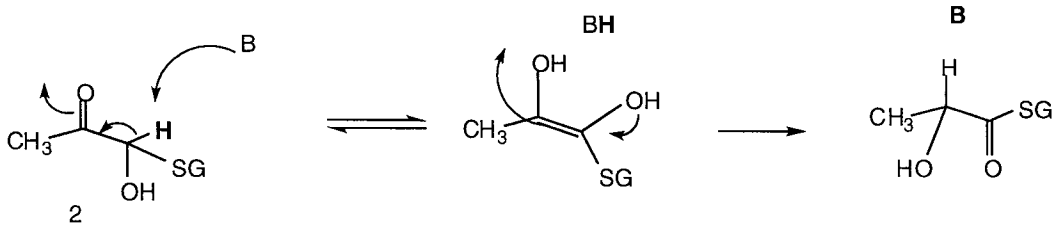
Answers to PS 6 Chem 5.50

1. Once the thiohemiacetal is formed (2), the mechanism can involve either a proton abstraction and a cis or trans ene-diol intermediate or a hydride transfer mechanism.



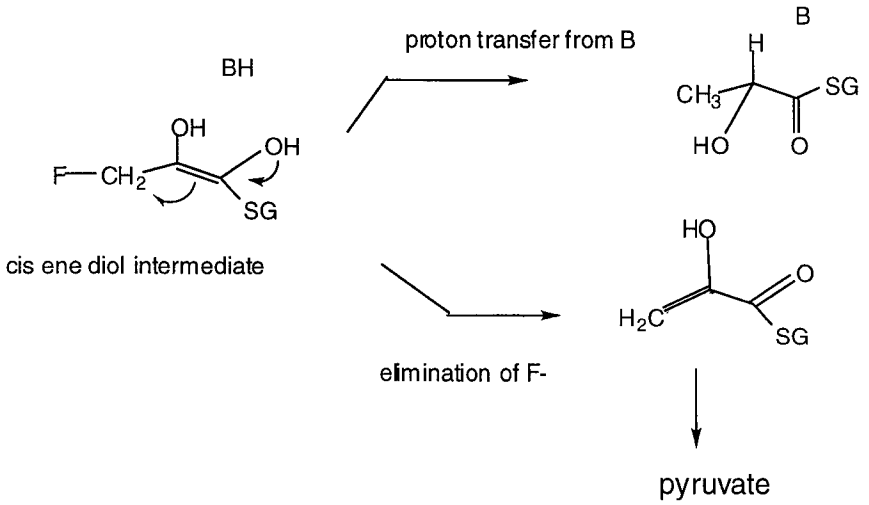
cis ene diol intermediate

hydride mechanism

The lack of exchange of tritium from the substrate to the solvent or the solvent to the substrate, does not permit a distinction to be made between these two mechanisms. The hydride mechanism requires no exchange. In the ene diol mechanism, the exchange is possible depending on the accessability of the BH in the active site to solvent and the lifetime of the cis-enediol intermediate. Thus if exchange is observed, then an enediol mechanism is required. No exchange could be rationalized by a closed active site and rapid proton transfer to the intermediate. In the this case the proton would be transferred from C-1 to C-2.

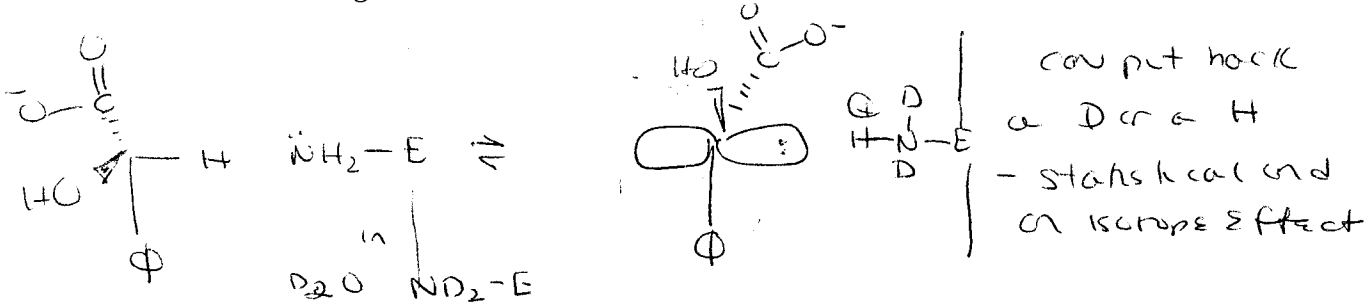
The results with fluoromethyl glyoxal are best rationalized through an enediol or ene diolate intermediate. The intermediate as shown below can partition between protonation by BH and the elimination of fluoride. (This type of partitioning is similar to the use of fluoroalanine with alanine racemase where one could potentially observe a partitioning between protonation and elimination though the carbanion intermediate.) If the Base is deuterated (BD), then there is an isotope effect on transfer of the proton to the ene diol slowing down the formation of the product resulting from that protonation. On the other hand, the elimination of fluroide is not effected by BD. Thus the amount of

pyruvate (the product of the elimination reaction) increases, consistent with the ene diol intermediate.



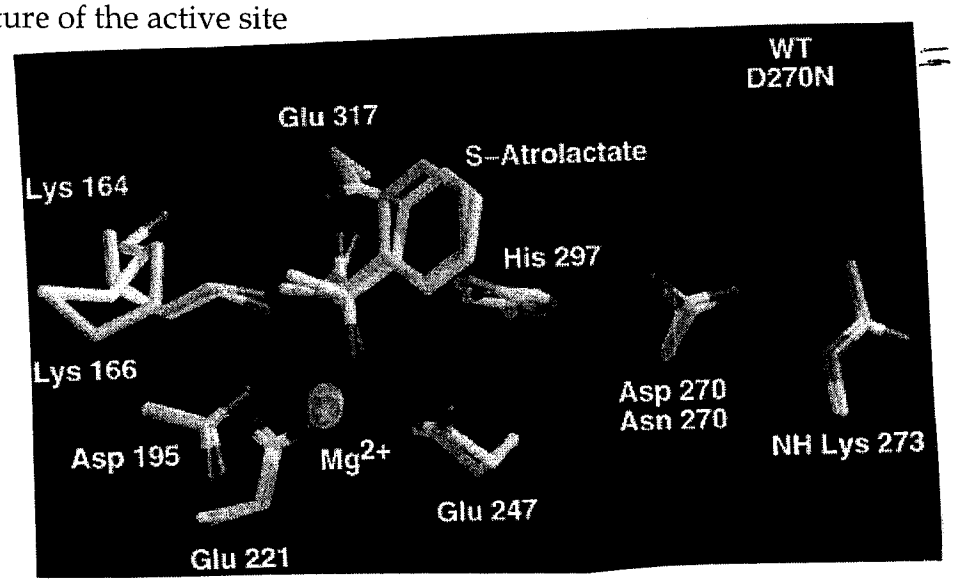
2. Mandelate racemase was discussed briefly in class as a founding member of the enolase superfamily. There are two possible mechanisms: a single base and a two base mechanism. In the two base mechanism, the structure and many studies suggest that lys 166 participates as an S specific acid/base catalyst and His297 participates as the R specific acid/base catalyst. A single base mechanism is also possible with histidine or lysine serving initially as a general base to remove the proton. A reorganization is then required of either the substrate or the enzyme's active site, followed by protonation with the same residue (now protonated), but on the opposite face of the carbanion intermediate. Given the difficulty of abstracting these very non-acidic hydrogens, the lifetime of the carbanion intermediate would be very short lived. This lifetime might make any re-organization mechanism difficult. In fact, the two base mechanism is the accepted mechanism from the data that you have been given. Strong support for the two base mechanism comes from use of mutants of H and K. In each case, one sees no racemization reaction, but one does see an exchange of the appropriate proton with the media. Thus the proton can be removed, and in the case of lysine reprotonated with one of its three protons that has exchanged with solvent.

Exchange but no racemization



Below is the structure of the active site of MR taken from Biochemistry 1996, 35, 5662-9. A key step in the mechanism is stabilization of the carbanion intermediate through electrostatic interactions with the carboxylate of the substrate and Mg^{2+} and K164 within the active site. In addition, the presence of two lysines (164 and 166) could depress the pKa of K166 sufficiently so that it is deprotonated, a requirement for it to function as a GBC.

Structure of the active site



The H297N mutant, can catalyze exchange of the proton of the S isomer of mandelate with the solvent, but cannot catalyze the exchange of the α proton with the R isomer. In addition, as noted above, no racemization is observed. These results suggest that H297 works specifically on the R enantiomer of mandelate. A similar experiment has been carried out with a K166R mutant. In this case, only the R mandelate can undergo exchange of the proton with one from solvent and racemization is not observed. No reaction occurs with the S mandelate.

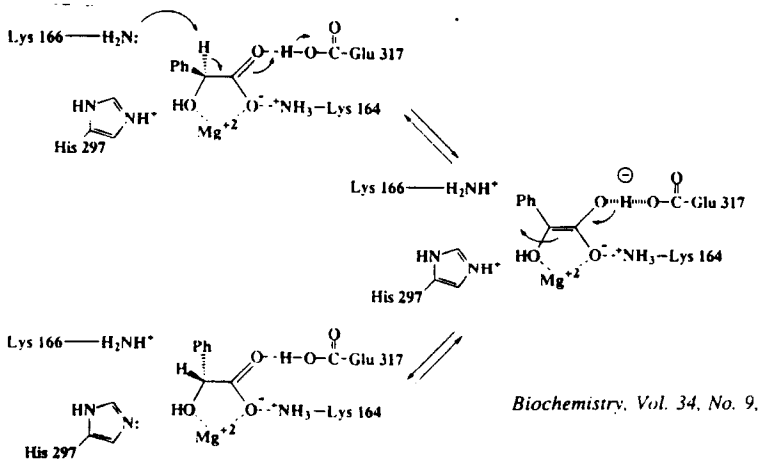
pH rate profiles have also been carried out on MR as indicated by the accompanying figures. For each enantiomer, the pH dependence of k_{cat} is described by a bell shaped curve, suggesting the participation of two ionizable groups. The rise phase or ascending limb suggests the requirement for a basic group with a pKa of 6.4 (value obtained from fits to the data) and descending phase suggests the requirement for an acidic catalyst with a pKa of 10.0. The results show that the pKa values are identical using R or S mandelate as the substrate. In the case of the H297N mutant, one only observes the rise phase, suggesting that K166 would be the group with a pKa of 6.4. Remember these are apparent pKas, not microscopic pKas and do not necessarily

reflect just groups involved in catalysis. The xray structure is very important in thinking about the pKas. The K166 and the H297 are very close to the essential Mg^{2+} and the K164. These charged groups can perturb the pKa of the groups involved in catalysis. In addition D270 that is negatively charged is adjacent to H297.

In the S to R direction, the pKa of Lys is proposed to be 6.4 and K is proposed to function as the base catalyst. The pKa perturbation could be the result of its juxtaposition to two positively charged residues, K164 and Mg^{2+}. In the R to S direction, the pKa of 6.4 would be associated with H297. Lack of its perturbation was rationalized by its interaction with D270.

How can one account for the apparent pKa of 10 in the pH rate profile in the R to S and S to R direction? Recall that changes in rate determining steps, denaturation of the protein, changes in conformation as discussed in the case of chymotrypsin can all effect pH rate profiles. The actual basis for the pKa at 10 is still being investigated.

The exchange data in Table 1 also support the two base mechanism. In the R to S direction at 5% extent of reaction, tells you that you have an intermediate, the carbanion and that the protons on the K and H can exchange with solvent. You would really need to look at the time course of wash in and wash out to obtain a quantitative model.



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If you have trouble finding the mandelate racemase sequence let me know and we can look for it together. If you use it to do a BLAST search you do find muconate lactonizing enzyme and enolase, although there is not much similarity. The ClustalW alignment (I was unable to print out the results) shows almost no similarity between MR and enolase. One gets 17% sequence similarity by just comparing any two proteins.