Arginine Kinase from Nautilus pompilius, a Living Fossil

SITE-DIRECTED MUTAGENESIS STUDIES ON THE ROLE OF AMINO ACID RESIDUES IN THE GUANIDINO SPECIFICITY REGION*

Received for publication, April 6, 2000, and in revised form May 9, 2000
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M002926200

Tomohiko Suzuki†, Hideki Fukuta, Hiromi Nagato, and Masahiro Umekawa

From the Laboratory of Biochemistry, Faculty of Science, Kochi University, Kochi 780-8520, Japan

Arginine kinases were isolated from the cephalopods Nautilus pompilius, Octopus vulgaris, and Sepioteuthis lessoniana, and the cDNA-derived amino acid sequences have been determined. Although the origin and evolution of cephalopods have long been obscure, this work provides the first molecular evidence for the phylogenetic position of Cephalopoda in molluscan evolution. A crystal structure for Limulus arginine kinase showed that four amino acid residues (Ser63, Gly64, Val65, and Tyr68) are hydrogen-bonded with the substrate arginine. We introduced three independent mutations, Ser63 → Gly, Ser63 → Thr, and Tyr68 → Ser, in Nautilus arginine kinase. One of the mutants had a considerably reduced substrate affinity, accompanied by a decreased Vmax. In other mutants, the activity was lost almost completely. It is known that substantial conformational changes take place upon substrate binding in arginine kinase. We hypothesize that the hydrogen bond between Asp62 and Arg183 stabilizes the closed, substrate-bound state. Site-directed mutagenesis studies strongly support this hypothesis. The mutant (Asp62 → Gly or Arg183 → Gly), which destabilizes the maintenance of the closed state and/or perhaps disrupts the unique topology of the catalytic pocket, showed only a very weak activity (0.6–1.5% to the wild-type).

Phosphagen (guanidino) kinases constitute a family of highly conserved enzymes, which catalyzes the reversible transfer of phosphate from phosphagen such as creatine phosphate to ADP. Creatine kinase (CK),† the most widely studied member of this family, plays a central role in both temporal and spatial ATP buffering in cells that display high and variable rates of energy turnover (1, 2). The crystal structures have been determined for chicken mitochondrial CK (3) and muscle and brain CKs (4, 5). The remaining major members of this family (arginine kinase (AK), glycocyamine kinase (GK), lombricine kinase (LK), taurocyamine kinase (TK)), have not been investigated as extensively as CK but likely play a similar physiological role (2, 6, 7).

Arginine kinase (AK) is widely distributed in the lower and higher invertebrate groups and is present in many lower chordates but absent in the vertebrates (6, 8). Interestingly, AK evolved at least twice during evolution of phosphagen kinases: first, at an early stage of phosphagen kinase evolution (its descendants are molluscan and arthropod AKs) and second, from CK at a later time in metazoan evolution (9). Conventional wisdom would suggest that AK is the most primitive member of the phosphagen kinase family and that the other members, including CK, arose from tandem gene duplications and subsequent divergence (6, 10, 11).

From an earlier amino acid sequence alignment of Cks, GK, LK, and AKs, we proposed that a region displaying remarkable amino acid deletions (referred to as the guanidino specificity (GS) region) was a possible candidate for the guanidino recognition site (12). This GS region is overlapped partly by the so-called flexible loop in the crystal structures of chicken mitochondrial CK (3) and Limulus AK (13). There is a proportional relationship between the size of the deletion in the GS region and the mass of guanidine substrate used (12). Among the amino acid residues in the GS region, we noted previously that the seventh residue, Asp, is conserved in all AK sequences but not in other phosphagen kinases. This residue is not associated with the substrate binding (13), but we assumed that it has a special role in the recognition of the substrate arginine (14).

Mollusks constitute one of the most diverse animal phyla. Living representatives are classically divided into seven classes, of which major ones are Bivalvia, Gastropoda, and Cephalopoda. There are several hypotheses concerning molluscan phylogeny based on morphological features, but the results are still controversial. Sequence comparisons using 18S RNA, 12S rRNA, or mitochondrial DNA have been used to solve the complicated phylogeny of molluscan classes except Cephalopoda (15–19). However, molecular analyses have not resulted in a clear determination for the phylogeny. Thus, the origin and evolution of cephalopods have long been obscured by the poor fossil record and molecular data.

Nautilus pompilius is the only living representative of the large extinct group of nautiloids, ammonites and belemnites, and is sometimes referred to as a living fossil, like Latimeria and Lingula. In this report, first, we determined the cDNA-derived amino acid sequences of AKs from three cephalopods N. pompilius, Octopus vulgaris, and Sepioteuthis lessoniana (squid) to elucidate the evolution of molluscan AKs. Next, we examined the functional role of several amino acid residues on the GS region of Nautilus AK by site-directed mutagenesis. In particular, we focused on functional significance of Asp62, which is not associated with the substrate binding but is conserved in all AK sequences.

Throughout this report, the sequence numbering of Limulus AK (20) is used. The actual sequence number in Nautilus AK, used in the mutagenesis studies, can be obtained by subtracting 6 from the Limulus numbering.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Biochemistry, Faculty of Science, Kochi University, Kochi 780-8520, Japan. Tel.: 88-844-0111; Fax: 88-844-8356; E-mail: suzuki@sc.kochi-u.ac.jp or suzuki@cc.kochi-u.ac.jp.

‡ The abbreviations used are: CK, creatine kinase; AK, arginine kinase; GK, glycocyamine kinase; LK, lombricine kinase; TK, taurocyamine kinase; GS region, guanidino specificity region; TSAC, transition state analog complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s).
ARGinine Kinase from N. pompilius

Isolation of Arginine Kinases—All procedures were carried out at 4–8 °C. The body wall muscle (10 g) of Nautilus, Sepioteuthis, or Octopus was homogenized with 50 ml of 10 mM Tris acetate buffer (pH 8.1) containing 0.1 mM diithiothreitol. The tissue extract was fractionated with 60–80% saturated ammonium sulfate. The precipitate was dissolved in a minimum volume of the same buffer, and applied to a Sephadex G-75 column (3 × 115 cm) equilibrated with the same buffer. The fractions possessing AK activity were pooled and applied to a DEAE-5PW column (7.5 × 75 mm, Tosoh) equilibrated with 20 mM Tris acetate buffer (pH 8.1) containing 0.2 mM dithiothreitol. The column was washed with the same buffer and then eluted with a linear gradient of 0–500 mM NaCl in 20 mM Tris acetate buffer. The purified AK was stored on ice or at 0–50 °C in 80 mM Tris acetate buffer containing 0.1 mM dithiothreitol, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The sample was incubated in acetate buffer (pH 8.1) containing 0.2 mM dithiothreitol. The column was stored on ice or at 0–50 °C in 80 mM Tris acetate buffer containing 0.1 mM dithiothreitol. The tissue extract was fractionated before electrophoresis.

Enzyme activity was assayed spectrophotometrically at 25 °C (7). Protein concentration was estimated from the absorbance at 280 nm (the absorbance 0.77 at 280 nm in a 1-cm cuvette corresponds to 1 mg of protein/ml).

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in 15% acrylamide gels containing 0.087% of protein/ml. (the absorbance 0.77 at 280 nm in a 1-cm cuvette corresponds to 1 mg of protein/ml).

The open reading frame of 1053 base pairs (bp) of Nautilus AK was purified by affinity chromatography using amylose resin (New England BioLabs). The enzyme assay was done using the purified fusion protein.

The PCR products were digested with DpnI, and the target 7000-bp DNA was recovered by EasyTrap (version 2, Takara). After blunting and kination, the DNA was self-ligated. The mutated protein was expressed as described above. The mutated cDNA insert was sequenced completely, and it was confirmed that there is no unexpected mutation.

RESULTS

Arginine kinases (AKs) from the body wall muscles of Nautilus, Octopus, and Sepioteuthis were purified to homogeneity using ammonium sulfate fractionation, Sephadex G-75 gel filtration and DEAE-5PW chromatography. SDS-PAGE, in the presence of a reducing agent, showed that the isolated AKs were highly purified, and the molecular masses were about 40 kDa. The AKs were confirmed to be monomers on the gel filtration column. No N-terminal amino acid of the AKs was detected by protein sequencing, indicating that N termini were blocked. The isolated enzyme was pyridylethylated and digested with lysyl endopeptidase. The peptides were purified by reverse-phase chromatography, and some peptides were sequenced (Fig. 1, A–C).

The enzymatic parameters,  for arginine and the open reading frame for Nautilus AK is 1053 nucleotides in length and encodes a protein with 350 amino acid residues; those for Octopus AK, 1044 nucleotides and 347 residues; those for Sepioteuthis AK, 1047 nucleotides and 348 residues. The validity of the cDNA-derived amino acid sequences was supported by chemical sequencing of internal lysyl endopeptidase peptides (81 amino acid residues determined for Nautilus AK, 43 residues for Octopus AK, and 1306 residues for Sepioteuthis AK). The molecular masses were calculated to be 39,276, 39,176, and 39,040 Da, respectively, consistent with the values estimated by SDS-PAGE. The nucleotide and amino acid sequences has been submitted to the DDBJ (accession numbers AB042330 for Nautilus, AB042331 for Octopus, and AB042332 for Sepioteuthis).

The recombinant maltose binding protein/Nautilus AK (wild type) gave enzymatic parameters  (0.68 mM) and (7.64 μmol of P/min/mg of protein) similar to those of the native enzyme. Those for the mutated Nautilus AKs are listed in Table I.

| Mutation | Primer name | Sequence |
|----------|-------------|----------|
| Asp62 → Gly | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Asp62 → Glu | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Ser65 → Gly | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Ser65 → Thr | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Tyr97 → Ser | NaAK.mF3 | TCTCCGCGCTGTGAACCAATGTGTT |
| Arg196 → Gly | NaAK.mR5 | GCTGATTCTAAGAACAGCGTGG |

MATERIALS AND METHODS

TABLE I

Primers used for site-directed mutagenesis of Nautilus AK

| Mutation | Primer name | Sequence |
|----------|-------------|----------|
| Asp62 → Gly | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Asp62 → Glu | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Ser65 → Gly | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Ser65 → Thr | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Tyr97 → Ser | NaAK.mF3 | TCTCCGCGCTGTGAACCAATGTGTT |
| Arg196 → Gly | NaAK.mR5 | GCTGATTCTAAGAACAGCGTGG |

| Mutation | Primer name | Sequence |
|----------|-------------|----------|
| Asp62 → Gly | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Asp62 → Glu | NaAK.mF1 | TCTAGCGCTGTGAACCAATGTGTT |
| Ser65 → Gly | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Ser65 → Thr | NaAK.mR1 | TCCCAACACACACTTCCAGGTGTT |
| Tyr97 → Ser | NaAK.mF3 | TCTCCGCGCTGTGAACCAATGTGTT |
| Arg196 → Gly | NaAK.mR5 | GCTGATTCTAAGAACAGCGTGG |
ARGinine Kinase from N. pompilius

We described before that the amino acid sequences of molluscan AKs could be used as a molecular clock to elucidate the divergence time of molluscs, because the enzymes appear to be derived from a single gene strain and have a moderate evolutionary rate (24). A maximum likelihood clock-like tree constructed from the 36 amino acid sequences allows us to estimate the divergence time of the interested molluscan species. If we assume the divergence between Mollusca and Arthropoda occurred about 550 million years before present (25), the radiation time of Polyplacophora, Gastropoda, and Cephalopoda is estimated to be 290 million years before present (data not shown).

Role of Amino Acid Residues of the GS Region in AK Function

Substrate-binding Residues—Amino acid sequences around the GS region of phosphagen kinases are shown in Fig. 4. This GS region is overlapped partly by the so-called flexible loop in the crystal structure of chicken mitochondrial CK, which has been speculated to move nearer to the active site to exclude water during catalysis (3). Clearly, there is a proportional relationship between the size of the deletion in GS region and the mass of guanidine substrate used. Namely, LK and AK, which use relatively large guanidine substrates, lombricine and arginine, have a five-amino acid deletion in this region, CK has one amino acid deletion, and GK has no deletion. It is possible that the introduction of an amino acid deletion as in the GS region in Fig. 4 shortens the flexible loop and allows the active site to accommodate a larger guanidine substrate.

Recently, a crystal structure for the transition state analog complex (TSAC) of the AK from the horseshoe crab Limulus has appeared (13). The structure showed that four amino acid residues (Ser63, Gly64, Val65, and Tyr68 in Limulus AK sequence), located in the GS region (see Fig. 4), are hydrogen-bonded with the amino and carboxylate groups of the substrate arginine. These residues are conserved in all three AK sequences from Nautilus, Octopus, and Sepioteuthis, suggesting that the substrate-binding mechanism of cephalopod AKs is the same as that of Limulus AK.

Among molluscan AKs, the four residues associated with substrate binding are conserved in most AKs. However, residue Ser64 or Ser65 is replaced by Ser, Lys, or Cys in the two-domain AKs from the clams Pseudocardium, Corbicula, and Solen and the sea anemone Anthopleura. Thus it appears that the two-domain AKs may have a unique substrate-binding system.

We introduced two independent mutations, Ser65 → Gly and Ser63 → Thr, in the 63rd position of Nautilus AK (Table II). At least one of the mutant proteins (Ser63 → Gly) had a considerably reduced substrate affinity ($K_m$ = 3.45 mM) compared with the wild type ($K_m$ = 0.68 mM), accompanied also by a decreased V$_\text{max}$ (less than 5% to the wild-type). In the case of Ser63 → Thr mutation, the AK activity was lost almost completely, probably due to a steric hindrance.

The Tyr68 is conserved in all AKs, including two-domain clam AKs, except in the sea cucumber Stichopus AK, which evolved not from the AK gene but from the CK gene (9). Stichopus AK has a completely different substrate-binding system (see the structure of Stichopus AK in Fig. 4), which will be described elsewhere. The aromatic side chain of Tyr forms a hydrogen-bond with the amino group of the substrate arginine in Limulus AK, and thus the Tyr68 appears to be the most important residue in substrate binding. Consistent with this, the enzyme activity of Nautilus Tyr68 → Ser mutation was lost.

**TABLE II**

Enzymatic parameters of molluscan AKs

All parameters were obtained at 25 °C under the same conditions. Values in parentheses are the relative value (%) to Nautilus recombinant wild type (WT) AK. The parameters are the average of at least two runs.

| Species | Origin | $K_m$ for arginine | V$_\text{max}$ or V* |
|---------|--------|-------------------|---------------------|
| Solen   | Native | 1.48              | 5.9                 |
| Meretrix| Native | 0.43              | 4.5                 |
| Aplysia | Native | 0.6               | ND                  |
| Octopus | Native | 2.82              | 4.3                 |
| Sepioteuthis | Native | 1.74              | 10.4                |
| Nautilus| Native | 1.02              | 10.4                |
| Recombinant | 0.65 (100) | 7.64 (100) |

* Data for Solen, Meretris, and Aplysia are unpublished results by T. Suzuki, T. Taniuchi, Y. Unemi, and T. Higashi.
* For the very weak enzyme activity, the exact $V_\text{max}$ could not be obtained. These values are the observed rates in the presence of 9.5 mM arginine.
* Two-domain AKs.

**DISCUSSION**

Evolution of Cephalopod AKs and Phylogenetic Position of Nautilus

The cDNA-derived amino acid sequences of Nautilus, Octopus, and Sepioteuthis AKs were aligned with that of Limulus (horseshoe crab) AK (20) in Fig. 2, which is the only AK whose crystal structure is known (13). The sequence of Nautilus AK showed high percentage identity (69–73%) with those of Octopus and Sepioteuthis AKs. The identity between Octopus and Sepioteuthis AKs is 83%. These cephalopod AK sequences still showed high percentage identity (69–73%) with those of the arthropod (horseshoe crab) AK (20) in Fig. 2, which is the only AK whose sequence has appeared (13). The structure showed that four amino acid residues (Ser63, Gly64, Val65, and Tyr68 in Limulus AK sequence), located in the GS region (see Fig. 4), are hydrogen-bonded with the amino and carboxylate groups of the substrate arginine. These residues are conserved in all three AK sequences from Nautilus, Octopus, and Sepioteuthis, suggesting that the substrate-binding mechanism of cephalopod AKs is the same as that of Limulus AK.

Among molluscan AKs, the four residues associated with substrate binding are conserved in most AKs. However, residue Ser64 or Ser65 is replaced by Ser, Lys, or Cys in the two-domain AKs from the clams Pseudocardium, Corbicula, and Solen and the sea anemone Anthopleura. Thus it appears that the two-domain AKs may have a unique substrate-binding system.

We introduced two independent mutations, Ser65 → Gly and Ser63 → Thr, in the 63rd position of Nautilus AK (Table II). At least one of the mutant proteins (Ser63 → Gly) had a considerably reduced substrate affinity ($K_m$ = 3.45 mM) compared with the wild type ($K_m$ = 0.68 mM), accompanied also by a decreased $V_\text{max}$ (less than 5% to the wild-type). In the case of Ser63 → Thr mutation, the AK activity was lost almost completely, probably due to a steric hindrance.

The Tyr68 is conserved in all AKs, including two-domain clam AKs, except in the sea cucumber Stichopus AK, which evolved not from the AK gene but from the CK gene (9). Stichopus AK has a completely different substrate-binding system (see the structure of Stichopus AK in Fig. 4), which will be described elsewhere. The aromatic side chain of Tyr forms a hydrogen-bond with the amino group of the substrate arginine in Limulus AK, and thus the Tyr68 appears to be the most important residue in substrate binding. Consistent with this, the enzyme activity of Nautilus Tyr68 → Ser mutation was lost.

**FIG. 1.** Nucleotide and derived amino acid sequences of cDNAs of N. pompilius (A), O. vulgaris (B), and S. lessoniana (C) AKs. Arrows indicate primers used for amplification. Amino acid sequences determined chemically are shown by dotted lines.
Role of Asp\textsuperscript{62}—We noted previously that the Asp\textsuperscript{62} in the GS region is conserved in most AK sequences, including Stichopus AK, which evolved from the CK gene, but not in other phosphagen kinases CK, GK, and LK (9, 14) (see Fig. 4). The TSAC crystal structure of Limulus AK indicates that Asp\textsuperscript{62} is not associated with the substrate binding, and the authors have not referred to any functional role (13). On the other hand, we assumed previously that Asp\textsuperscript{62} has a special role in the recognition of the substrate, arginine (14), and the first domain of Pseudocardium two-domain AK might not retain a complete enzyme activity, because the Asp\textsuperscript{62} was replaced by a Gly residue (Fig. 4).

The TSAC structure of Limulus AK indicates that it consists of two domains; an N-terminal small \(\alpha\)-helical domain (N-domain) of residues 1–111 and a C-terminal domain (C-domain) possessing an 8-stranded antiparallel \(\beta\)-sheet flanked by 7 \(\alpha\)-helices of residues 112–357 (13). One of the two substrates, ATP or ADP, is accommodated in the C-domain, and another substrate, arginine or arginine phosphate, contacts mainly with the N-domain. The catalytic center, where reversible transfer of the phosphate occurs, is located in the C-domain. We found that in the TSAC structure of Limulus AK, viewed by SwissPdbViewer (PDB Id: 1BG0), the side chain of Asp\textsuperscript{62} in the N-domain is hydrogen-bonded with that of Arg\textsuperscript{193} in the C-domain (Fig. 5), and that Arg\textsuperscript{193} is conserved in all AKs. Here we hypothesize that the hydrogen bond between Asp\textsuperscript{62} and Arg\textsuperscript{193} plays a crucial role in AK function, namely, that the two domains are maintained in a favorable topology by the hydrogen bond, where the two kinds of substrates are accessible enough for reaction.

If the 3-dimensional structure of Nautilus AK is homologous with that of Limulus AK (13), site-directed mutagenesis studies using Nautilus AK strongly support the above hypothesis. The references for sources of sequences: Pseudocardium (11); Liophora and Battilus (9); Nordotis (8); Corbicula.D2, Solen.D2, Aplysia, and Cellana (Suzuki et al.\textsuperscript{7}).
mutant of Stichopus AK. In addition, the results of mutation Asp62→Gly provides strong evidence for the functional role of Asp62. This mutation, like the former two mutations, could be expected not to change the substrate affinity for arginine, but it could be expected to have ability to form a hydrogen bond with Arg193. Enzymatic parameters are consistent with this idea; the $K_m$ (0.67 mM) is the same as that (0.68 mM) of wild-type, and the $V_{max}$ is reduced largely but not so much as that of the Asp62→Gly or Arg193→Gly mutations, probably due to a partial formation of hydrogen bond between Glu62 and Arg193. Zhou et al. (13) have suggested that it is the precise orientation of the substrates in the catalytic pocket of AK that dominates the forces driving catalysis. Substantial conformational changes take place upon substrate binding in CK (26). The recent crystal structures of CK (3–5) and AK (13) provide very compelling insight into the nature of these structural changes. The extent domain and flexible loop movements during catalysis have recently been modeled by Forstner et al. (27) for CK and by Zhou et al. (28) for AK. Our results and the above models show that, upon substrate binding, the flexible loop bearing Asp62 moves nearer to the active center to exclude water during catalysis, and the hydrogen bond between Asp62 and Arg193 is formed to link the N- and C-domain of AK (Fig. 5). This bond stabilizes the closed, substrate-bound state. The mutant (Asp62→Gly or Arg193→Gly) destabilizes the maintenance of the closed state and/or perhaps disrupts the unique topology of the catalytic pocket; therefore, these mutants show only a very weak activity (0.6–1.5% to the wild-type, see Table II). The mutant (Asp62→Glu), while allowing hydrogen bonding between Glu62 and Arg193, may also disturb the topology of the catalytic pocket.

Our results are highly consistent with prevailing views on the catalytic mechanism of phosphagen kinases. Maintenance of the precise three-dimensional structure of the active site is critical for optimum efficiency of enzyme function. Thus, we conclude that Asp62 (or Arg193) is one of the key residues in AK function.

**Acknowledgments**—We thank Dr. Ross Ellington for critical reading of the manuscript and giving us helpful suggestions and Takuya Hi-gashi for performing experiments on amino acid sequencing of Nautilus AK.

**REFERENCES**

1. Meyer, R. A., Sweeney, H. L., and Kushmerick, M. J. (1984) *Am. J. Physiol.* 246, C365–C377.
2. Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., and Eppenberger, H. M. (1992) *Biochem. J.* 281, 21–40.
3. Fritz-Wolf, K., Schneyder, T., Wallimann, T., and Kabsch, W. (1996) *Nature*
Arginine Kinase from N. pompilius

1. 381, 341–345
2. Rao, J. K., Bujacz, G., and Wlodawer, A. (1998) FEBS Lett. 439, 133–137
3. Eder, M., Schlattner, U., Becker, A., Wallimann, T., Kaehl, W., and Fritz-Wolf, K. (1999) Protein Sci. 8, 2258–2269
4. Watts, D. C. (1971) in Biochemical Evolution and the Origin of Life (Schoffeniel, E., ed) pp. 150–173, North-Holland, Amsterdam
5. Ellington, W. R. (1989) J. Exp. Biol. 143, 177–194
6. Watts, D. C. (1975) Symp. Zool. Soc. Lond. 36, 105–127
7. Suzuki, T., Kamidochi, M., Inoue, N., Kawamichi, H., Yazawa, Y., Furukohri, T., and Ellington, R. W. (1999) Biochem. J. 340, 671–675
8. van Thoai, N. (1968) in Homologous Enzymes and Biochemical Evolution (van Thoai, N., and Roche, J., eds) pp. 199–229, Gordon and Breach, New York
9. Suzuki, T., Kawasaki, Y., Furukohri, T. (1997) Biochim. Biophys. Acta 1348, 152–159
10. Zhou, G., Somasundaram, T., Blane, E., Parthasarathy, G., Ellington, W. R., and Chapman, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8449–8454
11. Suzuki, T., Kawasaki, Y., Nishimura, Y., Soga, T., Kamidochi, K., Yazawa, Y., and Furukohri, T. (1998) Biochim. Biophys. Acta 1388, 253–259
12. Winnepenninckx, B., Backeljau, T., and De Wachter, R. (1995) Mol. Biol. Evol. 13, 641–649
13. Winnepenninckx, B., Backeljau, T., and De Wachter, R. (1996) Mol. Biol. Evol. 13, 1306–1317
14. Steiner, G., and Muller, M. (1996) J. Mol. Evol. 43, 58–70
15. Adamkiewicz, S. L., Harasewych, M. G., Blake, J., Saudek, D., and Bult, C. J. (1997) Mol. Biol. Evol. 14, 619–629
16. Winnepenninckx, B., Backeljau, T., and De Wachter, R. (1994) Nautilus 2, (suppl.) 98–110
17. Strong, S. J., and Ellington, W. R. (1995) Biochim. Biophys. Acta 1246, 197–200
18. Suzuki, T., and Furukohri, T. (1994) J. Mol. Biol. 237, 353–357
19. Strimmer, K., and von Haeseler, A. (1996) Mol. Biol. Evol. 13, 964–969
20. Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, U. S. A.
21. Suzuki, T., Ban, T., and Furukohri, T. (1997) Biochim. Biophys. Acta 1340, 1–6
22. Goodman, M., Pedwaydon, J., Czelusniak, J., Suzuki, T., Gotel, T., Moens, L., Shishikura, P., Walz, D., and Vinogradov, S. (1990) J. Mol. Evol. 27, 236–249
23. Forstner, M., Kriechbaum, M., Laggner, P., and Wallimann, T. (1996) J. Mol. Struct. 383, 217–227
24. Forstner, M., Kriechbaum, M., Laggner, P., and Wallimann, T. (1998) Biophys. J. 75, 1016–1023
25. Zhou, G., Ellington, W. R., and Chapman, M. S. (2000) Biophys. J. 78, 1541–1550