Human protein C (PC) is a vitamin K-dependent serine protease zymogen present in normal plasma. PC was activated with soluble thrombin, thrombin-Sepharose, trypsin-Sepharose or Russell's viper venom. When activated protein C (APC) at 10 μg/ml was added to normal plasma for 3 min prior to assays, clotting was greatly inhibited. In prothrombin time, activated partial thromboplastin time, and partial thromboplastin time assays, the clotting times were prolonged from 24 to 68 sec, 57 to 616 sec, and 247 to > 1200 sec, respectively. Both calcium and phospholipid were needed for this potent anticoagulant activity. The thrombin time was unaffected. To test which coagulation factors in plasma were inactivated by APC, APC was added to normal human plasma for 3 min and residual clotting factor activities were assayed using appropriate deficient substrate plasmas. Only Factor V and Factor VIII activities were decreased by APC. PC and DFP-inhibited APC were not anticoagulant and did not decrease Factors V and VIII. APC hydrolyzed synthetic peptide substrates. The amidolytic activity of APC was inhibited by normal human plasma (50% of APC activity was lost in 10 min). APC inhibitory activity was not detectable in plasmas from 4 unrelated patients with combined Factor V/VIII deficiency whereas it was present in normal amounts in plasmas from patients with simple Factor V deficiency or Factor VIII deficiency. Thus, it appears that APC may regulate blood coagulation by enzymatically inactivating two critical cofactors, Factor Va and VIIIa. Moreover, APC itself may be regulated by a previously undescribed plasma protein inhibitor. It is suggested that the molecular basis for combined Factor V/VIII deficiency disease that exhibits simple autosomal recessive inheritance is a deficiency of APC inhibitor.