Phthalate dioxygenase (PDO) catalyzes the dihydroxylation of phthalate to form the 4,5-dihydro-cis-dihydrodiol of phthalate. Catalysis in PDO occurs at the mononuclear iron site, which receives electron from a Rieske redox-active iron-sulfur cluster. The Rieske and mononuclear centers are about 40 Å apart in each PDO monomer; however, in a head-to-tail stack arrangement the Rieske center can be less than 15 Å apart from the mononuclear sites of the adjacent subunits (see Figure). Interactions between the Rieske and the mononuclear centers are apparently facilitated by the bridging Aspartate 178 that in other enzymes was implicated in electron transfer, iron binding, and substrate dihydroxylation. In PDO substitution of Aspartate 178 with Asparagine or Alanine resulted in changes in the UV-VIS spectrum associated with the decrease in pKₐ of the Histidine(s) ligating the iron in the Rieske center. Rieske cluster ligands of the variants are more exposed to solvent, as evidenced by the pH effect on the UV-VIS spectra and by comparison of the rates of reduction of the Rieske center by phthalate dioxygenase and by sodium dithionite. Also, reduction of the Rieske center in the mutant enzymes failed to activate the mononuclear center for catalysis as shown by the decreased signals of Fe-NO complexes detected in EPR. While substrate binding perturbs the Rieske absorption spectrum in WT PDO, no such perturbation was observed in the mutant enzymes. At the same time, Kₘ values for substrate were essentially unaffected by the D178A,N substitutions, with the binding exhibiting a combination of phases characteristic for the sample that has mononuclear centers only partially reconstituted with iron. PDO with D178A,N substitution was still capable of substrate dihydroxylation similar to that of the WT PDO when the mutant enzyme was fully reconstituted with Fe(II), but was greatly diminished in the “as purified” enzyme. Thus, although D178A,N substitutions did not affect the substrate binding and its dihydroxylation, they disrupted the