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Importance of Historical Contingency in the Stereochemistry of Hydratase-Dehydratase Enzymes

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There are two stereochemical classes of hydratase-dehydratase enzymes. Those that catalyze the addition of water to α,β-unsaturated thioesters give *syn* addition-elimination stereochemistry, whereas those that catalyze the addition of water to conjugated carboxylate substrates give *anti* stereochemistry. This dichotomy could reflect different adaptive advantages or contingencies of separate evolutionary histories. Determination of the nonenzymatic stereochemistry of deuterium oxide addition to fumarate and to *S*-crotonyl *N*-acetylcysteamine has provided direct evidence for the importance of the contingencies of evolutionary history, rather than chemical efficiency, in the pathways of these hydratase-dehydratase enzymes.

The diversity inherent in the primary, secondary, and tertiary structures of proteins fosters catalysis of unparalleled precision and efficiency. The specificity that enzymes show in substrate recognition and the rate at which the subsequent chemical transformations are performed are extraordinary. With billions of years of evolutionary history, can we safely assume that enzymes will always use the most favorable pathways for the transformations that they catalyze? Dorit *et al.* have argued that modern protein diversity represents only a very limited exploration of sequence space—an exploration constrained by the success of earlier motifs—and that some proteins may well lie at local optima (*1*). Do the pathways of some natural enzymatic processes represent local rather than global optima?

Because of ambiguities in our understanding of the precise mechanistic and kinetic details of most complex enzymatic reaction pathways, it is difficult to find convincing evidence pertaining to this important question by examining the rates of enzymatic reactions; however, stereochemistry offers a more promising opportunity. The stereospecificity of enzyme-catalyzed reactions has been a fruitful source of information about the mechanisms of enzymatic catalysis, but stereochemical investigations have also generated interesting puzzles. One of these concerns the *syn* or *anti* stereochemistry of addition-elimination reactions, which play fundamental roles in most metabolic pathways.

All hydratase-dehydratase reactions in which the abstracted proton of the substrate is α to a carboxylate group, such as fumarate hydratase, aconitate hydratase, and enolase, proceed with *anti* stereochemistry. Seven examples are known (*2*, *3*). In general, *syn* pathways are not favored in most nonenzymatic elimination reactions because of the eclipsed geometry necessary in the transition state, and *anti* elimination is favored by stereoelectronic effects (*4*); therefore, this *anti* stereochemical pattern is no surprise. However, there is also a group of eight dehydratases that produce the *syn* elimination of water, including enoyl–coenzyme A (CoA) hydratase, fatty acid synthetase, and β-hydroxydecanoyl thioester dehydratase; in these reactions, the proton abstracted is α to a carbonyl group of a thioester or a ketone (*2*, *3*). It has been shown that enoyl-CoA hydratase favors the *syn* pathway by more than 8.8 kcal/mol (*5*).

Functional theories often argue that evolutionary selection pressures have produced enzymes that are optimally adapted to catalyze their specific reactions. Indeed, some enzymes act with a catalytic efficiency close to chemical perfection (*6*). In an effort to provide an adaptive explanation for the *syn* stereochemistry in addition-elimination reactions, it has been suggested that the acidity of the proton attached to the α carbon might influence which is the most efficient pathway. Because the α protons of thioesters ($pK_a \sim 22$ to 23, where K_a is the acid dissociation constant) are more acidic than those of carboxylate salts or carboxylic acids ($pK_a \geq 25$), it has been argued that this increased acidity could tilt the stereochemistry toward *syn* addition-elimination, with catalytic group economy in having a single acid-base enzymatic group that could interact both with the α proton and with the β leaving group (*2*, *7*).

However, the stereochemical preference need not have a basis in mechanistic advantage. There are two other evolutionary possibilities. In the first, the stereochemistry may be functionally significant, and in each stereochemical class of dehydratases the pattern simply represents divergent evolution from two ancestral progenitors with conservation of active site structure. A second possibility is that the functional difference is insignificant and therefore neutral to natural selection. Selective pressures might not be strong enough to favor one stereochemical outcome over the other, because the energetic difference between the *syn* and *anti* elimination pathways involving unhindered acyclic substrates is rarely, if ever, more than 3 kcal/mol (*4*).

We chose to try to understand this *syn-anti* dichotomy by determining the innate chemical stereoselectivity for the addition of water across the conjugated double bond of a substrate from each stereochemical class, under nonenzymatic conditions. The two substrates that we used are disodium fumarate (**1**) and *S*-crotonyl *N*-acetylcysteamine (**3**), shown in Fig. 1. Of course, fumarate is the actual substrate of fumarate hydratase, and **3** is identical to the substrate of enoyl-CoA hydratase near the site of the addition reaction. Although the actual substrate is the CoA thioester, Lynen found that **3** is also catalytically processed by the enzyme (*8*), which produces *syn* elimination on the *S*-pantetheine thioester of crotonate as well (*9*). In addition, β-hydroxydecanoyl thioester dehydratase gives *syn* elimination from the *N*-acetylcysteamine substrate (*10*).

The stereoselectivity of conjugate addition to α,β-unsaturated esters can be quite high, on the order of 10:1 *anti*/*syn* for the addition of deuterated ethyl alcohol

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(EtOD) and Me_3CSD (Me, methyl) to ethyl crotonate (*11*). In all cases that we have studied, the products of base-catalyzed conjugate addition and H-D exchange (at the α position of the β-substituted butyrate derivatives) have the same diastereomeric composition (*12*). This is powerful evidence that H-D exchange and conjugate addition reactions proceed through a common enolate-anion intermediate. Thus, in principle, the substrate diastereoselectivities that we need could be found either by exchange or by addition reactions.

It is likely that fumarate hydratase and enoyl-CoA hydratase also catalyze their reactions by pathways involving enolate or enolic intermediates. All of the enzymatic *anti* addition-elimination reactions so far examined have been reported to proceed by stepwise pathways involving enolate intermediates (*13*). There is also good evidence for enolates in the H-D exchange of thioesters in D_2O with 3-quinuclidinone buffers (*5*, *14*). Enoyl-CoA hydratase may proceed by a concerted pathway (*5*, *15*), although Gerlt and Gassman have argued for a stepwise pathway in which concerted general acid-base catalysis provides protonation of the carbonyl oxygen as the α-proton is abstracted, giving an enolic intermediate (*7*, *16*). Even if the mechanisms of the enzymatic and nonenzymatic processes whose stereochemistries we are comparing are subtly different, there is no reason to expect any fundamental change in the reaction stereoselectivities.

Addition and exchange reactions of fumarate and malate were run in D_2O under N_2 with 0.50 M **1** or disodium malate (**2**) and 0.005 to 0.50 M KOD over a temperature range of 50° to 100°C (*17*). The deuteroxide-catalyzed reaction of **2** in D_2O is remarkably clean, the only product being H-D exchange at the α carbon, C-3 (*18*). In the conjugate addition of D_2O to **1**, direct H-D exchange on fumarate competed to a small degree, but not sufficiently to confuse the issue. As shown in Table 1, the diastereoselectivity of deuteration at C-3 of malate was 57% of the 2*R**,3*S** diastereomer over the entire range of reaction conditions, including changes in temperature and pH. Thus, there is a slight bias for *anti* addition of D_2O to fumarate.

The necessary thioester substrates, **3** and *S*-β-hydroxybutyryl *N*-acetylcysteamine (**4**), were synthesized from *N*-acetylcysteamine and crotonyl chloride (*19*) and β-butyrolactone (*8*), respectively. Addition and exchange reactions were run in D_2O under N_2 with 0.25 M **3** or 0.5 M **4** and 0.1 or 0.5 M KOD over a temperature range of 23° to 50°C (*20*), and the results are shown in Table 2. The reaction of **3** with KOD-D_2O is complex. A number of products were formed in addition to deuterated **4**, including small amounts of rearranged alkenes. In all reactions, base-catalyzed hydrolysis of the thioesters accompanied H-D exchange and D_2O conjugate addition; 15% exchange of **4** was accompanied by approximately 30% hydrolysis.

The hydrolysis that accompanies H-D exchange was used to determine the structure–chemical shift correlation for the deuterated thioester **4**. Earlier work had provided the nuclear magnetic resonance (NMR) chemical shift correlation for 2-deuterio-3-hydroxybutanoic acid (*21*), showing the deuteron of the 2*R**,3*R** diastereomer upfield (δ 2.24, H_2O). Deuteroxide-catalyzed H-D exchange of the α protons of β-hydroxybutyrate in D_2O is a clean but very slow reaction; 7% exchange occurs in 3 hours at 100°C with 0.5 M KOD. Therefore, the H-D exchange observed in the β-hydroxybutyrate recovered from the reaction of **4** must have occurred before hydrolysis of the thioester. The fact that the deuterated thioester **4** and its hydrolysis product, β-hydroxybutyrate, both have 81% of the 2*R**,3*R** diastereomer adds additional weight to the structure correlation. Because formation of the 2*R**,3*R** configuration arises from an *anti* process, there is a 4.3:1 bias toward *anti* addition of D_2O to the thioester **3**.

Our data show that conjugate addition of D_2O to the α,β-unsaturated thioester **3** produces 3.2 times as much *anti* addition of water as does the addition of D_2O to fumarate. This is exactly opposite to the stereochemistry of the enzymatic *syn-anti* dichotomy, where fumarate hydratase gives *anti* addition-elimination and enoyl-CoA hydratase gives *syn* addition-elimination of water. Because enoyl-CoA hydratase apparently uses the *syn* addition-elimination pathway for reasons other than chemical efficiency, this is direct evidence for the importance of historical contingency in enzymatic catalysis (*22*).

It is difficult to distinguish experimentally between convergent evolution and functional conservation during divergent evolution, and evolutionary proposals relating to the kinetics and stereochemistry of enzymatic reactions have not been without controversy (*23*). Benner *et al.* have argued that the stereospecificities of some classes of enzymatic reactions, including polar addition-elimination reactions, suggest that they were the targets of natural selection, whereas the stereospecificities of others suggest that they were not (*24*). It now seems that the reactions of at least some hydratase-dehydratase enzymes must be removed from the former class. Dorit *et al.* have pointed out that it seems unlikely that the corner of sequence space occupied by modern proteins represents the best of all possible worlds, a selective optimum reached after a careful evolutionary walk through all of sequence space (*1*). In the case of the *syn-anti* dichotomy of hydratase-

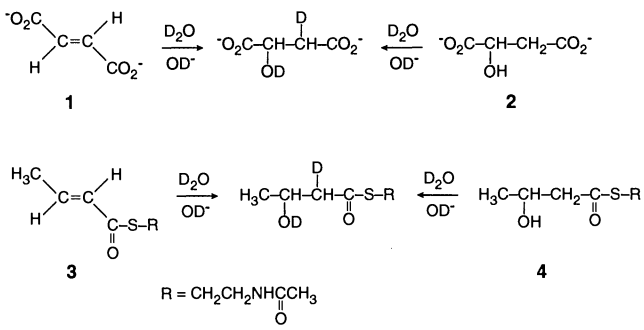

Fig. 1. Base-catalyzed addition of D_2O to fumarate (**1**) and *S*-crotonyl *N*-acetylcysteamine (**3**) and the related H-D exchange reactions of malate (**2**) and *S*-β-hydroxybutyryl *N*-acetylcysteamine (**4**).

Table 1. Diastereoselectivity of H-D exchange on malate and D_2O addition to fumarate, given as the percentage (±SD) of the *anti* addition product, the 2*R**,3*S** isomer of malate deuterated at C-3. In the structure–chemical shift correlation for 3-deuteriomalate, the deuteron of the 2*R**,3*S** isomer is the downfield portion of the AB pattern (*25*). Concentrations pertain to KOD.

Substrate	0.50 M, 100°C	0.05 M, 100°C	0.005 M, 100°C	0.50 M, 75°C	0.50 M, 50°C
2	57.3 ± 1.2	57.5 ± 0.4	57.6 ± 1.7	58.3 ± 0.1	58.3 ± 0.4
1	56.6 ± 1.3				

Table 2. Diastereoselectivity of H-D exchange and D_2O addition on *N*-acetylcysteamine thioesters of β-hydroxybutyrate and crotonate, given as the percentage (±SD) of the *anti* addition product, the 2*R**,3*R** diastereomer of **4** deuterated at C-2. Concentrations pertain to KOD.

Substrate	0.5 M, 23°C	0.1 M, 23°C	0.1 M, 50°C
4	82.0 ± 1.4	82	79 ± 1.4
3	80.5 ± 5.2		

dehydratase enzymes, this seems borne out in a convincing manner. The enzymatic *syn* addition-elimination of water with thioester substrates is not the most chemically efficient pathway but appears to depend instead on historical contingency.

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12. We have also examined the effect of solvent on these reactions. The deuteroxide-catalyzed H-D exchange of ethyl β-hydroxybutyrate in D_2O gives a 5.7:1 ratio of the 2*R**,3*R**:2*R**,3*S** diastereomers, in a pattern consistent with our other results. The 2*R**,3*R** notation refers to the absolute configurations at C-2 and C-3 and to the fact that this diastereomer is present as a racemic mixture.
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20. Because of concomitant hydrolysis, the pH of reaction mixtures dropped substantially from pH 13 to 9 over a 30-min period; in addition reactions, we added KOD to bring the pH back to 13 in order to produce sufficient adduct. Final purification of **4** was done on silica gel with a 3% methanol–97% ethyl acetate eluant. We separated the hydroxythioester and hydroxy-acid from inorganic salts by extraction of the crude lyophilized product with methanol, followed by further extraction with CH_2Cl_2 to dissolve the thioester. After purification of **4** on silica gel, ^2H NMR analysis (61 MHz) was carried out in a 3:2 or 4:1 benzene-methanol mixture, which provided a baseline between the integrated peaks (δ 2.58 and δ 2.43; C_6D_6 = δ 7.15). Exchange ranged from 15 to 37%, averaging 30% of one proton (seven reactions). Addition data were averaged from five reactions.
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Interaction of Papillomavirus E6 Oncoproteins with a Putative Calcium-Binding Protein

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Human papillomaviruses (HPVs) are associated with the majority of cervical cancers and encode a transforming protein, E6, that interacts with the tumor suppressor protein p53. Because E6 has p53-independent transforming activity, the yeast two-hybrid system was used to search for other E6-binding proteins. One such protein, E6BP, interacted with cancer-associated HPV E6 and with bovine papillomavirus type 1 (BPV-1) E6. The transforming activity of BPV-1 E6 mutants correlated with their E6BP-binding ability. E6BP is identical to a putative calcium-binding protein, ERC-55, that appears to be localized in the endoplasmic reticulum.

Infection with "high-risk" HPV, such as types 16, 18, and 31, can lead to malignancy, the most common of which is cervical cancer. Two viral transforming genes, *E6* and *E7*, are selectively retained and expressed in these cancers. Other HPVs such as types 6 and 11 are referred to as "low-risk" viruses because these are generally limited to benign genital and cervical papillomas that rarely progress to cancer. The high-risk HPV E6 genes induce immortalization of primary human epithelial cells either alone or in cooperation with *E7* [reviewed in (*1*)]. High-risk HPV E6 proteins bind the cellular factor E6-AP in vitro, and together these proteins bind and promote the ubiquitination and degradation of p53 (*2*, *3*). In cultured cells the introduction of HPV-16 E6 leads to increased p53 turnover (*4*, *5*), inhibits p53-regulated transcription (*6*, *7*), and blocks p53-induced G_1 growth arrest (*4*, *8*).

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Several observations suggest that papillomavirus E6 genes encode p53-independent transformation functions. HPV-16 E6 transforms NIH 3T3 cells but trans-dominant p53 mutants did not (*9*). We have found that HPV-16 E6 induces anchorage-independent growth of p53-deficient cells (*10*). The E6 genes from HPV-5 and HPV-8, BPV-1, and cottontail rabbit PV have oncogenic properties, yet these E6 proteins do not interact with p53 (*11*). To identify additional cellular proteins that interact with HPV-16 E6, we screened (*12*) a HeLa cell complementary DNA (cDNA) library (*13*) using the yeast two-hybrid system (*14*). After screening ~10^6 colonies on X-Gal plates, we isolated a HeLa cDNA encoding a protein referred to as E6BP (E6-binding protein) that specifically interacts with HPV-16 E6 (*12*).

Sequence analysis of the E6BP cDNA revealed a 210–amino acid open reading frame encoding a protein with four potential calcium-binding motifs, the EF hand (*15*), and a putative endoplasmic reticulum (ER) retention peptide (HDEL) at the COOH-terminus. E6BP is identical in sequence to ERC-55, a protein recently isolated on the basis of its reactivity with human auto-immune antiserum (*16*). The E6BP cDNA encodes a truncated version of