Takadiastase Adenosine Deaminase, Calf Duodenal Adenosine Deaminase, and Rabbit Muscle Adenosine Monophosphate Deaminase. A Comparison of Physical Properties and Amino Acid Composition*

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ABSTRACT: Hydrodynamic properties indicate that takadiastase adenosine deaminase, now purified to apparent homogeneity, has a molecular weight of approximately 217,000. Urea reversibly denatures this enzyme, causing it to dissociate into subunits of molecular weight approximately 105,000. Calf duodenal adenosine deaminase has a molecular weight of approximately 52,000, considerably higher than previous estimates based on gel filtration. Urea denatures this enzyme irreversibly, causing an increase in frictional coefficient from 1.4 to 2.1 without change in molecular weight. Rabbit muscle adenosine monophosphate deaminase has a molecular weight of approximately 270,000, and is also denatured irreversibly in urea. Mercurials, previously found to bind to the adenosine deaminases in competition with nucleosides, produce no apparent change in hydrodynamic properties of these enzymes.

Amino acid analyses are presented for each protein and indicate the presence of cysteine–half-cystine residues in numbers that are equal to the number of *p*-mercuribenzoate-titratable groups in both adenosine deaminases.

Of the numerous enzymes which are known to bind purines and pyrimidines with specificity, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) has received particular study with regard to mechanisms of substrate binding and catalysis. Adenosine deaminases from both *Aspergillus oryzae* and calf duodenum bind unsubstituted purine ribonucleoside tightly (Cory and Suhadolnik, 1965; Wolfenden *et al.*, 1967), and catalyze hydrolytic displacement of nitrogen, oxygen, and halogen leaving groups from C-6 of purine ribonucleosides (Wolfenden, 1966; Baer and Drummond, 1966; Chassy and Suhadolnik, 1967).

A common feature of these enzymes is the apparent participation of enzyme sulfhydryl groups in substrate binding, and perhaps also in catalysis. Thus mercuric ion and organic mercurials appear to compete with substrate binding by adenosine deaminase from *A. oryzae*, adenosine deaminase from calf duodenum, and cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) from *Escherichia coli* (Wolfenden *et al.*, 1967; Ronca *et al.*, 1967). Changes in ultraviolet spectra indicate that PMB[1] is bound to enzyme sulfhydryl groups. Inhibition by mercurials is competitive, and heterocyclic substrates and inhibitors protect against binding of mercurials by these enzymes. Comparable behavior is shown by another enzyme of this type, 5′-adenylic acid deaminase (AMP-aminohydrolase, EC 3.5.4.6) from rabbit back muscle, which is subject to allosteric inhibition by GTP. Mercurials "desensitize" the enzyme to allosteric inhibition, and equilibrium binding studies indicate that mercurials interfere with the actual binding of GTP (Y. Tomozawa and R. Wolfenden, in preparation).

To provide structural information concerning these enzymes, we have investigated their amino acid composition and physical properties. Pfrogner (1967) has recently described an adenosine deaminase from calf spleen which has not been studied mechanistically. This paper compares the properties of pure preparations of the nonspecific adenosine deaminase of *A. oryzae* (Takadiastase) (also active on 5′-AMP), the specific adenosine deaminase of calf duodenum (Brady and O'Connell, 1962), and the specific 5′-AMP deaminase of rabbit back muscle (Smiley *et al.*, 1967).

Experimental Procedure

Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia, Inc. Hydroxylapatite dry power was obtained from Bio-Rad, Inc. Urea (Ultra-Pure) was obtained from Mann Laboratories, Inc.

Adenosine deaminase activities were determined at 25° as described previously and converted to international units (micromoles per minute) using the known K_m values (Wolfenden *et al.*, 1967). Protein concentrations were measured by the method of Lowry *et al.*, (1951), using crystalline bovine serum albumin as a standard.

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: PMB, *p*-mercuribenzoate.

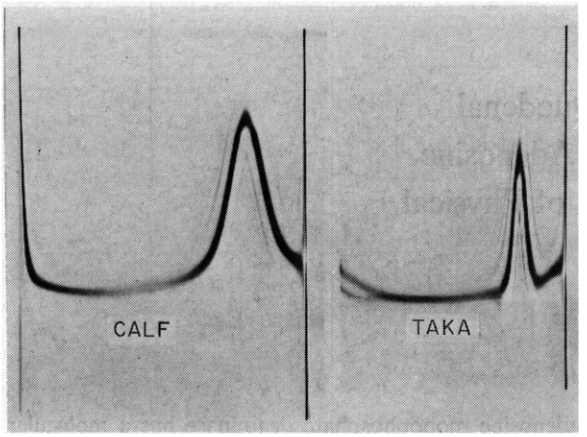

FIGURE 1: Sedimentation velocity profiles (schlieren optics) of takadiastase adenosine deaminase (left), and calf duodenal adenosine deaminase (right), at concentrations of approximately 3 mg of protein/ml in potassium phosphate buffer (0.01 M, pH 6.5) containing KCl (0.1 M). Direction of sedimentation is from right to left.

TABLE I: Purification of Takadiastase Adenosine Deaminase.

	Total Units (μmoles/min at 25°)	μmoles/min mg at 25°	Protein (mg)
Takadiastase powder extract	176,000	0.64	275,000
Ethanol precipitate extract	113,000	24	4,700
DEAE-Sephadex fractions	33,000	180	183
Hydroxylapatite fractions	22,000	533	41

Takadiastase adenosine deaminase was prepared by a modification of an earlier procedure (Wolfenden *et al.*, 1967), using takadiastase powder of higher initial deaminase activity (Takadiastase Y′, lot 1), the generous gift of Dr. Hiroshi Okazaki, Sankyo Co., Ltd., Tokyo, Japan. Chromatography on DEAE-Sephadex A-50 (Minato *et al.*, 1965) was performed before hydroxylapatite chromatography, permitting larger quantities of enzyme to be purified.

Takadiastase Y′ powder (500 g) was dissolved in water (4 l.) at 0°, the temperature at which subsequent steps were performed. Cold acetone (5 l.) was added with stirring, and the mixture was allowed to stand for 3 hr. The precipitate was removed by centrifugation and extracted with water (450 ml). Cold ethanol (450 ml) was added with stirring, and the mixture was allowed to stand for 5 hr. The precipitate was extracted with water (120 ml), dialyzed for 48 hr against potassium phosphate buffer (0.01 M, pH 6.5), applied to a column of DEAE-Sephadex A-50 (100-ml bed volume), and eluted with a linear gradient, the first reservoir containing potassium phosphate buffer (0.01 M, pH 6.5, 300 ml), the second reservoir containing KH_2PO_4 (0.5 M, 300 ml). Enzyme activity was eluted between 210 and 350 ml of eluent. Active fractions were combined and dialyzed for 24 hr against sodium acetate buffer (0.02 M, pH 5.33), applied to a column of hydroxylapatite (40-ml bed volume), and eluted with a linear gradient, the first reservoir containing sodium acetate (0.02 M, pH 5.33, 300 ml), the second reservoir containing 300 ml of the same buffer but including 0.44 M ammonium sulfate. Purification, summarized in Table I, yielded an enzyme of specific activity (533 μmoles/min per mg at 25°) somewhat higher than by the previous procedure. This was not increased by rechromatography on hydroxylapatite, and the protein was homogeneous by gel electrophoresis.

Calf duodenal adenosine deaminase, analytical reagent grade, was obtained as an ammonium sulfate suspension from Boehringer Mannheim Corp. This preparation (430 μmoles/min per mg at 37°) was equivalent in specific activity to that reported by Brady and O'Connell (1962) for the pure enzyme, and was homogeneous by comparison of weight- and number-average molecular weights (see Results and Discussion). On an analytical DEAE-cellulose column it was eluted as a single peak of constant specific activity equal to that of the original material (Brady and O'Connell, 1962).

Crystalline AMP deaminase was prepared according to the procedure of Smiley *et al.*(1967), and was similar in specific activity (90 μmoles/min per mg at 30°) and sedimentation behavior to their preparation.

Sedimentation experiments were performed with a Spinco Model E centrifuge at 20°, using schlieren optics and protein concentrations of 3–4 mg/ml. Sedimentation velocity was measured at 59,780 rpm by the usual method (Schachman, 1957). Sedimentation equilibrium experiments were performed with a short column (3 mm) using a Beckman double-sector cell (van Holde and Baldwin, 1958) at 20°. With calf duodenal adenosine deaminase equilibrium was achieved after 48 hr at 13,080 rpm. No further change in the slope, used for calculation of molecular weight (Schachman, 1957), occurred after these time periods, and there was no loss of enyzme activity during these experiments. Diffusion coefficients were obtained using a Beckman capillary type double-sector synthetic boundary cell (Schumaker and Schachman, 1957), with the centrifuge operated at 8800 rpm. Diffusion coefficients were calculated according to Svedberg (1940). The partial specific volume of each protein was calculated from its amino acid composition (McMeekin and Marshall, 1952).

Amino acid analyses were performed with the Beckman automatic analyzer according to Moore and Stein (1963), on protein samples (1 mg) dialyzed against water, hydrolyzed in evacuated, sealed tubes in HCl (6 N, 1 ml) for 24 and 72 hr, and reoxidized for 4 hr at room temperature in potassium phosphate

TABLE II: Molecular Properties of Deaminases.

Source:	*Aspergillus oryzae*		Calf Duodenum		Rabbit Muscle
Substrate Specificity:	Adenosine and AMP		Adenosine Only		AMP Only
Denaturant (M urea)	None	8	None	8	
$s_{20,w}$ (sec $\times 10^{13}$)	11.9	7.5	3.4	2.2	11.1
$D_{20,w}$ (cm^2 sec^{-1}) $\times 10^7$	4.5	6.45	6.3	4.2	3.75
$\bar{v}$[a] (cc/g)	0.722		0.746		0.731
MW_{sD}	217,000	103,000	52,000	50,300	270,000
MW_{eq}			52,450		
Stokes' radius (Å)[b]	44	33	34	51	56
f/f_0[b]	1.12	1.08	1.38	2.07	1.32
Cys residues/mole[a]	14.0		10.0		32
SH residues/mole[c]					
Rapid	13–14		1.9		6.2
Slow	0		7–8		*c*

[a] Calculated from amino acid composition (see Table III and text). [b] Calculated from the classical equations, $a = kT/6\pi\eta D$ and $f/f_0 = a/(3v M/4N)^{1/3}$, where a = Stoke's radius, k = Boltzmann's constant, T = absolute temperature, η = viscosity of the medium, $\bar{v}$ = partial specific volume, M = molecular weight, N = Avogadro's number, and $D = D_{20,w}$. [c] Determined by titration with PMB (see text). Slowly reacting residues were not determined in the case of rabbit muscle AMP deaminase, since turbidity began to appear after titration of rapidly reacting residues (see text).

buffer (pH 6.5, 0.1 M) after removal of HCl. Serine and threonine data were extrapolated to zero time. Analysis for cysteine content was made in separate amino acid analyses for cysteic acid after performic acid oxidation according to Hirs (1967). Tryptophan/tyrosine ratios were determined by ultraviolet spectrophotometry in alkali (Fraenkel-Conrat, 1957), and tryptophan content was calculated on the basis of amino acid analysis for tyrosine.

Polyacrylamide gel electrophoresis was performed according to Jovin *et al.* (1964), with the lower gel containing Tris buffer (pH 8.9). Gels were stained with Coomaisie brilliant blue R 250 according to Fazekas de St. Groth *et al.* (1963).

Titrations of reactive sulfhydryl groups with PMB were made according to Boyer (1954), using absorbancy at 250 mμ in potassium phosphate buffer (0.01 M, pH 6.5).

Results

Rabbit muscle AMP deaminase was a crystalline preparation equivalent in specific activity to that of Smiley *et al.* (1967), and appeared homogeneous in sedimentation velocity experiments. Takadiastase adenosine deaminase, whose sedimentation velocity profile is shown in Figure 1, was eluted from hydroxylapatite as a single peak of constant specific activity, and migrated as a single band upon polyacrylamide electrophoresis (Figure 2). Calf duodenal adenosine deaminase, which was obtained commercially, was equivalent in specific activity to the pure preparation described by Brady and O'Connell (1962). Its sedimentation velocity profile is shown in Figure 1, and there was good agreement between the number-average molecular weight (calculated from sedimentation and diffusion coefficients) and weight-average molecular weight (obtained by equilibrium sedimentation) as shown in Table II. However polyacrylamide electrophoresis of this enzyme yielded four bands (Figure 2), each of which contained enzymatic activity. Brady and O'Connell (1962) originally demonstrated four active isozymes of this enzyme on starch gel electrophoresis under similar conditions, and Cory *et al.* (1967) more recently observed four isozymes of calf serum adenosine deaminase.

Hydrodynamic properties of the enzymes were

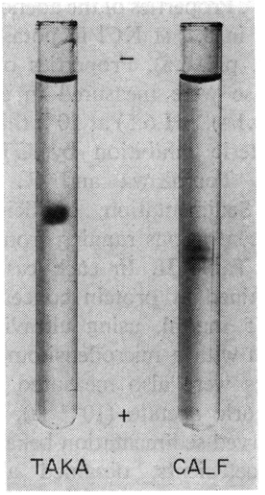

FIGURE 2: Polyacrylamide electropherograms of takadiastase adenosine deaminase (right), and calf duodenal adenosine deaminase (left), stained with Coomaisie brilliant blue (see text). Migration occurred in the downward direction toward the anode as indicated.

TABLE III: Amino Acid Analysis of Deaminases[a] (Moles/10,000 g of Protein).

Source:	*Aspergillus oryzae*	Calf Duodenum	Calf Spleen[b]	Rabbit Muscle
Substrate Specificity:	Adenosine and AMP	Adenosine	Adenosine	AMP
Lys	2.78	6.38	5.72	7.57
His	2.67	2.46	2.07	3.05
Arg	4.50	3.43	4.26	4.87
Asp	12.15	8.04	9.34	8.98
Thr	4.43	4.86	5.28	4.49
Ser	5.97	5.23	5.99	6.11
Glu	11.50	10.80	11.76	8.87
Pro	3.76	5.10	5.67	4.96
Gly	6.24	5.15	5.01	3.96
Ala	8.60	6.23	5.63	5.20
Cys[c]	0.646	1.92	12.8	1.24
Val	5.57	5.85	6.45	3.91
Met	1.61	1.71	1.34	2.58
Ile	3.49	3.14	3.20	4.02
Leu	8.32	7.80	8.25	8.37
Tyr	1.57	3.85	2.94	4.61
Phe	4.11	2.95	3.17	4.64
Trp[d]	2.08	1.03	1.07	0.75

[a] Extrapolated from values for duplicate samples hydrolyzed for 24 and 72 hr in 6 N HCl at 110°. [b] Calculated from the date of Pfrogner (1967). [c] Determined as cysteic acid in separate samples oxidized with performic acid according to Hirs (1967), then hydrolyzed for 24 hr. [d] Calculated from tyrosine analysis and tyrosine/tryptophan ratio determined by ultraviolet absorption in alkali (Fraenkel-Conrat, 1957).

measured at 20°. Properties of the adenosine deaminases were measured in 0.1 M KCl in potassium phosphate buffer (0.01 M, pH 6.5). Properties of rabbit muscle AMP deaminase were measured in ammonium succinate buffer (0.1 M, pH 6.5) at 20°; this enzyme shows maximal allosteric inhibition by GTP under these conditions (Y. Tomozawa and R. Wolfenden, in preparation). Sedimentation coefficients, measured at protein concentrations ranging from 2 to 4 mg/ml, are shown in Table II. In each case similar values were also obtained at protein concentrations of approximately 0.5 mg/ml, using ultraviolet optics and tracing the film with a microdensitometer. Sedimentation coefficients were also measured in the presence of methylmercuric acetate (10^{-3} M), which gave no change in observed sedimentation behavior.

Diffusion coefficients, obtained at protein concentrations of 2–4 mg/ml with a synthetic boundary cell and Schlieren optics, are shown in Table II. The molecular weight of the calf duodenal enzyme was also determined by the sedimentation equilibrium method, with the result shown in Table II.

Table II also compares the hydrodynamic properties (corrected for viscosity and density) of the adenosine deaminases in the presence and absence of 8 M urea. Whereas the molecular weight of the calf duodenal enzyme was unchanged, the molecular weight of the takadiastase enzyme was decreased in urea by a factor of 2. At an intermediate urea concentration (5 M), both a slowly and a rapidly sedimenting species of the takadiastase enzyme were observed, in approximately equal proportions. All three enzymes lost activity in urea. The takadiastase enzyme recovered 80% of activity when urea was removed by dialysis after 2-weeks' exposure. However the activity of calf duodenal adenosine deaminase and rabbit muscle AMP deaminase was not restored by removal of urea, and indeed both mammalian proteins coagulated when urea was removed by dialysis.

Partial specific volumes (Table II) were calculated from the amino acid compositions of the proteins, which are shown in Table III. Amino acid compositions were obtained from samples hydrolyzed in duplicate for 24 and 72 hr and extrapolated to zero time. Cysteine, the amino acid of greatest interest, was determined in duplicate independent analyses for cysteic acid after performic acid oxidation.

Titration with PMB was performed according to the method of Boyer, as described previously (Wolfenden *et al.*, 1967). Calf duodenal adenosine deaminase was found to contain 1.95 rapidly reacting sulfhydryl groups per 52,000 molecular weight (half-time for reaction <30 sec with 2×10^{-5} M PMB chloride in 0.01 M phosphate buffer, pH 6.5); 7–8 more groups reacted slowly under these conditions, with half-time approximately 200 min. Takadiastase adenosine deaminase was found to react instantaneously with 14 moles of PMB/220,000 g of protein; no slowly reacting residues were found to be present. Rabbit muscle AMP deaminase reacted rapidly with only 6.2 moles of PMB/mole of enzyme. Subsequent changes in optical density were obscured by precipitation of the protein, so that slowly reacting residues could not be determined.

Discussion

Sedimentation and diffusion coefficients indicate a molecular weight of approximately 52,000 for the calf enzyme, 217,000 for the takadiastase enzyme, and 270,000 for the rabbit muscle enzyme (Table II). The value obtained for the calf enzyme, supported by the sedimentation equilibrium method, is considerably higher than previous estimates in the neighborhood of 35,000 based on elution from Sephadex G-75 and G-100 (Andrews, 1964; Brady and O'Sullivan, 1967). The gel filtration behavior of proteins is probably more closely related to Stokes' radius than to molecular weight (Ackers, 1964; Siegel and Monty, 1966), so that the theoretical basis of the previous estimates is somewhat uncertain. Possibly the protein is slowly eluted from Sephadex because it is retarded by electrostatic or other anomalous interactions with the dextran.

There is considerable scatter in sedimentation coefficients which have previously been reported for calf

adenosine deaminases: 1.82 S for the spleen enzyme at 3.4° (Pfrogner, 1967), 2.15 S for the duodenal enzyme at 9° (Brady and O'Connell, 1962), and 3.9 S for the serum enzyme at 20° (Cory *et al.*, 1967). It seems probable that these enzymes may be closely related in structure; the serum and duodenal enzymes resemble each other in electrophoretic behavior (Cory *et al.*, 1967), whereas the spleen and duodenal enzymes are similar in amino acid composition (Table III). Even after correction for temperature by the usual equation there is a large range in sedimentation coefficients in the above examples. Cory *et al.* have demonstrated that the physical properties of calf serum adenosine deaminase are unusually sensitive to temperature, and indeed the physical complexity of the calf enzymes appears to be confirmed by the resolution of isozymes. In the present investigation all physical properties were determined under a fixed set of conditions, so that the results should be internally consistent.

The adenosine deaminases are distinctive in their behavior in the presence of urea. The calf duodenal enzyme undergoes a decrease in both sedimentation and diffusion coefficients in proportion, so that the molecular weight is almost unchanged. The frictional coefficient increases from 1.38 to 2.07, corresponding to a probable increase of fivefold or more in axial ratio (Oncley, 1941). These observations indicate that urea causes gross unfolding of the molecule without dissociation into subunits, and confirm Brady and O'Sullivan's (1967) explanation of the effect of urea on the elution of this enzyme from Sephadex. Urea denatures this enzyme irreversibly, as was demonstrated earlier by Brady and O'Sullivan (1967). With this enzyme, as with rabbit muscle AMP deaminase, removal of urea by dialysis results in precipitation of the protein. In contrast, urea causes the enzyme from takadiastase to dissociate into subunits of half the original molecular weight, with negligible change in frictional coefficient. Urea denaturation of the fungal enzyme is reversible, with 90% of the original activity recovered after 2-weeks' exposure to 8 M urea at 0°.

The three enzymes differ markedly in amino acid composition. The calf enzyme has the highest percentage cysteine content, corresponding to ten residues per molecule. Of these, two reacted rapidly with PMB, while the remaining eight reacted slowly. Ronca *et al.* (1967) recently reported the presence of approximately 1 mole of rapidly reacting sulfhydryl per 35,000 g of protein, which could be protected against PMB by purine substrate analogs. The takadiastase enzyme is lower in percentage cysteine content, which corresponds to 14 residues/molecule, or 7/subunit. All of these reacted rapidly with PMB. In this enzyme the binding of mercurials appears to occur in competition with the binding of purine substrate analogs, and in both the calf duodenal enzyme and the takadiastase enzyme mercurials are competitive inhibitors (Wolfenden *et al.*, 1967). An important result of the present observations is that the cysteine content of both of these enzymes accounts for their reactivity with PMB. In addition, the mercurials are found to be without effect on the hydrodynamic properties of these enzymes.

When the amino acid composition of the calf duodenal enzyme is compared with that of the enzyme from calf spleen as determined by Pfrogner (1967) (Table III), the content of cysteine, phenylalanine, tryptophan, glycine, and isoleucine are almost identical, suggesting that they may be closely related. Differences appear in the content of basic and acidic amino acids; in addition, the spleen enzyme has not been resolved electrophoretically into multiple components, and appears to have a somewhat lower specific activity than the dueodenal enzyme (Pfrogner, 1967).

Rabbit muscle AMP deaminase contains approximately 32 cysteine/half-cystine residues per molecule, of which only 6 are rapidly titrated with PMB. These may be significant for the binding of nucleotides, since we have found that mercurials desensitize this enzyme to allosteric inhibition by GTP and also prevent binding of GTP in equilibrium binding experiments (Y. Tomozawa and R. Wolfenden, in preparation).

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Purification and Mechanism of Action of Proline Racemase*

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ABSTRACT: Proline racemase was purified to near homogeneity from *Clostridium sticklandii*. The racemization of proline is accompanied by deuterium incorporation from the solvent into the α position of proline. In the initial phase of the reaction, the rate of deuterium incorporation is essentially equal to the rate of product formation, regardless of which isomer is used as substrate. These results indicate the participation of two equivalent hydrogen acceptor sites. No cofactor requirement could be detected.

The structures of inhibitors and the substrate suggest the possible involvement of a metal ion at the active center. The enzyme is a sulfhydryl enzyme and is inactive in the oxidized form. It is rapidly activated by reducing agents such as mercaptans or $NaBH_4$. When the racemization of L-proline is carried out in D_2O, the optical rotation is initially negative. As the reaction proceeds, it becomes positive, and finally zero when equilibrium is reached. This "overshoot" is attributed to a primary isotope effect.

A partially purified preparation of proline racemase was first described by Stadtman and Elliot (1957). The sulfhydryl nature of the enzyme was established and some information concerning cofactor requirements was obtained. Proline racemase appeared to differ from other amino acid racemases in that no requirement for pyridoxal phosphate could be shown. In that respect it resembles hydroxyproline epimerase which has recently been purified (Adams and Norton, 1964). We have now obtained a highly purified preparation of proline racemase and have investigated some aspects of the mechanism of action of this enzyme. Initially we showed that the α-carbon–hydrogen bond is broken in the course of racemization. Subsequent experiments were designed to establish the chemical mechanism by which the α-carbon–hydrogen bond is broken and how the inversion of configuration is brought about. The latter problem is concerned with the mechanism whereby hydrogen is transferred from one side of the α-carbon to the other and can be considered separately from the chemical mechanism by which the bond is dissociated and re-formed. Two mechanisms were considered for the hydrogen-transfer process: a "one-hydrogen-acceptor" and a "two-hydrogen-acceptor" mechanism. In the first mechanism hydrogen is transferred from the substrate to a hydrogen acceptor at the active site with the concomitant formation of an intermediate derived from the substrate. The intermediate could be a carbanion or an oxidized form of the substrate depending upon the chemical mechanism by which the α-carbon–hydrogen bond is broken. In a subsequent step hydrogen is transferred from the acceptor to the intermediate so that either the original substrate isomer or the product isomer is formed. If a single hydrogen-acceptor site participates, it must be flexible enough to interact with the α-hydrogen of both isomers, or the substrate must change its position relative to the hydrogen acceptor during the course of the racemization. The alternative mechanism is the "two-hydrogen-acceptor" mechanism. Here two equivalent sites are involved. The sites are so located that one interacts with the α-hydrogen of one substrate–isomer and the other site with the α-hydrogen of the other isomer. Inversion of configuration is achieved by transferring the α-hydrogen from the substrate to one of the acceptor sites and hydrogen from the other acceptor site to the substrate-derived intermediate. The question concerning the number of hydrogen acceptor sites has not been experimentally investigated for any amino acid racemase. The general problem has been considered by Rose (1966) for enzymes which carry out proton shifts. Evidence in favor of the two-site mechanism is avail-

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