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NOTE

Thermal stability and biochemical properties of isocitrate dehydrogenase from the thermoacidophilic archaeon *Thermoplasma acidophilum*

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Abstract Isocitrate dehydrogenase [IDH; EC 1.1.1.42] from the thermoacidophilic archaeon *Thermoplasma acidophilum* (*Ta*IDH) showed high thermal stability with an apparent melting temperature, T_m, of 82.2 and 84.5°C at pH 7.5 and 5.8, respectively. Based on structural alignment of *Ta*IDH with IDH from *Aeropyrum pernix* (*Ap*IDH) and *Archaeoglobus fulgidus* (*Af*IDH) residues forming an aromatic cluster in the clasp-domain thought to strengthen the dimer interface in *Ap*IDH and *Af*IDH were identified in the former enzyme. Moreover, *Ta*IDH had a shortened N-terminus that may protect the enzyme from thermal denaturation. The enzyme activity of *Ta*IDH was highest at 70°C. The pH-activity profile was bell-shaped with an optimum shifted to a lower pH compared to *Af*IDH. The activity of *Ta*IDH was influenced by changes in pH with a three-fold reduction in activity when the pH was shifted from the pH-optimum at 7.5 to pH 5.8. However, the specific activity at pH 5.8 was still high when compared with *Af*IDH. The reduction in activity at pH 5.8 was not due to instability of the enzyme as the T_m of *Ta*IDH was higher at pH 5.8 than at 7.5 and the enzyme retained 91% of its activity after incubation at 1 h at pH 5 and 60°C. The difference in the pH-profile of *Ta*IDH in comparison with *Af*IDH may thus be related to the pK_as of their catalytic residues involved in the initial proton abstraction and the final proton donation during the catalysis of oxidative decarboxylation of isocitrate to 2-oxoglutarate and reduced coenzyme.

Keywords Isocitrate dehydrogenase · *Thermoplasma acidophilum* · Thermal stability · Thermoactivity · Acidophilic

Abbreviations

IDH	Isocitrate dehydrogenase
*Ta*IDH	*Thermoplasma acidophilum* IDH
*Af*IDH	*Archaeoglobus fulgidus* IDH
*Pf*IDH	*Pyrococcus furiosus* IDH
*Ec*IDH	*Escherichia coli* IDH
DSC	Differential scanning calorimetry
T_m	Melting temperature

Isocitrate dehydrogenase (IDH) belongs to the metal-dependent (Mg^{2+} or Mn^{2+}) β-decarboxylating dehydrogenases, an enzyme in the tricaboxylic acid cycle which catalyses the oxidative decarboxylation and subsequent dehydrogenation of D-isocitrate to α-ketoglutarate and CO_2 using NAD^+(EC 1.1.1.41) or $NADP^+$(EC 1.1.1.42) as cofactor (Hurley et al. 1991). IDHs are broadly distributed throughout Bacteria, Eukarya and Archaea (Steen et al. 2001) and based on primary sequence identity, IDHs have previously been divided into three distinct phylogenetic subfamilies, subfamily I $(NAD(P)^+$-IDHs from archaea and bacteria), subfamily II $(NAD(P)^+$-IDHs from eukarya and bacteria) and subfamily III (NAD^+-IDHs from eukarya) (Steen et al. 2001). The crystal structures of $NADP^+$-dependent IDH from *Escherichia coli* (*Ec*IDH) (Hurley

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et al.1989), *Bacillus subtilis* IDH (Singh et al. 2001), *Archaeoglobus fulgidus* (*Af*IDH) (Stokke et al. unpublished data) and *Aeropyrum pernix* (*Ap*IDH) (Karlström et al. 2005) have revealed a high structural similarity between intra-family members of subfamily I. However, the thermal properties among these IDHs vary significantly with *Ap*IDH and *Af*IDH being most thermostable with an apparent melting temperature (T_m), of 109.9 and 98.5°C, respectively, in comparison with the T_m of 52.6°C of *Ec*IDH. Structural comparisons of *Ap*IDH and *Af*IDH with *Ec*IDH have revealed fixation of the N-terminus, shortening of surface loops, inter-domain ionic networks and aromatic clusters for stabilizing the dimer interface as putative major mechanisms for increasing the thermal stability of the two former enzymes (Karlström et al. 2005; Stokke et al. unpublished data).

Data regarding the thermal properties of IDH from *Thermoplasma acidophilum* (*Ta*IDH) will provide valuable information in the mid-range temperature area between the IDHs from the hyperthermophilic *A. pernix* and *A. fulgidus* and the IDH from the mesophilic *E. coli*.

Wild-type *T. acidophilum* DSMZ 1728 cells were cultivated aerobically at 59°C in medium 158 as described by DSMZ. Genomic DNA was isolated using AquaPure Genomic DNA Isolation Kit from BioRad (Bio-Rad Laboratories Ltd., UK) according to the manufacturer's instructions. The putative *idh* gene from *T. acidophilum*, as amplified by PCR using the following primer sets: 5′-CACCATGGCATATATTC AAGTGAAGGAGG-3′ and 5′-AAGCCTTTAGT GAACAGGTTTTTTCATCCTGTTG-3′, was found to contain a high percentage of rare *E. coli* codons, AGG and AGA, coding for arginine. Hence, expression of *Ta*IDH in *E. coli* BL21 was performed in the presence of a plasmid (pSJS1240), which expressed the argU and ileX genes encoding rare tRNAs (Kim et al. 1998). Twelve of the total 17 arginines in *Ta*IDH were encoded by AGG, whereas none of the total 17 arginines were encoded by AGG in *Ec*IDH. The overexpression of recombinant *Ta*IDH in *E. coli* strain BL21/pSJS1240 was performed by growing transformed cells in LB broth containing ampicillin (100 μg/ml) and spectinomycin (50 μg/ml) at 37°C to OD_{600} = 0.7–0.8 and subsequent expression (3–4 h) after addition of 1.0 mM isopropyl-beta-D-thiogalactopyranoside. The purification of recombinant *Ta*IDH was performed as previously described for other archaeal IDHs (Steen et al. 2001) and resulted in a high yield of purified enzyme (Table 1). However, the amount of purified recombinant *Ta*IDH of 3 mg pure protein/liter culture was considerable lower than previously obtained for other archaeal IDHs (Steen et al. 2001). This result indicates a low expression level of *Ta*IDH in *E. coli* despite the presence of the plasmid pSJS1240. The enzyme was purified to homogeneity as assessed by SDS-PAGE with Coomassie blue staining.

All amino acid residues involved in binding of isocitrate in *Ec*IDH and *Ap*IDH from subfamily I were conserved in *Ta*IDH (Fig. 1). Purified recombinant *Ta*IDH showed no activity when NAD^+ was used as cofactor in concentration up to 2 mM, but showed preference for $NADP^+$ with a K_m of 111 μM. This result is supported by the conservation (Fig. 1) of amino acids involved in cofactor specificity in the $NADP^+$-dependent *Ec*IDH (Hurley et al. 1991; Dean and Golding 1997). This result contradicts the data reported on enzyme activity measurements in crude extract from *T. acidophilum* indicating *Ta*IDH as having dual-cofactor spesificity (Potter 1993).

*Ta*IDH had highest specific activity at 70°C (Fig. 2a), i.e., 11°C higher than the growth optimum of the host organism (Table 2). At 70°C *Ta*IDH showed a half-life ($t_{1/2}$) of 88 min, which decreased to 24 min when the temperature was increased to 75°C (Fig. 2b). The thermal stability of *Ta*IDH was furthermore estimated by differential scanning calorimetry (DSC). As previously found for other IDHs (Steen et al. 2001), the thermal unfolding of *Ta*IDH was found to be an irreversible process. Hence, only an apparent midpoint T_m could be determined. At pH 7.5, a T_m of 82.2°C was found for *Ta*IDH and the value shows that *Ta*IDH has a thermal stability midway between *Ap*IDH and *Af*IDH, respectively, and *Ec*IDH (Table 2). Interestingly, at pH 5.8, close to the reported intracellular pH of the host organism (Searcy 1976), *Ta*IDH revealed a T_m of 84.6°C, i.e., 2.3°C higher than at pH 7.5 (Fig. 2c). The acid tolerance of *Ta*IDH, compared to *Af*IDH, was further estimated by incubating the enzymes for 1 h at pH 3, 4 and 5 and measuring the residual activity. *Ta*IDH was found to be moderate acid toler-

Table 1 Purification of recombinant *Ta*IDH expressed in *E. coli* BL21 (pSJS1240)

	Total protein/liter culture (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	187.3	1836	27	100
Heat treatment (60°C)	140	1821.25	23.5	99.2
Red Sepharose affinity chromatograpy	3	1190	396.7	64.8

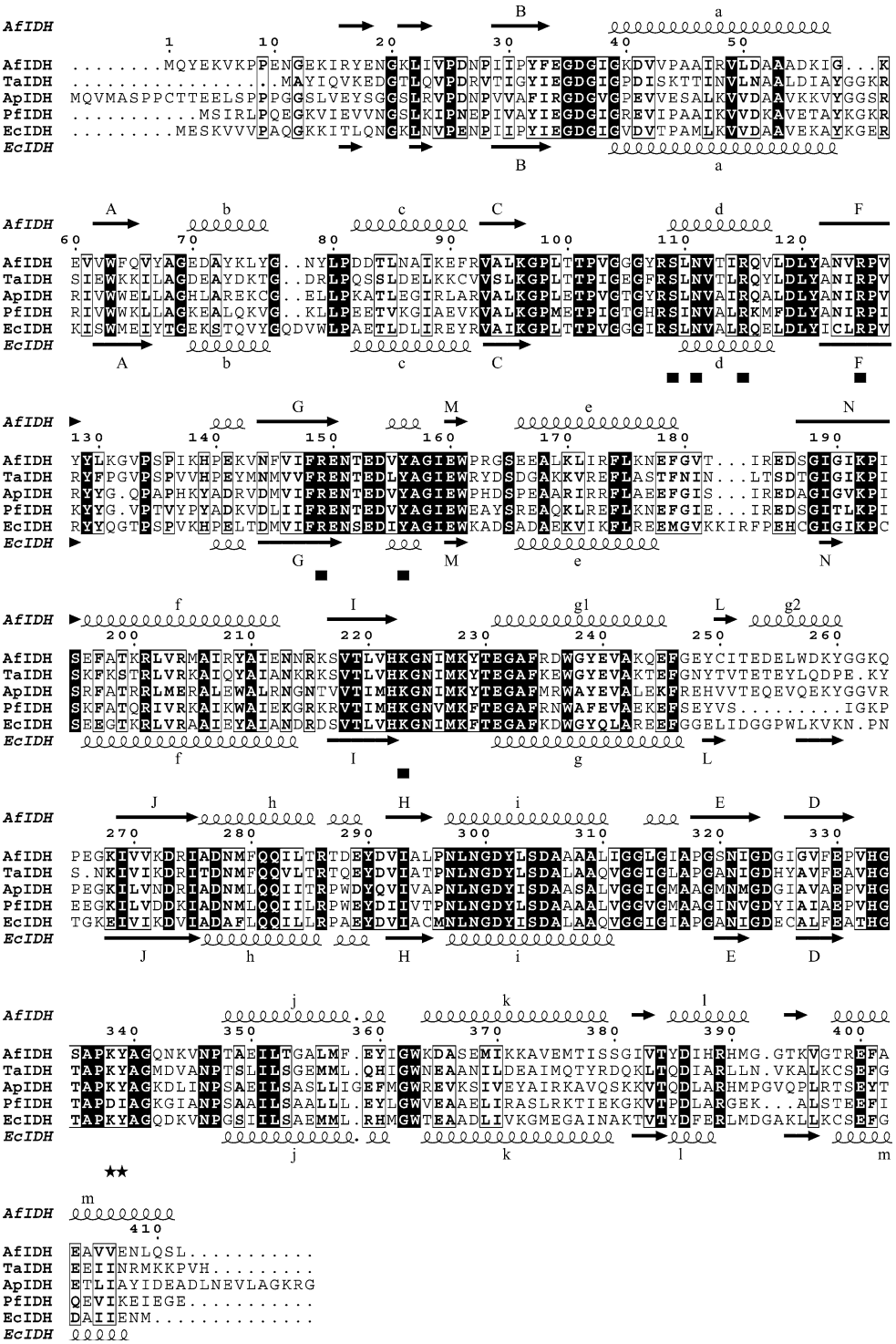

Fig. 1 Sequence alignment of IDH sequences from archaea; *Af*IDH (CAB09535), *Ta*IDH (NP_393595), *Ap*IDH (NP_147421) and *Pyrococcus furiosus* IDH (*Pf*IDH; NP_577931), and bacterial *Ec*IDH (NP_415654). The sequence alignment was made in clustalW and secondary structure assignments were added in ESPript (Gouet et al. 1999). Secondary structure assignments was given the nomenclature as implemented in *Ec*IDH (Hurley et al. 1989), *Ap*IDH (Karlström et al. 2005) and *Af*IDH (Stokke et al. unpublished data). Amino acids responsible for binding of isocitrate and the discrimination of cofactor $NADP^+$ in *Ec*IDH are marked with *boxes* and *stars*, respectively

ant with 81% residual activity remaining after 1 h at pH 4.0. In comparison, *Af*IDH showed only 67% residual activity. Both enzymes were inactive after 1 h at pH 3.0 (not shown).

Structure analysis of homodimeric *Ap*IDH and *Af*IDH revealed a conserved aromatic cluster in the clasp-domain formed by three aromatic residues from each monomer (Karlström et al. 2005; Stokke et al. unpublished data). This aromatic cluster is thought to strengthen the subunit interactions and contribute to the high thermal stability observed for these enzymes. An aromatic cluster may also be formed in *Ta*IDH as the residues involved are conserved in its primary structure; Trp154, Phe167 and Phe172 (Fig. 1). The

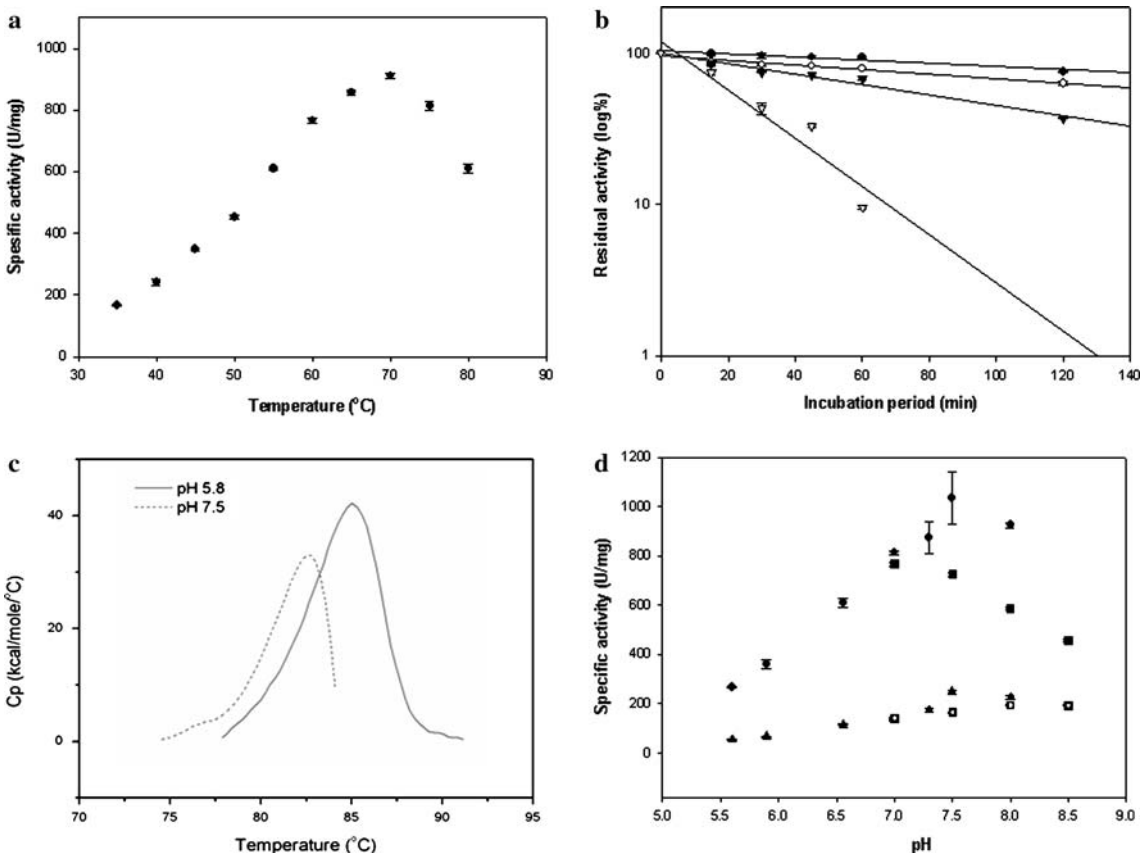

Fig. 2 **a** Temperature optimum for activity of *Ta*IDH as determined spectrophotometrically by monitoring the conversion of $NADP^+$ to NADPH at 340 nm and varying temperatures from 35 to 80°C in 50 mM Tricine/KOH pH 7.0. **b** Inactivation of *Ta*IDH incubated in a glycerol bath at given temperatures; 60 (*filled circle*), 65 (*open circle*), 70 (*filled inverted triangle*) and 75°C (*open inverted triangle*). Aliquots were removed at given time intervals, 0–120 min, snap-cooled on ice and residual activity was determined at 60°C in 50 mM Tricine/KOH pH 7.5. **c** Apparent T_m of *Ta*IDH, as determined by *DSC* in 50 mM potassium-phosphate, 0.1 M NaCl, pH 5.8 and 7.5, respectively. The calorimetric scans were carried out between 20 and 100°C with a scan rate of 1 K/min. A second scan was run to estimate reversibility. Apparent T_ms were determined from the transition midpoint upon unfolding, due to the irreversible nature of the enzyme. **d** pH-profile for activity of *Ta*IDH with *Af*IDH as control. The profiles were determined with two buffer systems; 20 mM sodium-phosphate pH 5.6–8.0 (*filled circle*, *Ta*IDH and *filled triangle*, *Af*IDH) and 50 mM Tricine/KOH pH 7.0–8.5 (*filled square*, *Ta*IDH and *circle inside filled square*, *Af*IDH)

Table 2 Thermal stability of IDH from mesophilic and (hyper)thermophilic microorganisms

Organisms	T_{growth} (°C)	T_{opt} (°C)	T_m (°C)[a]
P. furiosus	100	≥95	103.7[b]
A. pernix	90–95	≥95	109.9[b]
A. fulgidus	83	90	98.5[b]
T. acidophilum	59	70	82.2
E. coli	37	50	52.6[c]

[a] 50 mM potassium phosphate pH 7.5, 0.1 M NaCl

[b] Steen et al. (2001)

[c] Karlström et al. (2005)

residues involved in a 4-membered ionic network from the clasp-domain of one subunit to the small domain of the adjacent subunit in *Af*IDH were however, not conserved in the sequence of *Ta*IDH. However, the loop shortening observed between helix e and strand N (Fig. 1) in the clasp-domain of *Af*IDH compared to *Ec*IDH was also observed in the sequence of *Ta*IDH. One of the strategies of the hyperthermophilic IDHs to maintain the integrity of the structure at high temperature has been anchoring of the N-terminus. In *Ap*IDH, a disulfide bond at the N-terminus was confirmed by mutagenesis to be involved in the stability above 100°C, as disruption of this interaction reduced the T_m from 109.9 to 100.3°C (Karlström et al. 2005). In the structure of *Af*IDH, no disulfide bond was observed. However, the N-terminus of *Af*IDH was shown to be shorter than in *Ap*IDH. Furthermore, an aromatic cluster in the N-terminus of *Af*IDH was suggested to stabilize this region. The N-terminus of *Ta*IDH was shown to be shorter than both the mesophilic *Ec*IDH and the hyperthermophilic *Pf*IDH,

*Af*IDH and *Ap*IDH with 9, 7, 10 and 18 amino acids, respectively (Fig. 1). Hence, a substantial shortening in this area could protect the N-terminus and aid in the protection of *Ta*IDH from thermal degradation. Previous studies on citrate synthase (CS) have shown no extra interactions in the N-terminus of *T. acidophilum* CS to be essential for its thermal stability (Bell et al. 2002). In *Ap*IDH, a seven-membered network was located between the large and the small domain at the opposite side of the active site (Karlström et al. 2005). Analysis of *Af*IDH found three of these residues conserved in an ionic network. Sequence comparisons revealed the same three residues conserved in *Ta*IDH (Asp112, Arg194 and Asp315). However, the three residues were also conserved in *Ec*IDH. A structure of *Ta*IDH is being pursued in order to investigate the molecular elements involved in the thermal stability of this enzyme.

Thermoacidophiles are highly adapted to the high temperature and the harsh environment of low pH (0–4) by maintaining an intracellular pH close to neutral (Darland et al. 1970; Searcy 1976; van de Vossenberg et al. 1998; Macalady et al. 2004). Activity measurements of *Ta*IDH at various pH-values revealed a bell shaped pH-activity profile (Fig. 2d) with an optimum shifted to a lower pH compared with *Af*IDH. At 60°C *Ta*IDH showed the highest activity at pH 7.5. However, the activity in 20 mM sodium-phosphate revealed large differences between pH 7.3–7.5. In comparison, the pH-optimum for activity of *Af*IDH has previously been found to be pH 8.6 (Steen et al. 1997). Furthermore, the pH-profile of *Ta*IDH and *Af*IDH revealed the activity of *Ta*IDH as highly buffer- and pH-dependent compared to *Af*IDH (Fig. 2d). At pH 5.6, close to the physiological intracellular pH of *T. acidophilum* at pH 5.5 (Searcy 1976), *Ta*IDH still sustained a high specific activity as compared to *Af*IDH. The pH-optimum of activity for *Ta*IDH is approximately 2 pH units above the intracellular pH of the host organism (Searcy 1976). Similar observations have also been found for glucose dehydrogenase from *P. torridus* (Angelov et al. 2005). The decreased activity at pH 5.6 compared to pH 7.5 is not ascribed to an inactivation of *Ta*IDH since incubation at pH 5 and 60°C retained approx 91% initial activity of the enzyme. The bell shaped pH-activity profile of *Ta*IDH could be explained by the ionization states of two catalytic residues involved in the conversion of isocitrate to α-ketoglutarate. The oxidative decarboxylation of isocitrate to α-ketoglutarate and NADPH proceeds in two steps. In the initial step, isocitrate is oxidized to oxalosuccinate by the removal of a proton from the hydroxyl oxygen to a base and the transfer of a hydride to $NADP^+$. In *Ec*IDH, the base was suggested to be Asp283 (Hurley et al. 1991). However, in porcine IDH and *Ap*IDH an active-site water molecule was suggested to accept this proton as part of a proton relay to the solvent (Ceccarelli et al. 2002; Karlström et al. 2005). Second, the β-carboxylate of oxalosuccinate is lost as CO_2 followed by the stereospecific protonation of the β-carbon to form α-ketoglutarate. In *Ec*IDH, Tyr160 and Lys230′ was shown to be hydrogen-bonded to the β-carboxylate of isocitrate and acted as acid catalyst that protonated C3 after decarboxylation (Hurley et al. 1991). The observed shift in pH-optimum in *Ta*IDH compared to *Af*IDH could be a result of a pK_a change of the two catalytic residues Asp283 and Lys230 (*Ec*IDH numbering). This was, however, not as extreme as for extracellular acidophilic enzymes, but rather in agreement with previously observed characteristics for intracellular enzymes from acidophilic organisms (Richter and Schafer 1992; Nemoto et al. 2003; Hansen et al. 2004; Angelov et al. 2005).

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