Table I. Backbone ^1H, ^{13}C, and ^{31}P Chemical Shifts of $d(Gm^5C)_{20}$ in the Z Form and Differences in Shifts, Δ_{B-Z}, Relative to the B Form

	^1H		^{13}C	
	Z DNA	Δ_{B-Z}[a]	Z DNA	Δ_{B-Z}[a]
G1′	6.20	−0.28	87.53	−2.57
C1′	5.58	+0.05	88.95	−2.26
G2′	2.65	−0.13	40.47	+0.43
G2″	2.70	+0.07		
C2′	1.57	+0.42	44.25	−3.83
G2″	2.56	−0.24		
G3′	4.86	+0.09	80.02	+0.12
C3′	4.83	−0.04	76.70	+0.39
G4′	4.11	+0.23	86.47	+1.26
C4′	3.74	+0.40	86.40	−0.76
G5′	4.11	−0.01	68.14	+0.52
G5″	4.11	−0.01		
C5′	3.74	+0.40	67.11	+0.26
C5″	2.54	+1.60		

	^{31}P	
	Z DNA	Δ_{B-Z}[a]
Gpm^5C	−2.62	−1.19
m^5CpG	−4.68	+1.18

[a] The chemical shifts in the B form (100 mM NaCl, 5 mM phosphate buffer, 45 °C) were measured and assigned by the same procedure as described for the Z structure; $\Delta_{B-Z} = \delta_B - \delta_Z$; chemical shifts are given in ppm.

The C1′ and G1′ proton signals were assigned previously[3] and Figure 1 identifies the corresponding carbon resonances. Figure 1 also shows two pairs of correlations in the 2′/2″ region, one pair of correlations in the 5′/5″ region, and a correlation to a 5′-carbon for which the two protons are not resolved. The G2′, G2″, C2′, and C2″ protons are assigned from a 2D NOE spectrum (supplementary material) thus identifying the corresponding ^{13}C resonances. The 3′-protons show a small difference in chemical shift and are assigned through correlation to 2′-, 2″-, and 1′-protons in the 2D NOE spectrum. The G3′ resonance is shifted slightly downfield (≈0.03 ppm), which permits unambiguous assignment of the 3′-^{13}C signals. The crowded 4′–5′/5″ region in the 2D NOE spectrum between 3.7 and 4.1 ppm does not allow assignment of two 5′/5″ geminal pairs to a particular sugar moiety directly. However, an intense NOE 5′/5″ crosspeak at 2.54 and 3.74 ppm is clearly separated and was observed, although unidentified, in previous studies.[6,7]

The sugar specific assignment of the 5′/5″ signals is provided unambiguously by 2D ^1H–^{31}P chemical shift correlation (Figure 2). The proton-detected ^1H–^{31}P experiment with a constant time evolution period was applied as described previously.[12] The small difference of the chemical shifts between the two 3′-protons confirms the ^{31}P resonance assigment made by comparison of two phosphorothioate analogues.[15] Moreover, the correlations of the low-field ^{31}P Gpm^5C resonance (2.62 ppm) to the protons at 3.74 and 2.54 ppm can originate only from the interactions with 4′- and 5′/5″-protons in the O5′ part of the phosphodiester linkage, assigning these signals to the m^5C residue. The 4′ ^1H and ^{13}C signals separated by 0.07 and 0.37 ppm, respectively, are identified by comparing the crosspeak intensities with C3′, G3′, and C5″ protons in the 2D NOE spectrum. The ^1H, ^{13}C and ^{31}P chemical shifts obtained for the Z form of $d(Gm^5C)_{20}$ and the differences, Δ_{B-Z}, between the B and Z forms are summarized in Table I.

Although the values and comparison with those in B DNA will be discussed in detail elsewhere[17] their surprising nature deserves some comment. In the Z structure the configuration of the guanosine residue is changed from 2′-endo to 3′-endo and the glycosidic torsion angle from anti to syn compared to B DNA. This would suggest that the largest changes in the chemical shifts would be observed for signals originating from this sugar moiety. However, the assignment presented above shows that the most pronounced effects are observed in the m^5C nucleotide. In the proton spectrum the C2′, C4′, and C5′ signals are shifted substantially upfield (>0.40 ppm) with the largest change observed for the C5′ proton. The chemical shift difference (1.20 ppm) between C5′ and C5″ protons in Z DNA is unusual as is the large separation of C2′ and C2″ signals (0.99 ppm). The upfield shifts of the C2′ and the C5′ protons are most likely due to ring current effects; C2′H is located on top of the six-membered ring of the preceding guanine base and C5′H is located below the five-membered ring of the following guanine base. The change in the chemical shifts of C2′ and C2″ protons is accompanied by the largest Δ_{B-Z} in the ^{13}C spectrum (+3.83 ppm). Differences in ^{13}C chemical shifts greater than 1 ppm for C1′, G1′, and G4′ signals are also observed. These changes in ^{13}C chemical shift cannot be fully accounted for by ring current effects and may be caused in part by the change in orientation of the bases (and their dipole moments) with respect to the sugar carbons.[18] Comparison of the difference values (Table I) shows no correlation in the direction of changes in ^1H and ^{13}C chemical shifts during the B to Z transition. The observed values in the Z form are substantially different from those of B DNA and allow direct identification of the Z structure by NMR.

Acknowledgment. We thank Rolf Tschudin for continuous technical support and Dr. Laura Lerner for useful suggestions during the preparation of the manuscript.

Supplementary Material Available: 2D phase-sensitive NOE spectrum of $d(Gm^5C)_{20}$ in the Z DNA conformation (2 pages). Ordering information is given on any current masthead page.

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Reversal of Substrate Charge Specificity by Site-Directed Mutagenesis of Aspartate Aminotransferase

Ciaran N. Cronin,[†,‡] Bruce A. Malcolm,[†] and Jack F. Kirsch*[†,‡]

Department of Biochemistry and Center for Advanced Materials, Lawrence Berkeley Laboratory University of California, Berkeley, California 94720

Received November 24, 1986

The alteration of enzymes by site-directed mutagenesis to produce proteins with improved catalytic properties toward refractory substrates is an important goal of modern DNA technology. The most prominent example to date is found in the recent studies on subtilisin where changes at position 166 in the substrate binding pocket have produced a number of enzymes with increased catalytic efficiencies toward substrates that are less reactive with the wild-type enzyme.[1]

Aspartate aminotransferase (AATase; EC 2.6.1.1), a pyridoxal phosphate (PLP) dependent enzyme, catalyzes the reversible transamination of α-amino to α-keto acids, with the concomitant conversion of enzyme-bound PLP to pyridoxamine phosphate (PMP) (Scheme I).[2] The preferred substrates for this reaction are the dicarboxylic amino acids L-aspartate (I) and L-glutamate (II). We report here the results of a site-directed mutagenesis experiment that generates an inversion of the substrate charge specificity of *E. coli* AATase from the anionic I and II to the cationic amino acids L-lysine (III) and L-arginine (IV).

Crystallographic data[3] indicate that, in eukaryotic AATases,

* To whom correspondence should be addressed.
† Department of Biochemistry.
‡ Center for Advanced Materials.

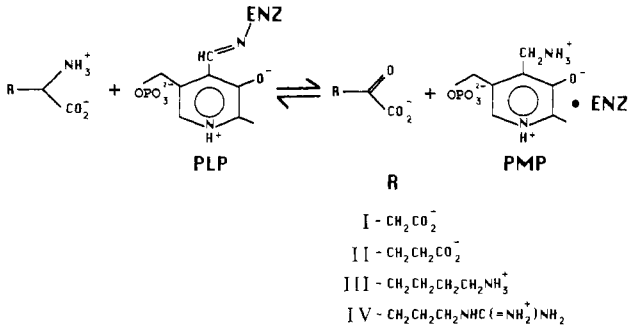
0002-7863/87/1509-2222$01.50/0

Table I. Substrate Specificities of *E. coli* Wild-Type and R292D Mutant Aspartate Aminotransferases

substrate	k_{cat}/K_M, M^{-1} s^{-1} wild type	k_{cat}/K_M, M^{-1} s^{-1} R292D mutant[a]	selectivity ratio $[k_{cat}/K_M(R292D)]/[k_{cat}/K_M(wild\ type)]$
L-arginine	0.0276 ± 0.0008[a]	0.429 ± 0.026	15.5 ± 1.0
L-lysine	0.0183 ± 0.0002[a]	0.156 ± 0.013	8.5 ± 0.7
L-aspartate	18500 ± 500[b]	0.0695 ± 0.0029	(3.8 ± 0.2) × 10^{-6}
α-ketoglutarate	14900 ± 400[b]	0.0593 ± 0.0016[c]	(4.0 ± 0.2) × 10^{-6}

[a] Pseudo-first-order rate constants for the enzymic half-reaction were determined under single turnover conditions by monitoring the increase in the A_{330} due to the formation of the PMP form of the enzyme. The pseudo-bimolecular rate constants were obtained from the slope of a linear plot of the pseudo-first-order rate constant vs. the substrate concentration. In the case of L-arginine, which showed some degree of saturation of both enzymes, the pseudo-bimolecular rate constants were calculated by fitting each data set to a rectangular hyperbola, using a computer program based on the method of Wilkinson.[11] Conditions: 100 mM TAPS–KOH, pH 8.5, 25 °C, 11.5 μM enzymes and 2.5–300 mM substrates (titrated to pH 8.5 with KOH). The ionic strength of each reaction mixture was adjusted to 1.0 by KCl. [b] Determined by steady-state kinetics using 2.5 nM enzyme, 2–10 mM α-ketoglutarate (K_M = 9.4 ± 0.7 mM), 1.7–20 mM L-aspartate (K_M = 7.5 ± 0.5 mM), and 6.3 mM $(NH_4)_2SO_4$ (contributed by the coupling enzyme solution). The production of oxaloacetate was coupled to the oxidation of NADH (0.15 mM) by using pig heart cytoplasmic malate dehydrogenase (10 units/mL). In this assay the contribution of the substrates to the ionic strength was neglected (±5% maximum). Other conditions used were identical with those described for the half-reaction assay. [c] Determined as described in footnote *a* with the exception that the increase in the A_{360}, due to the formation of the PLP form of the enzyme, was monitored and with the additional presence of 0.01 mM PMP. The PMP form of the enzyme used was that produced by the reaction described in footnote, *a*, followed by dialysis against 5 mM KH_2PO_4–KOH buffer, pH 7.0, containing 0.1 mM PMP. Enzyme concentrations were determined by using A_{205} = 31 mL mg^{-1} cm^{-1}.[12]

Scheme I



arginine residues at positions 292[4] and 386 are involved in substrate binding by ionic pairing with, respectively, the side-chain and α-carbon carboxylate groups of each substrate. The conservation of all active-site residues in the *E. coli* enzyme[5-7] indicates an identical role for the two arginines.

Preliminary crystallographic data have been obtained with the *E. coli* AATase,[8] and a recently obtained 3.0-Å map shows the same general folding pattern as the eukaryotic isozymes.[9] The hypothesis that Arg292 (R292) is largely responsible for the side-chain substrate specificity may be tested readily by introducing other amino acids into that position. It was expected, in particular, that the construct R292D (arginine replaced with aspartate) might invert the substrate charge specificity by counterion attraction to produce an enzymatic activity without precedent in nature, i.e., a lysine/arginine transaminase.

Some of the kinetic properties of the wild-type and R292D[10] aminotransferases are summarized in Table I. The theoretical objective in constructing the R292D mutant has been realized qualitatively in that, when compared to wild type, the mutant enzyme displays 16-fold and 9-fold higher k_{cat}/K_M values respectively toward arginine and lysine as substrates; moreover, the introduction of the mutation depresses the rate of aspartate transamination by over 5 orders of magnitude. Whereas wild-type AATase has an Asp/Arg selectivity ratio of 6.6 × 10^5 (18 500/0.028), this value is only 0.16 (0.070/0.43) for the mutant enzyme, demonstrating that R292D in fact prefers arginine as substrate. The mutant enzyme also catalyzes the amination of α-ketoglutarate, thus regenerating the PLP form of the enzyme, albeit at a slower rate in comparison to wild type (Table I).

Although R292D is a relatively specific cationic amino acid transaminase, its low value of k_{cat}/K_M of 0.43 M^{-1} s^{-1} for arginine argues that it is far from being optimally engineered for its new task. The finding that R292D has a rather high K_M (0.80 ± 0.29 M) toward L-arginine implies that the observed rate at high substrate concentrations will be closer to that of the wild type with L-aspartate than is observed at lower substrate concentrations. For example, at 1 mM substrate the rate ratio between wild-type AATase (with L-aspartate) and R292D (with L-arginine) is 38 000 whereas at 1 M substrates this ratio decreases to 720. These results show that the residue occupying position 292 in the amino acid sequence is of critical importance in dictating substrate specificity. However, the recent observation[7] that R292 is present also in the *TyrB* gene product, an aromatic amino acid transaminase, points to additional substrate relationships yet to be evaluated, although the presence of arginine at this position in the latter enzyme is somewhat expected in view of the use of α-ketoglutarate by all of these enzymes to regenerate the PLP containing enzyme form.

Acknowledgment. The genetic engineering for AATase was sponsored by NIH Grant GM35393 and the protein characterization studies were supported by the U.S. Department of Energy under Contract DE-AC03-76SF00098.

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