A tight regulation of proton transport in the inner mitochondrial membrane is crucial for physiological processes such as ATP synthesis, heat production, or regulation of the reactive oxygen species as proposed for the uncoupling protein family members (UCP). Specific regulation of proton transport is thus becoming increasingly important in the therapy of obesity and inflammatory, neurodegenerative, and ischemic diseases. We and other research groups have shown previously that UCP1- and UCP2-mediated proton transport is inhibited by purine nucleotides. Several hypotheses have been proposed to explain the inhibitory effect of ATP, although structural details are still lacking. Moreover, the unresolved mystery is how UCP operates in vivo despite the permanent presence of high (millimolar) concentrations of ATP in mitochondria. Here we use the topographic and recognition (TREC) mode of an atomic force microscope to visualize UCP1 reconstituted into lipid bilayers and to analyze the ATP–protein interaction at a single molecule level. The comparison of recognition patterns obtained with anti-UCP1 antibody and ATP led to the conclusion that the ATP binding site can be accessed from both sides of the membrane. Using cantilever tips with different cross-linker lengths, we determined the location of the nucleotide binding site inside the membrane with 1 Å precision. Together with the recently published NMR structure of a UCP family member (Berardi et al. *Nature*, **2011**, *476*, 109–113), our data provide a valuable insight into the mechanism of the nucleotide binding and pave the way for new pharmacological approaches against the diseases mentioned above.