# Effect of substrate binding on the *E. coli* MNM deamidase structure

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Keywords: (Nicotinamide mononucleotide (NMN), NMN-deamidase, steady-state fluorescence)

Nicotinamide mononucleotide (NMN) is a nucleotide that is mostly recognized for its role as an intermediate of nicotinamide adenine dinucleotide (NAD) biosynthesis. This particular molecule has demonstrated several beneficial pharmacological activities in preclinical studies, including a marked protective role in cardiac disorders, diabetes, Alzheimer's disease and complications associated with obesity; an anti-ageing activity in mouse models has also been reported [1]. Hence, measuring NMN levels in cells and biological fluids with a rapid and highly sensitive method is crucial for a better understanding of its role in both cellular metabolism and disease.

Currently, the detection and quantification of NMN is mainly performed by HPLC-UV methods; methods based on the use of ESI coupled with selected reaction monitoring (SRM), and fluorescence based methods have also been reported [2-3]. Nevertheless, no biosensors for this molecule have been described.

Recently, we have identified and functionally characterized a bacterial NMN-deamidase (namely YgaD*wt*), which catalyzes the hydrolysis of the carbamide bond in the nicotinamide moiety of NMN, yielding nicotinic acid mononucleotide. In addition, we have produced two mutants (S29A and K61Q) that completely lost catalytic activity [4].

In this study, we explored the usefulness of such mutants as molecular recognition elements to develop an NMN biosensor. For this purpose we studied the binding of NMN to the proteins through fluorescence spectroscopy measurements.

Fluorescence steady-state experiments were carried out on an ISS-K2 fluorometer. The protein samples were excited at 295 nm (maximum absorbance = 0.10 OD). The emission spectra were recorded between 310 nm and 410 nm. All measurements were performed at 25°C in 50 mM Hepes pH 7.5, 300mM NaCl, 1mM TCEP. The titration experiments were performed on YgaD*wt* and the two mutants, S29A and K61Q, both in the absence and in the presence of increasing amounts of NMN (ranging from 0.18 μM to 3000 μM).

The fluorescence emission spectra of YgaD*wt* and the two mutant protein forms, both in the absence and in the presence of NMN, displayed similar maximum emission peaks centered at 331 nm. On the contrary, as a consequence of the increase of NMN concentration, a strong quenching of fluorescence intensity was observed. In particular, the intrinsic fluorescence of the three NMN deamidase samples decreased rapidly starting from 3μM of ligand (protein:NMN ratio, 1:1). At NMN concentration of 3000 μM, the fluorescence intensity of YgaD*wt* was reduced of 72%. Similarly, S29A showed a reduction in fluorescence of 79.2% and K61Q a reduction of 78.6% (Figure 1).

These results indicate that S29A and K61Q, even enzymatically inactive, retain the ability to bind NMN. In addition, these two mutated protein forms show similar affinity toward the ligand as compared to the affinity of the YgaD*wt* protein (dissociation constant values in the μM order, consistent with the Km value of YgaD*wt* of 7±2 μM [4]).

Preliminary experiments showed no effect of nicotinamide adenine dinucleotide (NAD), supporting the specificity of fluorescence intensity quenching induced by NMN binding.

Experiments are ongoing to assess possible effects of other metabolites involved in NAD biosynthetic pathways, and to further investigate the possibility of exploiting NMN-deamidase inactive mutants for NMN sensing.

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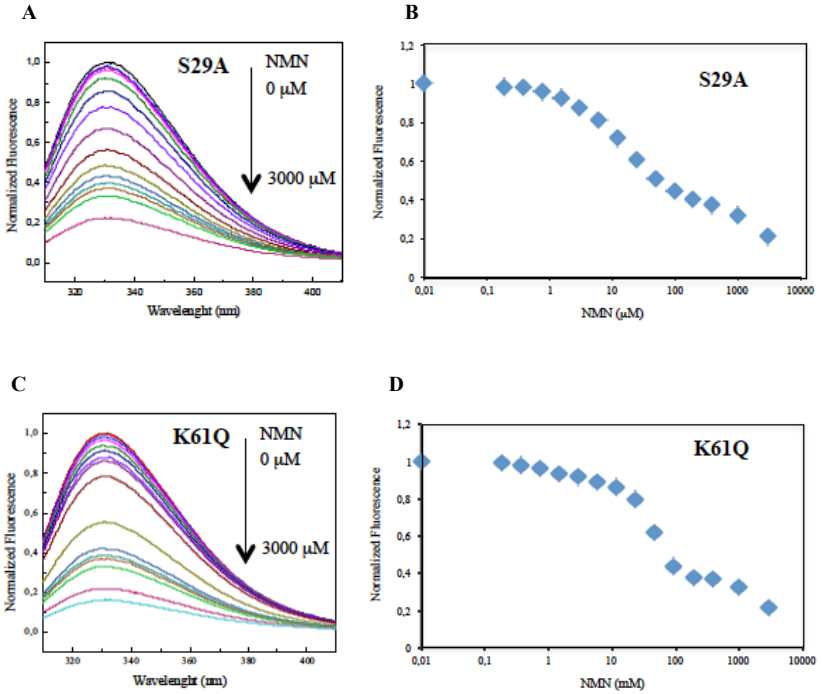


Figure 1. Fluorescence steady-state measurements of S29A and K61Q: effect of increasing amount of NMN on the fluorescence emission spectra of S29A (A) and K61Q (C). Variation of the maximum fluorescence emission value as function of NMN concentration (B and D).