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Review

Progress in the Reaction of Pyridine Nucleotide-Dependent Enzymes. Part II.

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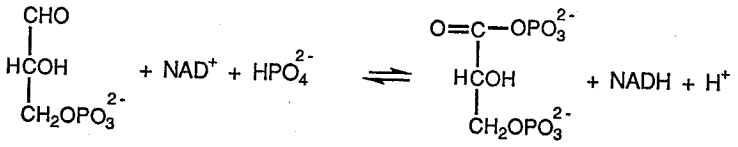
1. DEHYDROGENATION OF ALDEHYDES

2.1 Glycerakdehyde-3-phosphate Dehydrogenase

2.1.1 Introduction

Glyceraldehyde-3-phosphate dehydrogenase [GAPDH; EC 1.2.1.12][1] is an NAD^+-dependent enzyme which reversively catalyzes the oxidative pohospholylation of glyceraldehyde to 1,3-diphosphoglycerate, playing an important part in the metabolism of carbohydrates.



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This apparantly complex reaction proceeds in a multi-step manner.[2-6] 1) Initial step is the formation of a hemithioacetal intermediate between a substrate and a sulfhydryl group from the enzyme, a rational strategy for dehydrogenation of aldehydes. The essential and highly reactive sulfhydryl group has been well characterrized.[7-16] 2) Subsequently, a rapid dehydrogenation by NAD^+ from the hemithioacetal forms an acyl-enzyme intermediate.[17] 3) Then, follow the dissocia-

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tion of the resulted NADH and 4) association of the next $NAD.^+$ 5) Finally, phosphorolysis of the high energy bond of thioester reultsin the formation of the acylphosphate product in the presence of newly bound NAD^+.[18-21]

The overall result is the oxidation of aldehyde with efficient preservation of free energy. The rate-determining step of the oxidative phospholylation is NADH release at high pH and phosphorolysis of the acyl-enzyme at low pH. For the reverse, the rate-determining step is the process associated with the binding of NADH.[3] The requirement for coenzyme substitution in the cycle may be playing a significant role *in vivo* in the metabolic control of glycolysis.

GAPDH's from various sources including lobster muscle,[3,18,18,15-31] rabbit muscle,[16,17,32-37] stergeon muscle,[3,16,19,38-41] pig muscle,[4,19,35,42] yeast,[43-50] *Bacillus stearothermophilus*,[51-54] and some other bacteria[55-58] have been extensively studied. The enzyme is, so far as known, tetrameric with a molecular weight of about 145,000. Although the enzyme is usually[44] composed of chemically identical subunits,[22,42,52,55] allostericity in the ligand binding has been well documented. Muscle and bacterial enzymes exhibit negative cooperativity,[58] whereas yeast enzyme shows complex response to the NAD^+ binding including positive cooperativity.[59-61] X-ray structures at high resolution have been determined for the enzymes from lobster muscle[23-28] and from *B. stearothermophilus*.[51] Muscle enzymes have been extensively used for kinetic studies. Recent interests have been focused on the mechanism to make the enzyme exert the cooperativity.[32-35,38-40] Crystallographic studies with lobster muscle[25,27-29] and bacterial[51,54,57] enzymes have also been much concerned with the problem of molecualr symmetry in connection with the allostery. The yeast enzyme has been employed particularly in studies to prove the mode of the substrate binding[46,47] as well as of the cooperativity.[45] In addition, multiple forms of yeast GAPDH have been reported.[44,49]

2.1.2 Molecular Structure

The X-ray structures that have been determined on the lobster (*Homarus americanus*) enzyme involve the E-NAD^+ holo complex,[23-2] E-8-Br-NAD^+,[26] abortive ternary complexes (E-NAD^+-citrate[26] and E-NAD^+-trifluoroacetone[27]), "meso" complex containing about one NAD^+ per tetramer,[26] and apoenzyme.[28] Employing the enzyme from *B. stearothermophilus*, the structure of the holo and apo enzymes[51] and further of a partially ligated E-NAD^+ complex with one NAD^+ per tetramer ($E(NAD^+)_1$)[54] have also been investigated in detail.

Each subunit of GAPDH is divided into two domains[23,51] as in LADH, *i.e.*, the coenzyme binding domain (residues 1–147) and the catalytic domain (148–334). These domains are separated by the acitve site cleft with the essential Cys-149 in its center (Fig. 1). The coenzyme binding domain is again very similar to those in other dehydrogenases. The conformation of enzyme-bound NAD^+ is in an open form and is essentially the same as those in LDH and LADH except for a 180° rotation around the ribose-nicotinamide glycoside bond, which causes the B side specificity in the hydride-equivalent transfer in GAPDH reaction. Importantly, NAD^+ binds at sites close to subunit interfaces to form an intersubunit acitve site,[23,51,62] whereas LDH

has a catalytic site within a subunit and this site faces the outside of the molecule. The Q-axis dimers are associated in opposite ways in GAPDH and LDH.

AVKVGINGFGRIGRNVFRAALKNPDIEVVAVNDLT NADGLAHLLKYDSVHGRLDAEVVVNDG
HKVGINGFGRIGROVFRILHSRG VEVALINDLT NDKTLAHLLKYDSIYHRFPGEVAYDDQ
VKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLHYMVYMFEYDSTHGKFHGTVKAEDG
SKIGIDGFGRIGRLVLRAALSCG AQVVAVNDPFIALEYMVYMFKYDSTHGVFKGEVKHEDC
VRVAIDGFGRIGRLVMRIALSRPBVZVVASBBPFIBLDYAAYMFKYDSTHGRYAGEVSHDDK

DVSVNGKEIIVKAERNPENLAWGEICVDIVVESTGRFTKREDAAKHLEAGAKKVIISAPAKVE
YLYVDGKAIRATAVKDPKEIPWAEAGVGVVIESTGVFTDADKAKAHLEGGAKKVIITAPAKGE
KLVIDGKAITIFQERDPANIKWGDAGTAYVVESTGVFTTMEKAGAHLKGGAKRVIISAPSKAD
ALVVDGKKITVFNEMKPENIPWSKAGAEYIVESTGVFTTIEKASAHFKGGAKKVVISAPSKAD
HIIVDGKKIATYQERDPANLPWSSGDSVIAIDSTGVFKELDTAQKHIDAGAKKVVITAPSKST

NITVVMGVNQDKYDPKAHHVISNASCTTNCLAPFAKVLHQEFGIVRGMMTTVHSYTNNQRILD
DITLVMGVNHEAYDPSRHHIISNASCTTNSLAPVMKVLEEAFGVEKALMTTVHSYTBBZRLLD
APMFVMGVNHEKYDPNSLKIVSNASCTTNCLAPLAKVIHDHFGIVEGLMTTVHAITATQKTVD
APMFVCGVNLEKYSPKDMTVVSNASCTTNCLAPVAKVLHENFEIVEGLMTTVHAVTATQKTVD
APMFVMGVDEEKYTPSDLKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSLTATQKTVD

LP HKDLRGARAAAESIIPTTTGAAKAVALVLPELKGKLNGMAMRVPTPNVSVVDLVAELEKE
LP HKDLRRARAAAINIIPTTTGAAKATALVLPSLKGRFDGMALRVPTATGSISDITALLKRE
GPSGKLWRDGRGAAQNIIPASTGAAKAVGKVIPELDGKLTGMAFRVPTPNVSVVDLTCRLEKP
GPSAKDWRGGRGAAQNIIPSSTGAAKAVGKVIPELDGKLTGMAFRVPTPDVSVVDLTVRLGKE
GPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVBVSVVDLTVKLDKE

VTVEEVNAALKAAAEGELKGILAYSEEPLVSRNYNGSTVSSTIDALSTMVIDGKMVKVVSWYD
VTAEEVNAALKAAAEGPLKGILAYTEDEIVLZBIVHDPHSSIVDAKLTKALGNMXXKVFAWYD
AKYDDIKKVVKQASEGPLKGILGYTEDQVVSCDFNDSTHSSTFDAGAGIALNDHFVKLISWYD
CSYDDIKAAMKTASEGPLQGFLGYTEDDVVSSDFIGDNRSSIFDAKAGIQLSKTFVKVVSWYD
TTYDEIKKVVKAAAEGKLKGVLGYTEBAVVSSBFLGSBHSSIFDASAGIQLSPKFVKLVSWYD

NETGYSHRVVDLAAYINAKGL	*B. stearothermophilus*
NEWGYANRVADLVELVLRKGV	*T. aquaticus*
NEFGYSNRVVDLMVHMASKE	Pig Muscle
NEFGYSQRVIDLLKHMQKVDSH	Lobster Muscle
NEYGYSTRVVDLVEHVAKA	Yeast

Fig. 1. Amino acid sequences for various GAPDH's..

A; Ala, C; Cys, D; Asp, E; Glu, F; Phe, G; Gly, H; His, I; Ile, K; Lys, L; Leu, M; Met, N; Asn, P; Pro, Q; Gln, R; Arg, S; Ser, T; Thr, V; Val, W; Trp, Y; Tyr, X; unidentified.

An irregular S-shaped loop region (S-loop; 178–201) in the catalytic domain, extending across the R-axis into the neighboring subunit, is characteristic for GAPDH and responsible for the construction of the intersubunit active site. Some residues such as Pro-188 in the S-loop are situated in the position to lock" the adenosine moiety of the coenzyme in the adjacent active site. Thus, the cooperativity in the coenzyme binding[58-61] and the half-of-the-sites reactivity toward some acylating and alkylating reagents specific to the active site[53-65] might be related closely to this structural feature. In addition, the S-loop is also in contact with several amino acid residues across the P-axis as a small part of the most extensive interfacial contact of subunits that relate to the P-axis.[23,51] The sequences of the S-loop region, which forms the core of the tetramer as mentioned above, have been highly conserved within three enzymes from mesophiles, but much differ from those in two enzymes from thermophilic bacteria, although the latter two are very similar to each other. On the other hand, residues involved in subunit interaction across the P and Q axes are highly conserved even between the enzymes from mesophiles and those from thermophiles.

All residues implicated in substrate binding and cataysis are conserved except the residue 181 among at least five sequenced enzymes[53] (Fig. 1), and thus we could assume essentially the same molecular mechanism for these various GAPDH's. These mainly include the following: 1) Cys:149 and His-176; for substrate binding and catalysis. His-176 is located near the essential Cys-149 and is believed to act as a multifunctional acid-base catalyst. The well documented high reactivity of Cys-149 seems to be attributable to the formation of ion pair with His-176 with a pKa value of 5.5 in the apoenzyme.[13] In the holo complex of GAPDH (E-NAD^+), further modulation of the Cys-149 state might be introduced possibly by the formation of a charge transfer complex with nicotinamide,[23,51,56] which, assuming the occurrence, has been usually believed as a main cause of the "Racker band", or a characteristic absorption band at $\lambda_{max}=360$ nm.[67] Recent experimental results from the affinity labeling by a coenzyme analog, 3-chloroacetylpyridine adenine dinucleotide, are, however, apparently inconsistent with the attribution: the analog which reacts with Cys-149 exerts a "Racker band"-like spectrum although some other model compounds do not have such abosrption. A possible participation of Try-317 has been discussed as the origin of "Racker band".[16] Even if the latter is the case, however,

it seems still reasonable to assume the existence of electrostatic interaction between Cys-149 thiolate and positively charged nicotinamide-ring of the coenzyme in the course of the GAPDH reaction. Furthermore, His-176 is, of course, also expected to interact with the hydroxyl group of the intermediate hemithioacetal to extract a proton from the substrate in the oxidation step as seen in LDH reaction. 2) Try-311, Ser-238[23] and Thr-174[51] may be for catalysis through hydrogen bonding with His-176 (See 2.1.3). The formation and breakage of these bonds involving His-176 is believed to be critical in catalysis. 3) Thr-179 and Asp-181 (Thr-181 in mesophiles) and probably Lys-191 and Arg-231; for substrate phosphate binding. The site which is apparently created by the presence of 2′-hydroxyl group of the NAD^+-ribose[23,51] is one of the two anion binding sites seen in both lobster[23,56] and *Bacillus*[51] enzymes, and estimated as the substrate phosphate site by model building.[23,51] Arg-231 and Lys-191 are likely to participate in the charge balancing. Arg-231 might have a significant catalytic role as supposed by Rossmann and Grau (See 2.1.3). Thr-179, Asp-181, and Lys-191 are all in the S-loop and Arg-231 is surrounded by side chains of the S-loop. Thus, the S-loop is a linker between the coenzyme binding site in one subunit and the catalytic site in another subunit. These subunits face each other with respect to the R-axis. 4) Ser-148, Thr-150, and Thr-208; for the inorganic phosphate binding. The greater distance of the site from the nicotinamide than that of other anion binding sites reasoned the attribution. Ser-148 also interacts with the substrate C_2-hydroxyl group.

2.1.3 Substrate Binding and Mechanism for Catalysis

The structural information described in 2.1.2 has made it possible to speculate the molcular mechanism for each step in the overall ping-pong (enzyme substitution) reaction of GAPDH (See 2.1.1).

A model of hemithioacetal intermediate shows that the hydrogen atom on C_1 points the nicotinamide at a distance of less than 3.0 Å, with the substrate phosphate indeed falling into the substrate phosphate site".[23] The feature is consistent with the results from the studies on aldehyde binding.[47] K_m values for a series of aldehydes to yeast GAPDH are correlated by Taft ρ^* value of 1.7, which coincides with that for a model hemithioacetal formation between aldehydes and glutathione. Thus, there is no doubt that hemithioacetal is formed at the initial stage of the enzymic reaction. The study also revealed a specific affinity of the substrate phosphate for the enzyme with a positive deviation from the linear correlation. The phosphate group on the substrate C_3 not only contributes to the substrate specificity (reactivity)[68,69] in large extent in combination with the C_2-hydroxyl group but also enhances the stereoselectivity of the enzyme for this hydorxyl group[70]

In the resting holo enzyme, His-176 which probably forms an ion pair with Cys-146 seems to be in contact with Tyr-311 by hydrogen bonding[23] (Fig. 2a). The substrate binding might break the ion pair between Cys-149 and His-176 and a "charge transfer complex" between Cys-149 and nicotinamide-ring to form a reactive thiolate anion and protonated His-176 as demnostrated recently by Kellogg and his coworkers with a non-enzymic model system[71] (Fig. 2a→Fig. 2b). The protonated

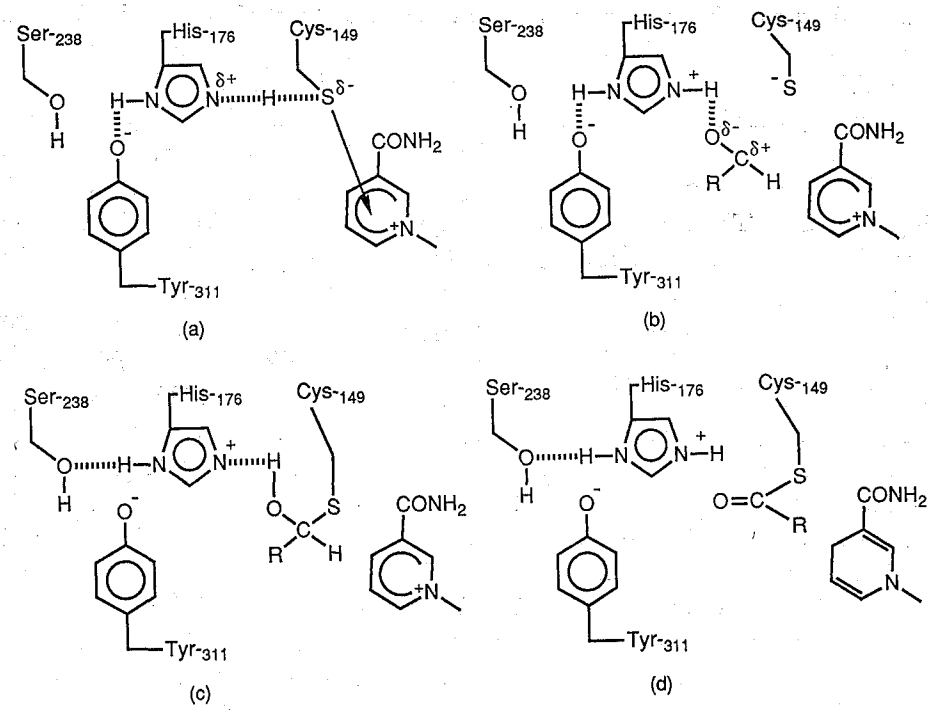


Fig. 2. Schematic illustration for the reaction mode with GAPDH.

His-176 in turn immediately protonates the carbonyl oxygen of the substrate aldehyde, which facilitats the nucleophilic attack at substrate C_1 by the Cys-149 thiolate to form a hemithioacetal tetrahedral intermediate (Fig, 2b→2c).

The hydride-equivalent transfer from the C_1 of the substrate to C_4 of the nicotinamide results in the formation of an acyl-enzyme intermediate. At the same time, His-176, which in this time is supposed to be coupled with Ser-238,[23] abstracts a proton from the C_1-hydroxyl group (Fig. 2c→2d).

After the displacement of the resulted NADH by NAD^+, the acyl transfer[46] from Cys-149 to the third substrate, phosphate, occurs. The acyl-enzyme and also acyl-enzyme-NAD^+ complex have at least two conformers, *i.e.*, reactive and unreactive ones toward the acyl transfer.[20,21] The "unreactive" conformer is probably active toward the reduction, instead. A conformational change thus has to occur prior to the acyl transfer.

The quintessence of the requirement for NAD^+ in the phosphorolysis step[18-21] might be the electrostatic effect which not only entices the phosphate toward the active center but also stabilizes the transition state by setting the nicotinamide close to Cys-146, where a negative charge develops. According to Biesecker and his coworkers, the attacking phosphate in the transition state can be stabilized by hydrogen bonds with Ser-148, Thr-150, the amide nitrogens of Cys-148 and Thr-150, and C_2-hydroxyl group of the substrate,[13-51] which may explain the fact that water is a poor substrate for the acyl transfer step: water reacts about 5×10^4 times less rapidly than

phosphate at pH 8.5.[72]

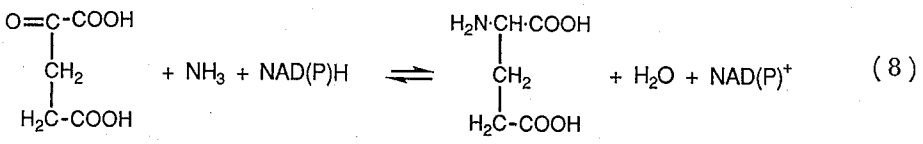
A possible role of Arg-231 to destabilize the Michaelis complex acyl-enzyme-NADH by charge repulsion was implicated in GAPDH action. The initial suggestion by Rossman and his co-workers[73] has been developed by Grau[74]. The proposed mechanism sets great store by a balance of charge repulsions as supposed for LDH reaction (See 1.2.3).

3. REDUCTION OF SHIFF-BASES

3.1 Glutamate Dehgdrogenase

3.1.1 Introduction

Glutamate dehydrogenase [GDH; EC 1.1.1.2–4][75] catalyzes the interconversion of α-ketoglutalate and L-glutamate.



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The enzyme serves in regulating carbohydrate and nitrogen metabolism. Therefore, it occupies an important position as a linker and is usually under the control of various metabolites. Three types of GDH which differ in coenzyme specificity have been known. NAD^+-specific [EC 1.4.1.2] and $NADP^+$-specific [EC 1.4.1.4] enzymes are widely distributed among microorganisms, fungi, and plants, with the former bing "biosynthetic" and the latter '·catabolic". GDH's from animals, some fungi (exclusively located in the mitochondria[75–77]), and from some bacteria utilize both NAD^+ and $NADP^+$ [EC 1.4.1.3]. Of the animal enzymes, the bovine liver enzyme has been the most studied. The enzyme is a hexamer comprised of six identical subunits of molecular weidth of about 56,000.[78,79] $NADP^+$-specific GDH[80–82] shows considerable homology to the bovine liver enzyme, whereas tetramer structure is often seen with NAD/-specific GDH's[83–87] The hexamer form of the bovine liver enzyme is composed of two identical triangular layers and is essential for catalytic activity.[88] The activity is asymmetrically distributed among the six subunits,[89] which is indicative of intersubunit acitve sites as seen in GAPDH. The GDH is thus an allosteric enzyme which is furhter regulated by purine nucleotides in a complex fashion. Here, ADP acts as an activator above pH 7 but GTP is a strong inhibitor.[90–98] NADH (NAD^+) in high concentration also depresses (stimulates) the enzymic activity.[91,100–104] The nucleotides bind to distinguishable but mutually interacting sites. The nucleotide binding sites of the enzyme include one site for ADP,[102] two for GTP[93] (in the presence of NADH) and two for NADH (one catalytic and one regulatory[103,104]) per subunit. The regulatory sites for the reduced coenzyme have more than ten times higher affinity for NADH than for NADPH.[100,102] The oxidized coenzyme seems to activate the enzyme by acting on the ADP sites.[101] Contrary to vertebrate enzymes, $NADP^+$-specific enzyme from *N. crassa* does not exhibit the regulatory responses to purine nucleotoide.[78,80] In addition, the bovine liver enzyme has notable tendency

ADREDRPN FFKMV EGFFDRGASIVEDKLVEDLKTRQTQEQKRNRVR GIL RIIKPC
CEAADKEDRPN FFKMV EGFFDRGASIVEDKLVEGLKARQSMEQRRHRVR GIL RIIKPC
SDLPSEPGFEQAYKELAYTLENSSLFQKHPGYR TAL TVASIP
MDQTYSLESFLNHVQKRDPNQTEFAQAVREVMTTLWPF LEQN PKYRQMSLLERLVEP

NHVLSLSFPIRRDDGS WEVIEGYRAQHSHQRTPCKGGIRYSTDVSVDEVKALASLMTYKCA
NHVLSVSFPIKRDDGS WEVIEGYRAQHSHQRTPCKGGIRYSLDVSVDEVKALASLMTYKCA
ERVIQFRVVWEDDNGN VQVNRGYRVQFNSALGPYKGGLRLHPSVNLSILKFLGFEQIFKNA
ERVIQFRVVWVDD RNQIQVNRAWRVQFSSAIGPYKGGMRFHPSVNLSILKFLGFEQTFKNA

VVDVPFGGAKAGVKINPKNYTDEDLEKITRRFTMELAKKGFIGPGVDVPAPNMSVGEREMSW
VVDVPFGGAKAGVKINPKNYTDEDLEKITRRFTMELAKKGFIGPGVDVPAPNMSVGEREMSW
LTGLSMGGGKGGADFDPKGKSDAEIRRFCCAFMAELHKH IGADTDVPAGDIGVGGREIGY
LTTLPMGGGKGGSDFDPKGKSEGEVMRFCQALMTELYRH LGADTDVPAGDIGVGGREVGF

IADTYASTIGHYDINAHACVTKPGISQGGIHGRISATGRGVFGHIENFIENASYMSILGMTP
IADTYASTIGHYDINAHACVTKPGISQGGIHGRISATGRGLFGHIENFIENASYMSILGMTP
MFGAYRKAANRFEGV LTGKGLSWGGSLIRPEATGGKLVYYVGHMLE YSGAG
MAGMMKKLSNNTACV FTGKGLSFGGSLIRPEATGYGLVYFTEAMLK RHGMG

GFGDKTFAVQGFGNVGLHSMRYLHRFGAKCVAVGESDGSIWNPDGIDPKELEDFKLQHGTIL
GFGDKTFAVQGFGNVGLHSMRYLHRFGAKCVAVGESDGSIWNPDGIDPKELEDYKLQHGTIM
SYAGKRVALSGSGNVANYAALKLIELGATVVSLSDSKGALVATGESGITVE IA VMAIKEA
FEGMRVSVSGSGNVAQYAIEKAMEFGARVITASDSSGTVV DESGFTKEKLARLIEIK A

GFPKAKIYE GSILEVDCDILIP AASEKQLTKSNAPRVK AKIIAEG
GFPKAQKLE GSILETDCDILIP AASEKQLTKANAHKVK AKIIAEG
RQSLTSFQHAGH LKWIEGARPW LHVGKVDIALPCATENQVSKEEAEGLLAAGCKFVAEG
SRDGRVADYAKEFGLVYLEGQQPWSLPV DIALPCATQNELDVDAAHQLIANGVKAVAEG

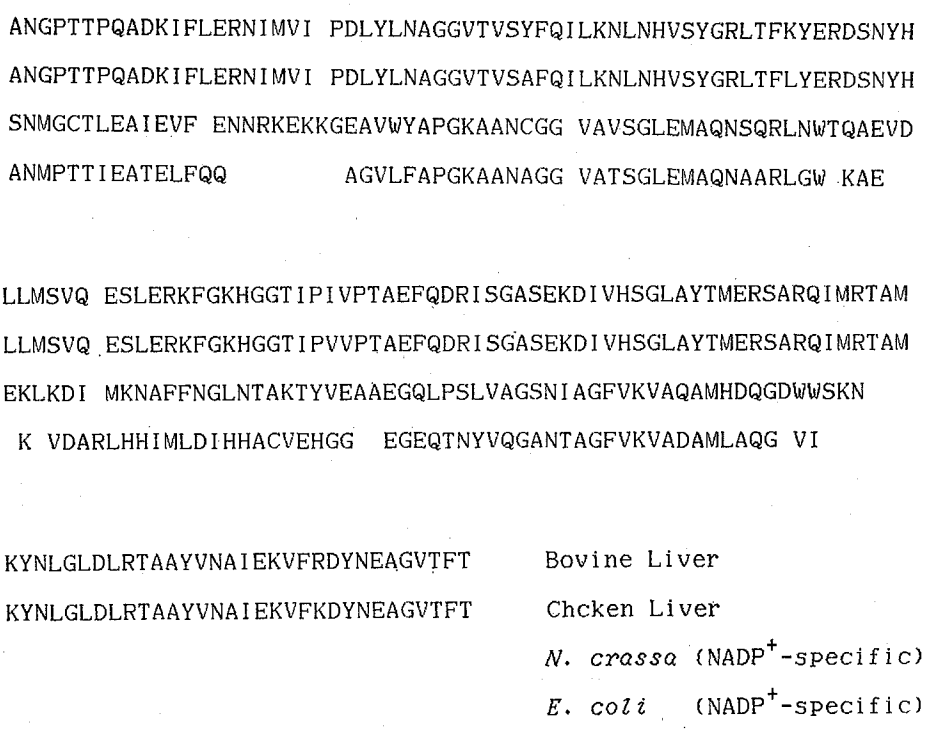


Fig. 3. Amino acid sequences for various GDH's.

to polymerize,[75,105)106)] which may play some role in the regulation of the activity. The GDH also seems to be involved in a network regulation system through complex formation with other enzymes such as aspartate aminotransferase and m-MDH.[107-110)]

Evidences that support the formation of a Shiff-base intermediate during the reaction catalyzed by GDH are as follows: 1) An isotope effect observed in the GDH-catalyzed rapid phase reaction with L-[2-D] glutamate.[111)] 2) The trapping of α-iminoglutarate on the surface of enzyme by the reduction with $NaBH_4$ or $Na_2S_2O_4$ to form L-glutamate in the absence of NADPH.[112)] 3) The ability of GDH to reduce a cyclic α-imino acid, Δ^1-pyrroline-2-carboxylic acid, to an α-amino acid, proline.[113)] 4) The rapid exchange of ^{18}O-labeled carbonyl oxygen atom of α-ketoglutarate with the oxygen in the solvent water thorugh the formation of quaternary E-NADPH-substrate-ammonia complex.[114)]

3.1.2 Molecular Structure and Mechanism for Catalysis

Although X-ray crystallographic studies on the bovine liver enzyme have been prevented by the failure to grow suitable crystals probably owing to its polymerizing nature, studies on rat and tuna liver enzymes started recently.[115,116)] The complete amino acid sequences of four GDH have been determined.[80,82,117-119)] As shown in Fig. 3 the highest homology of the primary structures has been found in the N-terminal half, and the predicted secondary structures of these enzymes are likely to be very similar to each other,[82)] although comparatively large differences are seen in

their primary structures between animal GDH and $NADP^+$-specific ones.

Chemical modifications have shown that bovine liver GDH has two very reactive lysine residues. These are Lys-27[120] and Lys-126[89,121-125] with pKa values of inactivation of about 8.2[120] and 7.7–7.8,[126] respectively. Lys-126 is conserved among four sequences, while Lys-27 is displaced by arginine (probably equivalent in the function) in *E. coli* GDH. Both residues have been implicated in substrate binding.[123,120] An arginine residue (Arg-81 in *N. crassa* $NADP^+$-specific GDH which may correspond to Arg-94 in bovine liver GDH and Arg-96 in *E. cori* GDH) has been implicated in coenzyme binding and probably resides at or near the nictoinamide binding site.[127-128] Affinity labelling has revealed the existence of a lysine and a tyrosine residues at the NADH reguratory site[104] as well as a tyrosine residue at one of GTP reguratory sites.[96] These affinity labellings of each site have also made it possible to estimate the distance between each two of the sites, *i.e.*, the distances of 33 Å between the catalytic and ADP sites, 23 Å between the catalytic and one of GTP sites, and 18 Å between the ADP and GTP sites have been measured by fluorescence energy transfer between chromophores site-specifically introduced in each pair of the sites.[966,97]

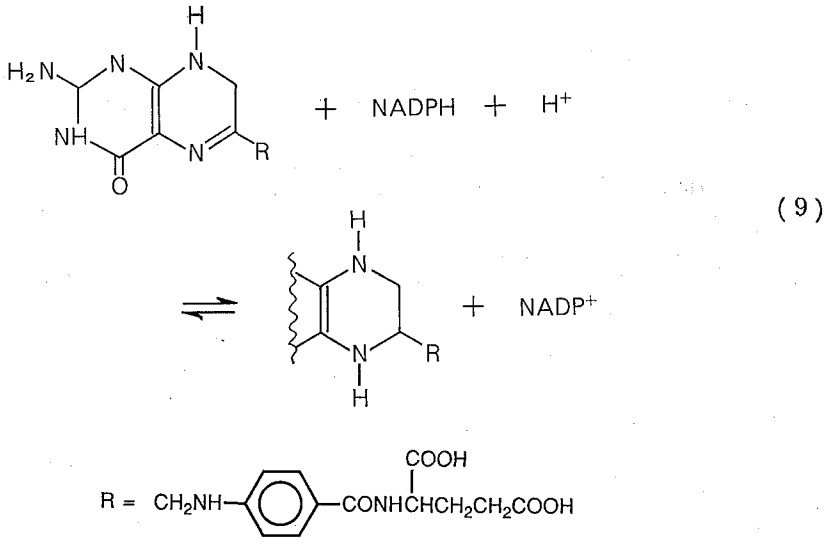
Kinetics on GDH reaction has been investigated with steady-state kinetics,[129-)36] transient kinetics[133-137] including cryoenzymology,[138-140] and equilibrium isotope exchange.[141] The ligand binding order is random for the glutamate oxidation (forward reaction) but apparently ordered for the reverse; reduction of α-ketoglutalate.[131] Transient kinetics have devotedly been concerned with the forward reaction. The initial burst phase which involves chemical reaction steps leads to the formation of a blue-shifted NADPH spectrum which is characteristic of E-NADPH complexes containing α-ketoglutarate. The spectrum is further converted to a red-shifted one characteristic of E-NADPH complexes lacking α-ketoglutarate. The rate-determining step for the forward reaction is usually believed to be the release of α-ketoglutarate from E-NADPH-ketoglutarate complex.[134] Alternatively, it is also possible that the release of NADPH from an abortive E-NADPH-glutamate complex becomes rate-determining step under some conditions with relatively high glutamate concentration.[133]

For the GDH reaction, a mechanism which involves the formation of a Schiff-base between α-ketoglutarate and a highly reactive lysine at the active site of the enzyme was suggested.[50] Rife and Cleland have, however, recently proposed an alternative mechanism which is composed of direct attack of ammonia on α-ketoglutarate to form a carbinolamine.[131,132] The carbinolamine forms α-iminoglutarate by the loss of water. Their argument based on the studies on the effect of pH with mono- and dicarboxylic keto and amino acid substrates as well as their inhibitory analogs. The experimental data from thermodynamic studies on lignad binding do not oppose the direct attack mechanism.[142]

3.2 Dihydrofolate Reductase

3.2.1 Introduction

Dihydrofolate reductase [DHFR; EC 1.5.1.3],[143] another example of Schiff-base reducing enzyme, catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF).



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THF is known to play an essential role as a one-carbon-unit carrier in the synthesis of thymidylate, purines, methionine, and other important metabolites. DHFR is thus in the position which can control the total DNA synthesis or, consequently, cell proliferation. So, DHFR activity must be carefully regulated *in vivo*. The control of activity includes autoregulation of the transcription,[144,145] gene amplification,[146)149] and so on. The expectation for antineoplasmic drugs have prompted the studies on various inhibitors against DHFR.[155,156] The study is also encouraged by the fact that the sensitivities of DHFR to drugs are rather different for enzymes from different sources, particularly between mamals and bacteria. The enzyme itself is usually a monomer of molecular weight of about 20,000, the smallest among the well characterized dehydrogenases. X-ray crystallographic structure at high resolution (1.7 Å) has been determined.[155,156] Today, pioneering works on site directed mutagenesis employing DHFR as one of the most suitable and facinating targets are in progress.[157,158]

3.2.2 Molecular Structure

Structures have been determined crystallographically on the E-methotrexate (MTX) binary complex for *E. coli* enzyme,[155,159] E-MTX-NADPH ternary complex for *L. casei* enzyme,[155,156,160-162] and E-phenyltriazine-NADPH ternary complex for chicken liver enzyme.[163]

Backbone structures of *E. coli* and *L. casei* enzymes have highly conserved feature each other.[155] Chicken liver enzyme also has overall backbone chain folding very

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VRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFQYFQRMTTVSSVEGKQNLVIMGRKTWFSIPEK
VRPLNCIVAVSQNMGIGKNGDLPWPPLRNEYKYFQRMTTTSSVEGKQNLVIMGRKTWFSIPEK
VRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLVIMGRKTWFSIPEK
VRSLNSIVAVCQNMGIGKDGNLPWPPLRNEYKYFQRMTSTSHVEGKQNAVIMGKKTWFSIPEK
  MISLIAALAVDRVIGMENAMPWN LPADLAWFKRNTL    N K  PVIMGRHTWESIG
   TAFLWAQNRDGLIGKDGHLPWH LPDDLHYFRAQTV     GK  IMVVGRRTYESFP K
  MFISMWAQDKNGLIGKDGLLPWR LPNDMRFFREHTM    D K  ILVMGRKTYEGMG K
  MLAAIWAQDENGLIGKEDQLPWR LPNDLKFFKQMTEA   N    TLVMGRKTFEGMG K

NRPLKDRINIVLSRELKEPPKGAHFLAKSLDDALELIEDPELTNKVDVVWIVGGSSVYKEAMN
NRPLKDRINIVLSRELKEPPQGAHFLAKSLDDALKLTEQPELKDKVDMVWIVGGSSVYKEAMN
NRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLIEQPELASKVDMVWIVGGSSVYEQAMN
NRPLKDRINIVLSRELKEAPKGAHYLSKSLDDALALLDSPELKSKVDMVWIVGGTAVYKAAME
 RPLPGRKNIILSSQPGTD DRV TWVKSVDEAIAACGDVP     EIMVIGGG RVYEQFLP
 RPLPERTNVVLTHQEDYQAQ GAVVVHDVAAVFAYAKQHLDQ  E LVIAGGAQIFTAFKD
 LSLPYRHIIVLTTQKDFKVEKNAEVLHSIDELLAYAKDIPE   D IYVSGGSRIFQALL
 RPLP

KPGHVRLFVTRIMQEFESDAFF PEIDFE   K  YKLLPEYPGVPLDVQEEKGIKYKFEVYE
KPGHIRLFVTRIMKEFESDTFF PEIDLE   K  YKLLSECSGVPSDVQEEKGIKYKFEVYE
EPGHLRLFVTRIMQEFESDTFF PEIDLG   K  YKLLPEYPGVLSEVQEEDGIKYKFEVYE
KPINHRLFVTRILHEFESDTFF PEIDYK   D  FKLLTEYPGVPADIQEEDGIQYKFEVYQ
K  AQKLYLTHIDAQVEGDTHF PDYEPDDWESVFSEFHDA     DAQNS  HSYCFEILE
D V DTLLVTRLAGSFEGDTKMIP LNWDDFTKVSSRTVED     TNPA  LTHTYEVWQ
 PETKIIWRTLIDAEFEGDT FIGEIDFTSFELVEEHEGI    VNQE NQYPHRFQKWQKMS
                                             MDEKNPYAH   QFETYQ
```

KNN	Bovine Liver
KNN	Porcine Liver
KKD	Mouse Lymphoma L1210
KSVLAQ	Chicken Liver
RR	*E. cori*
KKA	*L. casei*
KVV	*S. faecium II*
RKRK	*S. faecium I*

Fig. 4. Amino acid sequences forvarious DHFR's.

similar to the former two bacterial enzymes, although vertebrate enzymes including chicken liver enzyme remarkably differ in various physiacl and chemical properties from bacterial enzymes.[163]

Differently from NAD^+-dependent dehydrogenases so far known, the substrate and coenzyme binding in DHFR are carried out in overlapping portions of the amino acid sequence rather than in separate domains.[160] That is, the nicotinamide moiety of the coenzyme is bound within an intricately constructed cavity,[161] while 1,4-diaminopteridine portion of MTX binds to a wall in the same cavity.

Among all the known sequences, 10% of the residues (19 out of 186) are found to be identical. The amino acid sequences are shown in Fig. 4.

3.2.3 Substrate Binding and Mechanism for Catalysis

The reaction catalyzed by the enzyme from *E. coli* obeys to a random mechanism involving two dead-end complexes, *i.e.*, E-DHF-THF and E-$NADP^+$-DHF,[164] whereas that from *Streptococcus faecium* conforms to an ordered mechanism.[165] It has been proposed that Asp-26 (27) in *L. casei* (*E. coli*) enzyme (Glu-30 in vertebrate enzymes) acts as the proton source in DHFR reaction.[160] Kinetics on DHFR indicated that a group with a pKa value of about 6.6, which was attributed to Asp-26, must be protonated in order to make this enzyme catalytically active.[166] It was further suggested from pH studies on *E. coli* DHFR that unprotonated DHF reacts with the protonated E-NADPH complex to form a productive complex but it forms a dead-end complex with the unprotonated form of E-NADPH complex.[461] The protonation to the substrate is considered to preceed and facilitate (net) hydride transfer from NADPH to the C_6 carbon of DHF. The rate-determining step in the steady state of the overall reaction seems to be a step for the change of conformation associated with the proton transfer to DHF.[167]

From the mode of MTX (an inhibitor) binding, in which the carboxyl group of Asp-26 is hydrogen-bonded with the N_1 nitrogen of 2,4-diaminopteridine, a putative model for the binding of true substrate, DHF, has been proposed.[155] In the structure the pteridine-ring is turned upside down from that in enzyme-bound MTX. In the case of MTX, it has been well established that the pteridine-ring turns its

C_7-*re* face toward the nicotinamide of the coenzyme. On the contrary, a substrate, DHF, seems to bind with a 180° rotation of the pteridine-ring about C_6-C_9 bond with the C_7-*si* face turning to the nicotinamide.

This model gives the product THF with *S* configulation at the C_6 position, which is consistent with the absolute configulation determined for biological THF's.[168,169] In the model, 4-keto tautomer of the pteridine, which is known to be the stable form of dihydrofolate both in solution[170,171] and in the complex with the enzyme,[172] was best fitted into the protein structure fixed to that seen in the complexes containing MTX. The C_6 of the substrate is positioned itself about 3.5 Å apart from the nicotinamide C_4. Protonation to the substrate occurs at N_3 instead of N_1 in MTX. On the other hand, some chemically attracting models, in which the positions of N_1 and C_4 are exchanged each other and N_5 of the substrate is protonated, have been proposed.[166] Model building based on the structure elucidated by X-ray crystallography, however, could not support such a model without an extensive and unpredictable change in protein conformation.[155] This seems to be a problem to be elucidate in the future.

The importance of Asp-27 (*E. coli*) is directly evidenced by the fact that the change of the residue into Asn by a genetic modification technique led to an almost complete loss of the enzymic activity (about 0.1% of the original activity).[157,158]

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