

October 16, 2000

ID number _____

Biochemistry Cumulative Exam

Very often you will run into areas with which you are unfamiliar. You will need to be able to apply your knowledge to other systems and generalize. The more you know and the more deeply you understand biochemistry, the better you will be able to perform.

The Premise:

You are on a job interview at an up-and-coming biotechnology company, and you are talking with the leader of the project who will make the decision on whether or not to hire you. After spending a few minutes discussing what you did during your Ph.D. research, she gives you this paper, and says "I have to leave for a few minutes to meet briefly with my group — it should not take more than 20 minutes. That should give you enough time to read this paper — it's fairly short. It has some implication for a new direction we want to take in this project and I'd like to discuss that with you and hear your thoughts on it."

Obviously, this discussion will test how good you are at assimilating new facts, understanding them, and drawing some conclusions — skills that any scientist must develop to be successful. This exam will model the interview and see how well you are able to understand and apply your knowledge. (Note: this premise is not all unrealistic!)

If you get offered the job, then you pass. For the purpose of this exam, you have to convince me by your written words that you can understand the contents of this paper well enough to make some educated guesses. (I am aware that some of the material will be unfamiliar to most of you. In fact, I had not heard of "tryptophan tryptophylquinone" until I picked up this paper! However, even without the benefit of looking up the references to the paper, you ought to be able to draw out the meaning and the main points.) First read the paper (its only 3 pages) and then try to answer the questions. You may not use any other materials or tools (including **calculators** — you would not have that on a job interview, would you?) besides what is contained in this exam and in your brain.

PASS

("We'd like to offer you a good job with stock options and benefits.")

FAIL

("Don't call us — we'll call you.... maybe...")

F. The authors say that the recombinant protein "possesses an engineered six-histidine tag at the C-terminus of the β subunit to facilitate purification." What does this mean? What is a "six-histidine tag" and how does it facilitate purification? Why do you think they put it at the C-terminus?

G. Briefly summarize the main point of the paper in one paragraph. What do *the authors* think is the significant accomplishment of this work?

After that, you may mention any criticism you have about this paper.

2. Time's up. The director comes back in the room and says:
"Ah, I see you've read the paper. What did you think — pretty interesting, eh?
I was especially struck by the fact that the k_{cat} values don't really change when you look at the different substrates — it's all controlled by K_M . Do you find that surprising? Of course, it is convenient when you want to compare the specificity constants very quickly — its really just a matter of comparing the K_M 's."

Put your response to this here:

(Remember: your point is to show that you understand what is going on without taking an hour to say it. Write down what you would actually **say**.)

"In fact," she continues, "I was amazed that k_{cat} actually went up in their mutant. They got really lucky, don't you think?"

Your response:

"Well, I hope we get so lucky. In our case, we are trying to come up with a biological catalyst for the oxidation of butanoic acid to *trans*-2-butenic acid. We get the stereospecificity for the *trans* isomer from the enzyme, of course."

"Of course," you say.

Does this make sense? Explain in one sentence why or why not.

"Luckily, nature has already made this catalyst for us — it is used during β -oxidation of fatty acids, as I'm sure you're aware, to convert acyl-CoA into enoyl-CoA, specifically the *trans* isomer."

"Oh, yes," you say, "you are talking about this reaction..." and you proceed to draw the general scheme of the reaction on the chalkboard in her office.

Do this here:

"What we do," she says, "is mimic the vitamin CoA by N-acetyl-2-aminoethanethiol, commonly called N-acetyl cysteamine, and make the thioester to butanoic acid. We can remove the N-acetylcysteamine afterwards by hydrolysis. "

Taking up the chalk again, you write out this structure. "So, the substrate should look like this, right? Oh, I see how this part mimics Coenzyme A", you say while indicating that part of the molecule.

Do this here:

"Very good," she says. "As you know, acyl-CoA dehydrogenase uses a flavin for the oxidation reaction, and it is coupled into the mitochondrial electron transport chain. We now have a soluble version of this enzyme from a newly discovered bacterium, *Geobacoccus getrichquickus*, and we can use artificial electron acceptors as a sink for the electrons. That's not a problem. The problem is that the enzyme has a strong preference for long acyl chains and we want to make butanoic acid. In a way, we are trying to achieve the opposite of what the authors of this paper were trying to do. Our X-ray crystallography team has succeeded in getting a 2.5-Å resolution structure, and we can see that the active site, near the flavin, is right next to a channel in the protein defined mostly by the interaction of two domains of the protein. This is where we think the acyl chain binds. It is mostly made up of smaller residues, like Ala, Gly, Ser, etc. Of course, it should be obvious what we are thinking of doing."

"Yes," you say, "what you ought to do is....."

Finish the thought.

"That's right", she replies. "That would give us an enzyme with preference for shorter acyl chains, like butanoyl-CoA. However, the members of my team are divided — some think that, even if you get better specificity for the shorter chains, you might not get *higher affinity* for them. What do you think about that?"

Your response:

"So, now you know what the project is. We have the gene for the *G. getrichquickus* ACDH (acyl-CoA dehydrogenase) cloned in a vector to overproduce it in *E. coli*. We put a His-tag at the C-terminus for rapid purification. The system works: we can get lots of active enzyme relatively quickly. As you know, time is money. And we may have competition. We need to get a catalyst for butenoic acid production as soon as possible. Tell me what you would do if you were given this project."

Your response:

Conversion of Methylamine Dehydrogenase to a Long-Chain Amine Dehydrogenase by Mutagenesis of a Single Residue[†]

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ABSTRACT: Methylamine dehydrogenase (MADH) is a tryptophan tryptophylquinone (TTQ) dependent enzyme that catalyzes the oxidative deamination of primary amines. Amino acid residues of both the TTQ-bearing β subunit and the noncatalytic α subunit line a substrate channel that leads from the protein surface to the enzyme active site. Phe55 of the α subunit is located at the opening of the active site. Conversion of α Phe55 to alanine dramatically alters the substrate preference of MADH. The K_m for methylamine increases from 9 μ M to 15 mM. The preferred substrates are now primary amines with chain lengths of at least seven carbons. The K_m for 1,10-diaminodecane is 11 μ M, compared to 1.2 mM for wild-type MADH. Despite the large variation in K_m values, k_{cat} values are relatively unaffected by the mutation. Molecular modeling of substrates into the crystal structure of the enzyme active site and substrate channel provides an explanation for the dramatic changes in substrate specificity caused by this mutation of a single amino acid residue.

Methylamine dehydrogenase (MADH)¹ from *Paracoccus denitrificans* catalyzes the oxidative deamination of methylamine to formaldehyde and ammonia, and subsequent electron transfer to a type I copper protein, amicyanin (1, 2). The structure of MADH is that of an $\alpha_2\beta_2$ heterotetramer (3). Each β subunit possesses a tryptophan tryptophylquinone (TTQ) prosthetic group (2) which is formed by posttranslational modifications of Trp57 and Trp108 of the β subunit (3). The substrate amine forms a covalent adduct with the C6 carbonyl of TTQ (4, 5). The C6 of TTQ is exposed in a hydrophilic area of the enzyme active site, which is connected to the enzyme surface by a short hydrophobic substrate channel. This channel includes two amino acid residues from the noncatalytic α subunit,² α Phe55 and α His54. α Phe55 is located at the position where the substrate channel

opens into the active site, and from the crystal structure, it appears as though α Phe55 may act as a gate which restricts access into and from the active site (Figure 1). We report here that conversion of α Phe55 to alanine by site-directed mutagenesis dramatically alters the substrate preference of MADH, converting it from a methylamine dehydrogenase to a long-chain amine dehydrogenase. Molecular modeling studies provide an explanation for the observed changes that are caused by the α F55A mutation, and suggest approaches that may be useful for protein engineering in altering enzyme substrate specificity.

EXPERIMENTAL PROCEDURES

Native MADH was purified from *P. denitrificans* as described previously (6). Site-directed mutagenesis studies were performed with recombinant MADH which is heterologously expressed in *Rhodobacter sphaeroides* (7) and which possesses an engineered six-histidine tag at the C-terminus of the β subunit to facilitate purification (8). The kinetic properties of the recombinant tagged MADH are very similar to those of the native wild-type MADH (8). The amines that were used as substrates were purchased from Sigma and Aldrich.

Site-directed mutagenesis was performed on double-stranded pMEG976 (8) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and two mutagenic primers following a previously described procedure (8). The primers used to create the mutation to convert α Phe55 to alanine were 5'-GTCAACGACCCGGCGCATGCTGCAGCGGT-CACCCAGCAATTCG-3' and its complementary sequence.

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¹ Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.

² The larger α subunit of MADH, sometimes termed the H subunit, is encoded by the *mauB* gene. The smaller β subunit of MADH, sometimes termed the L subunit, is encoded by the *mauA* gene. The numbering system used here for the MADH α subunit is based on the refined crystal structure of MADH (3). The residue number is different from that found in Protein Data Bank files 2MTA and 2BBK. To convert to the numbering system which is used in this paper, one must add 13 to the residue number as listed in these PDB files. The numbering for the β subunit is unchanged.

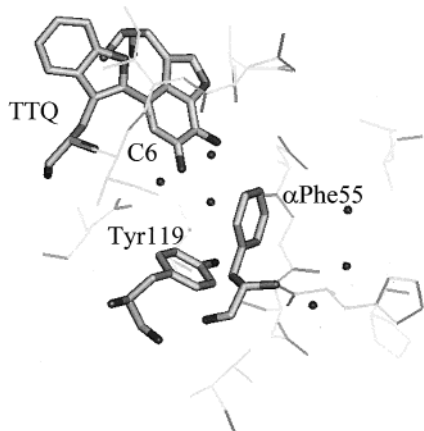


FIGURE 1: Positions of α Phe55 and β Tyr119 relative to TTQ and solvent molecules (dark spheres) present in the active site and substrate channel of MADH. The reactive C6 carbonyl of TTQ is also indicated. The coordinates for this MADH structure are available as PDB entry 2BBK (3).

The underlined bases are those which were changed to create the desired mutation, as well as to generate a new *PstI* site to facilitate screening for the mutation. The mutation was confirmed by sequencing 70 base pairs around the mutated site.

Steady-state kinetic assays (6) were performed in 10 mM potassium phosphate at pH 7.5 and 30 °C. The assay mixture contained 16 nM MADH, varied concentrations of substrates, 4.8 mM phenazine ethosulfate, and 170 μ M 2,6-dichlorophenolindophenol. The reaction was monitored at 600 nm to determine the rate of reduction of the latter. Data were fit to eq 1

$$v/E = k_{\text{cat}}[S]/(K_m + [S]) \quad (1)$$

where v is the measured initial rate, E is the MADH concentration, $[S]$ is the substrate concentration, k_{cat} is the turnover number, and K_m is the Michaelis constant.

Molecular modeling was performed using the QUANTA and CHARMM (Molecular Simulations) computer programs run on a Silicon Graphics O2 computer. The crystal structure of MADH that was used is PDB entry 2BBK (3).

RESULTS AND DISCUSSION

Steady-state kinetic analysis with a variety of amines as substrates revealed that the substrate specificity of α F55A MADH was dramatically different from that of native MADH (Table 1). The K_m value of methylamine increases 1700-fold from 9 μ M for native MADH to 15 mM for α F55A MADH. As the carbon chain length of the amine substrate increases from one to five, the corresponding K_m values increase for native MADH, whereas the K_m values decrease for the α F55A mutant (Figure 2). Monoamines longer than amylamine (1-aminopentane) are not soluble enough in aqueous solution to achieve concentrations necessary for the kinetic studies. Primary 1,*N*-diamines, which are more soluble than their corresponding monoamines, were tested as substrates to examine the effect of increasing the carbon chain length beyond five. For α F55A MADH, the K_m values are similar for amylamine and 1,5-diaminopentane. The K_m values decrease with increasing chain length, reaching a plateau at 1,7-diaminoheptane, with little change

Table 1: Substrate Specificities of Native and α F55A MADH^a

substrate	native MADH		α F55A MADH	
	K_m (μ M)	k_{cat} (s^{-1})	K_m (μ M)	k_{cat} (s^{-1})
methylamine	9 \pm 1	30 \pm 2	14900 \pm 1100	77 \pm 2
ethylamine	19 \pm 1	24 \pm 1	9200 \pm 1300	23 \pm 1
propylamine	36 \pm 2	27 \pm 1	1300 \pm 150	24 \pm 1
butylamine	870 \pm 59	22 \pm 1	240 \pm 28	34 \pm 1
amylamine	2500 \pm 290	17 \pm 1	47 \pm 5	20 \pm 1
1,5-diaminopentane	820 \pm 47	22 \pm 1	59 \pm 6	59 \pm 2
1,6-diaminohexane	720 \pm 83	17 \pm 3	21 \pm 73	43 \pm 6
1,7-diaminoheptane	380 \pm 46	27 \pm 1	7 \pm 1	32 \pm 1
1,8-diaminooctane	340 \pm 98	11 \pm 1	9 \pm 1	38 \pm 1
1,9-diaminononane	830 \pm 96	17 \pm 1	9 \pm 1	39 \pm 1
1,10-diaminodecane	1200 \pm 150	21 \pm 1	11 \pm 2	41 \pm 2

^a Assays were performed as described in the text. The standard errors of the fit of each data set are listed.

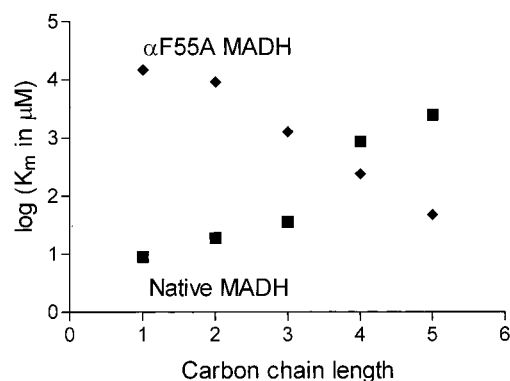


FIGURE 2: Relative substrate specificities of native and α F55A MADH. Data are taken from Table 1.

as the length increases to 1,10-diaminodecane. The K_m values of these 7–10-carbon diamines for α F55A MADH are approximately the same as the K_m value of methylamine for the native MADH. Despite the large variations in K_m values, the k_{cat} values for turnover in the steady state are relatively unaffected by the mutation and do not vary appreciably with substrate. Thus, this mutation does not appear to have changed the catalytic properties of MADH, beyond specifically altering its preference for substrates.

To understand how the alteration of a single amino acid residue can cause such dramatic changes in substrate preference, molecular modeling studies were performed. Substitution of α Ala55 for α Phe55 was carried out by computational mutation using the protein design function of QUANTA (Molecular Simulations). Modeling of the substrate 1,7-diaminoheptane into the native and mutant structures was carried out manually using the following criteria. The substrate nitrogen is placed within van der Waals contact of the C6 of TTQ, its expected position prior to nucleophilic attack (9). The carbon chain of the substrate is placed in the center of the substrate channel. In the wild-type structure, it is placed equidistant between α Phe55 and Tyr119 of the β subunit (Figure 3A). In α F55A MADH, it is placed equidistant between α Ala55 and Tyr119 (Figure 3B). This allows visualization of possible favorable interactions and likely unfavorable interactions between substrates of seven or fewer carbons and relevant amino acid residues. In native MADH, α Phe55 exhibits favorable van der Waals interactions with the substrate C1. Thus, with methylamine as a substrate, it will interact with the methyl group to help orient the substrate amino group for nucleophilic attack. This

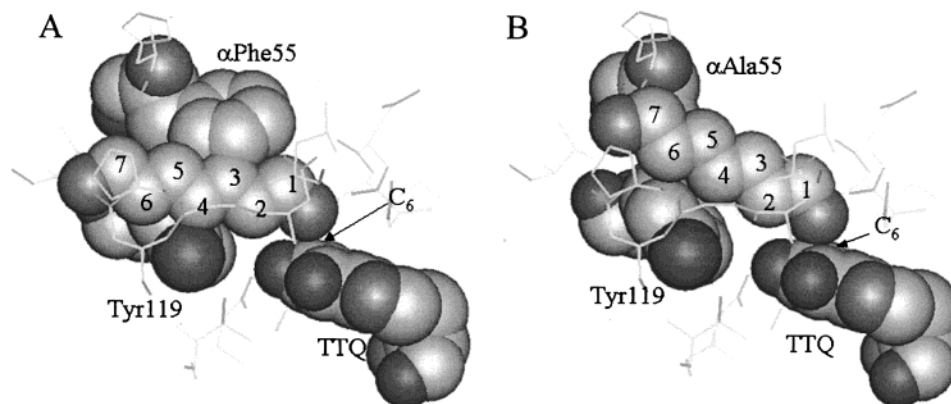


FIGURE 3: Molecular modeling of 1,7-diaminoheptane into the active site of (A) native MADH and (B) α F55A MADH. TTQ, α Phe55, Tyr119, and the diamine are drawn as space-filling models to highlight potentially favorable and unfavorable van der Waals interactions. Heteroatoms are indicated by darker shading.

stabilization is lost in the α F55A MADH, and this explains the very large increase in K_m for methylamine for α F55A MADH relative to that for native MADH.

For native MADH, a large increase in K_m occurs as the carbon chain length of the substrate is increased from three to five. As seen in Figure 3A, when the chain length increases to four carbons it is not possible to position the chain without causing unfavorable overlap of van der Waals radii with α F55A and Tyr119. For α F55A MADH, a corresponding large decrease in K_m occurs as chain length is increased from three to five. As seen in Figure 3B, when the chain length increases to four carbons it becomes possible to achieve favorable van der Waals interactions with Tyr119 since the space is no longer constricted by α Phe55. As the chain length increases to seven, additional favorable van der Waals interactions with Tyr119 and α Ala55 become possible. This explains the decrease in K_m values observed as the chain length of diamines increases to seven. Beyond a length of seven carbons, no additional stabilizing effects are evident from inspection of the structure. This accounts for the observed plateau in K_m values at this length.

It is noteworthy that mutation of a residue on the noncatalytic α subunit has such a profound and specific effect on activity, and addresses the question of why a cofactor dependent enzyme may require an additional subunit. The role of the α subunit of MADH is not simply to provide stability against denaturation. In fact, if the α and β subunits are resolved, the β subunit is much more stable than the α subunit or holoenzyme as judged by solubility and spectral properties.³ For MADH, the noncatalytic α subunit actually provides an amino acid residue which is critical for determining the substrate specificity of the enzyme. Since α Phe55 appears in the crystal structure to separate the active site from the substrate channel (Figure 1), it was not surprising that mutation to alanine allowed longer chain amines to be better substrates. It was, however, unexpected that the affinity for methylamine would be so greatly reduced. The molecular modeling studies provide a reasonable explanation for this observation. They also provide an explanation for the previous observation that the resolved β subunit, which retains the spectral features of MADH, is not reduced by methylamine (10). It is interesting to note that aromatic amine

dehydrogenase, another TTQ enzyme which prefers phenylethylamines, also has a very weak affinity for methylamine (i.e., $K_m = 38$ mM) that is similar to that for α Phe55 MADH (11). The structure of that enzyme is not known, but if its active site can accommodate aromatic amines, one would expect the much smaller methylamine to have access to TTQ. Its poor affinity for methylamine may reflect the absence of a residue which serves the function of α Phe55 in MADH. The characterization of α Phe55 as not only excluding large substrates from the enzyme active site but also helping to orient a preferred small substrate in a large active site describes an elegant and simple mechanism for determining substrate specificity. This may be applicable to other enzymes, and from a protein engineering perspective, it provides a simple approach for altering substrate specificity.

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³ V. L. Davidson, unpublished results.