Supplementary Figure 1. A) SDS-PAGE analysis of purified D135N* residues 1-340 (left) and D135N* 1-318 (right) showing that the minor, truncated fraction corresponds in size to the 1-318 fragment. B) Sequence of CaMKII D135N* 1-340. The red fragments were recovered from mass spec analysis of the truncated band excised from the gel while the underlined peptides were recovered from mass spec analysis of the major band corresponding to CaMKII 1-340. C) EPR spectra of representative sites in the regulatory domain introduced into the D135N* 1-340 and the D135N* 1-318 backgrounds. The superimposable lineshapes demonstrate that the truncation does not affect the dynamics of the spin label.

Supplementary Figure 2. Crystal structure of the C. elegans CaMKII kinase domain where two regulatory domains, each belonging to a different monomer, form a coiled coil (PDB 2BDW) (image created with PyMOL, DeLano Scientific). Sites 302 and 307 are at the dimer interface separated by 5 and 13 Å respectively. The EPR spectra for CaMKII labeled at each site are shown for concentrations up to 200 µM. The absence of concentration-dependent broadening indicates that this dimer is not stable in solution.
**Supplementary Figure 3.** EPR spectra along the regulatory domain. A) and B) The lineshapes in the R2 and R3 segments show distinct evidence of at least two populations of spin labels with vastly different mobilities. A mobile population marked by the blue arrow reflects spin labels undergoing large amplitude/fast motion. Another population marked by the red circle reflects spin labels in restricted environments. C) and D) In contrast, the EPR lineshapes in the R1 segment seem to arise from a predominant population of spin labels. The minor sharp component has contribution from unreacted labels. In the 285-293 segment, the mobility varies with a 3.7 periodicity indicative of an α-helix. Uniformly mobile lineshapes in the 278-284 segment are indicative of a loop structure.

**Supplementary Figure 4.** The EPR lineshapes in the CaM binding region were analyzed assuming two populations of spin labels with distinct motional parameters. Non-linear least squares fits of the experimental spectra yielded two component spectra at each site. The relative fraction of each component is shown in Figure 2B.
Supplementary Figure 5. EPR spectra of $(i, i+4)$ spin label pairs (black) along with the corresponding sum of singles (red) in the R1(A) and R3 (B) segments. The sum of singles spectrum was obtained from a digital sum of the single sites spectra which do not contain contributions from the distance-dependent dipolar interaction. Each double mutant spectrum was fit (dashed line) as described in the methods section to a distance distribution between the two spin labels shown in Figures 3 and 4.
Supplementary Figure 6. A) EPR spectra of sites in the R3 segment in the presence of ATP (purple) overlaid with their corresponding apo spectra (black). B) The ATP-bound lineshapes were analyzed using the two-population model of supplementary Fig. 4. The fast mobile component (green) and the slow immobile component (orange) for the ATP-bound spectrum are summed (gray dotted) to fit the experimental spectra (purple). The relative fraction of each component is shown in Figure 2B. C) The ATP induced spectral changes are eliminated if the highly conserved Lys42 in the ATP binding cleft is substituted by a methioine.
Supplementary Figure 7. A) EPR spectra of \((i, i+4)\) spin label pairs revealing opposite changes in distance in the R1 (286/290) and R3 (300/304) segments. Both the double mutant spectrum and that of the corresponding sum of singles are shown for the apo and CaM-bound intermediates. B) Spin label mobility changes in the R3 region (red) and C) in the R1 region upon Ca\(^{2+}\)/CaM binding.
Supplementary Figure 8. Effects of the T286E mutation on the dynamics of the regulatory domain. A) Comparison of EPR lineshapes in the T286E mutant (green) to the CaM-bound intermediate (red) in R1. B) Binding of CaM to the T286E mutant leads to EPR lineshapes in the R1 segment consisting primarily of a highly mobile component. C) EPR lineshapes in the CaM binding region. The spectrum of site 299 is consistent with increased contact between the spin label and CaM.
Supplemental Figure 9. Shifts in the R3 equilibrium induced by binding of autocamtide-2 (AC-2) or CaMKIIN. EPR spectra for site 307 are shown in the apo state (black) and in the T286E phosphomimic background (blue). A) 20 fold excess of substrate autocamtide-2 (purple) or inhibitor CaMKIIN (red) do not cause significant spectral changes but B) increase the fraction of mobile spin labels in the phosphomic background.

Reference