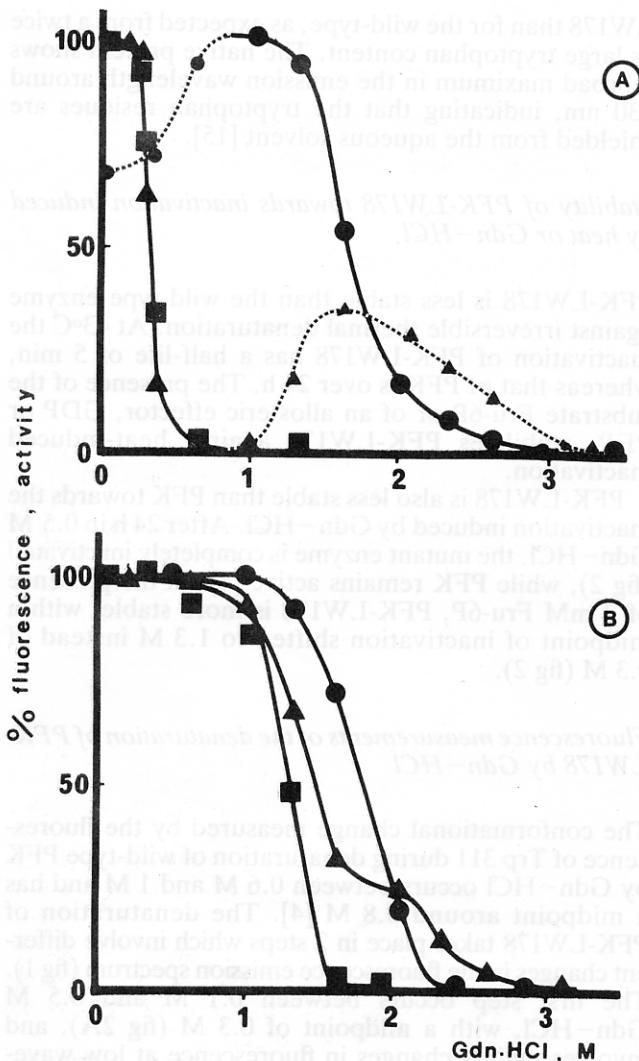
PFK-LW178 is less stable than the wild-type enzyme against irreversible thermal denaturation. At 43°C the inactivation of PFK-LW178 has a half-life of 5 min, whereas that of PFK is over 24 h. The presence of the substrate Fru-6P or of an allosteric effector, GDP or PEP, stabilizes PFK-LW178 against heat-induced inactivation.

PFK-LW178 is also less stable than PFK towards the inactivation induced by Gdn-HCl. After 24 h in 0.5 M Gdn-HCl, the mutant enzyme is completely inactivated (fig 2), while PFK remains active [4]. In the presence of 2 mM Fru-6P, PFK-LW178 is more stable, with a midpoint of inactivation shifted to 1.3 M instead of 0.3 M (fig 2).

#### Fluorescence measurements of the denaturation of PFK-LW178 by Gdn-HCl

The conformational change measured by the fluorescence of Trp 311 during denaturation of wild-type PFK by Gdn-HCl occurs between 0.6 M and 1 M and has a midpoint around 0.8 M [4]. The denaturation of PFK-LW178 takes place in 2 steps which involve different changes in the fluorescence emission spectrum (fig 1). The first step occurs between 0.1 M and 0.5 M Gdn-HCl, with a midpoint of 0.3 M (fig 2A), and involves mainly changes in fluorescence at low wavelength, around 310 nm (fig 1). This first step is accompanied by the disappearance of activity (fig 2A). In the presence of 2 mM Fru-6P, this first step is markedly shifted towards higher Gdn-HCl concentrations (fig 2B). Because this first step is similar to the change observed in wild-type PFK, it is probably due to the exposure of Trp 311 upon the dissociation of dimeric PFK into monomers [4].

The second step in the unfolding of PFK-LW178 by Gdn-HCl occurs between 1.3 M and 2.5 M Gdn-HCl, with a midpoint of 1.8 M (fig 2A), and involves fluorescence changes in the whole spectrum (fig 1). This second step is insensitive to the presence of 2 mM Fru-6P (fig 2B). Since this second step is absent in wild-type PFK, it is probably due to a change in the environment of the new residue Trp 178. This change, which takes place in a monomeric state of PFK, is likely to reflect the exposure to solvent of Trp 178 upon the unfolding of a single chain.



**Fig. 2.** Denaturation of PFK-LW178 by Gdn-HCl. PFK-LW178 at a chain concentration of 1.4  $\mu$ M is incubated for 24 h at 20°C in a buffer containing 0.1 M sodium phosphate, 1 mM MgCl<sub>2</sub>, 2 mM DTT, and a variable concentration of Gdn-HCl, at pH 7.6, in (A) the absence of Fru-6P, or (B) the presence of 2 mM Fru-6P. Fluorescence intensities are measured in the same buffer. The fluorescence changes are conveniently expressed using the ratio between intensities emitted at 2 different wavelengths [4]. The biphasic character of the unfolding of PFK-LW178 is more apparent by choosing the ratios between the intensities F emitted at 330 nm and 310 nm (▲), and between those at 330 nm and 350 nm (●). In each case, the changes are normalized between 100% for the native protein and 0% for the completely unfolded protein. The dashed lines and smaller symbols shown in (A) correspond to the minor variations of each of these ratios,  $F_{330}/F_{310}$  and  $F_{330}/F_{350}$ . The residual enzymatic activity of PFK-LW178 is measured as described in *Materials and Methods*, immediately after diluting the denaturant at least 50-fold and is indicated by (■).

## Conclusions

The structure of PFK is not markedly distorted by the replacement of a leucine by a tryptophan at position 178 since the mutant PFK-LW178 is enzymatically active. The modified protein is, however, less stable than the wild-type against the denaturation induced by heat (see above) or Gdn-HCl (fig 2). The binding of Fru-6P stabilizes PFK-LW178 against both types of denaturation. This stabilization by Fru-6P suggests that PFK-LW178 denatures by the same mechanism as that of PFK, beginning by the dissociation of the A interface. The Fru-6P binding site lies across this A interface, with the fructose moiety interacting with residues from one subunit and the phosphate group with two arginines, Arg 162 and Arg 243, from the other subunit [3]. The extra interactions between subunits due to bound Fru-6P make this A interface more stable [6].

The unfolding of PFK-PW178 can be described by the simple scheme proposed earlier for wild-type PFK [4-6]: tetramer  $\rightarrow$  dimer  $\rightarrow$  folded monomer  $\rightarrow$  unfolded.

The 2 tryptophans of PFK-LW178 become exposed at different steps: the original Trp 311 is exposed when the dimer dissociates into monomers, and the new Trp 178 when the monomer unfolds. Site-directed mutagenesis has allowed a fluorescent probe specific for the intramolecular folding or unfolding to be introduced, thus making possible the kinetic investigation of this particular step.

## Acknowledgments

The authors are indebted to D Deville-Bonne and G Le Bras for their helpful cooperation. W. Teschner was supported by an EMBO postdoctoral fellowship and MC Serre by a thesis fellowship from the Ministère de la Recherche et de la Technologie. This work benefitted from grants from the CNRS (LP 2401) and the Université Paris 6 (UER 58).

## References

- 1 Blangy D (1968) *FEBS Lett*, 2, 109-111
- 2 Hellinga HW, Evans PR (1985) *Eur J Biochem* 149, 363-373
- 3 Shirakihara Y, Evans PR (1988) *J Mol Biol* 204, 973-994
- 4 Teschner W, Garel JR (1989) *Biochemistry* 28, 1912-1916
- 5 Deville-Bonne D, Le Bras G, Teschner W, Garel JR (1989) *Biochemistry* 28, 1917-1922
- 6 Le Bras G, Teschner W, Deville-Bonne D, Garel JR (1989) *Biochemistry* 28, 6836-6841
- 7 Lau FTK (1988) PhD Thesis, Imperial College, University of London
- 8 Dente L, Cesarini G, Cortese R (1983) *Nucleic Acids Res* 11, 1645-1655
- 9 Serre MC, Garel JR (1990) *Eur J Biochem* 189, 487-492
- 10 Daldal F (1983) *J Mol Biol* 168, 285-305
- 11 Lau FTK, Fersht AR, Hellinga HW, Evans PR (1987) *Biochemistry* 26, 4143-4148
- 12 Kotkalarz D, Buc H (1982) *Methods Enzymol* 90, 60-70
- 13 Fraenkel DG, Vinopal RT (1973) *Ann Rev Microbiol* 27, 69-100
- 14 Serre MC, Teschner W, Garel JR (1990) *J Biol Chem* (in press)
- 15 Brand L, Witholt B (1967) *Methods Enzymol* 11, 776-856