Stereochemistry of NAD(P)-Coenzyme in the Reaction Catalyzed by Glycerol Dehydrogenase

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The coenzyme stereospecificities in the reaction of glycerol dehydrogenases (GDH) from various microbial sources have been determined. It is found that the nicotinamide-coenzymes (NADH and NADPH) employed by GDHs from eucaryotes exert the *pro-S* specificity, whereas those employed by procaryotes exert the *pro-R* specificity. The stereospecificity of the coenzyme seems to have a correlation with chemical evolution of GDH as well as biological evolution of the host organism.

Accumulating a lot of results on stereochemistry of oxido-reductases, Bentley proposed an experimental generalization, so called Bentley's rule,[1] which is now widely accepted.

The rule says that:

(1) The stereospecificity of a particular reaction is fixed and does not depend on the source of the enzyme.

(2) The stereospecificity of a particular reaction is the same in those cases in which both NAD^+ and $NADP^+$ can be used as coenzymes.

(3) If an enzyme utilized a range of substrates, the stereospecificity with respect to hydrogen transfer will remain the same with all substrates.

In addition to the above rule, it is interesting to note that both L- and D-lactate dehydrogenases, different enzymes (1.1.1.27 and 1.1.1.28) each other, use the same *pro-R* hydrogen of NADH to reduce pyruvic acid.[2-5]

The "rule 2" is striking because NADH usually has a different biological role from NADPH. For example, NADH is used in the degradation of fatty acids, whereas NADPH is employed in their synthesis.[6]

Benner is to be honored for his insight into summarizing chaotic stereochemistry of NAD(P)-coenzymes. He classified NAD(P)-dependent dehydrogenases into *pro-R* and *pro-S* specific ones[7-9] with respect to the reactivity of their natural substrates: less reactive substrates are reduced by more reactive *pro-S* hydrogen of NAD(P)H and vice versa. The proposal is quite interesting and suggestive from the viewpoint of chemical evolution of enzymes,[10] but, at the same time, was subjected to the criticism.[11-13] There appeared other proposals.[13,14] The biochemical stereochemistry-reactivity relationship has obtained an analogy in organic chemistry and the idea has been unambiguously proved to be valid.[15]

Although it is difficult to obtain a scientifically unambiguous proof for a proposal on biological and/or chemical evolution, it will be worthwhile to shed light on the stereochemistry of enzymatic reactions to understand the evolutionary process of proteins and other biologically important materials as well as its role for the host organism.

Bentley himself pointed out that there are some exceptions for the "rule 1." Namely, the enzymes associated with electron transport processes exert different specificity.[1] The second example for the violation of the "rule 1" is seen in alcohol dehydrogenase (1.1.1.1): those from yeast and horse liver use the *pro-R* hydrogen of NADH, whereas that from *Drosophila melanogaster* uses the *pro-S* hydrogen.[9] The difference in stereochemistry of the coenzyme is explained by means of physiological roles of these enzymes for the survival of host organisms and has been nominated as an evidence to support the Functional Model of chemical evolution.[9] The Historical Model should not allow such a divergence in stereochemistry of isozymes.

We would like to report in this paper that the third example of the violation exists in a series of glycerol dehydrogenases (GDH, 1.1.1. 6). Such a number of violation seems to suggest that Bentley's "rule 1" is no more a correct prediction and should be withdrawn.

Results and Discussion

GDH catalyzes the oxidoreduction between glycerol and dihydroxyacetone and the equilibrium constant of this reaction lies in the borderline of the *pro-R* and *pro-S* specificities based on Benner's classification.[8] Although GDH has been classified by Benner into the *pro-R* specificity, experimental results reported in literatures appear messy: NADP-dependent GDH from *Mucor javanicus* shows the *pro-S* specificity,[16] which is opposite to the stereochemistry in the reaction with NAD-dependent GDH from *Aerobacter aerogens*[17] and *Bacillus megaterium*.[13] Although Bentley's "rule 2" predicts that NAD and NADP-dependent GDHs behave similarly, the rule has no guarantee to be universal and it is desirable to find an example of NAD-dependent GDH which exibits the *pro-S* specificity in order to claim the enzyme of this class to be the third example of scattered stereospecificity.

Three other GDHs that are dependent on NAD-coenzyme were, therefore, subjected for investigation. They are GDHs from *Geotrichum candidum, Cellulomonas species,* and the one from a Bacterium (or

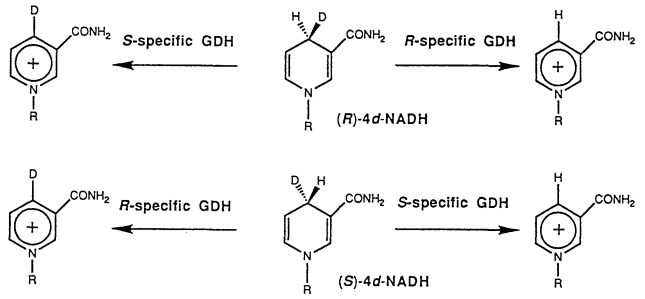
Bacteria).[18] GDH from *Bacillus megaterium* was also employed for the present study to confirm the validity of our results by comparing them with those from others.

The reduction of dihydroxyacetone with (4*R*)- or (4*S*)-NADH-4-*d* in D_2O at pH 7 was run in an NMR sample tube in the presence of a GDH, and the reaction was monitored on a 200 MHz ^1H NMR spectrometer. The stereospecificity of the reaction was determined by observing the presence or absence of a doublet at 8.9 ppm, which is the signal from the proton at the 4-position of NAD^+.

The results are summarized in Table 1 together with those from literatures, from which it is obvious that the enzyme from *Geotrichum candidum* is the first example for a *pro-S* specific NAD-dependent GDH and that GDHs from different sources exert different stereospecificity.

Based on the Fundamental Model for the chemical evolution of an enzyme, it is possible to predict that GDHs from *Mucor* and *Geotrichum*, which are *pro-S*-specific, are evolved as dihydroxyacetone reductase and the other GDHs, which are *pro-R*-specific, are evolved as glycerol oxidases, based on redox potentials of these chemicals.[8,10] However, since most of GDHs from these microbes are not characterized, we do not know if these enzymes can be categorized into large/small-molecular-weight subunit of enzymes,[14] and/or into Zn^{2+}-dependent/independent enzymes.[13] At the same time, we think that the classification of enzymes by their molecular weight has no scientific background because we cannot predict any biological role for the molecular-weight of an enzyme.

Table 1. Stereospecificity of NAD-Coenzyme in the Reduction of Dihydroxyacetone Catalyzed by GDH



Source of GDH (1.1.1.6)	Signal at δ=8.9 ppm NADH-4-*d* Starting		Stereo-specificity
	From 4*R*	From 4*S*	
Mucor javanicus[a]	—	—	*pro-S*
Geotrichum candidum[b]	No	Yes	*pro-S*
Cellulomonas species[c]	Yes	No	*pro-R*
Bacillus megaterium[d]	Yes	No	*pro-R*
Aerobacter aerogens[e]	Yes	No	*pro-R*
Bacterium (or Bacteria)[f]	Yes	No	*pro-R*

a) Ref. 16. b) Amano Co. c) Toyobo Co. d) Toyo Jozo Co. and Ref. 13. e) Ref. 17. f) Oriental Yeast Co. and Ref. 18.

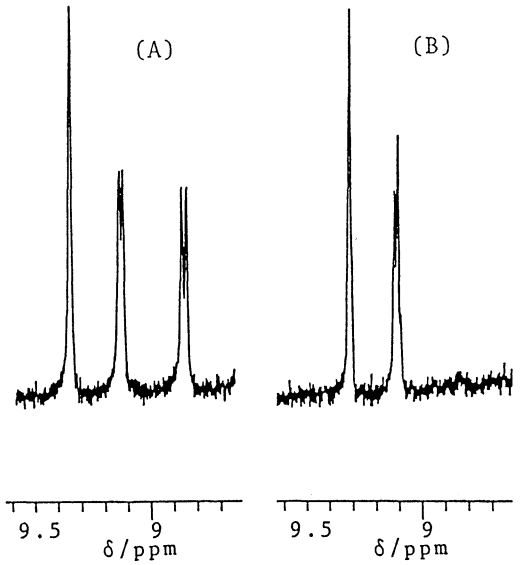


Fig. 1. ^1H NMR spectra of the reaction mixture of (*R*)-NADH-4-*d* with GDH from (A): *Cellulomonas species* (B): *Geotrichum candium.*

In addition to these possibilities, we can propose the third classification: the microbes *Mucor* and *Geotrichum* belong to a group of eucaryotes and others to procaryotes. Eucaryotes and procaryotes might have different GDH to satisfy their intrinsic (biological) survival conditions. In other words, the GDHs have received biological pressure from their host organisms in their evolutionary processes and the biological pressure acted differently in eucaryotes from procaryotes. We have reported a chemical evidence and theory to support the Functional Model.[15,19] The present and other[9] evidence seem to suggest that the biological pressure from the host organism is quite important to make a biochemical reaction run on a particular potential energy surface of the reaction. In this sense, our proposal for the classification of enzymes (eucaryote/procaryote) seems more reasonable than those based on molecular-weight and/or requirement of a cofactor. Of course, we have to answer to many questions before the validity of our proposal is confirmed; how eucaryotes and procaryotes are different each other from the viewpoint of biochemistry; how different the biological pressure from these entirely different organisms on their own biochemical processes; and so on. At the same time, many other examples are necessary for any of the proposals.

Experimental

Instruments. ^1H NMR spectra were recorded on a Varian VXR-200 spectrometer in D_2O with sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 as an internal reference. UV spectra were obtained on a Union Giken SM-401 spectrometer.

Glycerol Dehydrogenases. GDHs from *Geotrichum candidum* (246 unit ml^{-1}) and *Bacillus megaterium* (42 unit mg^{-1}) were kindly supplied by Amano Pharmaceutical Co. and Toyo Jozo Co., respectively. Those from *Cellulomonas species* (50 unit mg^{-1}) and Bacterium (or Bacteria) (100 unit mg^{-1}) were purchased from Toyobo Co. and Oriental Yeast Co, respectively.

(4*R*)-NADH-4-*d*. A 200 ml round-bottomed flask containing 100 mg of β-NAD^+ (purchased from Kohjin Co.), 10.0 mg (21 units) of horse liver alcohol dehydrogenase (HLADH) purchased from Sigma Co., 1 ml of ethanol-d_6, and 100 ml of pH 9 phosphate buffer solution (50 mM, M=mol dm^{-3}) was kept in an ice-bath for 3 h. The mixture was concentrated under reduced pressure and the residue was subjected to column chromatography on 100 ml DEAE-Toyopearl 650 (22 mmϕ×160 mm length). NAD^+ was eluted by pH 8 phosphate buffer (1.0 mM) monitoring the absorption at 260 nm. Then, NADH-4-*d* was eluted by subjecting 0.2 M ammonium carbonate-pH 8 phosphate buffer (1.0 mM) solution monitoring the absorption at 340 nm. The eluent containing NADH-4-*d* was collected and ammonium carbonate was removed under reduced pressure from the freezed substrate. The remained powder (971 mg) contained 68 mg (68% yield) of (4*R*)-NADH-4-*d*. The deuterium content and enantiomeric excess in the product were measured on ^1H NMR spectrometer to be both more than 95%. No signal corresponding to the 4-*pro-R* proton was observed.

(4*S*)-NADH-4-*d*. NAD^+-4-*d* prepared from NAD^+ and alkaline potassium cyanide in D_2O according to the literature method[20] was subjected to the reduction with ethanol mediated by HLADH as described above. The deuterium content and enantiomeric excess in the (4S)-NADH-4-*d* thus obtained and employed for the reaction were 81% and more than 95%, respectively.

Reduction of Dihydroxyacetone. The reduction of dihydroxyacetone (7.2 mg) with (4*R*)-NADH-4-*d* (7.7 mg) was done in an NMR sample tube with 0.7 ml of D_2O at pH 7.0 as the solvent in the presence of GDH (5—30 unit). The reaction was monitored on a 200 MHz ^1H NMR spectrometer. As shown in Fig. 1, the signal from the proton at the 4-position of NAD^+ appears at around δ=8.9 ppm when the enzymatic reaction proceeds with the *R*-specificity, whereas the reaction with the *S*-specificity does not show this signal because the deuterium at the 4-position of NADH is abstracted in the former case but the protium is abstracted in the latter. The reduction with (4S)-NADH-4-*d* was monitored in the same way as described above. Of course, the appearance/disappearance of the signal at δ=8.9 ppm is reversed herein from the above mentioned reactions. However, since the deuterium content in this coenzyme is only 81%, the signal at 8.9 ppm did not disappear completely.

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