Cognitive and Motor Impairments Associated with SIV Infection in Rhesus Monkeys

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Cognitive and motor deficits are now recognized as significant clinical features of infection with the human immunodeficiency virus (HIV). Juvenile rhesus macaques infected with simian immunodeficiency virus (SIV) were found to exhibit cognitive and motor deficits characteristic of HIV infection. Impairment on a motor skill task was the most reliable indicator of infection. Various cognitive impairments were also evident. These deficits were related to SIV infection of the brain but not to inflammatory lesions at a particular locus. The results suggest that the SIV-infected rhesus macaque is a valuable model for understanding the cause of HIV-associated central nervous system dysfunction and for developing a treatment.

YOUNG RHESUS MONKEYS INOCULATED with SIV, which is morphologically, antigenically, and genetically related to HIV, undergo a chronic wasting disease characterized by loss of weight and occurrence of opportunistic infections and diarrhea (*1*). Some strains of SIV, such as Delta B670, are also associated with a central nervous system (CNS) infection and with AIDS-like CNS pathology (*2*). We investigated whether SIV-infected rhesus monkeys, like HIV-infected humans, display cognitive or motor deficits. If so, the behavioral status of SIV-infected monkeys might be used as a bioassay both to guide the search for causative agents of virally induced CNS dysfunction and to measure the efficacy of CNS-directed therapy.

Rhesus monkeys [*Macaca mulatta*; $n = 15$; age = 12.0 ± 0.8 months (mean ± SD)] were trained to perform a battery of tasks designed to evaluate cognitive and motor abilities. Cognitive abilities were assessed with three tasks administered on an automated apparatus (*3*): (i) delayed matching-to-sample with novel stimuli on every trial, a test of visual recognition memory (*4*); (ii) delayed matching-to-sample with two repeatedly used stimuli, a test of recency memory (*5*); and (iii) visual discrimination learning and retention, a measure of stimulus-response association (*6*). We assessed motor skill by measuring the ability of each monkey to retrieve food from a rotating turntable (7). These tasks were chosen not only because they tax the neuropsychological functions (for example, learning, memory, and fine motor control) reported to be compromised in some HIV-positive individuals (*8*), but also because the different tasks are known to depend on different neural substrates (*9*). Consequently, the nature of observed neuropsychological changes could be related to the locus of any neuropathological changes in the SIV-infected monkeys. Home-cage behavior was monitored closely (*10*) to determine if the infected animals showed changes in behavior that might not be evident from formal testing.

The monkeys were trained and their home-cage behavior was evaluated until they had attained a stable performance on the recognition and recency memory tasks, after which they were inoculated with the Delta B670 strain of SIV_{smm} ($n = 10$) or were sham-inoculated ($n = 5$) (*11*). Starting the next week, the performance of each monkey on the various tasks (Table 1), as well as its home-cage behavior, was regularly assessed.

During the course of the study, blood samples were collected weekly from each animal in order to monitor its virologic and immune status (*12*). The body weight, body temperature, and general physical condition of each monkey were also noted. Because any decline in cognitive or motor performance might simply reflect lethargy due to systemic illness, the clinical exam, as well as measures of home-cage behavior and daily food intake, were used as indicators of the health and motivational state of each animal.

Eight of the ten monkeys inoculated with the virus were productively infected as determined from repeatedly obtained positive assays for serum antigenemia and virus rescue. Neither of the other two inoculated monkeys and none of the five controls was infected according to these criteria for the ~10 months of the experiment that followed inoculation. For statistical purposes, only the eight productively infected monkeys were compared with the five controls. Data were subjected to both parametric and

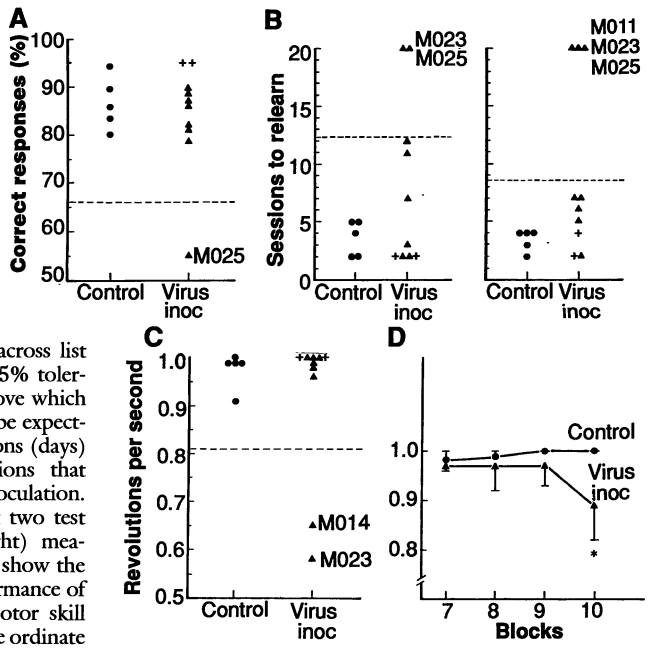

Fig. 1. Performance on cognitive and motor tasks after inoculation with SIV. In (A through C), data are shown for blocks when impairment was first evident and performance was stable. (**A**) Mean scores of control and virus-inoculated (virus inoc) monkeys on delayed matching-to-sample with trial-unique stimuli on blocks 4 through 6. There was no interaction of group and list factors, so the data have been collapsed across list items. Dashed line indicates 95% tolerance limit, that is, the level above which 95% of the population would be expected to fall (*13*). (**B**) Mean sessions (days) to relearn visual discriminations that were initially learned before inoculation. (Left) Measured over the first two test blocks after inoculation; (right) measured in block 5. Dashed lines show the 99% tolerance limit. (**C**) Performance of individual monkeys on the motor skill task for blocks 4 through 6. The ordinate indicates the speed of a rotating turntable at which each monkey could retrieve food on 50% of the trials. Dashed line indicates the 99% tolerance limit. (**D**) Group mean scores (± SD) on the motor skill task for blocks 7 through 10. Data from monkeys M014 and M023, who were impaired in (C), are not included in (D); scores shown are for the remaining virus-infected animals ($n = 6$). By block 10 there was a significant group difference (Mann-Whitney U test, $U = 4.15$, $^*P < 0.05$). The two animals that were inoculated but uninfected obtained a mean score of 1.0 for each of the four blocks illustrated. The scores of the control animals are at or near ceiling for the motor skill task, and, therefore, the difference between virus-infected and control groups may be underestimated. (●), Sham-inoculated controls; (▲), SIV-infected monkeys; (+), virus-inoculated but uninfected monkeys.

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nonparametric analyses. Within 2 to 6 months of inoculation, four of the eight infected animals were impaired on one or more of the behavioral tasks (Fig. 1), as shown when the scores of individual virus-infected monkeys were compared to control group scores by the method of tolerance limits (*13*). One monkey (M025) was impaired in both recognition and recency memory, and in discrimination learning and retention as well. Another animal (M023) was impaired in both discrimination learning and retention and in motor skill. Two other monkeys exhibited impairment in one ability only, one (M011) in discrimination retention and the other (M014) in motor skill. During this time, the scores on all tasks of the other four infected monkeys were within or near the range of the control monkeys. By the last block of testing, which was about 10 months after inoculation, all but one of the SIV-infected monkeys had slightly, but nevertheless significantly, reduced scores on the motor skill task (*14*). The two inoculated but uninfected monkeys scored within the range of the control animals on all tasks throughout the study. Viral infection led to no consistent changes in home-cage behavior (*15*).

Histological evaluation of the brains of the infected monkeys revealed a variety of pathological alterations, ranging from minimal perivascular lymphocytic inflammation

Table 1. Testing schedule after inoculation with SIV. Testing was organized into ten 4-week blocks; each behavioral measure was evaluated in each monkey during each block. Tests were given 5 days per week. After every 8 weeks of behavioral testing, the animals were put on rest and fed ad libitum for a week.

Block	Week	Test
1	1 and 2	90 trials of recognition memory task (delayed matching-to-sample, trial unique stimuli) concurrent with 20 different visual discrimination trials
	3 and 4	80 trials of recency memory task (delayed matching-to-sample, two stimuli) concurrent with 20 different visual discrimination trials; plus 40 trials of motor skill task
2	5 through 8	Repeat of preceding 4-week cycle
	9	On rest; no testing

Table 2. Time course of cognitive and motor impairments and of clinical signs in the ten virus-inoculated monkeys. The retention of visual discriminations occurred only in blocks 1 and 2 (retention 1). and in block 5 (retention 2), and the rate of learning of new problems was assessed in blocks 3, 4, and 6 through 10. For neuropathological evaluation, one hemisphere from each monkey was fixed in formalin, and selected blocks from both hemispheres were embedded in paraffin and stained for histological examination. Immunocytochemistry for SIV antigen was performed on paraffin-embedded brain sections with an affinity-purified rabbit polyclonal antiserum to SIV (*16, 17*). One of the uninfected monkeys (M010) had mild lymphocytic inflammation of the stroma of the choroid plexus, a change noted in studies of uninfected rhesus macaques (*17*). L, lymphadenopathy; S, splenomegaly; F, fever; R, rash; and LA, loss of appetite. Asterisk (*) denotes inoculated but uninfected monkeys. Numerals in parentheses indicate the test block in which the impairment or the clinical sign occurred or when the animal was killed. Arrows (→) indicate that the impairment or the clinical sign continued until the experiment was terminated or the animal was killed, whichever occurred first.

Animal	Test impaired	Neuropathology	Immunocytochemistry for SIV in brain	Clinical signs	Cause of death
M007	Motor skills (10)	Perivascular inflammation (slight)	Not detected	L (1 →), S (4, 5, 7, 9), F (7, 8), and LA (9 →)	Wasting (>10)
M008	Motor skills (10)	Perivascular macrophage (focal), lymphocytic meningitis, and choroid plexitis	Probable positive cells in choroid plexus	L (6, 9, 10)	Scheduled (>10)
M010*	None	Choroid plexitis (mild, background)	Not detected	None	Scheduled (>10)
M011	Motor skills (8 →), discrimination learning (7), and discrimination retention (5)	Macrophage infiltrates, perivascular lymphocytes, lymphocytic meningitis, and choroid plexitis	Positive in macrophages, in brain, and in cells in leptomeninges	L (4, 6 →), S (7 →), and F (8 through 10)	Scheduled (>10)
M014	Motor skills (4 through 6)	Perivascular inflammation (slight)	Not detected	L (1 through 3, 5, 7), R (1, 7), and LA (6 →)	Wasting (8)
M019*	None	No lesions	Not detected	R (8)	Scheduled (>10)
M023	Motor skills (4 →), discrimination learning (3, 4, 6 →), and discrimination retention (2, 5)	Perivascular inflammation (severe), multinucleated giant cells, white matter pallor, and gliosis	Positive cells in choroid plexus and leptomeninges	L (2 →), R (4 →), and S (5 →)	Seizure (8)
M025	Recognition (4 →), recency (6 through 9), discrimination learning (3, 4, 6 →), and discrimination retention (2, 5)	Perivascular inflammation (slight) and lymphocytic meningitis	Not detected	L (4 →) and S (5 →)	Pneumonia (10)
M030	Motor skills (10)	Perivascular inflammation	Rare positive cell in putamen	L (2, 5 →) and R (7)	Scheduled (>10)
M031	Motor skills (10)	Lymphocytic meningitis and choroid plexitis (focal)	Not detected	L (1, 3 →), S (4, 5, 7 →), R (1 →), and F (5, 10)	Scheduled (>10)

and choroid plexitis to typical SIV meningoencephalitis, with macrophages and multinucleated (syncytial) giant cells (Table 2) (*2*). Analysis of the locus and the extent of the lesions revealed no anatomical findings pathognomonic for the motor or cognitive impairments (Table 2), even though we examined the brains of the infected monkeys very near the time at which the CNS dysfunction became manifest. The results are consistent with the notion that the motor and cognitive impairments associated with SIV infection are due to global immunological, neurochemical, or trophic changes in the CNS rather than to discrete virus-induced lesions.

These data demonstrate that SIV-infected rhesus monkeys, like HIV-infected humans, develop cognitive and motor impairments during disease progression. These changes are specific to SIV infection and are unlikely to be due to a general lack of motivation or poor health. Animals exhibited significant behavioral deficits well before either evidence of opportunistic infection or signs of progressive clinical disease (Table 2) (*16–18*). In each case, monkeys that were impaired on one task performed normally on another. The inoculated monkeys, unlike sick monkeys, always willingly jumped from their home cage to the test cage, routinely completed all test sessions, and exhibited normal home-cage behavior.

The CNS dysfunction observed in SIV-infected rhesus macaques closely resembles that seen in HIV-infected humans: the types of neuropsychological impairment observed and the proportion of individuals affected were similar to those reported for HIV-infected humans, and the pattern of neuropsychological impairment was variable (*8, 19*). Within the time frame of the experiment, motor skill deficits were more frequent than cognitive impairments in the infected monkeys. Because some of the animals were killed before the terminal stages of disease, we conclude that motor skill deficits either are more frequent, or occur earlier, than cognitive deficits in the complete course of immunodeficiency disease. Although no clinical studies have directly addressed this issue, our observations suggest that they should. In addition, because the motor and cognitive dysfunction was not related to either the location or the extent of inflammatory lesions in the CNS, it seems likely that the neuropsychological impairments in both monkeys and humans are caused by indirect effects of SIV and HIV infection, respectively (*20*). If so, an understanding of HIV-associated CNS dysfunction may well depend on the identification of specific neurochemical or immunological abnormalities that arise with the onset of behavioral impairments in SIV-infected monkeys. The neuropsychological status of SIV-infected macaques may also serve as a bioassay to test potential CNS-targeted therapeutics.

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3. The test apparatus consisted of an IBM PC/AT clone connected to a color monitor fitted with a touch screen (Microtouch Systems, Inc., Wilmington, MA) and an automatic pellet dispenser (BRS/LVE, Laurel, MD). The system design was adapted from Gaffan *et al.*, [*Q. J. Exp. Psychol.* **36B**, 173 (1984)], and the software was modified by D. Gaffan, J. K. Parkinson, and E. A. Murray for the equipment used in this study. In brief, two-dimensional colored stimuli, each comprising two ASCII characters of different colors and different sizes that were superimposed, were generated by the computer. The monkey's response was to simply touch a given stimulus, the location of which was recorded by the touch screen and computer. Correct responses were rewarded with the delivery of a single 190-mg food pellet (Bio-Serv, Inc., Frenchtown, NJ).
4. On each trial, three different sample stimuli were presented in the center of the screen, one at a time, separated by an interval of 2.5 s. The monkeys were required to touch each stimulus in turn for the trial to proceed, but no rewards were given. After an 8-s delay, the three sample stimuli were presented in reverse order, each paired with a novel stimulus; the two members of the pair appeared on the left and right sides of the screen (the left-right position of the correct stimulus was random). A correct (matching) response resulted in the delivery of food reward, as well as visual feedback consisting of the matching stimulus remaining on the screen for 1 s and, at the same time, the reappearance of the sample stimulus in the center of the screen. An incorrect response resulted in a blank screen. The intertest intervals were 2.5 s, yielding sample-test intervals of 8, 13, and 18 s. This procedure was repeated with a novel set of 6 stimuli for every trial until 30 such lists were completed in a test session. Lists were separated by 12 s.
5. The recency memory task is a version of delayed matching-to-sample that uses only two stimuli per test session; the interference engendered with the use of highly familiar stimuli makes the task difficult for monkeys. Either member of the pair appeared randomly as a sample on a given trial in each daily session. As in the recognition task, the sample appeared in the center of the screen and disappeared when the monkey touched it. After a 5-s delay, the animals could obtain a food pellet on the choice test by touching the stimulus that had just appeared as the sample. The left-right position of the correct stimulus followed a random order. The visual feedback for correct responses was the same as that provided for the recognition memory task (*4*). Eighty such trials were given in each session, and a new pair of stimuli was used each day.
6. Twenty different visual discrimination problems were administered, across days, for one trial each day. On each trial, two simultaneously presented stimuli occupied the left and right sides of the screen. One of these stimuli was arbitrarily designated positive (correct) and the other negative. A response to the positive stimulus resulted in the delivery of a food reward, and responses to either the positive or the negative stimulus resulted in a blank screen. The trials were separated by 12 s. The 20 discriminations were presented daily, in the same pairings, with the same reward value, and in the same order. The left-right position of the positive stimulus was random both within and across days. Testing after inoculation included evaluation of the retention of the problems learned before inoculation and of the rate of learning of new problems of the same type.
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9. Both versions of delayed matching-to-sample depend on the integrity of a limited set of interconnected brain regions, primarily the medial temporal lobe limbic structures (amygdala, hippocampus, and subjacent periallocortex), medial diencephalic structures, and ventromedial prefrontal cortex, whereas the discrimination learning task does not; by contrast, the specific version of visual discrimination learning used here, and probably the motor skill task as well, depends on the integrity of certain portions of the basal ganglia, whereas the matching-to-sample tasks do not. M. Mishkin, B. Malamut, J. Bachevalier, in *Neurobiology of Learning and Memory*, G. Lynch, J. L. McGaugh, N. M. Weinberger, Eds. (Guilford Press, New York, 1984), pp. 65–77; L. R. Squire, *Science* **232**, 1612 (1986); M. Mishkin and T. Appenzeller, *Sci. Am.* **256**, 80 (June 1987).
10. The mean duration and frequency of normal locomotion, interaction with a ring attached to the top center of the home cage, environmental exploration, social contact, self grooming, and stereotypies that occurred during several 5-min observation periods per test block (Table 1) were recorded with a lap-top computer.
11. Each of the 15 monkeys was assigned to one of two groups that were matched for final performance levels before inoculation on recognition memory [controls, $n = 5$, 84.9 ± 6.2% correct responses (mean ± SD); virus-inoculated monkeys, $n = 10$, 85.4 ± 7.8%], recency memory (controls, 86.7 ± 9.6% correct responses; virus-inoculated monkeys, 87.0 ± 5.4%), and motor skill (controls, 0.60 ± 0.19 revolutions per second; virus-inoculated monkeys, 0.57 ± 0.13 revolutions per second). Anesthesia was induced with an initial intramuscular dose of 0.3 ml of a mixture of acepromazine maleate and ketamine hydrochloride (1:10 v/v; 10 mg/ml and 100 mg/ml, respectively); supplemental doses of 0.15 ml were used as needed. Ten monkeys were injected intravenously with ten rhesus infectious doses of the Delta B670 strain of SIV in 1.0 ml of culture medium (RPMI1640), and five monkeys were injected with 1.0 ml of culture medium alone. The monkeys were ~18 months of age [18.5 ± 0.9 months (mean ± SD)] at the time of inoculation or sham inoculation.
12. Dose and route of administration of anesthetic as in (*11*). The p26 antigen in serum was assayed by enzyme-linked immunosorbent assay (ELISA, Coulter Corp.), and virus rescue was determined by p26 production after culture of rhesus peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin-stimulated human PBMCs, assayed by ELISA.
13. Except where noted in Fig. 1, no significant group differences emerged from the analyses of variance (ANOVAs) and nonparametric tests. Significant differences were uncovered, however, by the method of tolerance limits [B. Ostle, *Statistics in Research* (Iowa State Univ. Press, Ames, IA, ed. 2, 1963), pp. 98–101], a statistic that is based in part on the mean and SD of the control scores and that indicates the range of scores within which a specified percentage of a statistical population can be expected to fall. For comparison with a commonly used statistic of dispersion, the tolerance limits (one-sided) calculated here are well outside 2 SDs of the mean of the control scores.
14. Because this impairment became manifest only late in the experiment, one of the virus-infected monkeys (M011) was compared with four of the controls for an additional block and was found to be still impaired.
15. We analyzed each measure of home-cage behavior (*10*) using both ANOVAs with repeated measures and the tolerance limit method (*13*). The ANOVA revealed a significant interaction of group and block for one measure, the duration of the monkey's interaction with the ring, but no other comparisons attained significance. The interaction appeared to be

due to the controls scoring lower than normal during block 5 but scoring higher than normal for block 6, whereas the reverse was true for the SIV-infected monkeys for the same blocks. In any event, the interaction cannot account for the pattern of cognitive and motor impairments. The tolerance limit method yielded no consistent findings.

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18. With few exceptions, the monkeys displayed food intake, body temperatures, and body weights that were in the normal range; M007, M011, and M031 were occasionally febrile, and M007 and M014 showed occasional, brief (1 to 2 days) losses of appetite during the course of training after inoculation, but these episodes generally postdated, and so cannot account for, the cognitive and motor deficits. For the most part, the SIV-infected monkeys succumbed to diarrhea, wasting, or opportunistic infection only very late in the course of disease, about a week before they became moribund and were euthanized (Table 2).

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Converting Trypsin to Chymotrypsin: The Role of Surface Loops

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Trypsin (Tr) and chymotrypsin (Ch) have similar tertiary structures, yet Tr cleaves peptides at arginine and lysine residues and Ch prefers large hydrophobic residues. Although replacement of the S1 binding site of Tr with the analogous residues of Ch is sufficient to transfer Ch specificity for ester hydrolysis, specificity for amide hydrolysis is not transferred. Trypsin is converted to a Ch-like protease when the binding pocket alterations are further modified by exchange of the Ch surface loops 185 through 188 and 221 through 225 for the analogous Tr loops. These loops are not structural components of either the S1 binding site or the extended substrate binding sites. This mutant enzyme is equivalent to Ch in its catalytic rate, but its substrate binding is impaired. Like Ch, this mutant utilizes extended substrate binding to accelerate catalysis, and substrate discrimination occurs during the acylation step rather than in substrate binding.

CHYMOTRYPSIN (CH) AND TRYPSIN (Tr) have extensive sequence identity (*1*) and seemingly superimposable main chain structures (*2, 3*) yet have very different substrate specificities. Until recently, the substrate specificity of these pancreatic serine proteases was believed to be a simple function of the steric and electrostatic properties of the S1 binding site (*2, 4*). The S1 binding sites of Tr and Ch are nearly identical in structure and primary sequence (Figs. 1 and 2). Chymotrypsin has a hydrophobic S1 binding pocket formed by residues 189 through 195, 214 through 220, and 225 through 228 (*5*); this feature ostensibly explains Ch's specificity for large hydrophobic residues. The preference of Tr for Lys and Arg results from the presence of Asp^{189} (Ser in Ch) at the bottom of the S1 binding pocket (*6, 7*). The structural basis of substrate specificity in these enzymes is more complex than these simple mechanistic postulates would imply. Mutation of Asp^{189} to Ser (D189S) in Tr does not switch the substrate specificity of Tr to that of Ch but creates a poor, nonspecific protease (*8, 9*) (Table 1). We have further elucidated the structural determinants of specificity and activity in Tr and Ch by showing that the S1 binding pocket determines the specificity of ester hydrolysis, whereas specific amide hydrolysis requires both the proper S1 binding site and more distal binding site interactions. These interactions are profoundly influenced by surface loops that do not directly contact the substrate.

Ester hydrolysis is intrinsically less specific than amide hydrolysis. The hydrolysis of hydrophobic oligopeptide amide substrates by Tr is 10^5-fold less efficient than by Ch, but only a 10- to 100-fold difference in k_{cat}/K_m (k_{cat}, catalytic rate constant; K_m, Michaelis constant) exists between Tr and Ch for the hydrolysis of *N*-acetylphenylalaninyl-*p*-nitrophenylester (AcF-pNP) and succinyl-AlaAlaProPhe-thiobenzyl ester (sucAAPF-SBzl) (Tables 1 and 2). The k_{cat} values are similar, whereas K_m differs 10- to 100-fold. This apparent lack of specificity for ester hydrolysis may be a consequence of k_{cat}/K_m approaching the rate of diffusion, which would effectively limit selectivity (*10*). The steady-state kinetic constants for the hydrolysis of both AcF-pNP and sucAAPF-SBzl by D189S are equivalent to those of Ch, indicating that the Asp^{189} to Ser mutation in Tr is sufficient to change the specificity of ester hydrolysis.

Unlike ester hydrolysis, specific amide hydrolysis by pancreatic serine proteases is influenced by the length of the oligopeptide substrate, and specificity is largely determined by the extended substrate binding sites (*11*). The steady-state kinetic parameters for the hydrolysis of single amino acid and oligopeptide amide substrates by Ch, Tr, and D189S are compared in order to assess the contribution to amide substrate specificity of the S1 binding site and the extended binding sites (Table 2). Chymotrypsin hydrolyzes the oligopeptide amide substrates succinyl-AlaAlaProPhe-7-amino-4-methylcoumarin (sucAAPF-AMC) and succinyl-AlaAlaProPhe-*p*-nitroanilide (sucAAPF-pNA) 10^5-fold faster than acetylphenylalaninamide (AcF-NH_2), as measured from k_{cat}/K_m. Trypsin hydrolyzes sucAAPF-AMC only tenfold faster than AcF-NH_2; thus, Tr hydrolyzes sucAAPF-AMC 10^5-fold more slowly than Ch. This difference in catalytic activity for extended oligopeptide substrates contrasts dramatically with the modest differences between Tr and Ch in AcF-NH_2 hydrolysis. Trypsin cannot utilize extended substrate binding to accelerate hydrolysis of Ch-specific substrates. Clearly, the extended binding sites contribute substantially to substrate specificity. Efficient hydrolysis of amides requires both the correct P1 residue and an extended substrate (*11*). The specificity-determining transition state must include the substrate P2, P3, and P4 residues, as well as the P1 residue.

The hydrolysis of AcF-NH_2 was also used to probe the function of the S1 binding pocket independent of the extended binding sites. Trypsin hydrolyzes AcF-NH_2 100-fold more slowly than Ch (k_{cat}/K_m, Table 2). The k_{cat} value is 10^3-fold less than that of Ch; surprisingly, K_m is 10-fold lower. Perhaps Tr binds AcF-NH_2 nonproductively. The D189S mutant Tr partially reconstructs Ch amide specificity. The k_{cat} for hydrolysis of AcF-NH_2 is improved 50- to 100-fold to 5% of the Ch value. However, K_m is also increased 100-fold over that of Tr, so k_{cat}/K_m does not change. Thus, the S1 binding site of D189S is significantly compromised relative to Ch.

The complete replacement of the S1 bind-

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