CHEMISTRY LETTERS, pp. 1749-1752, 1987.

Stereospecificity of the Hydride Transfer Reaction Catalyzed by Isopropylmalate Dehydrogenase of Thermophilic Bacteria Thermus thermophilus

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Nuclear megnetic resonance studies on the NAD-dependent reaction catalysed by isopropylmalate dehydrogenase from T. thermophilus HB8 revealed that pro R specific (A specific) hydride transfer from the substrate to the nicotinamide ring is involved during the said oxido-reduction.

The enzyme, threo-D_S-3-isopropylmalate dehydrogenase (IPMDH, E.C.1.1.1.85) which is responsible in the leucine biosynthesis, has been attracting much attentions from various aspects, as mentioned in the preceeding paper.[1] We have been involved in biochemical as well as genetic studies on the IPMDH from thermophilic bacteria Thermus thermophilus HB8 to clarify general insights of thermostable proteins. Another feature of our current interests is from evolutionary standpoints. It has been generally accepted that nicotinamide-cofactor-linked-enzymes reduce stereospecifically the cofactors (NAD or NADP) by a transfer of a hydrogen of the substrate to the nicotinamide ring and the stereospecificity of this hydride transfer reaction is always the same, irrespective of the sources of the enzymes, as far as the same chemical reaction is involved,[2,3] and this stereochemical homology has long been argued including from the evolutionary bases.[4-6] An exception to this generality has been recently demonstrated.[7] Thus, an alcohol dehydrogenase of Drosophila melanogaster catalyses a hydride transfer to the pro S site of the dihydropyridine ring (B specific). All other alcohol dehydrogenases [E.C.1.1.1.1] from various sources so far known are pro R specific (A specific). Similar diversity, but slightly different in the strict chemical sense, has been found in an enoyl reductases being involved in the fatty acid biosynthesis.[8] It is intriguing then to study whether the dehydrogenases from thermophiles are stereochemically the same to

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those of mesophiles and of other organisms or not. We undertook a stereochemical study on the reaction catalysed by IPMDH from T. thermophilus HB8 and were able to demonstrate for the first time the stereospecificity of the reaction.

The IPMDH of T. thermophilus HB8 was purified to homogeneity,[9] and was subjected to the present study. The experimental procedures were adopted principally from the method of Arnold et al. with slight modifications.[10,11] The stereochemistry of the hydride (deuteride) transfer reaction from the substrate IPM to the nicotinamide ring of NAD was monitored by the ^{1}H-NMR spectroscopy.

The labeled substrate, racemic threo-[2-^{2}H]-3-isopropylmalic acid and unlabeled substrate were prepared as described in the preceeding paper.[1] The enzyme reaction was carried out as follows: 4.18×10^{-5} mol of NAD (Oriental Yeast Co. Ltd.) and 1.02×10^{-4} mol of either labeled or unlabeled IPM were dissolved in 6.0 mL of 0.20 M carbonate buffer (pH 10.2, 0.10 M $KHCO_3$-0.10 M K_2CO_3) containing 1 M KCl and 0.20 mM $MgCl_2$. The reaction was started by adding 0.3 mg of the purified enzyme, at which time the total volume was about 6.1 mL. The reaction was continued at 50 °C until the ratio of A_{260}/A_{340} decreased to less than 2.8, which corresponds to the NADH/NAD ratio of over 5.5. The pH of the mixture was raised to 12 by addition of 2 M LiOH solution and each reaction mixture was lyophilized. To the residue was added 0.60 mL of deuterium oxide (99.95 atom%, Merck & Co. Ltd.) to extract the NADH formed. The mixture was filtered and then cooled in an iced water bath to precipitate inorganic salts out. The aqueous extract was again filtered and then the filtrate was subjected to the ^{1}H-NMR analysis on a JEOL JNM-GX270 spectrometer operating at 270 MHz. The chemical shifts were calculated from the HDO signal as a reference (δ 4.80 ppm).

Pertinent regions of the resulting spectra are shown in Fig. 1. Based on the signal intensity measurements, the biologically active (2R, 3S)-IPM was virtually completely oxidized into 2-oxoisocaproic acid under these conditions, since the intensities of the C-4 methylene group of the NADH (δ 2.74 and 2.85 ppm) and that of the remaining C-3 methine hydrogen (δ 2.32 ppm) of the unreactive (2S, 3R)-IPM were 2.09 to 1.00. Actually as described above, the substrate used was a racemate so that the inactive enantiomer was remained unchanged throughout the reaction. In the methyl signal region, a doublet due to the isopropyl group of 2-oxoisocaproic acid was observed at δ 1.01 ppm, and two doublets due to the isopropyl group of the unreacted (2S, 3R)-IPM were observed at δ 1.02 and 1.08 ppm. The multiplet signals around δ 2.0 ppm were assigned to the C-4 protons of both of IPM and 2-oxoisocaproic acid. The hydrogens of the C-3 methylene group of 2-oxoisocaproic acid were probably replaced by deuterium atoms under these conditions (pD 12).

The crucial signals to the stereochemical discussions were a pair of broad doublet at δ 2.74 and 2.85 ppm (^2J = ca.18 Hz) in the spectrum A and the former was assigned to the pro S proton signal and the latter to the pro R proton according to the literature data.[10,11] Disappearance of the pro R proton was clearly demonstrated in the spectrum B, when the deuterated IPM was employed as a substrate. The C-3 proton signal of the deuterated IPM was naturally observed at δ 2.31 ppm as a doublet.

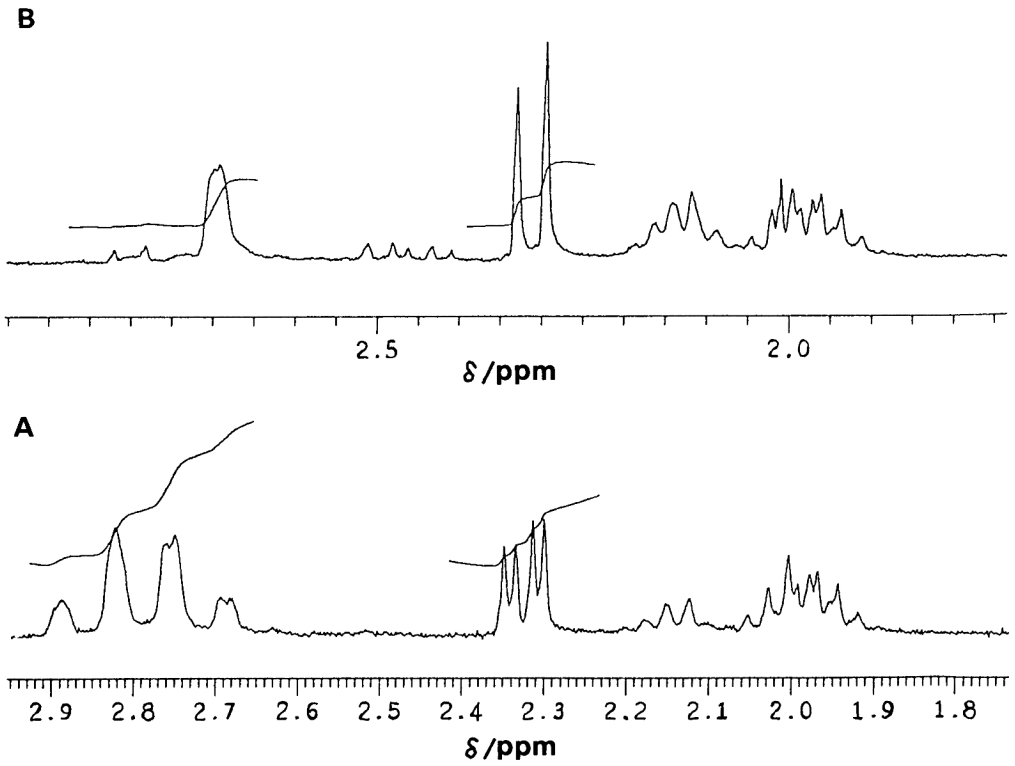
B
2.5
2.0
δ/ppm
A
2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8
δ/ppm

Fig. 1.

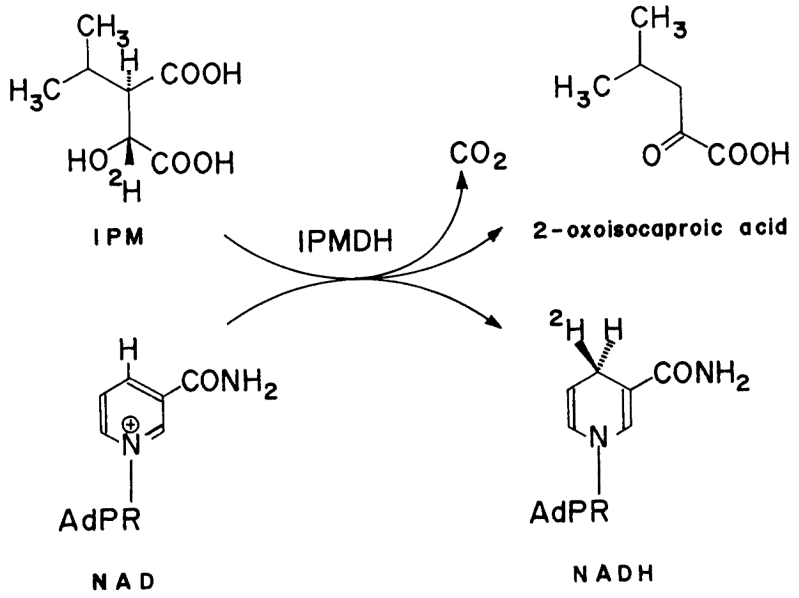
IPM
IPMDH
CO2
2-oxoisocaproic acid
NAD
NADH
AdPR
AdPR

Fig. 2.

It now appears that the IPMDH of a thermophile T. thermophilus HB8 transfers a hydride from the C-2 position of IPM to the pro R position (A site) of the dihydropyridine ring of NAD (Fig. 2) and this is the first stereochemical assignment of the proton transfer reaction catalysed by IPMDH, although the same enzyme has been purified from various sources. The reaction mechanism of this enzyme reaction has been supposed to be quite similar to that of isocitrate dehydrogenases (E.C.1.1.1.41 and E.C.1.1.1.42) involved in the TCA cycle. According to the literatures,[1,2] both NAD-linked and NADP-linked isocitrate dehydrogenases catalyse pro R (A site) specific hydride transfer reactions. Now, the stereospecificity has been in fact determined to be the same for both enzymes, which is of significance from the evolutionary respects.

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 60060004) from the Ministry of Education, Science and Culture, and by Special Coordination Funds from Science and Technology Agency, Japanese Government.

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(Received June 6, 1987)