Mitochondrial membrane uncoupling protein 3 (UCP3) is not only expressed in skeletal muscle and heart, but also in brown adipose tissue (BAT) alongside UCP1, which facilitates a proton leak to support non-shivering thermogenesis. In contrast to UCP1, the transport function and molecular mechanism of UCP3 regulation are poorly investigated, although it is generally agreed upon that UCP3, analogous to UCP1, transports protons, is activated by free fatty acids (FFAs) and is inhibited by purine nucleotides (PNs). Because the presence of two similar uncoupling proteins in BAT is surprising, we hypothesized that UCP1 and UCP3 are differently regulated, which may lead to differences in their functions. By combining atomic force microscopy and electrophysiological measurements of recombinant proteins reconstituted in planar bilayer membranes, we compared the level of protein activity with the bond lifetimes between UCPs and PNs. Our data revealed that, in contrast to UCP1, UCP3 can be fully inhibited by all PNs and IC50 increases with a decrease in PN-phosphorylation. Experiments with mutant proteins demonstrated that the conserved arginines in the PN-binding pocket are involved in the inhibition of UCP1 and UCP3 to different extents. Fatty acids compete with all PNs bound to UCP1, but only with ATP bound to UCP3. We identified phosphate as a novel inhibitor of UCP3 and UCP1, which acts independently of PNs. The differences in molecular mechanisms of the inhibition between the highly homologous transporters UCP1 and UCP3 indicate that UCP3 has adapted to fulfill a different role and possibly another transport function in BAT.