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JB Reflections and Perspectives

Kenji Soda—researching enzymes with the spirit of an alpinist

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Like an alpinist continuously seeking virgin peaks to climb, Kenji Soda has investigated a variety of unique enzymes for which there was little or no information available; and by doing so he opened up a variety of new fields in enzyme science and technology. In particular, he has promoted the study of enzymes requiring vitamin B-derived cofactors such as FAD, NAD(P) and pyridoxal 5′-phosphate, shedding light on their reaction mechanisms, enzymological properties, crystal structures and potential practical applications. Highlighted in this review are the studies of enzymes acting on D-amino acids and sulphur/selenium-containing amino acids and those from thermophilic and psychrophilic bacteria.

Keywords: D-amino acid/pyridoxal 5′-phosphate/selenium/stereochemistry/thermostable enzyme.

Abbreviations: BCAT, branched-chain L-amino acid aminotransferase; DAAT, D-amino acid aminotransferase; DTT, dithiothreitol; LeuDH, leucine dehydrogenase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

Kenji Soda (1933–) graduated from the Graduate School of Agricultural and Biological Chemistry, Kyoto University, where he obtained his doctoral degree in 1961. He was appointed Instructor in the Department of Agricultural and Biological Chemistry, Kyoto University in 1962 and, after studying in the laboratory of Prof. Alton Meister at Tufts University in Boston, MA for nearly 2 years, became Associate Professor at the Institute for Chemical Research of Kyoto University in 1963. He was then appointed Professor of Microbial Biochemistry in 1981 and became Professor Emeritus of Kyoto University on his retirement in 1996. He continued to study and teach at Kansai University as Professor of Biomolecular Engineering until his complete retirement in 2003. He served as President of the Japanese Biochemical Society from 1992 to 1993 and of the Vitamin Society of Japan from 1999 to 2003. He is still very active in both academic and public societies (Fig. 1).

Soda began his research career during his graduate course at Kyoto University, where he discovered lysine ε-aminotransferase (EC 2.6.1.36) in a bacterium belonging to the genus *Flavobacterium* and identified its reaction product as a heterocyclic compound, Δ^1-piperideine 6-carboxylic acid (*1*). Since then he has investigated a large number (>60) of enzymes mainly involved in amino acid metabolism. As summarized in Table I, the enzymes studied are mostly dependent on vitamin B-derived cofactors such as FAD, NAD(P) and pyridoxal 5′-phosphate (PLP); span all of the EC classes; and are derived from a wide variety of organisms, including bacteria, yeast and mammals. The genes encoding many of these enzymes have been cloned, sequenced and efficiently expressed in *Escherichia coli* cells, and the X-ray crystal structures have been determined for several. Although at first glance these enzymes may appear to have no relation to one another, most are classified into the same groups, based on several research streams. The largest group includes the PLP-dependent enzymes, which catalyse versatile reactions categorized over EC classes (2 to 5). Two major research streams have arisen from studies of enzymes belonging to this group, one for the enzymes acting on D-amino acids (particularly from a stereochemical viewpoint) and another for enzymes acting on sulphur/selenium-containing amino acids (particularly in relation to enzymic discrimination between sulphur and selenium). The second largest group includes the NAD(P)-dependent amino acid dehydrogenases, from which an important research stream involving the study of enzymes from thermophilic (and later psychrophilic) bacteria emerged. In this review, these three major research streams are described in some detail and provided with some historical perspectives. Unfortunately, other noteworthy studies, including those on FAD-dependent enzymes and cofactor-independent dehalogenases, are not discussed here solely due to the limitation of space.

In addition to his scientific activities, Soda has been an enthusiastic mountaineer. He entered the mountaineering club in Okazaki high school and then devoted most of his youthful days to mountaineering (Fig. 2). Even after his appointment to an academic position, when it became difficult to spare enough time for mountaineering, he continuously supported young

Fig. 1 Kenji Soda, lecturing in the 62nd Annual Conference of the Vitamin Society of Japan held at Morioka in June 11, 2010 (by courtesy of Dr H. Taguchi).

alpinists as President of Kyoto University Alpine Club and as the director of Academic Alpine Club of Kyoto. He has learned that scientific research and alpinism share a number of common aspects: *i.e.* in science one must first select a target to research (preferably in relatively unexplored area), while in mountaineering one must select a mountain to climb (preferably a virgin peak); then one must plan the experiments or explore the route to the summit and work hard to achieve the aim of the study or to gain the peak; and finally one must publish scientific articles or report the record of the climb. It appears that Soda's attitude toward enzyme research has been considerably affected by alpinism and his desire to climb virgin peaks.

Enzymes Acting on D-Amino Acids

Because the Chinese-originated 'Kanji' characters, one of the main scripts used in the Japanese writing system, are ideograms, Japanese names written in Kanji have their inherent meanings. Soda often jokes, 'My (family) name, "Soda", means "left-and-right rice field". Thus, I was destined to study D-amino acids, which are the mirror images of L-amino acids, and their racemization'. Not merely joking, he has indeed practiced this in his research activity, and as described below he is undoubtedly a pioneer in the D-amino acid studies in Japan.

Amino acid racemases (EC sub-subclass 5.1.1) are the only enzymes that can catalyse the *de novo* synthesis of free D-amino acids. With the exception of phenylalanine racemase (EC 5.1.1.11), which requires ATP for catalysis, amino acid racemases are divided into two groups: PLP-dependent and cofactor-independent. Following the first paper describing arginine racemase (EC 5.1.1.9) in 1967 (*2*), Soda's group successively studied various PLP-dependent amino acid racemases, including amino acid racemase with low substrate specificity (EC 5.1.1.10) (*3*), α-amino ε-caprolactam racemase (EC 5.1.1.15) (*4*) and alanine racemase (EC 5.1.1.1) (*5*), as well as cofactor-independent racemases such as aspartate racemase (EC 5.1.1.13) (*6*) and glutamate racemase (EC 5.1.1.3) (*7*). In general, enzymes exhibit strict stereochemical selectivity for their substrates and the reactions they catalyse. Consequently, the capability of amino acid racemases to act equally on both enantiomers of amino acids has been of particular interest to Soda from mechanical, stereochemical and structural points of view.

The catalytic reaction of amino acid racemase is apparently very simple, proceeding through

Table I. Representative enzymes studied by Kenji Soda.

Cofactor	Category (EC class or subclass)	Enzyme
PLP	Aminotransferase (EC 2.6)	D-Amino acid aminotransferase
		ω-Amino acid: pyruvate aminotransferase
		Aspartate aminotransferase
		Kynurenine aminotransferase
		Lysine ε-aminotransferase
		Ornithine δ-aminotransferase
		Taurine aminotransferase
	Racemase (EC 5.1)	Alanine racemase
		α-Amino ε-caprolactam racemase
		Arginine racemase
		Amino acid racemase with low-substrate specificity
	Decarboxylase (EC 4.1)	Aspartate β-decarboxylase
		Meso-α,ε-diaminopimelate decarboxylase
		Lysine decarboxylase
		Methionine decarboxylase
		Ornithine decarboxylase
	Hydrolase (EC 3.7)	Kynureninase
	Lyase (EC 4.3, 4.4)	Glucosaminate dehydratase
		S-Alkylcysteine α,β-lyase
		Methionine γ-lyase
		Selenocysteine β-lyase
		D-Selenocystine α,β-lyase
NAD(P)	Dehydrogenase (EC 1)	Alanine dehydrogenase
		Leucine dehydrogenase
		Meso-α,ε-diaminopimelate dehydrogenase
		Phenylalanine dehydrogenase
		Valine dehydrogenase
		α-Hydroxyglutarate dehydrogenase
		Formate dehydrogenase
		Alcohol dehydrogenase
FAD	Oxidase (EC 1.4, 1.7)	Lysine α-oxidase
		Nitroalkane oxidase
	Oxygenase (EC 1.13)	2-Nitropropane dioxygenase
$AdoB_{12}$	Lyase (EC 4.2)	Diol dehydratase
None	Miscellaneous	*S*-(β-Aminoethyl)cysteine ω-*N*-acetyltransferase
		Asparaginase
		Glutaminase
		N-Acylamino acid acylase
		Dipeptidase
		Aspartate racemase
		Glutamate racemase
		L-2-Haloacid dehalogenase
		DL-2-Haloacid dehalogenase
		L-α-Amino ε-caprolactam hydrolase
		Poly-γ-glutamate synthetase

Fig. 2 Kenji Soda, standing on a summit of Japan Central Alps in July, 1987 (*left*) and walking in a small village of the Yunnan Province, China, in May, 1991 (*right*).

(i) abstraction of the α-proton from an amino acid substrate to produce an anionic intermediate without chirality at the α-carbon atom and (ii) re-protonation of the anionic intermediate from the opposite direction to form the antipodal amino acid. Alternatively, proton abstraction and re-protonation may proceed in concert without forming an anionic intermediate. Based on the concept proposed by Snell and Braunstein ~60 years ago [see Ref. (*8*) for a review], the cofactor in PLP-dependent racemases was thought to stabilize the anionic intermediate by forming a quinoid (keto-imine) species, thereby lowering the pK_a of the α-proton of α-amino acids. However, the reaction mechanism of amino acid racemase is not so straightforward when the structure of the enzyme active site is taken into consideration. There are two possible mechanisms by which to achieve racemization. A 'one-base' mechanism (also called the 'swinging-door' mechanism) utilizes a single amino acid residue in the active site as a base that abstracts the α-proton from both D- and L-enantiomers of a substrate. In this case, the plane involving the anionic intermediate must swing within the active site so that the opposite side of the α-carbon atom is oriented towards the single catalytic base. On the other hand, a 'two-base' mechanism utilizes two different active site residues, alternately abstracting the α-proton from either a D- or L-amino acid and returning it to form the opposite enantiomer. A classical biochemical method for distinguishing the two mechanisms is to examine the distribution of a hydrogen isotope (typically, deuterium) within the substrate and product. In the one-base mechanism, the ^{2}H-label at the α-carbon atom of the substrate amino acid should be partially, if not fully, retained in the product enantiomer after a single turnover of the racemase reaction (internal return). In the two-base mechanism, by contrast, the ^{2}H-label would be mostly lost without being transferred to the product. When this method was used to analyse the reactions of α-amino ε-caprolactam racemase (*9*) and amino acid racemase with low-substrate specificity (*10*), significant internal return of the ^{2}H-label was observed, suggesting these two enzymes employ the one-base mechanism. On the other hand, assisted by the later availability of the X-ray crystal structures and advanced molecular biological techniques (*e.g.* site-directed mutagenesis), the reaction catalysed by alanine racemase from the thermophilic bacterium *Bacillus stearothermophilus* was unequivocally shown to proceed through the two-base mechanism (*11*, *12*). During the reaction, two residues, Lys39 and Tyr265, located opposite cofactor PLP within the active site, abstract and return the α-proton of D- and L-alanine, respectively. Although the one-base mechanism proposed earlier remains to be reinvestigated, these elegant studies by Soda's group were performed >30 years after the discovery of arginine racemase activity in bacterial cells (*2*). This time-scale well represents Soda's attitude and continuous and strong interest in this research.

A D-amino acid can also be produced from an α-keto acid and another D-amino acid through a reaction catalysed by an aminotransferase (EC sub-subclass 2.6.1), which also utilizes PLP as an essential cofactor. In 1974, D-amino acid aminotransferase (DAAT) (EC 2.6.1.21) was first purified from *B. sphaericus* and its enzymological properties characterized (*13*). Fifteen years later, a thermostable DAAT was purified from the thermophilic bacterium *Bacillus* sp. YM-1, which was isolated from sauna dirt collected from a public bathhouse by a bright graduate student in Soda's laboratory (*14*). He also cloned the gene encoding DAAT, which was efficiently expressed in *E. coli* cells with the aid of gene cloning techniques rapidly being developed around that time (*15*). The X-ray crystal structure of the recombinant enzyme was determined several years later (*16*). A number of aminotransferases acting on L-amino acids had been reported at that time. Thus, L-aspartate, L-alanine, L-lysine, as well as branched-chain L-amino acids such as L-leucine and L-valine, served as specific amino donors for each particular enzyme, though the amino acceptors were limited to α-ketoglutarate and pyruvate. By contrast, DAAT is the only enzyme known to act on D-amino acids with broad substrate specificity for a variety of D-amino acids and α-keto acids. Upon sequence determination of the *DAAT* gene (*15*), however, it was unexpectedly noted that DAAT has significant sequence similarity to branched-chain

L-amino acid aminotransferase (BCAT) (EC 2.6.1.42). Moreover, structure-based classification of PLP-dependent enzymes (*17*) puts both DAAT and BCAT in the fold-type IV group, while most other aminotransferases acting on L-amino acids belong to the fold-type I group. Hence, an important and interesting question to be subsequently addressed in Soda's group was: how do these two structurally and evolutionarily related enzymes recognize their amino acid substrates with opposite stereochemical configurations?

To answer this question, a stereochemical analysis was conducted to investigate the hydrogen transfer process during the transamination reaction, which consists of two half-reactions mediated by the cofactor PLP. In the first half-reaction, which proceeds through the formation of a Schiff-base intermediate between an amino acid substrate and PLP, the proton abstracted from the α-carbon atom of the substrate is transferred to the 4′-carbon atom of PLP, forming a quinoid intermediate that is then hydrolysed to the product α-keto acid and the pyridoxamine 5′-phosphate (PMP)-form of the cofactor. In the second half-reaction, which proceeds through the formation of the same intermediate between another α-keto acid substrate and PMP, one of the two prochiral hydrogen atoms at the C4′ position of bound PMP is returned to the Cα position of the substrate, producing a new amino acid and regenerating PLP. These proton transfers are assumed to be performed by a single active-site base (usually a PLP-binding Lys residue), although the transferred proton may be rapidly exchangeable with the solvent during the reaction. Therefore, the process should proceed only on one side (either the *re*- or *si*-face) of the plane of the Schiff-base/PLP π-electron conjugate system (defined as the 'suprafacial' proton transfer) (Fig. 3). To investigate the stereospecificity of the proton transfer in aminotransferase reactions, a neat and simple method using two stereospecifically ^{3}H-labelled PMPs, (4′*S*)-[4-^{3}H]PMP and (4′*R*)-[4-^{3}H]PMP, was developed (*18*, *19*). The aminotransferase to be examined was first converted to the PLP-free (apo) form and then incubated with either one of the ^{3}H-labelled PMPs. Finally, the enzyme was added to an α-keto acid to initiate the half-reaction of transamination. By measuring the ^{3}H-label released into the solvent after the reaction, the stereochemistry of the proton transfer can then be easily determined; the ^{3}H-label in (4′*R*)-PMP is released during the *re*-face reaction, whereas the ^{3}H-label in the (4′*S*)-isomer is released during the *si*-face reaction (Fig. 3). When the reactions of various aminotransferases and other PLP-dependent enzymes acting on L-amino acids were examined comprehensively, it was found that the ^{3}H-label was only released from (4′*S*)-PMP, indicating that these enzymes have *si*-face stereospecificity for proton transfer, in agreement with the results obtained for individual enzymes in previous studies (*19*). By contrast, the ^{3}H-label was released exclusively from (4′*R*)-PMP in the reactions catalysed by DAAT and BCAT, showing that these two enzymes have *re*-face stereospecificity. This suggests the proton-transferring catalytic base is located on the *re*-face side in both DAAT and BCAT. But to position the α-hydrogen atom of L- and D-amino acids perpendicularly with respect to the *re*-face (*i.e.* to achieve the maximum overlap of the σ and π electrons), the binding site for the substrate α-carboxyl group must be in topologically opposite locations in the two enzymes (*18*). Comparison of the active site structures of the two enzymes determined by X-ray crystallography supported this prediction (*19*).

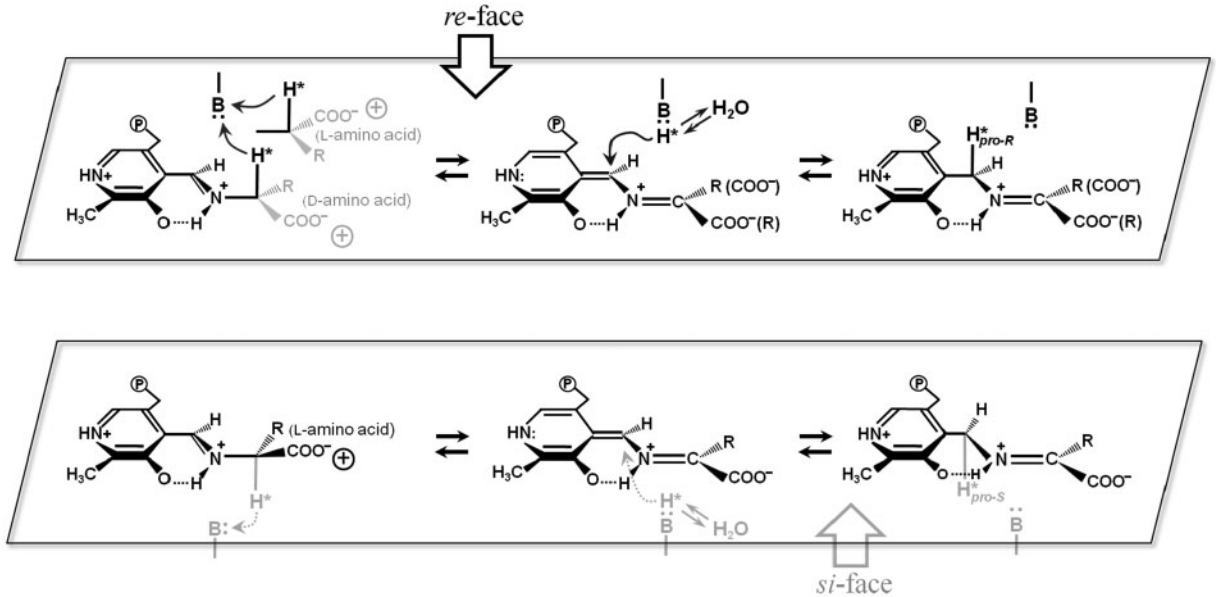

Fig. 3 Stereospecificity of hydrogen transfer during transamination. The conformation of the substrate amino acid around the Cα atom is fixed by electrostatic interaction of the carboxyl group with the positively charged amino acid residue (⊕) of the enzyme. The α-hydrogen atom of the substrate amino acid, which forms an external Schiff-base with PLP, is abstracted by the catalytic base (–B:) of the enzyme, and transferred to the C4′ of the cofactor to produce a quinoid intermediate. The hydrogen transfer proceeds either above (*re*-face, shown in black) or below (*si*-face, shown in grey) the plane of the Schiff-base. The proton on the catalytic base is subject to solvent exchange.

Interestingly, another PLP-dependent enzyme, aminodeoxychorismate lyase (EC 4.1.3.38), which is not an aminotransferase but exhibits significant sequence similarities with DAAT and BCAT, and also belongs to the same fold-type IV group, also catalysed the *re*-face proton transfer in its abortive transamination (*20*). Moreover, alanine racemase, which employs the two-base mechanism described earlier, catalysed both *si*-face and *re*-face hydrogen transfers in the half-reaction of abortive transamination with L- and D-alanine, respectively (*19*). Altogether, these and other studies of PLP-dependent enzymes acting on D- and L-amino acids (*18–24*) convey the important concept that whereas the stereospecificity of inherent reactions was strongly conserved during the molecular evolution of these enzymes, the stereo- and structural specificities for substrates were subject to reversion and diversion through subtle changes in the active site structure of the enzymes.

Before and even for a long time after Soda initiated the D-amino acid studies, D-amino acids were considered to occur only in microorganisms and certain plants and were believed to have no physiological functions in animals. At present, however, much attention is being paid to D-amino acids because of the discovery of several kinds of free and protein-bound D-amino acids in various eukaryotic organisms, including mammals, and identification of their important physiological functions. For example, D-serine has been shown to serve as a co-agonist of the *N*-methyl-D-aspartate receptor and to be involved in higher brain functions such as memory and learning (*25*). In addition, D-aspartic acid participates in important functions such as regulation of hormone secretion (*26*) and is involved in mammalian fertilization processes (*27*). As a result, enzymes responsible for the synthesis and degradation of D-amino acids are now being extensively studied by many researchers. To further facilitate these studies and to exchange mutual information, the 'D-Amino Acid Research Society of Japan' was recently established, and Soda was asked to serve as its first president.

Enzymes Acting on Amino Acids Containing Sulphur or Selenium

The extensive studies by Soda's group focusing on identifying novel enzymes involved in amino acid metabolism are complimented by their study of enzymes acting on sulphur- and selenium-containing amino acids. In 1977, methionine γ-lyase (EC 4.4.1.11) was purified from *Pseudomonas ovalis* and characterized (*28*), and was subsequently isolated from other bacterial species (*29*, *30*). Collectively, these enzymes catalyse a variety of PLP-dependent reactions, including α,γ-elimination and γ-replacement in L-methionine and its analogues (L-homocysteine, L-ethionine and *O*-acetyl-L-homoserine) as well as α,β-elimination and β-replacement in L-cysteine and its analogues (*S*-methyl-L-cysteine and *O*-acetyl-L-serine). Moreover, the enzyme was also shown to act on L-vinylglycine, which acts as a suicide substrate to inactivate several PLP-dependent enzymes, and to catalyse the γ-addition reaction with various alkanethiols to produce the corresponding *S*-substituted L-homocysteines. In applied fields, the versatile reactivity of methionine γ-lyase is very advantageous for synthesizing various sulphur-containing amino acids from cheaply available thiol compounds and L-methionine (*31*). At present, studies of methionine γ-lyase are focusing on exploitation of the enzyme as a drug target for the treatment of infectious diseases caused by parasitic protozoa and anaerobic periodontal bacteria (*32*). In addition, methionine γ-lyase has been utilized to develop therapeutic interventions for various cancers, by introducing recombinant enzymes to deplete L-methionine, which is essential for the growth of cancer cells (*32*).

The unique capability of methionine γ-lyase to act on L-selenomethionine and L-selenocysteine triggered the study of novel enzymes related to selenium-containing amino acids. With atomic number 34, selenium belongs to the same group as sulphur (group 16) in the periodic table and has just begun to be recognized as an essential micronutrient in mammals. Most notably, it has been identified in the active site of several enzymes, where it occurs as a selenocysteine residue (*33*). Because selenocysteine is not included in the genetic codon table, it was believed that there must be a specific co- or post-translational mechanism for incorporating selenocysteine into proteins (selenocysteine was later called the '21st amino acid' encoded by the termination codon TGA), but the biosynthesis and metabolism of free selenium-containing amino acids (selenomethionine and selenocysteine) had not been well-studied. Soda and his colleagues began investigating how selenated amino acids are synthesized and metabolized in rat liver. An important new finding that came out of this study was that selenocysteine could be synthesized from selenomethionine via a pathway analogous to its sulphur counterparts; *i.e.* the *trans*-sulphuration pathway involving cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1) (*34*). This pioneering observation is consistent with recent findings indicating that most enzymes are not able to distinguish between sulphur and selenium atoms contained in their substrate molecules. Thus, selenomethionine is easily incorporated into proteins in place of methionine; this property is utilized for the non-specific selenium labelling of proteins in the multiwavelength anomalous diffraction (MAD) method applicable for X-ray crystallography.

During the study of selenocysteine biosynthesis, another, far less efficient, pathway to selenocysteine formation was detected using a rat liver homogenate system. This suggested that there must be a novel enzyme that decomposes selenocysteine in liver homogenate. In 1982, a unique PLP-dependent enzyme, selenocysteine β-lyase (EC 4.4.1.16), was discovered in mammalian tissues (pig liver) and bacterial cell extracts (from *Citrobacter freundii*), and was identified as the first enzyme that acted exclusively on a selenated amino acid (*35*, *36*). Characterization also revealed that the mammalian and bacterial enzymes differ

greatly with respect to their physicochemical properties and their amino acid compositions (*36*, *37*). Nevertheless, the two enzymes share very similar enzymological properties, catalysing the degradation of L-selenocysteine to L-alanine and selenium, but not acting on L-cysteine at all. Thus, in marked contrast to methionine γ-lyase, selenocysteine β-lyase distinguishes selenium from sulphur in its substrate. Through the action of this enzyme, L-selenocysteine is stoichiometrically converted to hydrogen selenide and L-alanine when there is an excess amount of a reducing agent, dithiothreitol (DTT), in the reaction mixture. However, this does not necessarily mean that the primary product of the enzyme reaction is hydrogen selenide. Therefore, the stoichiometry of the enzyme reaction was carefully investigated by varying the concentrations of L-selenocystine and DTT in the presence of a large amount of enzyme to instantly decompose all of the L-selenocysteine formed from L-selenocystine (*38*). When the DTT-to-L-selenocystine molar ratio in the reaction mixture was 1 or <1, formation of red elemental selenium was observed, while no hydrogen selenide was formed at all. The amount of alanine formed was essentially twice the amount of selenocystine consumed. By contrast, when the molar ratio was >1, hydrogen selenide was produced. These experiments clearly show that selenocysteine β-lyase catalyses the removal of elemental selenium from L-selenocysteine, and that the formation of hydrogen selenide is due to non-enzymatic reduction of the product, elemental selenium, by DTT. Later on, the cDNA encoding mouse selenocysteine β-lyase was cloned (*39*), and more recently the X-ray crystal structure of the rat liver enzyme was determined (*40*). The results of the mechanistic studies of selenocysteine β-lyase raised a fundamental question about the physiological role of the enzyme in mammalian tissues. Although the results of a number of studies have implicated this enzyme in selenium recycling for selenoprotein biosynthesis, further studies will be required to better define the cellular function of selenocysteine β-lyase. Aside from its potential role in selenoprotein synthesis, recent studies also indicate selenocysteine β-lyase is up-regulated in acute glomerulonephritis and may be involved in the pathophysiology of hepatocellular carcinoma (*41*).

In 1988 another novel enzyme, D-selenocystine α,β-lyase (EC number, not given yet), was found in *Clostridium sticklandii* (*42*). This PLP-dependent enzyme catalysed α,β-elimination of D-selenocystine to produce pyruvate, ammonia and elemental selenium. In addition to D-selenocystine, D-cystine, D-lanthionine, meso-lanthionine and D-cysteine could also serve as substrates, but D-selenocysteine was inert. This enzyme also catalysed the β-replacement reaction between D-selenocystine and various thiol compounds to yield the corresponding *S*-substituted D-cysteines. Thus far, the gene encoding D-selenocystine α,β-lyase has not been cloned, and much about its structure and physiological function remains unknown.

Almost a decade after the discovery of selenocysteine β-lyase by Soda's group, an enzyme homologous to mammalian selenocysteine β-lyase was reported by Zheng *et al.* (*43*). They studied the function of a protein called NifS, which is required for the construction of [Fe-S] clusters for nitrogenase in the diazotrophic bacterium *Azotobacter vinelandii*. The NifS protein was identified as a PLP-dependent cysteine desulphurase (EC 2.8.1.7), which catalyses the same type of reaction as selenocysteine β-lyase, but acts on both L-selenocysteine and L-cysteine indiscriminately (*43*, *44*). Since then, these enzymes from bacteria and eukaryotes have been extensively studied, and their structures, reaction mechanisms and physiological functions have been elucidated (*45–50*). Notably, it was shown that cysteine desulphurase decomposes L-cysteine to L-alanine and sulphane sulphur (a trivial name for polysulphides) via the formation of an enzyme-bound persulphide intermediate. The persulphide sulphur is subsequently incorporated into the biosynthetic pathways of sulphur-containing molecules such as [Fe-S] clusters, thiamin, thionucleosides (in tRNA), biotin, lipoic acid and molybdopterin (*49*). The formation of an enzyme-bound selenopersulphide intermediate has also been demonstrated in the reaction of selenocysteine β-lyase and implicated in the delivery of an activated form of selenium for an enzyme involved in the biosynthesis of selenoproteins (*40*). It is thus becoming clear that cysteine desulphurase and selenocysteine β-lyase share a similar chemical mechanism for their catalytic reactions.

Enzymes from Thermophilic and Psychrophilic Bacteria

While performing basic biochemical studies on the various enzymes described earlier, Soda always had in mind their application as biocatalysts. For example, leucine dehydrogenase (LeuDH) (EC 1.4.1.9) purified from *B. sphaericus* (*51*) was shown to be applicable to the spectrophotometric determination of branched-chain amino acids and their α-keto acid analogues (*52*), and to the clinical assay of leucine aminopeptidase in human serum (*53*). Based on the information about LeuDH reported by Soda's group, a German group developed a novel type of enzyme membrane reactor for the continuous production of L-leucine coupled with an NADH regeneration system driven by formate dehydrogenase (*54*). However, industrial use of this membrane reactor system was restricted due to the high cost of large-scale purification of LeuDH. Thus, until the end of 1970s, two serious problems associated with the high cost of enzyme preparation stood in the way of more extensive applications of enzymes: their instability and low productivity. To overcome enzyme instability, starting in the1980s Soda and his colleagues began exploring thermostable enzymes produced by thermophilic bacteria and found high levels of LeuDH activity in a moderate thermophile *B. stearothermophilus* (recently renamed as *Geobacillus stearothermophilus*), from which the enzyme was purified and characterized (*55*). As expected, the *B. stearothermophilus* enzyme was very stable when incubated for 1 h at a high temperature (~70°C), was also stable over a wide range of

pHs (5.5–10.0), and could be stored in solution at 4°C for more than a month without appreciable loss of activity. Indeed, the high stability of the enzyme significantly prolonged its half-life in the membrane reactor system and markedly improved production of L-leucine (*56*).

To improve enzyme productivity, Soda's group began using the genetic engineering techniques that had been rapidly developing since the 1970s. A simple but brilliant idea occurred to them: if the gene encoding a thermostable enzyme was efficiently expressed in *E. coli* cells, the gene product could be easily purified by heating the cell extract, as most of the heat-labile proteins derived from the host cells would be denatured and precipitated. This idea was first tested with LeuDH from *B. stearothermophilus*, and it was found that the enzyme was produced 50-fold more efficiently when heterologously expressed in *E. coli* cells than when expressed in the original *B. stearothermophilus* cells (based on the specific activities of LeuDH in the crude extracts) (*57*). In addition, the enzyme was purified very rapidly with a high yield using heat treatment (70°C, pH 5.4, for 30 min) and two conventional column chromatography steps. This dramatically reduced the time needed for enzyme purification from >3 months to <1 week. Hence, an innovative method for the efficient production of highly purified enzymes from thermophilic bacteria was established (*58*), which not only reduced the cost of enzymes useful for various biotechnological applications but also promoted further application of thermostable enzymes (*59*, *60*) and stimulated basic biochemical and structural study of numerous other enzymes (*5*, *11*, *12*, *14–16*, *61*, *62*), some of which have already been described.

Studies of thermostable enzymes produced by thermophilic bacteria also gave rise to another related research stream. If, as was expected, psychrophilic bacteria living in a cold environment expressed enzymes capable of high catalytic activity at low temperatures, where enzymes from mesophiles and thermophiles would exhibit almost no activity, it might be possible to apply these enzymes in food processing and related fields. Thus, alanine racemase (EC 5.1.1.1) was first purified from the psychrotroph *P. fluorescens*, isolated from raw milk, and compared with corresponding enzymes from mesophilic and thermophilic bacteria (*63*). Although most of their enzymatic properties were nearly the same, the psychrophilic enzyme was extremely thermolabile and exhibited a high level of activity at temperatures as low as 0°C (*63*, *64*). Subsequently, aldehyde dehydrogenase (EC 1.2.1.5) and aspartase (EC 4.3.1.1), purified from *Cytophaga* sp. KUC-1, a typical psychrophile living in Antarctic seawater, were unexpectedly found to be thermostable and to show their highest levels of activity at ~55°C, yet they also showed significant activity under the cold conditions (*65*, *66*). These thermostable, cold-active enzymes are expected to be ideal catalysts for use in clinical analyses, food processing and as additives in laundry detergents. The higher contents of branched-chain, hydrophobic amino acid residues (Ile, Val and Leu) in enzymes from psychrophiles may account for the low thermostability and/or high activity at low temperatures. More detailed information about the structural basis of these properties must wait for their X-ray crystallographic analysis (*67*). In addition, structural comparison of psychrophilic and thermophilic enzymes should provide valuable information that can be used for the development of more practically useful enzymes in the near future.

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Conflict of interest
None declared.

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