J. Biochem. 119, 1014-1018 (1996)

Conversion of the Coenzyme Specificity of Isocitrate Dehydrogenase by Module Replacement[1]

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Received for publication, February 8, 1996

The coenzyme specificity of isocitrate dehydrogenase from an extreme thermophilic bacterium was converted from NADP-dependent to NAD-dependent by replacing a "module" involved in the coenzyme binding site. The conversion was not possible with the replacement of a few residues that interact with the coenzyme. In addition, the module-replaced mutant dehydrogenase was as stable as the original, wild type enzyme. The results support a previous hypothesis that a module is a structural and functional unit of a protein.

Key words: coenzyme specificity, exon-shuffling hypothesis, module, molecular recognition, protein engineering.

Isocitrate dehydrogenase [ICDH; (2*R*,3*S*)-isocitrate-$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] in the TCA cycle, together with 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85), belongs to a new family of enzymes called "decarboxylating dehydrogenases." These enzymes act on structurally similar substrates containing a malate moiety, and catalyze chemically equivalent reactions; dehydrogenation at carbon-2 of the malate moiety to form a carbonyl group from a hydroxyl group, and decarboxylation at carbon-3. The primary and three-dimensional structures of these enzymes resemble each other (*1, 2*). It has been speculated that ICDH and IPMDH evolved from a common ancestral enzyme, and exhibit no evolutionary relationship to well-known dehydrogenases such as alcohol dehydrogenase, lactate dehydrogenase and malate dehydrogenase (*3*).

NADP-dependent ICDH and NAD-dependent IPMDH have been cloned, and sequenced, from an extreme thermophile, *Thermus thermophilus* HB8 (*3-5*). The X-ray crystallographic structure of the IPMDH has been determined (*1*). The amino acid sequences of these enzymes exhibit homology, 35.5% of the amino acid residues being identical, suggesting that their coenzyme specificities can be altered by substitution of a relatively limited number of amino acid residues, particularly ones involved in the recognition of the presence or absence of a phosphate group at the 2′-position of the adenine ribose ring in a nicotinamide nucleotide. For *Escherichia coli* ICDH, the X-ray crystallographic structure of the NADP-enzyme complex was determined at 2.5 Å resolution (*2*), and Arg292′, Tyr345, Tyr391, and Arg395 (the prime indicates the residue from the second subunit) have been suggested to interact with the 2′-phosphate group of NADP. Two of them, Arg292′ and Tyr345, are conserved in *T. thermophilus* ICDH, corresponding to Arg231′ and Tyr284, respectively, while the other two (Tyr391 and Arg395) are not conserved. In addition, Lys283, which is adjacent to Tyr284, is also conserved in NADP-dependent ICDHs. In NAD-dependent IPMDH and NAD-dependent ICDH of *Saccharomyces cerevisia* (*6*), Lys283 and Tyr 284 are replaced by Asp278 and Ile279 (numbering according to the thermophile IPMDH), respectively, and Arg231′ is replaced by a variety of residues (Asn, Gln, Tyr, Lys, Ala, Asp, and Trp) (*7*). Thus, Lys283, Tyr284, and Arg231′ are expected to be involved in the coenzyme discrimination between NAD and NADP by the thermophile ICDH (Fig. 1).

Interactions of Tyr284 and Arg231′ with the 2′-phosphate group have been proved by our mutational analyses (*8*). Recent X-ray crystallographic analyses of the thermophile IPMDH-NAD and IPMDH-ADP-ribose complexes revealed that Asp278 (corresponding to Lys283 of *E. coli* ICDH) of the thermophile IPMDH contributes to the affinity for NAD (*9*). The 2′-OH and 3′-OH groups of NAD form hydrogen bonds with Asp278, with a van der Waals contact between 2′-OH and Ile279. Probably replacement of Lys by Asp278 drastically alters the electrostatic field of the binding site in the case of NAD-dependent IPMDHs, making it more negative, which is unfavorable for the binding of the more acidic coenzyme NADP (*9*) (Fig. 1).

In this study, the coenzyme specificity of ICDH from *T. thermophilus* was converted from NADP-dependent to NAD-dependent by replacing a "module" containing these coenzyme discriminating residues. A part of the present study was preliminarily reported at an international meeting (*10*).

[1] This work was partly supported by grants from the Ministry of Education, Science, Sport and Culture of Japan (No. 02403029), and the Mitsubishi Science Foundation.

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Abbreviations: ICDH, isocitrate dehydrogenase; IPMDH, 3-isopropylmalate dehydrogenase.

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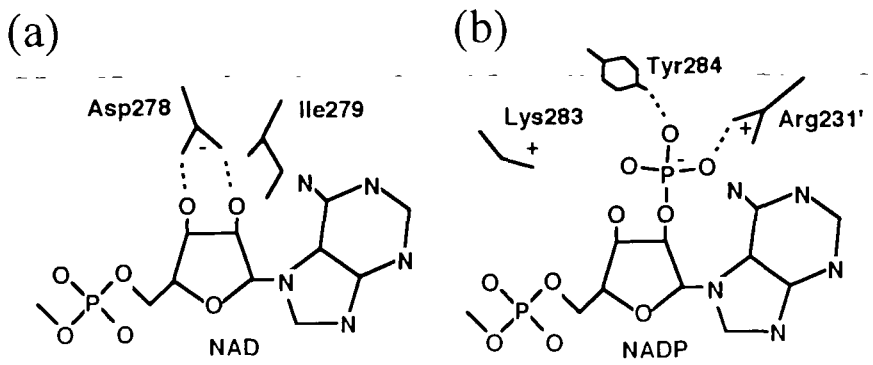

Fig. 1. **Putative schematic drawings of the coenzyme discrimination sites of (A) *T. thermophilus* IPMDH (*7, 9*), and (B) *T. thermophilus* ICDH (*2, 8*).**

MATERIALS AND METHODS

Construction of Mutant Enzymes—Site-directed mutagenesis was carried out according to the method of Kunkel (*11*). The oligonucleotide used for generating mutations was 5′-CTCAAACTGCTCCGGAGCTTTCACCAGCTG-3′ for Arg231Ala. 5′-TTCTTCCCGGCGATATCGGGGGCG-GA-3′ was used to replace Lys283 and Tyr284 of *T. thermophilus* ICDH by Asp and Ile, respectively, which are found in IPMDH from the same organism at the corresponding positions. Asn288, Val289, and Ile290 were converted to Gly, Ile, and Ala, respectively, with 5′-CGCGGTGGGGTT-CGCGATGCCCTTCCCGGCGAT-3′ as a mutation primer. The mutant whose Lys283, Tyr284, Asn288, Val289, and Ile290 were replaced by Asp, Ile, Gly, Ile, and Ala, respectively, was named EX5. In mutant EX6, an Arg231Ala mutation was introduced in addition to those in EX5. The gene of Lys283Asp/Tyr284Ile was used as the template for mutagenesis of EX5 and EX6. All mutations were confirmed by DNA sequencing. Expression and purification of the wild type and mutant enzymes were performed as described previously (*12*). All the enzymes used in this study were purified to homogeneity, as judged on SDS-polyacrylamide gel electrophoresis.

Steady-State Kinetic Analysis—The Michaelis constant, K_m, for isocitrate and the catalytic constant, k_{cat}, were determined in steady-state experiments at 60°C in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate-NaOH buffer (pH 7.8) containing 5.0 mM $MgCl_2$, 5.0 mM NAD, or NADP. The isocitrate concentration was varied in the range of 5-500 μM. The reaction was monitored by measuring the absorbance at 340 nm. In order to determine K_m for NAD(P), the concentration of the coenzyme was varied in the range of 5-5,000 μM, with a fixed isocitrate concentration (1.0 mM). No substrate inhibition occurred under these conditions.

Module Analysis and Computer Graphic Manipulation—The "module" structures of *T. thermophilus* IPMDH and *E. coli* ICDH were analyzed by the centripetal profile method reported previously (*13, 14*). Computer graphic manipulation was conducted on an IRIS-4D, using the MOLSCRIPT (*15*).

RESULTS

Point Mutation of the Coenzyme Discriminating Residues—To change the coenzyme specificity based on the above mentioned speculations, three mutant ICDHs, Tyr-284Ile, Lys283Asp/Tyr284Ile, and Arg231Ala/Lys283-Asp/Tyr284Ile, were constructed by site-directed mutagenesis (Tyr284Ile is a mutant enzyme in which Tyr284 of the thermophile ICDH is replaced by Ile, and Lys283Asp/Tyr284Ile and Arg231Ala/Lys283Asp/Tyr284Ile are double and triple mutated enzymes, respectively.). However, contrary to our expectations, these mutant enzymes were expressed as insoluble materials in *E. coli*, suggesting that the substitutions destroyed their three-dimensional structures.

Module Analysis—As an alternative approach for converting an enzyme function, the "module" structure of *T. thermophilus* IPMDH was analyzed by the centripetal profile method reported previously (*13, 14*). The enzyme can be divided into 21 modules (Fig. 2). Though X-ray analysis of the thermophile ICDH is underway (*16*), it can be assumed, based on the high sequence homology, that the enzyme contains a similar module structure. Tyr284 of *T. thermophilus* ICDH is located in module-19. In IPMDH, this module contains a loop structure between β-strand D and α-helix i (Fig. 3). The sequence of this region is highly conserved in ICDHs and IPMDHs (*3*). *E. coli* ICDH (*2*) was also analyzed (data not shown), and Tyr345 (corresponding to Tyr284 of the thermophile enzyme) was found in module-26, which contains the residues that interact with the adenosine moiety of NADP, such as His278, Ala281, Tyr284, and Asn291 (thermophile ICDH numbering; Fig. 4). In *T. thermophilus* IPMDH, His273, Ala276, Asp278, and Asn286 also interact with the adenosine moiety (*9, 17*). These corresponding modules seem to form a common adenosine binding pocket. The backbone structure of module-26 of the ICDH well resembles that of module-19 of the thermophile IPMDH (Fig. 4).

Module Replacement—Module-19 of the thermophile NADP-dependent ICDH consists of 18 residues, 5 of which are different from those in the sequence of module-19 of the thermophile NAD-dependent IPMDH (Fig. 3). Some of them do not directly interact with NADP. We constructed a module-replaced mutant of the ICDH (named EX5) by changing these 5 residues. Mutant EX5 is catalytically active and as stable as the original, thermophile enzyme, as shown in Fig. 5. The kinetic constants of EX5 are summarized in Table I. The K_m value for NADP increased to a great extent (about 180-fold), and that for NAD decreased by one order of magnitude, without a large change in the k_{cat} value. The K_m value for isocitrate as to NAD-dependent activity decreased about 4-fold.

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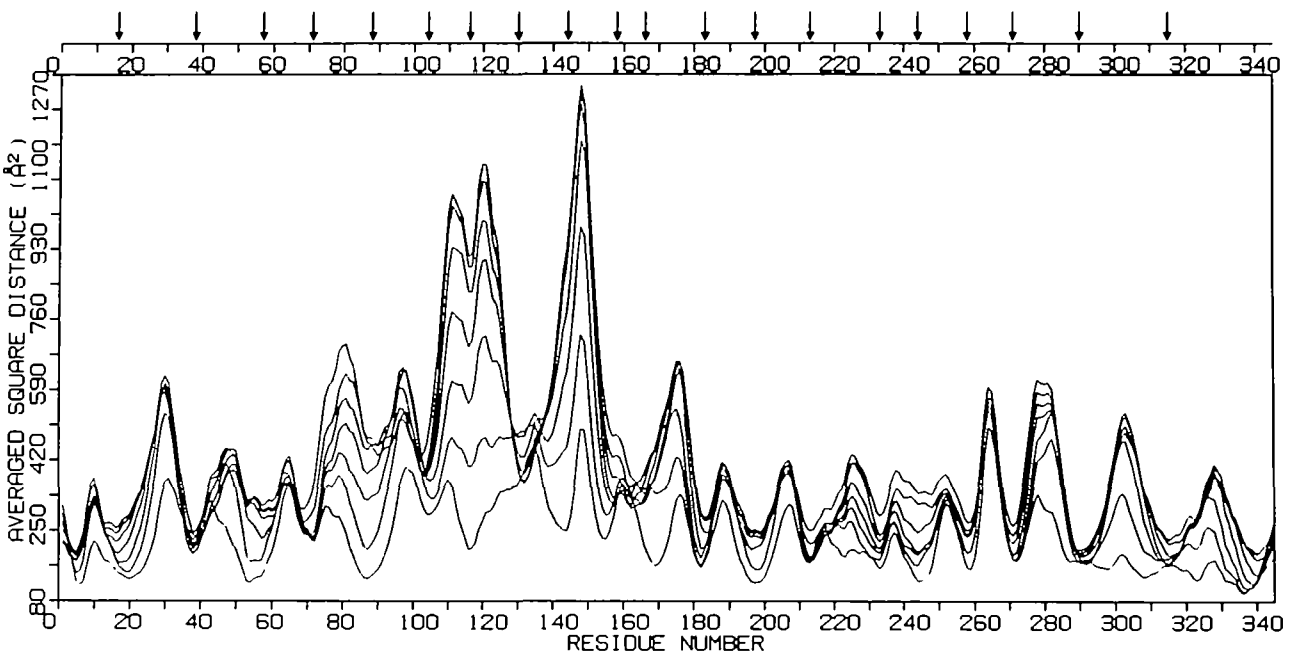

Fig. 2. **Centripetal profile (*13, 14*) of *T. thermophilus* IPMDH.** The vertical axis is the mean square distance between the Cα atom of the ith residue and the Cα atoms of the residues located within k residues along the polypeptide chain from the ith residue. Curves are drawn for $k=15$, 20, 25, 30, 35, 40, and 45 residues. The common stable minima among the curves, indicated by arrows, are employed as module boundaries; at least 20 boundaries, which yield 21 modules, can be identified.

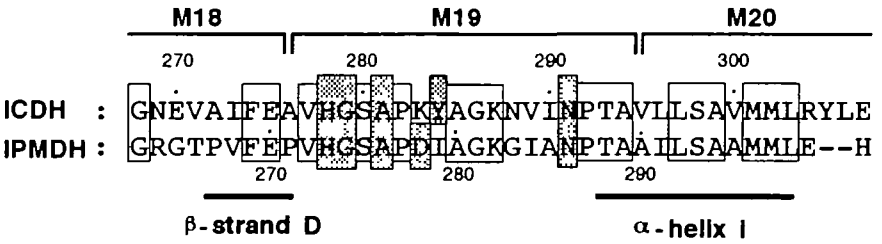

Fig. 3. **Amino acid sequence comparison of the region of module-19 containing a loop structure between β-strand-D and α-helix-i of IPMDH with that of ICDH from *T. thermophilus*.** The amino acid residues identical in the two enzymes are boxed. Shaded residues are assumed to interact with NAD(P), based on the mutational analysis and three-dimensional structures of the enzyme-coenzyme complexes (*2, 7-9, 17*). The secondary structures and module numbers are given under and above the sequences, respectively.

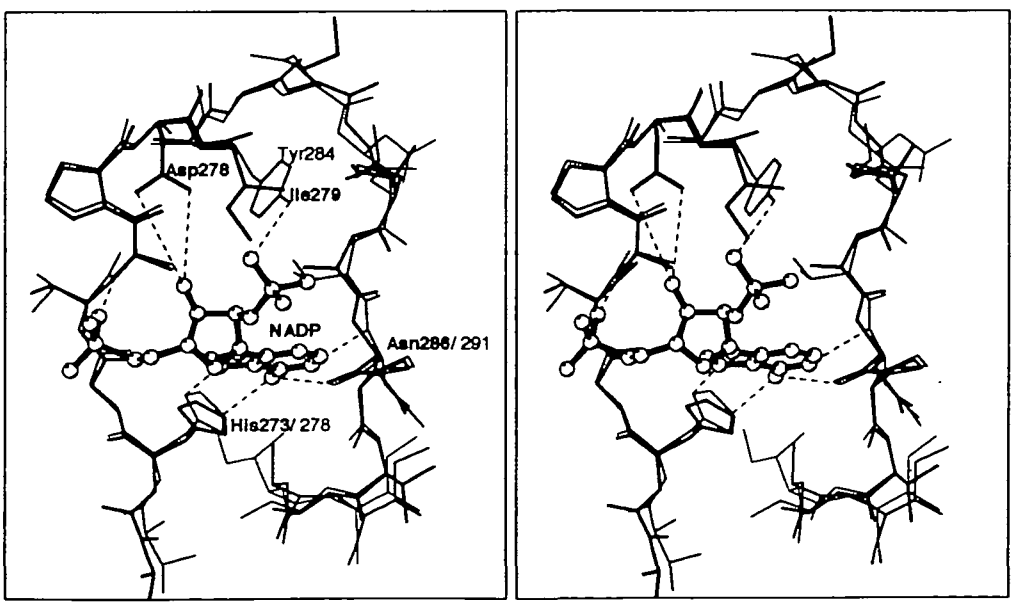

Fig. 4. **Comparison of the module-19 structure of *T. thermophilus* IPMDH (*1*) with module-26 of the *E. coli* ICDH-NADP complex (*2*).** The heavy lines show the backbone structure of IPMDH, and the light ones ICDH (thermophile ICDH numbering).

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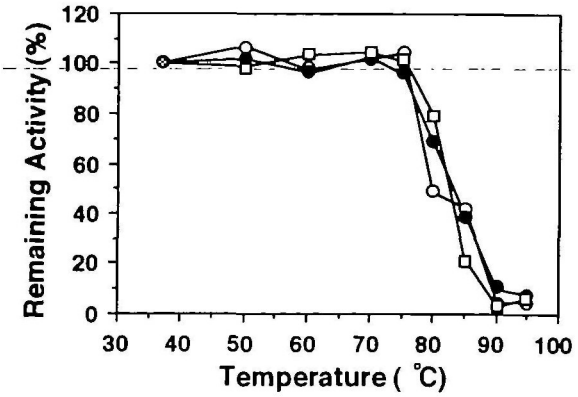

Fig. 5. **Remaining activity of the wild type (squares), EX5 (open circles) and EX6 (closed circles) enzymes after heat treatment.** Each enzyme (0.1 mg/ml in 50 mM sodium phosphate buffer, pH 7.5) was incubated for 10 min at each temperature, and then the remaining activity was determined. The value at 37°C was taken as 100% for each enzyme.

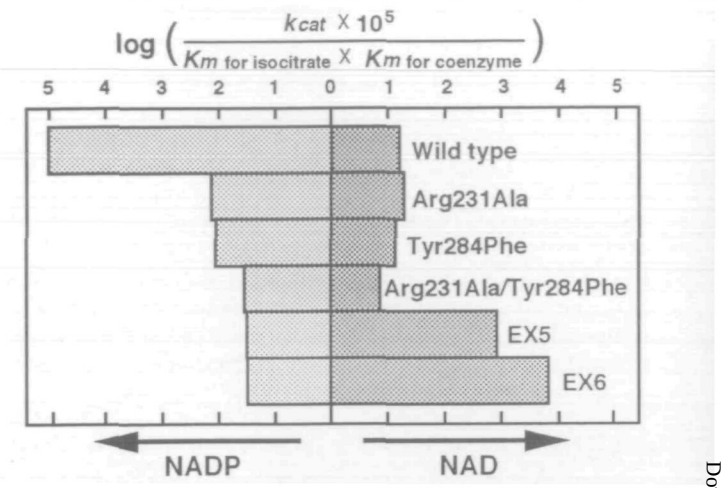

Fig. 6. **Coenzyme specificities of the wild type and mutant enzymes.** The overall catalytic efficiency is shown in log scale. The data for single and double mutated enzymes were reported previously (*8*).

TABLE I. **Kinetic parameters of wild type and mutant enzymes.**

Enzyme and coenzyme	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	
	Coenzyme	Isocitrate		Coenzyme	Isocitrate
Wild type					
NADP	7.2	8.9	71	9.9	8.0
NAD	3,100	81	41	0.013	0.51
EX5					
NADP	1,300	82	24	0.018	0.29
NAD	220	22	41	0.19	1.9
EX6					
NADP	2,900	62	39	0.013	0.63
NAD	36	12	32	0.88	2.7

Taking into account that Arg231′ is also involved in the coenzyme specificity, a mutant, EX6, was constructed in which an Arg231Ala mutation was added to those in EX5. With this mutation, the affinity for NAD was further improved 6 times (Table I). The k_{cat} value did not change significantly (Table I). The K_m value for isocitrate in the presence of NAD was close to that of the wild type enzyme in the NADP-dependent reaction (Table I). The overall catalytic efficiencies, defined as $k_{cat}/(K_m$ for isocitrate $\times K_m$ for coenzyme), of the wild type and mutant enzymes are compared in Fig. 6. The module-replaced mutants reduced the catalytic efficiency as to NADP-dependent activity by about three orders of magnitude, and increased that as to NAD-dependent activity by about two to three orders. The conversion was successfully achieved only with module replacement, *i.e.*, not with single, double or triple residue replacement.

DISCUSSION

Module replacement converted the coenzyme specificity of the thermophile ICDH from NADP-dependent to NAD-dependent. The preference for NAD was improved about 10^5 times by the module replacement. Additional residue substitution (EX6) further improved the preference by 10 times. However, even if in the best mutant, EX6, the overall NAD-dependent catalytic efficiency was about one-tenth of that of the wild type NADP-dependent activity. These results suggest that, in addition to the present module, other elements are also involved in the coenzyme recognition.

The three-dimensional structure of module-19 of *T. thermophilus* IPMDH well resembles the corresponding module (module-26) of *E. coli* ICDH (Fig. 4). In *E. coli* ICDH and *T. thermophilus* IPMDH, the residues which interact with the adenosine moiety of the coenzyme are concentrated in this module (Figs. 3 and 4). This suggests that module-19 is the nucleotide binding unit of a common ancestral enzyme of the family. The coenzyme specificities of ICDH and IPMDH would have diverged through minor replacements in this NAD(P) binding unit.

Engineering of the coenzyme specificity has been reported for some typical enzymes which have a common $\beta\alpha\beta$-fold in the coenzyme-binding domain (*18-20*). The present enzyme belongs to a distinct dehydrogenase family which lacks this conventional nucleotide binding fold. The successful conversion of the specificity has not been reported so far for enzymes in this family.

One of us (M.G.) proposed the "module" structure is a compact protein structural unit, which consists of about 10–40 continuous amino acid residues and can be identified by plotting the distances between the α-carbons of a protein (*21, 22*). Good correlations have been found between the module structures and exon regions of the genes of several proteins (*13, 21-24*). Such a close relationship between exons and module structures supported the exon shuffling hypotheses of Gilbert (*25, 26*), and Blake (*27*).

Functional conversion of homologous proteins, such as α-lactalbumin and lysozyme, or the α and β subunits of hemoglobin, has already been successfully achieved by exon exchange engineering (*28, 29*). By replacing the exon 2 region of goat α-lactalbumin with that of hen lysozyme, catalytic activity was conferred to α-lactalbumin. Usually, a bacterial gene coding for an enzyme including the *T. thermophilus* and *E. coli icd* genes does not contain introns. However, the present study suggests that, if acceptable framework structures for modules can be identified by

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structural analysis, the functional conversion of a bacterial enzyme is possible by module exchange, as demonstrated by some pioneering works on exon exchange engineering mentioned above.

In the present study, single and double mutated enzymes, Tyr284Ile and Lys283Asp/Tyr284Ile, did not retain their active conformations, even at the growth temperature of *E. coli*. In contrast, the EX5 and EX6 mutants in the present study were as stable as the wild type enzyme (Fig. 5). Lys283 and Tyr284 are present in module-19, indicating that partial substitution of the module resulted in a remarkable loss of stability. The present results are consistent with the idea that a "module" is both a structural and a functional unit in protein architecture.

Note added at the proof: Recently the coenzyme specificity of *E. coli* isocitrate dehydrogenase was converted by multiple mutations [Chen, R., Greer, A., and Dean, A.M. (1995) A highly active decarboxylating dehydrogenase with rationally inverted coenzyme specificity. *Proc. Natl. Acad. Sci. USA* **92**, 11666-11670]. A preliminary report of the present study has appeared in the proceedings of an international meeting held in November, 1994 (*10*).

REFERENCES

1. Imada, K., Sato, M., Matsuura, Y., Katsube, Y., and Oshima, T. (1991) Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus*, at 2.2 Å resolution. *J. Mol. Biol.* **222**, 725-738
2. Hurley, J.H., Dean, A.M., Koshland, D.E. Jr., and Stroud, R.M. (1991) Catalytic mechanism of $NADP^+$-dependent isocitrate dehydrogenase: Implications from the structures of magnesium-isocitrate and $NADP^+$ complexes. *Biochemistry* **30**, 8671-8678
3. Miyazaki, K., Eguchi, H., Yamagishi, A., Wakagi, T., and Oshima, T. (1992) Molecular cloning of the isocitrate dehydrogenase gene of an extreme thermophile, *Thermus thermophilus* HB8. *Appl. Environ. Microbiol.* **58**, 93-98
4. Tanaka, T., Kawano, N., and Oshima, T. (1981) Cloning of 3-isopropylmalate dehydrogenase gene of an extreme thermophile and partial purification of the gene product. *J. Biochem.* **89**, 677-682
5. Kirino, H., Aoki, M., Aoshima, M., Hayashi, Y., Ohba, M., Yamagishi, A., Wakagi, T., and Oshima, T. (1994) Hydrophobic interaction at the subunit interface contributes to the thermostability of 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*. *Eur. J. Biochem.* **220**, 275-281
6. Cupp, J.R. and McAlister-Henn, L. (1992) Cloning and characterization of the gene encoding the IDH1 subunit of NAD^+-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276**, 16417-16423
7. Miyazaki, K. and Oshima, T. (1994) Co-enzyme specificity of 3-isopropylmalate dehydrogenase from *Thermus thermophilus* HB8. *Protein Eng.* **7**, 401-403
8. Yaoi, T., Miyazaki, K., and Oshima, T. (1994) Roles of Arg231 and Tyr284 of *Thermus thermophilus* isocitrate dehydrogenase in the coenzyme specificity. *FEBS Lett.* **355**, 171-172
9. Kadono, S., Sakurai, M., Moriyama, H., Sato, M., Hayashi, Y., Oshima, T., and Tanaka, N. (1995) Ligand-induced changes in the conformation of 3-isopropylmalate dehydrogenase from *Thermus thermophilus*. *J. Biochem.* **118**, 745-752
10. Oshima, T., Yaoi, T., and Go, M. (1995) Module replacement converted coenzyme specificity of isocitrate dehydrogenase in *Tracing Biological Evolution in Protein and Gene Structures* (Go, M. and Schimmel, P., eds.) pp. 197-203, Elsevier, Amsterdam
11. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488-492
12. Miyazaki, K., Yaoi, T., and Oshima, T. (1994) Expression, purification, and substrate specificity of isocitrate dehydrogenase from *Thermus thermophilus* HB8. *Eur. J. Biochem.* **221**, 899-903
13. Go, M. and Nosaka, M. (1987) Protein architecture and the origin of introns. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 915-924
14. Noguti, T., Sakakibara, H., and Go, M. (1993) Localization of hydrogen-bonds within modules in barnase. *Proteins* **16**, 357-363
15. Kraulis, P.J. (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Cryst.* **24**, 946-950
16. Ohzeki, M., Yaoi, T., Moriyama, H., Oshima, T., and Tanaka, N. (1995) Crystallization and preliminary X-ray studies of isocitrate dehydrogenase from *Thermus thermophilus* HB8. *J. Biochem.* **118**, 679-680
17. Yaoi, T., Miyazaki, K., and Oshima, T. (1995) His273 of 3-isopropylmalate dehydrogenase from *Thermus thermophilus* HB8 is involved in the coenzyme binding. *Biochem. Biophys. Res. Commun.* **210**, 733-737
18. Scrutton, N.S., Berry, A., and Perham, R.N. (1990) Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* **343**, 38-43
19. Bocanegra, J.A., Scrutton, N.S., and Perham, R.N. (1993) Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* **32**, 2737-2740
20. Nishiyama, M., Birktoft, J.J., and Beppu, T. (1993) Alteration of coenzyme specificity of malate dehydrogenase from *Thermus flavus* by site-directed mutagenesis. *J. Biol. Chem.* **268**, 4656-4660
21. Go, M. (1981) Correlation of DNA exonic regions with protein structural units in haemoglobin. *Nature* **291**, 90-92
22. Go, M. (1983) Modular structural units, exons, and function in chicken lysozyme. *Proc. Natl. Acad. Sci. USA* **80**, 1964-1968
23. Go, M. (1985) Protein structures and split genes. *Adv. Biophys.* **19**, 91-131
24. Gilbert, W. and Glynias, M. (1993) On the ancient nature of introns. *Gene* **135**, 137-144
25. Gilbert, W. (1978) Why genes in pieces? *Nature* **271**, 501
26. Gilbert, W. (1987) The exon theory of genes. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 901-905
27. Blake, C.C.F. (1979) Exons encode protein functional units. *Nature* **277**, 598
28. Kumagai, I., Takeda, S., and Miura, K. (1992) Functional conversion of the homologous proteins α-lactalbumin and lysozyme by exon change. *Proc. Natl. Acad. Sci. USA* **89**, 5887-5891
29. Wakasugi, K., Ishimori, K., Imai, K., Wada, Y., and Morishima, I. (1994) "Module" substitution in hemoglobin subunits. Preparation and characterization of a "chimera $\beta\alpha$-subunit." *J. Biol. Chem.* **269**, 18750-18756

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