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Isolation and Characterization of a Neutral Deoxyribonuclease from the Testes of the Crab *Cancer pagurus*†

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ABSTRACT: A deoxyribonuclease has been isolated from the testes and deferent ducts of the crab *Cancer pagurus*. The purification procedure involves ammonium sulfate precipitation, gel filtration on Sephadex G-200, affinity chromatography on RNA core-Sepharose, and hydroxylapatite chromatography. The enzyme shows endonucleolytic activity and is obtained free of phosphomonoesterase, phosphodiesterase, exonuclease, and RNase activities. The enzyme requires divalent cations and has an optimum activity at neutral and alkaline pH. Monovalent ions and EDTA are inhibitors. The sedimentation constant of the enzyme is 5.7. K_m and V_m have been calculated for the degradation of five DNAs with different base compositions. The enzyme splits the phosphodiester bonds of native DNA by simultaneous diplotomic (formation of double-strand breaks) and haplotomic (formation of single-strand breaks) mechanisms; the first one predominated largely.

The use of several DNases with different specificities for studying DNA sequences is of great importance. Bernardi *et al.* (1973) have investigated four such enzymes and shown that they hydrolyze specific sets of short nucleotide sequences.

In the course of recent studies on *Cancer pagurus* DNA, the presence of numerous nicks in our final preparations as well as their low molecular weight (Sabeur *et al.*, 1974) led us to suspect the presence of potent DNases in the extracted organs.

Though not systematically investigated, the presence of DNases in marine animals had already been reported (Georgatsos and Antonoglou, 1964) and an alkaline DNase has been partially purified from the testes of the crab *Neptunus astatus* (Georgatsos, 1965).

The present paper describes the purification of a DNase extracted from the testes and deferent ducts of the crab *Cancer pagurus*. Some enzymatic and physicochemical properties of the enzyme have been investigated.

Materials and Methods

Nucleic Acids. DNA preparations were obtained from calf thymus by the method of Kay *et al.* (1952). Bacterial DNAs were extracted by the method of Marmur (1961). Crab DNA was prepared following the technic of Baranowska *et al.* (1968). Full deproteinization was achieved by repeated treatments with phenol. In the case of crab DNA, deproteinization was obtained only by shaking with chloroform–isoamyl alcohol (9:1, v/v) since phenol denatures the polyd(A-T) component of this DNA (Sabeur *et al.*, 1973). Poly d(A-T), a biosynthetic product, was purchased from Miles. RNA core has been prepared according to Hilmoe (1960) using yeast total RNA.

DNase Assay. For testing DNase activity during the purification, liberation of acid-soluble oligonucleotides from calf thymus was used. The reaction medium containing 5×10^{-2} M $MgCl_2$, 0.5 ml of DNA (stock solution), 0.5 ml of 0.1 M sodium cacodylate buffer (pH 7.5), and 0.1 ml of enzyme (diluted if necessary with 0.05 M Tris-HCl, pH 7.5) was incubated 30 min at 37°; the reaction was stopped by adding 0.5 ml of 12% $HClO_4$. After centrifugation, the extent of hydrolysis was measured by determining the A_{260} of the supernatant against an appropriate blank. Under these conditions, one unit of DNase was defined as the amount of enzyme which catalyzes the liberation of oligonucleotides having a corrected A_{260} equal to 1.00 in 1 min. This arbitrary unit will be systematically used in the course of this study as a representation of the enzyme amount added to incubation medium. The specific activity was calculated by dividing the activity of the enzyme by its A_{280}.

Stock solution contained 1000 μg/ml of calf thymus DNA in 0.05 × SSC, 10^{-3} M EDTA. For kinetic measurements, the same type of assay was used, but DNA stock solution was dialyzed against the appropriate buffer, prior to its incubation.

Nonspecific Phosphodiesterase Assay. Nonspecific phosphodiesterase activity of DNase was determined according to Hodes *et al.* (1967). The reaction medium containing 0.5 ml of stock solution (0.5 M sodium acetate buffer (pH 7.5)–4×10^{-3} M EDTA (pH 5.7)–0.3% Tween-80, 10:5:3), 0.4 ml of freshly prepared 2.5 mM calcium bis(*p*-nitrophenyl) phosphate, and 0.1 ml of enzyme solution was incubated 16 hr at 37°; then the reaction was stopped by adding 1 ml of 2 N NH_4OH and the extent of hydrolysis was measured by determining the A_{400} of the reaction medium against an appropriate blank. The non-

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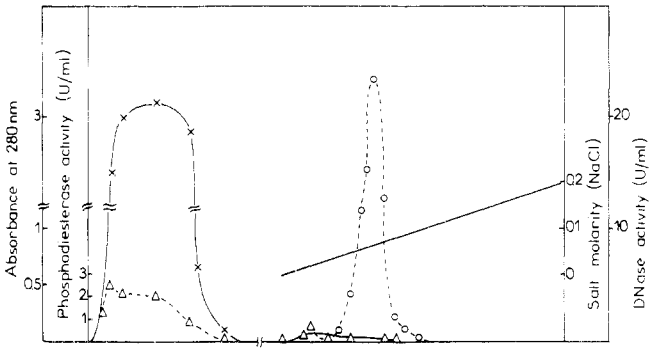

FIGURE 1: Chromatography on RNA core-Sepharose: (×) absorbance at 280 nm (left outer scale); (Δ) phosphodiesterase activity (left inner scale); (○) DNase activity (right inner scale). Total volume of the gradient: 500 ml (for details, see text).

specific phosphodiesterase unit was defined as the amount of enzyme which, in the above-described conditions, induces a corrected A_{400} increase equal to 1.00 for 1 hr.

RNase Assay. RNase was assayed according to Bernardi and Bernardi (1966), by measuring the liberation of acid-soluble oligonucleotides from tRNA. The definition of the activity unit and specific activity was the same as for DNase.

Phosphomonoesterase Assay. This was performed according to Chersi *et al.* (1966) with disodium *p*-nitrophenyl phosphate as a substrate.

Exonuclease Assay. Exonuclease activity was measured using the conditions described by Bernardi and Bernardi (1968), with 3′-phosphodeoxyribonucleotides as substrate.

Determination of Proteins. Protein concentrations in effluent solutions were estimated from the 280-nm absorbance (1 mg/ml = 1 A_{280}U).

RNA Core-Sepharose Column. RNA core was grafted on Sepharose activated by cyanogen bromide (Porath *et al.*, 1967) and substituted with ε-aminocaproic acid, in the presence of carbodiimide (Cuatrecasas, 1970). The grafted Sepharose was washed with water and 0.05 M Tris-HCl (pH 7.5)–1 M NaCl, and then equilibrated with 0.05 M Tris-HCl (pH 7.5).

Physicochemical Methods. The determinations of the sedimentation constants of native and degraded DNAs were performed at 44,000 rpm with a Beckman Model E ultracentrifuge, equipped with a uv light source and a monochromator, using cells with Kel F centerpieces. The drop of the molecular weight as a function of the degradation was estimated using Studier's (1965) equations:

$$s_{20,w}{}^{0} = 0.0882M^{0.346} \quad \text{in 1 M NaCl} \qquad (1)$$

$$s_{20,w}{}^{0} = 0.0528M^{0.400} \text{ in 0.9 M NaCl–0.1 M NaOH} \qquad (2)$$

The weight-average number of single-strand and double-strand breaks was calculated according to Sicard et al. (1972):

$$\text{neutral medium } d_w = 2\left[\left(\frac{(s_{20,w}{}^{0})_0}{(s_{20,w}{}^{0})_t}\right)^{2.89} - 1\right] \qquad (3)$$

$$\text{alkaline medium } s_w = 4\left[\left(\frac{(s_{20,w}{}^{0})_0}{(s_{20,w}{}^{0})_t}\right)^{2.50} - 1\right] \qquad (4)$$

where $(s_{20,w}{}^{0})_0$ and $(s_{20,w}{}^{0})_t$ are sedimentation coefficients, respectively, in neutral medium (3) and alkaline medium (4) at times 0 and t.

The ratio R of single-strand to double-strand breaks which was used to determine the relative importance of haplotomic (single-strand breaks on one or another strand, or double-hit mechanism) and diplotomic (simultaneous scission of both strands or single-hit mechanism) mechanisms was given by $R = s_w/d_w$.

The kinetic data of the initial degradation were treated according to Cavalieri and Rosenberg (1961)

$$\log [(1 - R)/R] = n \log T + \text{constant} \qquad (5)$$

where $R = M_t/M_0$, M_t and M_0 being the molecular weights at time t and at time 0, respectively, n is the apparent number of strands. When n is close to 1, the enzyme is assumed to split native DNA according to a diplotomic mechanism.

The sedimentation constant of the enzyme was determined by sedimentation in sucrose gradient (5–20%) using cytochrome *c* as a reference (Martin and Ames, 1961).

Results

Isolation and Purification of the Enzyme. Male crabs were obtained from Roscoff fisheries,[1] during the reproduction period (April–May). The testes and deferent ducts from living animals were collected and used immediately or refrigerated at −80° and kept at the same temperature. All purification operations were carried out at 4°. The gonads were homogenized for 2–3 hr in 0.15 M NaCl (200 g/400 ml). After centrifugation at 16,300*g* for 30 min, the supernatants were pooled. Ammonium sulfate (243 g/l.) was added with continuous stirring and EDTA was adjusted at 10^{-3} M using a solution at pH 7.5. The suspension was allowed to stand for 1 hr, then centrifuged, and the precipitate discarded. The supernatant was mixed with 240 g/l. of ammonium sulfate. After centrifugation, the pellet which contained DNase activity was dissolved in a minimum volume of Tris-HCl (0.1 M, pH 7.5) and dialyzed overnight against the same buffer. The dialyzed solution was submitted to a second fractionation with ammonium sulfate. The resulting active precipitate was dialyzed against Tris-HCl (0.05 M, pH 7.5) and centrifuged at 54,000*g* for 30 min.

Gel Filtration on Sephadex G-200. Aliquots of 250 ml of the 54,000*g* supernatant were loaded on a column of Sephadex G-200 (5.5 × 120 cm), equilibrated with Tris-HCl (0.05 M, pH 7.5). Fractions of 10 ml were collected. DNase activity was eluted at the end of a broad peak, containing excluded components, together with RNase, phosphomonoesterase, and phosphodiesterase activities. The fractions containing DNase activity were pooled and could be stored at 4° during several months without loss of activity.

RNA Core-Sepharose Affinity Chromatography. Aliquots of 100–150 ml were loaded on a column of RNA core-Sepharose (1 × 13 cm), equilibrated with Tris-HCl (0.05 M, pH 7.5). Then, the column was washed with the same buffer. Elution of the enzyme was obtained with a NaCl gradient (0–0.2 M) in Tris-HCl (0.05 M, pH 7.5)–$MgCl_2$ (10^{-3} M). Fractions (4 ml) were collected. Fractions with highest activities were pooled and dialyzed against potassium phosphate buffer (0.05 M, pH 6.8) (PPB).

Hydroxylapatite Column. The dialysate was loaded on a hydroxylapatite column (2.5 × 8 cm), equilibrated with PPB. Elution was performed with a linear gradient PPB 0.05–0.7 M (300 ml). The DNase was eluted at 0.15 M. The enzyme was free of phosphomonoesterase, phosphodiesterase, and RNase activities. The results of the purification steps are summarized in Table I.

When the A_{280} of the enzyme solution is below 0.05 a concentration step is needed. For such a purpose, Diaflo apparatus was used yielding two or three times concentration.

[1] La Langouste, 29211 Roscoff, France.

TABLE I: Purification Procedure.[a]

Step	Vol (ml)	Total Protein (mg)	Total Units $\times 10^{-3}$	Sp Act.
I. Extract	3500		28	
II. Ammonium sulfate precipitation				
1	460	26.7×10^3	19.8	0.74
2	380	22×10^3	22.8	1.03
III. Sephadex G-200 chromatography	1300	4×10^3	15.0	3.77
IV. RNA core-Sepharose chromatography	443	(22.15)	9.75	(440)
V. Hydroxylapatite chromatography	55	(2.75)	5.50	(2000)

[a] Results in parentheses are approximate values, because of the two low A_{280} values.

Difficulties encountered in the concentration had made it almost impossible to carry out satisfactory electrophoretic analysis of the purified enzymes. By gel acrylamide electrophoresis in Tris-glycine buffer (pH 8.6) (Davis, 1964), it was possible to find one active band in conditions when it was hardly possible to stain proteins.

The DNase dialyzed against Tris-HCl (0.05 M, pH 7.5) and concentrated two or three times can be kept at −20° during several weeks without a noticeable loss of activity.

Action of Divalent Cations. Crab DNase is strongly activated by divalent cations. For each ion, the linear proportionality between activity and enzyme concentration has been first determined with an arbitrary ion concentration and then verified with the optimum divalent metal concentration. Because high metal concentrations led to gelification, activation by Ni^{2+} and Co^{2+} was tested with suboptimal concentrations. The optimum divalent cation concentrations are very high: 2×10^{-2} M Mg^{2+}, 10^{-2} M Mn^{2+}, 10^{-1} M Ni^{2+}, 10^{-1} M Co^{2+}. These high concentrations are not due to the presence of the non specific ions of the buffer, contrary to previous results obtained with DNase I (Junowicz and Spencer, 1973), since the nature of the buffer, its molarity and pH are without effect on the ion concentration-activity curves. The most effective cations are $Mg^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Ba^{2+}$. Only a very low activation can be detected with Ca^{2+}.

pH and Divalent Metal Activation. With Mg^{2+}, Co^{2+}, and Mn^{2+}, the activity increased up to pH 7; there is a low decrease of activity between pH 7 and 9, except for Co^{2+}.

Influence of Ionic Strength. Increasing ionic strength of the medium induces a decrease of hydrolysis rate of DNA. This inhibitory effect depends on the nature of the buffer. At optimal concentration of Mg^{2+}, in 0.1 M Tris-HCl (pH 8), the activity is 66% of the activity in 5×10^{-3} M. In 10^{-1} M sodium cacodylate (pH 7), the activity is 85% compared to the activity in 5×10^{-3} M sodium cacodylate. To avoid the inhibitory effect of high ionic strength, a molarity of 5×10^{-3} M has been selected for all assays.

Inhibition of Enzyme Activity by EDTA and Monovalent Ions. A 90% inactivation of the enzyme is achieved by addition of EDTA at the same concentration as the concentration of the activating cation (Mg^{2+}). Addition of monovalent ions (Na^+ and K^+) has an inhibitory effect.

Action on Denatured DNA and on Polyribonucleotides. With heat-denatured DNA as a substrate, in the presence of Mg^{2+}, the activity of crab testes DNase is decreased by 90–95%, compared with the activity on native DNA. In the presence of Ca^{2+}, no activity is observed. When Mg^{2+} and Ca^{2+} are added simultaneously, the activity is threefold more than with Mg^{2+} alone. The same effect has been observed with *Paracentrotus lividus* DNase (Parisi and De Petrocellis, 1972).

In optimal conditions of activity, there is no hydrolysis of tRNA, poly(A), and poly(A-U).

Sedimentation Constants. The sedimentation coefficient determined through five experiments was 5.7, using a $s_{20,w}{}^0$ of 1.7 for cytochrome *c*.

Kinetics. K_m and V_m have been determined from Lineweaver-Burk plot (1934) with five different DNAs as substrates (Table II) in the presence of Mg^{2+}. The straight lines have been calculated from the experimental values of V and s by the method of least squares.

Mode of Action. Three different DNAs (calf thymus, *Escherichia coli* and crab *Cancer pagurus*) were hydrolyzed in presence of Mg^{2+} and Mn^{2+} as activators. The results shown that crab testes DNase degrades native DNA according to diplotomic and haplotomic mechanisms. The results obtained with the homologous DNA are given in Table III.

Discussion

The deoxyribonuclease which we have isolated from the testes and deferent ducts of the crab *Cancer pagurus* is a true endonuclease, devoided of phosphomonoesterase, phosphodiesterase, exonuclease, and RNase activities. In the purification procedure, the most efficient step is based on affinity chromatography (RNA core-Sepharose column) derivated from the denatured DNA affinity process described by Schabort (1972). With denatured DNA-Sepharose, the eluted DNase presents a A_{260}/A_{280} ratio which differs from the values generally found for proteins; this fact could result from an hydrolysis of the denatured DNA. A stepwise elution was carried out and the enzyme eluted at a molarity of 0.2 M, without Mg^{2+}. The change of the A_{260}/A_{280} ratio began at the end of the 0.1 M peak. Identical results are obtained in the presence of EDTA. The risk of a partial hydrolysis of the ligand might be avoided by using RNA core. But even with this type of ligand, the isolated

TABLE II

DNA Samples	G + C (%)	K_m[a]	V_m[b]	ΔG[c] (cal/mol)
Poly[d(A-T)]	0	5×10^{-4}	0.0625	−6407
Cancer pagurus	35	6.6×10^{-4}	0.0285	−6570
Proteus vulgaris	39	8×10^{-4}	0.0169	−6683
Calf thymus	44	8×10^{-4}	0.0208	−6683
Micrococcus luteus	72	9.7×10^{-4}	0.0112	−6798

[a] Equivalent nucleotides per l. [b] U of A_{260}/min. [c] Free energy of complex formation; buffer: Tris-HCl (5×10^{-3} M, pH 7.5)–$MgCl_2$ (2×10^{-2} M).

TABLE III: Degradation of Native Crab DNA by Crab Testes Deoxyribonuclease.[a]

Incubation Time (min)	Activator Ion: Mg^{2+} s_w	d_w	R	Mn^{2+} s_w	d_w	R
10	0.40	0		4.84	0.60	8.06
20	1.80	0.88	2.04	5.24	0.98	5.34
40	4.44	0.80	5.55	6.32	1.74	3.63
60	4.64	0.84	5.52	10.08	3.50	2.88
80	6.56	1.04	6.30	13.48	3.98	3.38

[a] Crab DNA is the total DNA which is composed of 75% of a main component (43% G·C base pairs) and 25% of poly[d(A-T)] (90% alternating A-T, 7% nonalternating A-T and 3% G-C); buffer: Tris-HCl, 5×10^{-3} M, pH 7.5 ($MgCl_2$, 2×10^{-2} M or $MnCl_2$, 10^{-2} M). The hydrolysis has been performed at 25°. DNase units: with Mg^{2+}, 0.55×10^{-4} U/ml; with Mn^{2+}, 1×10^{-2} U/ml.

DNase has a high A_{260}/A_{280} ratio. In the presence of Mg^{2+}, the results are slightly better because the binding of enzyme is weaker, so that it can be eluted with 0.1 M NaCl. However this disadvantage is compensated by the efficiency of this type of chromatography. The efficiency depends upon the batches of RNA core-Sepharose and one can obtain a 20- to 70-fold purification. The binding capacity is high and results are reproducible several times with the same batch. In the present work, the purification rate was exceptionaly high after the affinity chromatography.

The kinetics obey the Michaelis law. The affinity of the enzyme for the different DNAs is related to their base composition: the better substrate is biosynthetic poly[d(A-T)]; *Cancer pagurus* DNA which contains a "satellite" poly[d(A-T)] representing 25% of the total DNA is also a good substrate. On the contrary, *Micrococcus luteus* DNA is a relatively poor one. These differences can be related either to an uneven reactivity of the various nucleotides sets towards this DNase or to an unequal affinity of this DNase toward substrates of different conformation, since it is well known that very (A + T)-rich DNAs have a secondary structure which differs from that of low and moderate A-T content (Bram, 1971).

Crab testes neutral DNase degrades native DNA according to a diplotomic and a haplotomic mechanisms, but there is a predominance of the first one. In the case of a random degradation such as admitted for DNase I, the weight-average number of single-strand breaks necessary to halve the molecular weight of a DNA with $M_w = 6.6 \times 10^6$ has been calculated by Thomas (1956) and is equal to 133 in a haplotomic degradation and to 4 in a diplotomic degradation. With the crab DNase, in the presence of Mg^{2+} or Mn^{2+}, the number of single-strand breaks necessary to halve the molecular weight of different DNAs has been measured and is given in Table IV. It is larger than for a theoretical diplotomic mechanism but much smaller than for a pure haplotomic mechanism. It is of the same order of magnitude as the number found by Bernardi and Sadron (1964) for the hog spleen acid DNase.

The apparent number of strands calculated from the equation of Cavalieri and Rosenberg (1961) is equal to 0.97 ± 0.03; this crab DNase splits native DNA predominantly by a single-hit mechanism.

High sedimentation coefficient of the enzyme correlated with its behavior on gel filtration (Sephadex G-200 and Bio-Gel P-100) suggests a molecular weight higher than 10^5 daltons. This fact could support an oligomeric structure of the enzyme and account for its diplotomic action. This type of single-hit mechanism which seems to be normal in the case of DNase II (Bernardi and Sadron, 1964; Cordonnier and Bernardi, 1968; Laval and Paoletti, 1972) is an uncommon phenomenon in the case of DNase I, except when acting in special medium conditions. The fact that this enzyme is able to produce DNA fragments containing only a few nicks could be of interest for sequence analysis of DNA. This, indeed, implies a demonstrated base specificity of the enzyme. Preliminary results are in agreement with a useful specificity.[2]

TABLE IV: Number of Single-Strand Breaks (s_w) Inducing a 50% Decrease of the Neutral Weight-Average Molecular Weight of DNA Samples.

DNA Samples	$M_N \times 10^{-6}$	s_w	n[a]
	Mg^{2+} as activator		
Calf thymus	8.3	14	0.95
E. coli	11.2	16	0.95
Crab	4.7	14	0.93
	Mn^{2+} as activator		
Calf thymus	8.3	12	1.00
Crab	4.7	7	1.02

[a] n is the apparent number of strands, calculated from the slope of $\log[(1 - R)/R]$ *vs.* $\log t$.

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Glutamine Synthetase of *Bacillus stearothermophilus.* I. Purification and Basic Properties†

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ABSTRACT: To assess the amount of complex functional information that has evolved and is maintained in a thermophilic regulatory enzyme, glutamine synthetase from *Bacillus stearothermophilus* has been isolated and partially characterized. Enzyme synthesis is derepressed by limiting ammonia or glutamine in chemically defined media at 55°. Procedures for purification of the enzyme to homogeneity are described, including an affinity gel chromatography step. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate indicates a subunit of mol wt ≃51,000, and electron microscopy reveals a double hexagonal dodecameric subunit arrangement. The enzyme resembles that from *Bacillus subtilis* in amino acid content and lack of bound cofactors or adenylyl groups. Activity is stimulated by bound metal ions, $Mn^{2+} > Mg^{2+} \simeq Cd^{2+} > Co^{2+} >$ others, each of which induces a different pH-activity profile. Optimal activity occurs at $[Mn^{2+}] = [ATP]$. Binding constants and specificity for substrates at 55° are comparable to those for mesophilic enzymes. The plot of velocity *vs.* [Mn-ATP] is sigmoidal with Hill $n = 2.3$, whereas those for NH_3 and L-glutamate are hyperbolic. With saturating substrate levels, the Arrhenius plot is nonlinear, concave downward with an intersection point at 55°. These phenomena appear to indicate multiple enzyme conformational states.

Glutamine synthetase catalyzes the reaction

$$ATP + \text{L-Glu} + NH_3 \rightarrow \text{L-Gln} + ADP + P_i \quad (1)$$

Its activity is highly regulated in microorganisms by end-product metabolite feedback inhibition, since it occupies a key position in nitrogen metabolism (Shapiro and Stadtman, 1970). The present study is part of an investigation to determine how much of the complex functional information of the mesophilic enzymes has evolved and is maintained in thermophiles.

Despite extensive research on thermophilic proteins, the molecular basis for thermostability is not yet well defined. As reflected by recent reviews of this subject (Singleton and Amelunxen, 1973; Amelunxen and Lins, 1969; Howell *et al.,* 1969), the contributing factors appear to be both various and subtle.

In this paper we describe a procedure for preparing and purifying to homogeneity the glutamine synthetase from *Bacillus stearothermophilus.* This includes a new affinity gel method, using a glutamate side-arm ligand. The basic physicochemical and kinetic properties are reported. These results provide the basis for more detailed mechanistic studies. The accompanying paper (Wedler and Hoffmann, 1974) deals with our investigations on the regulation of enzyme activity and those forces lending thermostability to the protein.

Experimental Section

Materials. All biochemicals used were of the highest purity obtainable from Sigma Chemical Co. Proteins were from Sigma and Worthington. Inorganic salts and metal ions were of analytical grade from Fisher Scientific. A culture of *B. stearothermophilus,* strain 4S, was kindly supplied by Dr. Neal E. Welker, and was transferred every 6-8 weeks to 2% trypticase-agar (BBL) slants, grown at 55°, and stored at 4°.

Growth Media. Cells were grown first in a rich preinoculation broth, collected by centrifugation, washed with "M" buffer (Welker and Campbell, 1963), and transferred to minimal media, which contained (in g/10 l.) 57.6 glucose, 0.53 NH_4Cl, 1.05 L-Arg, 0.6 D,L-Met, 1.44 D,L-Val, 1.5 thiamine, 0.015 nicotinic acid, 0.00001 *d*-biotin, 5 potassium acetate, 10 KH_2PO_4, 10 NaCl, and 0.05 each of $FeCl_3 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, and $CaCl_2 \cdot 6H_2O$. Initially, starter cultures of the limiting medium were supplemented with 0.1% casein-amino acids, to reduce lag times in growth curves. Each volume was transferred to a

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