Biochimica et Biophysica Acta, 613 (1980) 1—9

BBA 68951

PHYSICOCHEMICAL AND CATALYTIC PROPERTIES OF THERMOSTABLE MALATE DEHYDROGENASE FROM AN EXTREME THERMOPHILE *THERMUS FLAVUS* AT-62

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(Received August 24th, 1979)

Key words: Malate dehydrogenase; Thermophile; Thermostable; (T. flavus)

Summary

Physicochemical and catalytic properties of thermostable malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37), isolated from an extreme thermophile, *Thermus flavus* AT-62, were studied. The enzyme had a molecular weight of 67 000 and consisted of two subunits with an identical molecular weight. The helical content of the enzyme was estimated to be about 25% from the circular dichroism spectrum. The amino acid composition of the thermophilic enzyme was similar to that of its mesophilic counterparts. Titration with 5,5′-dithiobis(2-nitrobenzoic acid) showed that the enzyme contained only one sulfhydryl group per subunit. Substrate inhibition by oxaloacetate was observed. The inhibition decreased with increasing temperature, but was still significant at 60°C. The enzyme was remarkably heat stable, without losing activity after incubation at 90°C for 60 min. The melting temperature of the secondary structure of the enzyme was 96°C.

Almost all enzymes and macromolecular structures of thermophile are known to be thermostable. The molecular mechanism of thermophily has been the subject of biological interest and important information on the problem has accumulated over the last years [1,2]. The free energy of stabilization of thermophilic proteins is estimated to be only 5—10 kcal per mol larger than that of their mesophilic counterparts [3,4]. An increased number of salt bridges in certain thermophilic proteins [5,6] and binding of metal ions in some others [7] have been shown to contribute to their thermostability. It has also been shown that thermophilic enzymes tend to have fewer sulfhydryl groups than their mesophilic counterparts so as to prevent oxidative inactiva-

tion at higher temperature [8]. Yutani and his collaborators [9,10], found that even an exchange of just one or two amino acid residues of α-subunit of tryptophan synthetase can bring about significant increase or decrease in its thermostability.

Many proteins, including hydrolytic enzymes and glycolytic enzymes from thermophiles have been studied extensively. However, studies on thermophilic enzymes involved in tricarboxylic acid cycle are still limited [1]. The purification, properties and catalytic mechanism of malate dehydrogenase from various sources have been the subject of extensive study in recent years. We undertook a study of a thermophilic malate dehydrogenase with the aim of understanding better the molecular mechanisms of thermostability of thermophilic enzymes by comparing the physicochemical and catalytic properties of this enzyme with those of the mesophilic enzymes.

We report here the physicochemical and catalytic properties of malate dehydrogenase from the extreme thermophile, *Thermus flavus* AT-62.

Materials and Methods

Chemicals

NAD^+ and NADH were purchased from Sigma Chemical Co. St. Louis, MO. *cis*-Oxaloacetic acid and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Tokyo Kasei Co. Ltd., Tokyo. L-Malic acid and sodium dodecyl sulfate (SDS) were from Wako Pure Chemical Industry Ltd., Osaka. Guanidine hydrochloride and diethyl pyrocarbonate were from Nakarai Chemical Ltd., Osaka. Pig heart mitochondrial malate dehydrogenase was the product of Boehringer Mannheim, F.R.G.

Thermophilic malate dehydrogenase

T. flavus AT-62 was grown and malate dehydrogenase purified using previously described procedures [11,12]. The purified preparation was homogeneous as judged by disc gel electrophoresis.

Enzyme assay

Malate dehydrogenase was assayed by following the decrease in absorbance of NADH at 340 nm in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratory Inc., Oberin, OH, U.S.A.) equipped with a Haake bath at 30°C. The standard assay mixture (3 ml) contained 33 mM potassium phosphate buffer (pH 7.0), 0.16 mM oxaloacetate, 0.15 mM NADH and catalytic amounts of the enzyme. One unit of enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 μmol NADH per min.

Protein determination

Protein concentration was determined by using an absorption coefficient $E^{1\%}_{280nm}$ of 10.6, which was obtained by measuring the absorbance at 280 nm and dry weight.

CD spectra and melting profile method

CD spectra were measured by a JASCO J-20 automatic recording spectro-

polarimeter with a temperature control system emloying a Haake bath. The data are expressed in mean residue ellipticity, with 108.3 for the mean residue weight of the enzyme.

The melting profile experiments were performed according to Fujita and Imhahori [4]. The CD signal at 223 nm was continuously recorded while the sample was heated at a constant rate (0.5—2.0°C per min). The temperature of the sample solution was monitored with a Takara SPD-1D thermister attached through the cap of the cell.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [13]. Isoelectric focusing was carried out according to the manufacturers instruction in a 110-ml electrofocusing column (LKB-Produkter).

Analytical ultracentrifuge

Sedimentation velocity analyses were performed at 20°C in a Hitachi UCA-1A ultracentrifuge at 55 000 rev./min with shlieren optics. Sedimentation equilibrium experiments were carried out using the short column technique according to Yaphanits [14]. The enzyme sample (1.2—6.5 mg/ml) was centrifuged at 8800 rev./min at 20°C. Before ultracentrifugal experiments enzyme solutions were exhaustively dialyzed against 5 mM Tris-HCl buffer (pH 7.2) containing 0.1 M KCl.

Gel filtration

The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-100 column. The column (2.2 × 80 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.2) containing 0.1 M KCl. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*.

Amino acid analysis

Lyophilized enzyme preparations were hydrolyzed with 6 N HCl in sealed, evacuated tubes at 110°C for 24, 48 and 72 h. Hydrolysates were analyzed on a JEOL-6AH amino acid analyzer. Corrections for zero time hydrolysis were made for threonine and serine from semilogarithmic extrapolation. Values determined with samples hydrolyzed for 72 h were used for valine and isoleucine. Tryptophan was determined by the spectrophotometric method of Goodwin and Morton [15]. Cysteine was determined as cysteic acid after performic acid oxidation.

Reaction with 5,5'-dithiobis(2-nitrobenzoic acid)

The enzyme was denatured in 0.1 M sodium phosphate buffer (pH 8.0) containing 1% SDS at 90°C for 5 min or in 0.1 M Tris-HCl buffer (pH 8.1) containing 6 M guanidine hydrochloride for 2 h at room temperature, then an excess of 5,5'-dithiobis(2-nitrobenzoic acid) was added. The number of sulfhydryl groups of the enzyme was estimated by determining at 412 nm the concentration of 2-nitro-5-thiobenzoate [16].

Results

Physicochemical properties

The sedimentation coefficient of the enzyme was determined at several protein concentrations (1.3—4.9 mg/ml). Extrapolation of the $s_{20,w}$ values to infinite dilution of protein concentration gave a $s^0_{20,w}$ value of 4.50 S. A molecular weight of 67 000 of the enzyme was found by using sedimentation equilibrium centrifugations. A partial specific volume of 0.745 cm^3/g, which was determined from the amino acid composition of the enzyme, was used in the calculation. A molecular weight of approx. 60 000 was obtained using gel filtration on a Sephadex G-100 column. During SDS-polyacrylamide gel electrophoresis the enzyme moved as a single band with a mobility corresponding to a molecular weight of 35 000. This value is approximately half of that obtained from ultracentrifugation and gel filtration studies. It is therefore concluded that the *T. flavus* enzyme is made up of two subunits with an identical molecular weight.

Fig. 1 shows far ultraviolet CD spectrum of the enzyme. In the wavelength range below 250 nm, negative bands at 209 and 223 nm are observed. The CD profile resembles that reported with the beef heart cytoplasmic enzyme [17]. From the ellipticity value at 209 nm, by using the relationship introduced by Greenfield and Fasman [18], the contant of α-helix was estimated to be about 25%. This value is smaller than that reported with the pig heart mitochondrial enzyme [19]. Isoelectrofocusing experiments gave a value of 5.25 for the isoelectric point of the enzyme.

Amino acid composition

The results of amino acid analyses are shown in Table I; the data are compared with those of the enzymes from *Pseudomonas testosteroni* [20], *Escherichia coli* [21], *Bacillus subtilis* [21] and pig heart mitochondria [22]. On the whole, the amino acid composition of the thermophilic enzyme is similar to that of the enzymes from the mesophilic sources. The number of sulfhydryl groups of the thermophilic enzyme was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid). The denatured enzyme rapidly reacted with the reagent and was shown to have 0.90—0.95 sulfhydryl groups per subunit. In contrast, the native enzyme (in 0.1 M sodium phosphate buffer, pH 8.0) did not react with the reagent.

Catalytic properties

Fig. 2 shows double reciprocal plots for oxaloacetate reduction. The enzyme is susceptible to substrate inhibition by excess of oxaloacetate. The inhibition decreased with increasing temperature. The threshold for inhibition by high oxaloacetate was 50 μM at 30°C, and 350 μM at 60°C. A similar substrate inhibition by oxaloacetate has been known to be a characteristic property of mitochondrial malate dehydrogenase from animal sources [23—25]. You and Kaplan reported that the *Pseudomonas* enzyme was also inhibited by excess oxaloacetate [20]. Fig. 3 shows pH dependence of the activity of the enzyme at 30 and 60°C. Apparent pH optima of 9.5 and 7.2 for oxaloacetate reduction were observed at 30 and 60°C, respectively. Corresponding pH optima for

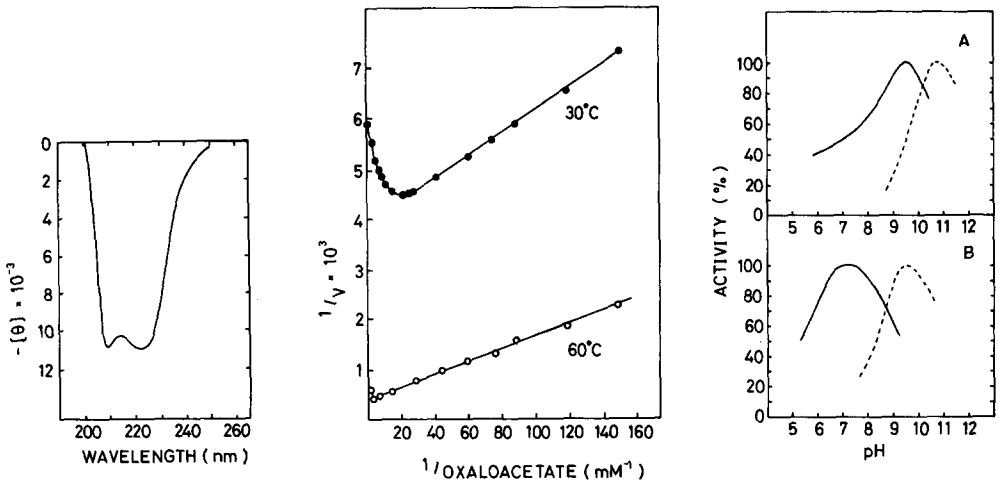


Fig. 1. CD spectrum of thermophilic malate dehydrogenase. The spectrum was measured in 20 mM potassium phosphate buffer (pH 7.0) in a 1-mm quartz cell at a protein concentration of 0.76 mg/ml at 25° C.

Fig. 2. Double reciprocal plots of initial velocity versus oxaloacetate concentration at 30 and 60° C. The assay mixture contained 33 mM potassium phosphate buffer (pH 7.0), 0.15 mM NADH and various amounts of oxaloacetate.

Fig. 3. Effect of pH on malate dehydrogenase activity at 30° C (A) and 60° C (B). Buffers employed were acetate (pH 4.5—6.0); potassium phosphate (pH 5.7—8.2); Tris-HCl (pH 7.2—9.5); and glycine-NaOH (pH 8.2—12.5). The assay mixture for oxaloacetate reduction contained 33 mM buffer, 0.15 mM NADH and 0.33 mM oxaloacetate. The assay mixture for malate oxidation contained 50 mM buffer, 0.5 mM NAD^+ and 6.0 mM malate. pH of the assay mixtures was directly measured using TOA model HM-5B pH meter at 30 and 60° C. ———, oxaloacetate reduction; - - - - - -, malate oxidation.

TABLE I

AMINO ACID COMPOSITIONS OF MALATE DEHYDROGENASE FROM *T. FLAVUS, P. TESTOSTERONI, E. COLI, B. SUBTILIS* AND PIG HEART MITOCHONDRIA

Amino acid	Residues per 70 000 × *g*				
	T. flavus	*P. testosteroni*	*E. coli*	*B. subtilis*	Pig heart mitochondria
Lys	33.3	36.6	43.5	41.9	56.7
His	7.5	9.8	6.0	1.8	13.3
Arg	35.4	16.9	19.3	27.9	15.4
Asp	49.5	67.7	51.9	61.2	49.0
Thr	27.3	30.6	38.1	46.6	46.2
Ser	17.6	29.5	35.1	37.8	38.5
Glu	68.9	58.0	73.9	73.1	48.3
Pro	35.1	30.7	30.1	29.7	52.5
Gly	58.5	64.6	72.8	67.9	61.6
Ala	92.1	107.2	71.8	51.9	74.2
Cys	2.0	3.5	5.8	0	15.4
Val	54.2	37.7	66.0	69.3	49.7
Met	22.3	21.4	10.3	8.5	11.2
Ile	44.7	33.0	36.1	59.5	40.6
Leu	51.0	63.3	77.6	77.0	58.1
Tyr	13.1	14.2	11.8	24.0	9.8
Phe	19.3	23.1	25.8	17.5	22.4
Trp	14.1	10.2	4.1	0	0

TABLE II

K_m VALUES FOR THERMOPHILIC MALATE DEHYDROGENASE

Assays in the direction of oxaloacetate reduction were performed in 33 mM potassium phosphate buffer (pH 7.0). Assays in the direction of malate oxidation were in 50 mM glycine-NaOH buffer (pH 10.0, 30°C).

Variable concentration substrate	Fixed concentration substrate	Apparent K_m (M)	
		at 30°C	at 60°C
NADH	0.33 mM oxaloacetate	$6.0 \cdot 10^{-6}$	$2.0 \cdot 10^{-5}$
Oxaloacetate	0.15 mM NADH	$9.8 \cdot 10^{-6}$	$3.0 \cdot 10^{-5}$
NAD^+	6.0 mM malate	$1.9 \cdot 10^{-5}$	$9.5 \cdot 10^{-5}$ *
Malate	2.0 mM NAD^+	$2.6 \cdot 10^{-5}$	$1.4 \cdot 10^{-4}$ *

* pH of reaction mixtures was 9.3 at 60°C.

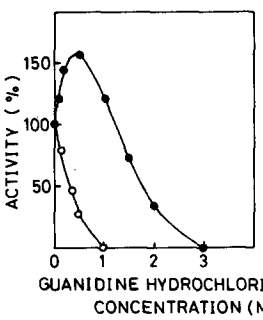
malate oxidation were 10.7 and 9.5. K_m values for the substrates and the coenzymes are summarized in Table II. The K_m values are 3—5-fold larger at 60°C than at 30°C. An Arrhenius plot of V for oxaloacetate reduction was linear to at least 75°C. An apparent energy of activation was calculated to be 14.5 kcal/mol.

Effects of diethyl pyrocarbonate

The thermophilic enzyme was inhibited by diethyl pyrocarbonate at pH 6.4. NADH protected the enzyme from the inactivation, whereas oxaloacetate had no effect. These results suggest that a histidyl residue is essential for malate dehydrogenase activity as is the case in porcine heart mitochondrial [26—28] and cytoplasmic [29] malate dehydrogenase.

Heat stability

There was no loss of enzymatic activity of thermophilic malate dehydrogenase after an incubation at 90°C for 60 min, whereas 50% of activity was lost



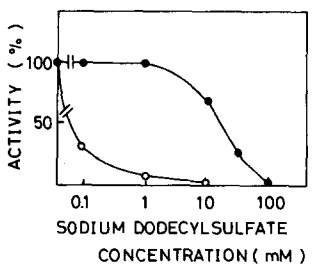


Fig. 4. Effects of guanidine hydrochloride on *T. flavus* (●) and pig heart mitochondrial (○) malate dehydrogenase. The enzymes were preincubated in 0.1 M Tris-HCl buffer (pH 7.2) containing various concentrations of guanidine hydrochloride at 30°C for 5 min, then the enzymatic reaction was started by the addition of substrates.

Fig. 5. Effects of SDS on *T. flavus* (●) and pig heart mitochondrial (○) malate dehydrogenase. The enzymes were preincubated in 0.1 M sodium phosphate buffer (pH 7.2) containing various concentrations of SDS at 30°C for 5 min, then the enzymatic reaction was started by the addition of substrates.

after 30 min at 96°C. The heat stability of the secondary structure of the enzyme was examined by the CD melting profile method. The melting temperature at which a half change in the CD signal occurs was observed to be 96°C. Changes observed in the CD signal were reversible below 90°C, while those observed above 93°C were irreversible.

Effects of denaturants

Effects of guanidine hydrochloride and SDS on the enzymatic activity were studied. The denaturants were directly added to the assay mixtures and the activity were assayed in the presence of them. The thermophilic enzyme was activated approx. 170% by 0.5 M guanidine hydrochloride, while the pig heart mitochondrial enzyme lost 70% of its initial activity by the same treatment (Fig. 4). As shown in Fig. 5, the thermophilic enzyme was also far more resistant to SDS than the pig heart mitochondrial enzyme.

Discussion

Murphey et al. [30] have divided malate dehydrogenase into two groups by size, the dimers and the tetramers. The former includes malate dehydrogenase from animal sources, *E. coli* and *P. testosteroni*, while the enzymes from *B. subtilis* and *B. stearothermophilus* belong to the latter group. From molecular weight analyses, the *T. flavus* enzyme obviously belongs to the former group.

The overall amino acid composition of the thermophilic enzyme does not differ significantly from the compositions of the enzymes from mesophilic sources. Several researchers have examined the amino acid compositions of thermophilic enzymes with regard to hydrophobicity [31—35]. They calculated the average hydrophobicity [36] and the polarity ratio [37], and reported that there was no definite correlation between these parameters of hydrophobicity and the thermal stability of proteins. As shown in Table III the differences in the values between thermophilic and mesophilic malate dehydrogenase are small. This is in accordance with previous observations which indicate that the molecular basis of thermal stability must be very subtle and it is not necessarily the same for all thermostable proteins.

Arrhenius plot of V for oxaloacetate reduction was linear up to 75°C. This suggests that there is probably no change in enzyme conformation over this

TABLE III

CALCULATED VALUES OF HYDROPHOBICITY PARAMETERS FOR MALATE DEHYDROGENASE FROM VARIOUS SOURCES

The values of parameters were calculated from the amino acid compositions given in Table I.

	Polarity ratio	Average hydrophobicity (kcal/mol)
T. flavus	0.715	1.170
P. testosteroni	0.729	1.092
E. coli	0.750	1.129
B. subtilis	0.872	1.186
Pig heart mitochondria	0.788	1.162

range of temperature. However, with some thermophilic enzymes, discontinuous slopes in Arrhenius plots of V have been reported [38—40]. Increases in K_m values for substrates, that is, a decrease in affinity for substrates with increasing temperature (Table II) have also been reported for glucose 6-phosphate isomerase [38] and fructose-1,6-diphosphate aldolase [39] from *B. stearothermophilus*.

T. flavus malate dehydrogenase exhibits similarities with the mesophilic enzymes in the amino acid composition, catalytic properties behaviour toward diethyl pyrocarbonate. On the other hand, a significant difference resides in heat stability. Murphey et al. [21] reported that the enzyme from *E. coli* was completely inactivated after an incubation at 50°C for 20 min. However, the *T. flavus* enzyme was still active after 60 min at 90°C. From the results of the CD melting profile experiments, the secondary structure of the enzyme was also stable up to 90°C. Furthermore, the thermophilic enzymes was more resistant to denaturants than the pig heart mitochondrial enzyme. Similarly, Fujita et al. [35] reported that glyceraldehyde-3-phosphate dehydrogenase from an extreme thermophile *T. thermophilus*, is more resistant to urea and SDS than its mesophilic counterparts.

Acknowledgement

The authors wish to express their thanks to the Central Research Laboratory of Ajinomoto Co., Ltd. (Kawasaki) for large scale cultivation of the organism.

References

1 Zuber, H. (eds.), (1976) Enzymes and Proteins from Thermophilic Microorganism, Birkhäuser Verlag, Basel
2 Friedman, S.M. (1978) Biochemistry of Thermophily, Academic Press, New York
3 Nojima, H., Ikai, A., Oshima, T. and Noda, H. (1977) J. Mol. Biol. 116, 429—442
4 Fujita, S.C. and Imahori, K. (1974) in Peptides, Polypeptides and Proteins (Blout, E.R., Bovey, F.A., Goodman, M. and Lotan, N., eds.), pp. 217—229, John Wiley and Sons, New York
5 Perutz, M.F. and Raidt, H. (1975) Nature 255, 256—259
6 Biesecker, G., Harris, J.I., Thierry, J.C., Walker, J.E. and Wonacott, A.J. (1977) Nature 266, 328—333
7 Saiki, T., Shinshi, H. and Arima, K. (1973) J. Biochem. 74, 1239—1243
8 Hoking, J.D. and Harris, J.I. (1976) in Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), pp. 121—133, Birkhäuser Verlag, Basel
9 Yutani, K., Ogasahara, K., Sugino, Y. and Matsushiro, A. (1977) Nature 267, 274—275
10 Yutani, K., Ogasahara, K., Suzuki, M. and Sugino, Y. (1979) J. Biochem. 85, 915—921
11 Saiki, T., Kimura, R. and Arima, K. (1972) Agric. Biol. Chem. 36, 2357—2366
12 Saiki, T., Iijima, S., Tohda, K., Beppu, T. and Arima, K. (1978) in Biochemistry of Thermophily (Friedman, S.M., ed.), pp. 287—303, Academic Press, New York
13 Weber, K. and Osborn, M. (1969). J. Biol. Chem. 244, 4406—4412
14 Yaphanits, D.A. (1960) Ann. N.Y. Acad. Sci. 88, 586—601
15 Goodwin, T.N. and Morton, R.A. (1946) Biochem. J. 40, 628—632
16 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
17 Wolfenstein, C., Englard, S. and Listowsky, I. (1969) J. Biol. Chem. 244, 6415—6419
18 Greenfield, N.J. and Fasman, G.D. (1969) Biochemistry 8, 4108—4116
19 Thorne, C.J.R. and Kaplan, N.O. (1963) J. Biol. Chem. 238, 1861—1868
20 You, K. and Kaplan, N.O. (1975) J. Bacteriol. 123, 704—716
21 Murphey, W.H., Barnaby, C., Lin, F.J. and Kaplan, N.O. (1967) J. Biol. Chem. 242, 1548—1559
22 Thorne, C.J.R. (1962) Biochim. Biophys. Acta 59, 624—633
23 Siegel, L. and Englard, S. (1961) Biochim. Biophys. Acta 54, 67—76
24 Wolfe, R.G. and Neilands, J.B. (1956) J. Biol. Chem. 221, 61—69
25 Davies, O.D. and Kun, E. (1957) Biochem. J. 66, 307—316

26 Gregory, E.M., Rohrbach, M.S. and Harrison, J.H. (1971) Biochim. Biophys. Acta 243, 489—497
27 Anderton, B.H. (1970) Eur. J. Biochem. 15, 562—567
28 Foster, M. and Harrison, J.H. (1974) Biochim. Biophys. Acta 351, 295—300
29 Holbrook, J.J., Lodola, A. and Illsley, N.P. (1974) Biochem. J. 139, 797—800
30 Murphey, W.H., Kitto, G.B., Everse, J. and Kaplan, N.O. (1967) Biochemistry 6, 603—610
31 Singleton, R., Jr. and Amelunxen, R.E. (1973) Bacteriol. Rev. 37, 320—343
32 Singleton, R., Jr. and Amelunxen, R.E. (1968) J. Biol. Chem. 244, 1623—1630
33 Ljungdahl, L., Brewer, J.M., Neece, S.H. and Fairwell, T. (1970) J. Biol. Chem. 245, 4791—4797
34 Biffer, J.H.F. and Williams, R.A.D. (1976) in Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), pp. 157—167, Birkhäuser Verlag, Basel
35 Fujita, S.C., Oshima, T. and Imahori, K. (1976) Eur. J. Biochem. 64, 57—68
36 Bigelow, C.C. (1967) J. Theor. Biol. 16, 187—211
37 Fisher, H.F. (1964) Proc. Natl. Acad. Sci. U.S.A. 51, 1285—1290
38 Muramatsu, N. and Nosoh, Y. (1971) Arch. Biochem. Biophys. 144, 245—252
39 Sugimoto, S. and Nosoh, Y. (1971) Biochim. Biophys. Acta 235, 210—221
40 Saiki, T., Mahmud, I., Matsubara, N., Taya, K. and Arima, K. (1976) in Enzymes and Proteins from Thermophilic Microorganisms (Zuber, H., ed.), pp. 169—183, Birkhäuser Verlag, Basel