Isolation and Properties of 6-Phosphogluconate Dehydrogenase from *Escherichia coli*. Some Comparisons with the Thermophilic Enzyme from *Bacillus stearothermophilus*†

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ABSTRACT: 6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), EC 1.1.1.44) of *Escherichia coli* MRE 600 has been isolated with the purpose of carrying out comparative studies with the thermostable enzyme previously isolated from *Bacillus stearothermophilus* (Veronese, F. M., Boccù, E., Fontana, A., Benassi, C. A., and Scoffone, E. (1974), *Biochim. Biophys. Acta 334*, 31). The purified enzyme appeared homogeneous by the criteria of disc gel electrophoresis with and without sodium dodecyl sulfate, ultracentrifugation, and gel filtration. The enzyme has enzymological and physicochemical properties similar to the enzyme isolated from other sources, including *B. stearothermophilus*. The *E. coli* enzyme has a mol wt of 100 000 ± 3000 and is composed of two apparently identical subunits. The amino acid composition of both the mesophilic and thermophilic enzyme has been determined and found to present large similarities. The *E. coli* enzyme shows a high degree of specificity for nicotinamide adenine dinucleotide (NADP) and it is inhibited by reduced NADP (NADPH). Cysteine residues are involved in the catalytic activity, since on incubation of the enzyme with *p*-chloromercuribenzoate or 5,5′-dithiobis(2-nitrobenzoic acid) strong inhibition occurs, activity being restored by treatment with excess of β-mercaptoethanol. The substrate 6-phosphogluconate protects partially the enzyme from inactivation. Both the mesophilic and thermophilic 6-phosphogluconate dehydrogenases are inactivated by Rose Bengal in the presence of light by similar kinetics and protected against photoinactivation by the enzyme substrate. The *E. coli* enzyme, on the other hand, showed distinct differences in stability against heat and unfolding agents in respect to the *B. stearothermophilus* enzyme. Heating at 50 °C or incubation in 8 M urea results in rapid inactivation. The gross structure of the mesophilic and thermophilic enzyme was very similar as judged by circular dichroic measurements. The far-ultraviolet circular dichroic spectrum had a negative band centered at about 220 nm. In both cases, the fluorescence emission spectrum indicates that the environment of the tryptophan residues is similar, since both enzymes show an emission maximum at 334 nm upon excitation at 295 nm. Circular dichroism measured at various temperatures between 25 and 80 °C showed the mesophilic enzyme to be conformationally stable below about 45 °C and the thermophilic enzyme below 60 °C. The secondary structure of the *E. coli* enzyme was very sensitive to the denaturing action of urea, since in 8 M urea it rapidly unfolded. Partial renaturation after urea treatment occurred on dilution with buffer or dialysis, as evidenced by spectral properties of the renatured enzyme. The results show that the mesophilic and thermophilic enzymes are very similar and that differences in thermal stability depend on subtle differences in the architectures of the proteins.

In recent years 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP[1] oxidoreductase (decarboxylating), EC 1.1.1.44), has been extensively studied because of its key role in the pentose-phosphate cycle. The enzyme has been isolated from various tissues of a number of species (Villet and Dalziel, 1969, 1972; Dyson et al., 1973; Pearse and Rosemeyer, 1974a,b; Betts and Mayer, 1975) and from microorganisms (Pontremoli et al., 1961; Rippa et al., 1966, 1970; Miller and Shepherd, 1972; Scott and Abramsky, 1973). The enzyme, regardless of its source, has mol wt 90 000–120 000 and is a dimer composed of subunits of equal molecular weight. Extensive studies have been made on the chemical groups involved in the activity of the enzyme (Rippa et al., 1966, 1970, 1972; Rippa and Pontremoli, 1968; Grazi et al., 1965) and a preliminary crystallographic study of the sheep liver enzyme has been reported (Adams et al., 1975). The enzyme from bacterial origin, however, has been studied only recently and the isolation and characterization of 6-phosphogluconate dehydrogenase from *Streptococcus faecalis* (Bridges et al., 1975) and from *Bacillus stearothermophilus* (Veronese et al., 1973, 1974; Pearse and Harris, 1973) has been reported.

This report describes a purification procedure of 6-phosphogluconate dehydrogenase from *Escherichia coli* and a characterization of the enzymatic, physical, and chemical properties that are pertinent for a comparative study with the thermostable enzyme obtained from *B. stearothermophilus*. It seemed worthwhile to examine in detail this mesophilic 6-phosphogluconate dehydrogenase because of the interest in the structural and catalytic properties of this enzyme in general and because of the importance of all *E. coli* proteins as components of a model cell whose biochemistry has been extensively studied. Furthermore, another purpose of our study was to carry out structural studies on a thermophilic and mesophilic enzyme, in an effort to contribute to the understanding of the mechanism of the thermostability of thermophilic proteins.

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[1] Abbreviations used are: NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; CD, circular dichroism.

Experimental Procedure

Materials

6-Phosphogluconate, NADP, β-mercaptoethanol, dithiothreitol, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Fluka, and Rose Bengal (tetraiodotetrachlorofluorescein) was from BDH (Poole, England). Urea (Fluka) was recrystallized from 95% ethanol and only fresh solutions were used. All other chemicals were reagent grade products obtained from the usual commercial sources and used without further purification.

Methods

Protein Concentration. In crude, cell-free extracts, protein concentration was estimated spectrophotometrically (Layene, 1957) and for purified enzyme solutions the value of $E_{280\,nm}^{0.1\%} = 1.0$ was used (Pearse and Rosemeyer, 1974a,b).

Enzyme Assay. The 6-phosphogluconate dehydrogenase activity was measured at room temperature. The standard assay mixture contained 0.3 μmol of NADP and 6-phosphogluconate in a final volume of 1 ml of 0.1 M Tris-HCl buffer, pH 8.0. The reaction was initiated by the addition of the enzyme and followed by the rate of NADPH formation at 340 nm. Activities were calculated from initial slopes and are reported in international units, i.e., μmol of NADPH formed min^{-1} ml^{-1}.

Amino Acid Determination. Enzyme samples were hydrolyzed in 6 N HCl in sealed tubes under vacuum at 110 °C for 24, 48, and 72 h. The amino acids obtained by hydrolysis were analyzed with a Jeol Model JLC-6AH amino acid analyzer using a single-column procedure. The cysteine content was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) in 8 M urea as described by Ellman (1959). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967).

Acrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out as previously described (Veronese et al., 1974). Protein was stained by immersion of the gels in a solution of Coomassie blue dye and 6-phosphogluconate dehydrogenase activity was detected by tetrazolium reduction (Tsao, 1962).

Sucrose Density Gradient Centrifugation. The sucrose gradients were prepared and run according to the procedure of Martin and Ames (1961). A Spinco Model L ultracentrifuge with a swinging bucket rotor SW 39 was used. The gradients ranged from 5 to 20% sucrose in 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM EDTA and 1 mM β-mercaptoethanol, and were centrifuged for 16 h at 38 000 rpm at 21 °C.

Sedimentation Velocity. The sedimentation coefficient was determined with a Beckman Model E analytical ultracentrifuge at 20 °C in 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM EDTA and 1 mM β-mercaptoethanol. The rotor speed was 52 000 rpm. Photographs were taken at 16-min intervals. The determined S value was reported to the reference conditions of water and 20 °C. The diffusion coefficient was determined from the same photographs used for the evaluation of the sedimentation coefficient according to the height-area method (Schachman, 1957). An apparent specific volume of 0.74 cm^3 g^{-1}, calculated from the amino acid composition, was used for computations.

Inactivation by SH Reagents. The reaction of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) (4×10^{-4} M) and *p*-chloromercuribenzoate (2×10^{-5} M) was carried out at room temperature in 0.1 M Tris-HCl buffer, pH 8.5, with an enzyme concentration of 0.8 mg/ml. The inactivation process was also studied in presence of the substrate 6-phosphogluconate and coenzyme NADP at 5×10^{-3} M concentration. Reactivation of the inhibited enzyme was carried out by adding excess β-mercaptoethanol to a final concentration of 10^{-3} M.

Photooxidation. Photooxidation was carried out in a vessel equipped with a water jacket. The temperature of the reaction mixture was maintained at 25 °C with the aid of an external circulating bath. The enzyme sample, 0.05 mg/ml in 0.05 M potassium phosphate buffer, pH 7.2, containing 1 mM dithiothreitol and 1 μM Rose Bengal, was stirred with a magnetic bar and illuminated with a 200-W lamp placed 10 cm above the surface of the solution. The control was treated in an identical manner except that the vessel was kept in the dark. Aliquots were removed from the sample and control at the desired time intervals and added directly to the standard assay mixture.

Circular Dichroism. Circular dichroism (CD)[1] spectra were measured with a Cary 61 automatic recording circular dichrograph calibrated with an aqueous solution of 10-camphorsulfonic acid (Cassim and Yang, 1970). The temperature of the cell compartment was controlled by circulating ethylene glycol through the cell-holder jacket with the use of a Haake bath. The temperature of the sample was determined using a thermistor attached through the cap of the cell. Measurements were made with a 1-mm path length quartz cell. A CD scale setting of 0.02 was used in all experiments. Spectra were corrected for baseline shifts by running samples of solvent buffer. The mean residue weight of amino acids was taken as 110 in the calculation of the molar ellipticity, $[\theta]$, which is expressed as mean residue ellipticity in deg cm^2/dmol.

Fluorescence Measurements. Fluorescence spectra were measured with a Perkin-Elmer, Model MPF-2A spectrofluorimeter connected to a Hitachi, Model QPD_{33} recorder. Protein fluorescence intensities were measured with excitation at 295 nm. Temperature control was accomplished by circulating water from a Haake thermostat bath through a hollow copper block that surrounded the sample cuvette. The temperature inside the cell was measured as indicated above in the CD measurements. In studying the effect of temperature on protein fluorescence, the temperature of the water bath was raised at a constant rate (3–5 °C/min), and the fluorescence was recorded continuously. Fluorescence-intensity values obtained using this method were in good agreement with those obtained when the protein solution was allowed to come to a steady state at all temperatures studied. To avoid bubble formation and consequent light scattering, the buffers used for preparing protein samples were degassed under vacuum. The buffers were routinely checked for fluorescent contaminants by recording the emission spectra following excitation at 295 nm.

Other Methods. Absorbance measurements at single wavelengths were carried out with a Hitachi Perkin-Elmer spectrophotometer, Model 139. pH measurements were determined with a Metrohm Model E 510 instrument equipped with a combined glass electrode.

Enzyme Purification. E. coli MRE 600 was purchased as frozen cell sludges from the Microbiological Research Establishment (Porton Down, England). The microorganism was grown in continuous culture at 35 °C, pH 6.8, as already described (Elsworth, 1968). About 500 g of *E. coli* paste was suspended in 800 ml of 10 mM Tris-HCl buffer, pH 7.2, 1 mM EDTA, and 1 mM β-mercaptoethanol using a blender. The

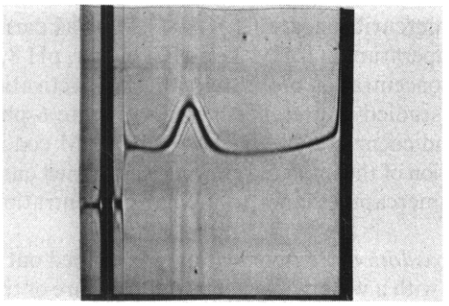

FIGURE 1: Sedimentation pattern of purified *E. coli* 6-phosphogluconate dehydrogenase. The protein was 6.5 mg/ml in 0.1 M potassium phosphate buffer, pH 6.8, containing 1 mM EDTA and 1 mM β-mercaptoethanol. The photograph was taken 64 min after reaching a speed of 52 000 rpm. The temperature was 20 °C.

suspension was treated with the Manton Gaulin homogenizer at 550 atm. The homogenate was diluted to 1.5 l. with Tris buffer and centrifuged at 25 000*g* for 60 min. From the supernatant crude extract, 6-phosphogluconate dehydrogenase was purified following essentially the procedure already reported for the isolation of the *B. stearothermophilus* enzyme (Veronese et al., 1974). A relevant modification was the heat treatment of the dialyzed enzyme after the ammonium sulfate fractionation, carried out under nitrogen stream at 50 °C for 15 min. The *B. stearothermophilus* enzyme was prepared as previously reported (Veronese et al., 1974) with the difference that the cells were disrupted and the enzyme was solubilized by a Manton Gaulin homogenizer, instead of by sonication. Some influence of the method of solubilization on the properties of the enzyme has been observed (Veronese et al., 1974). The enzymes were stored as an ammonium sulfate suspension at 0 °C. Solutions of the enzyme were prepared by centrifugation of an aliquot of this suspension, removal of the supernatant solution, and addition of a proper amount of buffer.

Results

Purity of the Enzyme Preparation. 6-Phosphogluconate dehydrogenase from *E. coli* MRE 600 was isolated by a procedure similar to that already described for the thermostable enzyme from *B. stearothermophilus* (Veronese et al., 1974). The results of a typical purification scheme are summarized in Table I. By this procedure the enzyme was obtained in 47% yield with an overall purification of about 1000-fold and a specific activity of 32.

The homogeneity of the enzyme preparation was tested by several criteria. The specific activity of the enzyme peak, obtained by gel filtration on Bio-Gel A-0.5 m (the last step of the purification procedure; Table I), was constant throughout the elution profile, as shown by the superimposition of the protein band and enzyme elution profiles. A single protein band was detected by disc gel electrophoresis at 7.5 and 10% gel concentration at pH 7.0 and 8.9. Gels were also stained for the enzyme activity (Tsao, 1962) and a single band was observed at a position corresponding to the protein band. Additional evidence of a homogeneous enzyme preparation was obtained from sedimentation velocity experiments (Figure 1). A single symmetrical peak was observed in Schlieren patterns obtained from a run at a protein concentration of 6.5 mg/ml in 0.1 M potassium phosphate buffer, pH 6.8, containing 1 mM EDTA and 1 mM β-mercaptoethanol.

Molecular Weight. 6-Phosphogluconate dehydrogenase from *B. stearothermophilus* was found to have a molecular weight of 100 000 by sucrose density gradient centrifugation and composed of two apparently identical subunits (Veronese et al., 1973, 1974; Pearse and Harris, 1973). The enzyme from *E. coli* and *B. stearothermophilus* was subjected to sucrose density gradient centrifugation in the same run but in different tubes. Since the enzyme from both sources was found to sediment at the same velocity, an analogous molecular weight of 100 000 can be assumed for the mesophilic and thermophilic enzyme. This figure is in agreement with the data obtained by sedimentation velocity experiments of the *E. coli* enzyme at 20 °C in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM β-mercaptoethanol, and 1 mM EDTA at 52 000 rpm. A molecular weight of 97 000 ± 5000 was calculated (Schachman, 1957) on the basis of a sedimentation coefficient of 5.9×10^{-13} s, a diffusion coefficient of 5.8×10^{-7} cm^2 s^{-1} and a partial specific volume of 0.74 cm^{-3} g^{-1} calculated from the amino acid composition of the protein (Table II). The subunit composition of the enzyme was determined by denaturation with sodium dodecyl sulfate in the presence of a reducing agent (Weber and Osborn, 1969). Subunits obtained in this manner were analyzed in polyacrylamide gels containing sodium dodecyl sulfate, and a single sharp band was obtained with a

TABLE I: Purification of 6-Phosphogluconate Dehydrogenase from *E. coli*.

Fraction	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recovery (%)
Crude extract	70 000	2370	0.034	100
Ammonium sulfate	25 380	2200	0.08	93
Heat treatment	16 500	1980	0.12	83
DEAE-cellulose	391	1683	4.3	71
Phosphocellulose	47	1345	28.3	57
Bio-Gel A-0.5 m	35	1120	32.0	47

TABLE II: Amino Acid Composition of 6-Phosphogluconate Dehydrogenase from *E. coli* and *B. stearothermophilus*.[a]

Amino acid	*E. coli* Amino Acid Residues/ 50 000	*E. coli* Nearest Integer	*B. stearothermophilus* Amino Acid Residues/ 50 000	*B. stearothermophilus* Nearest Integer
Lysine	29.2	29	33.1	33
Histidine	5.4	5	9.9	10
Arginine	18.1	18	20.7	21
Aspartic acid	50.8	51	39.3	39
Threonine	21.8	22	18.2	18
Serine	26.5	26	17.2	17
Glutamic acid	58.1	58	52.8	53
Proline	17.8	18	17.6	18
Glycine	43.6	44	40.3	40
Alanine	45.4	45	47.6	48
Cysteine	2.6	3	2.8	3
Valine	23.8	24	28.1	28
Methionine	6.9	7	9.6	10
Isoleucine	28.3	28	33.1	33
Leucine	36.3	36	37.7	38
Tyrosine	20.3	20	19.3	19
Phenylalanine	15.6	16	17.8	18
Tryptophan	4.7	5	4.6	5

[a] The values shown have been corrected for the destruction of serine, threonine, and tyrosine and for the rate of release of valine and isoleucine from 24-, 48-, and 72-h hydrolysis. The value of cysteine has been obtained by titration with 5,5′-dithiobis(2-nitrobenzoic) acid in 8 M urea (Ellman, 1959). Tryptophan has been determined spectrophotometrically by the method of Edelhoch (1967).

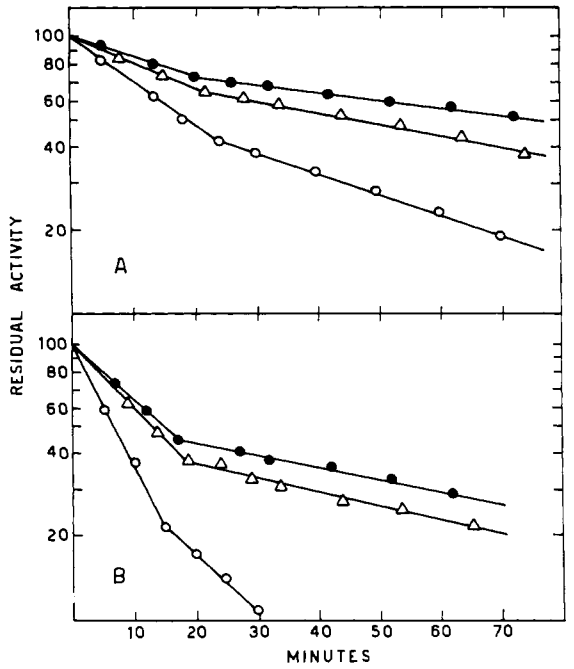

FIGURE 2: Kinetics of photoinactivation of 6-phosphogluconate dehydrogenase from *E. coli* (A) and *B. stearothermophilus* (B) with Rose Bengal as photosensitizer. The reaction mixture contained the enzyme at a concentration of 0.05 mg/ml in 0.05 M potassium phosphate buffer, pH 7.2, with 1 mM dithiothreitol and 1 mM Rose Bengal. The experiment was performed without substrates (O—O), or in presence of 3×10^{-4} M phosphogluconate (●—●), or NADP (△—△).

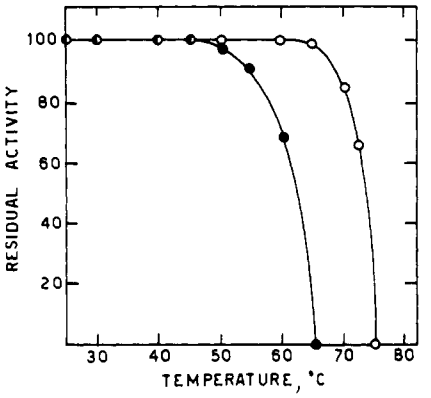

FIGURE 3: Effect of heat on the activity of the *E. coli* (●—●) and *b. stearothermophilus* enzyme (O—O). Solutions of the enzyme (0.1 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM dithiothreitol were incubated in closed vials for 15 min at the indicated temperature. The vials were cooled in ice, opened, and then assayed for activity.

mobility corresponding to a molecular weight of about 50 000 ± 1000, as determined using proteins of known molecular weight as markers. Thus, the native enzyme appears to exist as a dimer composed of monomers having the same molecular weight.

Amino Acid Composition. The amino acid composition of the *E. coli* enzyme is given in Table II, together with the previously unreported composition of the *B. stearothermophilus* enzyme. No striking differences in the amino acid compositions are evident, indicating strict similarities between the mesophilic and thermophilic enzyme. In particular, in both cases, 3 residues of cysteine and 5 residues of tryptophan per polypeptide chain of 50 000 molecular weight have been determined.

Enzymatic Properties. The pH optimum for the 6-phosphogluconate dehydrogenase activity was evaluated in 0.1 M triethanolamine-HCl buffers at 25 °C and was found to extend from 7.8 to 8.2, this range being similar to that found with the *B. stearothermophilus* enzyme (Veronese et al., 1974). The Michaelis constants at 25 °C for NADP and 6-phosphogluconate in 0.1 M Tris-HCl buffer, pH 8.0, were 3.3×10^{-5} and 5×10^{-5} M, respectively. The enzyme was found to be strictly specific for NADP, no activity being observed even at NAD concentration up to 1 mM.

NADPH acts as a competitive inhibitor of the *E. coli* 6-phosphogluconate dehydrogenase. From Lineweaver–Burk plots using different concentrations of NADPH and NADP a K_i value of 4.9×10^{-5} M was calculated. Also, the enzyme from *B. stearothermophilus* was found to be inhibited by NADPH, with a K_i value of 2.6×10^{-5} M.

Properties of the Active Site. Chemical modification studies of functional groups in enzymes are useful in identifying amino acid residues responsible for catalytic activity. 6-Phosphogluconate dehydrogenase in microorganisms (Rippa et al., 1966; Scott and Abramsky, 1973) and in mammalian systems (Silverberg and Dalziel, 1973; Dyson et al., 1973; Pearse and Rosemeyer, 1974a,b) has been shown to be affected by sulfhydryl reagents. Cysteine residues are at or near the active site of the enzyme, since protection by the substrate 6-phosphogluconate has been observed.

The *E. coli* enzyme is also readily inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) (5×10^{-4} M), the activity being protected by 6-phosphogluconate at 2 mM concentration, whereas the coenzyme NADP is less effective. Analogous inhibition occurred upon incubation of the enzyme with *p*-chloromercuribenzoate. Inactive enzyme prepared by reaction with these SH reagents can be completely reactivated by incubation in presence of an excess of β-mercaptoethanol. Inhibition, protection by substrate or coenzyme, and reversibility of the inhibition were very similar to these reported for the *B. stearothermophilus* enzyme (Veronese et al., 1974).

The photoinactivation process in the presence of Rose Bengal of the *E. coli* and the *B. stearothermophilus* enzyme was also studied. As shown in Figure 2, in both cases inactivation similarly occurred as a biphasic process and protection by 6-phosphogluconate and NADP was achieved.

Thermal Properties. Purified *E. coli* 6-phosphogluconate dehydrogenase in phosphate buffer, pH 6.8, in the presence of the reducing agent β-mercaptoethanol and EDTA remained fully active on storage at room temperature for 24 h. The thermostability of the enzyme was evaluated upon heating enzyme samples at different temperatures for 15 min (Figure 3). The mesophilic enzyme retains its activity upon heating up to about 50 °C, whereas rapid inactivation occurs near 60 °C. On the other hand, the *B. stearothermophilus* enzyme clearly shows higher stability against heat, being stable up to about 70 °C.

The effect of temperature on the catalytic process was also studied. The optimum temperature for the enzyme derived from *E. coli* (55–60 °C) was approximately 20 °C lower than the optimum temperature of the dehydrogenase from the thermophile (Veronese et al., 1974). This difference closely corresponds to the difference for optimal growth of the *E. coli* (37 °C) and *B. stearothermophilus* (60 °C) microorganisms. From an Arrhenius plot of the logarithm of velocity and the reciprocal absolute temperature, an activation energy of 11 160 cal/mol for the *E. coli* enzyme was calculated. Whereas the Arrhenius plot of the *B. stearothermophilus* enzyme showed a marked break near 43 °C (Veronese et al., 1974), the plot of the *E. coli* enzyme was linear from 20 to ~50 °C. Above this

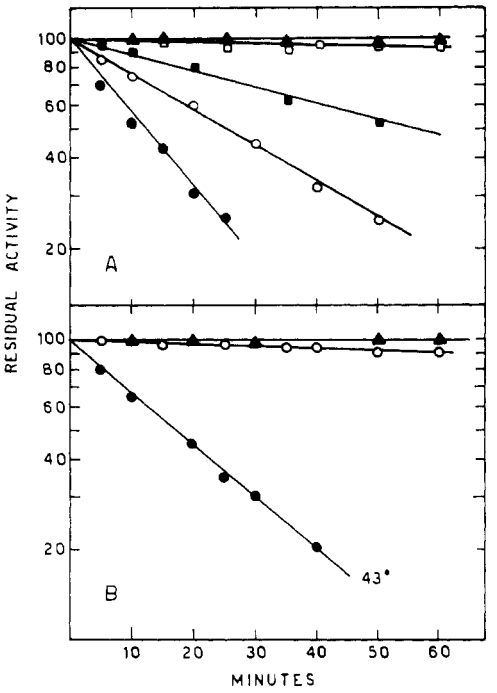

FIGURE 4: Influence of urea on the inactivation of 6-phosphogluconate dehydrogenase. The enzyme concentration was 0.05 mg/ml. (A) *E. coli* enzyme in 0–2 M (▲—▲), 4 M (□—□), 6 M (■—■), 7 M (○—○) and 8 M (●—●) urea at 35 °C. (B) *B. stearothermophilus* enzyme in 6 M (▲—▲) and 8 M urea (○—○) at 35 °C or in 8 M urea at 43 °C (●—●).

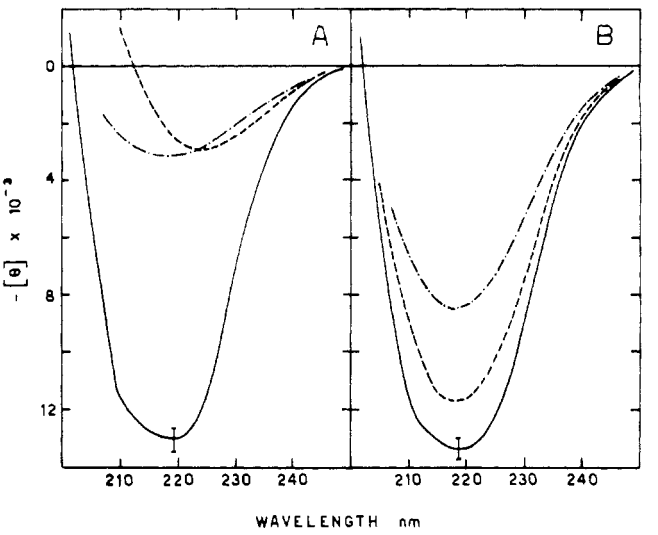

FIGURE 5: CD spectra of 6-phosphogluconate dehydrogenase from *E. coli* (A) and *B. stearothermophilus* (B) in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM EDTA, and 1 mM β-mercaptoethanol. The protein concentration was 0.04 mg/ml for the mesophilic enzyme and 0.05 mg/ml for the thermophilic one. Measurements were made at 25 °C (—), at 60 °C (- - -) and in 8 M urea (- · - ·) in phosphate buffer, pH 6.8. The spectra in urea have been obtained after 5 min of incubation at 35 °C.

temperature linearity was not observed, the enzyme being rapidly heat denatured.

Effect of Urea. The denaturation of the *E. coli* enzyme by urea has been studied in detail and compared with that of the *B. stearothermophilus* enzyme. The enzyme was incubated at 35 °C in urea solutions of increasing concentration and samples were removed on a time schedule for residual activity measurements. Figure 4 shows that the mesophilic enzyme is stable at 35 °C in concentrations of urea up to 4 M, being inactivated at higher molarities with a half-denaturation time of 50 min in 6 M, 25 min in 7 M, and 10 min in 8 M urea. The *B. stearothermophilus* enzyme is by far more stable, being nearly unaffected by 8 M urea with only 10% loss of activity after exposure to 8 M urea solution for 60 min. At higher temperatures, the thermophilic enzyme is more rapidly inactivated, with a half-denaturation time of 20 min when incubated in 8 M urea at 43 °C. The enzyme from both sources is inactivated by urea solutions by a first-order process.

Circular Dichroism Measurements. The CD spectra of 6-phosphogluconate dehydrogenase from *E. coli* and *B. stearothermophilus* between 200 and 250 nm are shown in Figure 5. In both cases, the spectrum at 25 °C is characterized by a negative band centered at about 220 nm. The ellipticity value at 220 nm ($-13\,000 \pm 300$ deg cm^2/dmol) was, within the experimental error, in complete agreement between the two proteins.

The CD spectra were also recorded at higher temperatures in order to examine the effect of heating on the secondary structure of the enzyme. As the temperature is increased, the intensity of the dichroic signal at 220 nm is reduced, the changes observed being larger with the mesophilic than with the thermophilic enzyme. As shown in Figure 5, at 60 °C extensive loss of structure of the *E. coli* enzyme occurs. The characteristics of the spectrum at 60 °C are not similar to those reported for random coils (Greenfield and Fasman, 1969). A broad band centered at about 225 nm occurs, with a crossover from negative to positive ellipticity occurring at 213 nm. On the other hand, the *B. stearothermophilus* enzyme at 60 °C retains more than 90% of its structure.

In 8 M urea solution, the *E. coli* enzyme showed weak negative ellipticity, and the effect of this denaturing agent was roughly analogous to the effect of heating at 60 °C (Figure 5). Spectra were recorded after 10-min incubation at 35 °C. Whereas the *E. coli* enzyme dissolved in 8 M urea appears to be largely unfolded, the *B. stearothermophilus* enzyme was clearly more resistant to the denaturing action of urea. It is evident from Figure 5B that important structural elements of the thermophilic enzyme still exist in 8 M urea, the intensity of the CD signal being about 33% less in urea than in buffer.

Fluorescence Measurements. The fluorescence emission spectra of both the mesophilic and thermophilic enzyme were measured after excitation at 295 nm. At this wavelength the fluorescence spectra of proteins result from the contribution of tryptophan residues only (Teale, 1960; Steiner et al., 1964; Brand and Witholt, 1967; Eisinger, 1969). Figure 6 reports the fluorescence emission spectra of the mesophilic and thermophilic enzyme, as well as of *N*-acetyltryptophanamide in water. Both proteins show the same maximum of emission at 334 nm, whereas the model compound shows a maximum of emission at 350 nm. As is well known, the wavelength of emission can be taken as an indication of the molecular environment around the emitting tryptophan of a protein (Steiner et al., 1964; Cowgill, 1966). Thus, the tryptophan environment on both proteins should be similar and moderately hydrophobic.

Upon heating, the fluorescence intensity gradually decreased in a monotonic way due to thermal quenching (Steiner and Edelhoch, 1962; Gally and Edelman, 1962; Brand and Witholt, 1967) (Figure 7), up to about 50 °C for the mesophilic enzyme and up to about 65 °C for the thermophilic one. Up to these temperatures the maximum wavelength of emission remained constant, indicating that no gross changes in structure occur in both proteins. In addition, since on cooling at room tem-

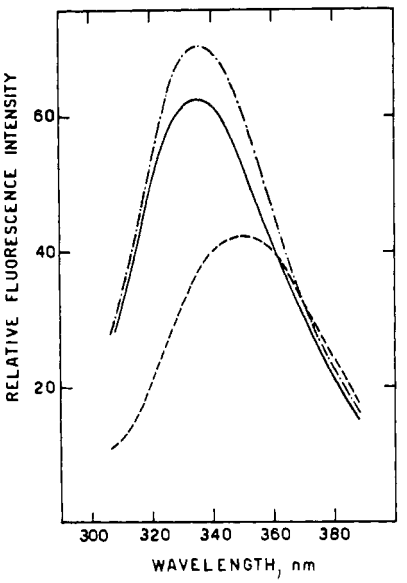

FIGURE 6: Emission spectra of 6-phosphogluconate dehydrogenase from *E. coli* (—) and *B. stearothermophilus* (- · - ·) in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM EDTA, and 1 mM β-mercaptoethanol. The protein concentration was 0.04 mg/ml for the mesophilic enzyme and 0.05 mg/ml for the thermophilic one. Spectra were recorded at 35 °C and excitation was at 295 nm. The emission of *N*-acetyltryptophanamide (- - -) in phosphate buffer, pH 6.8, is included for comparison.

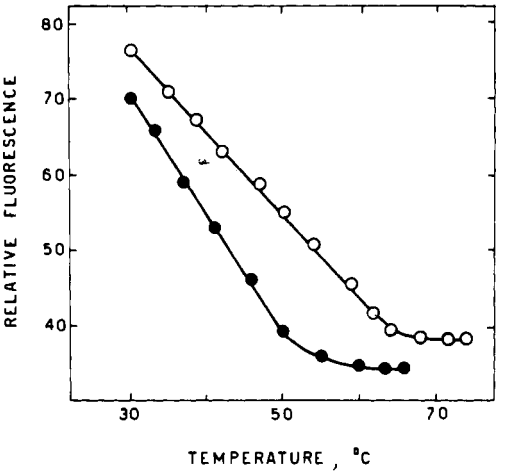

FIGURE 7: Effect of temperature on the emission fluorescence intensity of 6-phosphogluconate dehydrogenase from *E. coli* (●—●) and *B. stearothermophilus* (○—○). The experimental conditions are as those described under Figure 6 and the Experimental Procedure. The intensity of fluorescence was measured at 334 nm.

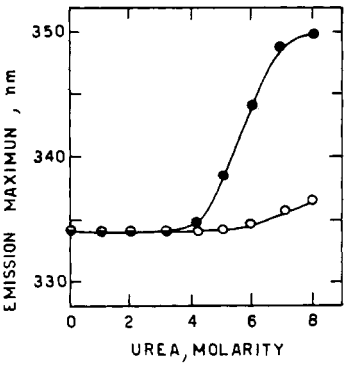

FIGURE 8: Effect of urea on the maximum wavelength of emission on 6-phosphogluconate dehydrogenase from *E. coli* (●—●) and *B. stearothermophilus* (○—○). Spectra were recorded after 10-min incubation at 35 °C in various concentrations of urea in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM EDTA, and 1 mM β-mercaptoethanol.

perature the intensity of fluorescence increased to reach exactly the starting value, this would indicate that no structure alteration occurs on heating up to about 50 and 65 °C for the mesophilic and thermophilic enzyme respectively. When the protein samples were heated at higher temperature, unfolding occurred, the maximum wavelength of emission being shifted to 350 nm.

The effect of urea on the structure of 6-phosphogluconate dehydrogenase was followed by fluorescence measurements. A red shift in fluorescence usually represents unfolding of a protein to a more open, flexible form. Figure 8 shows the change in the maximum wavelength of fluorescence emission in the *E. coli* and *B. stearothermophilus* enzyme in urea solutions of increasing concentrations. The data of Figure 8 represent values obtained after 10-min incubation at 35 °C. It is clear that with the *E. coli* enzyme a molecular transition occurs between 5 and 6 M urea, the maximum being shifted from 334 to 350 nm. The concentration range of urea over which the *E. coli* enzyme is denatured is typical for most globular proteins (Tanford, 1970). On the other hand, the *B. stearothermophilus* enzyme is much more resistant to the denaturing action of urea, since in 8 M urea solution the maximum of emission is shifted only by about 3 nm.

Discussion

The experiments that have been described in this communication have yielded some basic information on the properties of 6-phosphogluconate dehydrogenase from *E. coli*. The molecular weight of the *E. coli* enzyme has been found the same (about 100 000) by a variety of techniques, as has that found for the enzyme from *B. stearothermophilus*, and it appears to be a dimer and easily dissociated by sodium dodecyl sulfate into two apparently identical subunits. The molecular weight of the bacterial enzyme is similar to that reported for the enzyme extracted from *Candida utilis* (Rippa et al., 1967) and erythrocytes (Pearse and Rosemeyer, 1974a,b), but somewhat different from the enzyme from *Neurospora crassa* (110 000–120 000) (Rippa et al., 1970) and *Streptococcus faecalis* (106 000–115 000) (Bridges et al., 1975). The amino acid composition of the *E. coli* enzyme does not show distinctive differences in respect to the enzyme from *B. stearothermophilus*, as well as from other sources (Rippa et al., 1967; Silverberg and Dalziel, 1973; Pearse and Rosemeyer, 1974a,b; Bridges et al., 1975).

The catalytic properties are similar to those of the thermophilic enzyme (Veronese et al., 1974), apparent K_m values for NADP and 6-phosphogluconate being of the same order of magnitude. The enzyme is inhibited by NADPH, whereas fructose 1,6-diphosphate, which was shown to be a potent inhibitor of the *Neurospora* (Rippa and Pontremoli, 1968; Scott and Abramsky, 1973) and sheep liver (Dyson and D'Orazio, 1971) enzyme, does not show any effect. Another property that the bacterial 6-phosphogluconate dehydrogenase, both from *E. coli* and *B. stearothermophilus*, shares with the enzyme from other sources, is its sensitivity to sulfhydryl reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-chloromercuribenzoate. These reagents bring about complete inhibition, the rate of the reaction being reduced by addition of either NADP or 6-phosphogluconate, which indicates that these substrates bind in the vicinity of essential thiol(s). The catalytic activity is regenerated by treatment with a reducing agent such as β-mercaptoethanol.

Photochemical oxidation mediated by Rose Bengal has provided information on the presence of photooxidizable amino acids in the active center of 6-phosphogluconate dehydrogenase from *Candida utilis* (Rippa et al., 1970), and *Neurospora crassa* (Scott and Abramsky, 1973). Both enzymes are inactivated irreversibly, but the kinetics of the photooxidative reaction has been found different in the two cases, being a biphasic process in the *Neurospora* enzyme and a first-order process in the *Candida* enzyme. The kinetic behavior of the reaction, together with some different protecting effects on the photooxidation of the substrates NADP and 6-phosphogluconate, have indicated in the enzyme from the aforementioned sources subtle differences in the environment of the active site (Scott and Abramsky, 1973). The photooxidation studies, as applied to the *E. coli* and *B. stearothermophilus* 6-phosphogluconate dehydrogenase, indicate that strict similarities do exist at the active site, since in both cases a biphasic inactivation process takes place upon illumination in the presence of Rose Bengal and similar protecting effects are obtained by photooxidation of the enzyme in the presence of substrates.

In spite of the many similarities between the mesophilic and thermophilic enzyme discussed above, the *E. coli* 6-phosphogluconate dehydrogenase shows less stability toward heat than the thermophilic enzyme (Figure 3). The thermostability of an enzyme, as verified by measuring its residual activity after incubation at high temperatures, does not necessarily imply stability of its secondary and tertiary structure. Clearly, although structure of an enzyme is most likely a prerequisite of its activity, still the two parameters constitute different aspects of the problem (Fujita and Imahori, 1975). It seemed, therefore, interesting to investigate some conformational aspects of the *E. coli* and *B. stearothermophilus* enzyme, at lower and higher temperature. The secondary structure of both the mesophilic and thermophilic enzyme appears to be strictly similar at room temperature, as shown by CD measurements (Figure 5). When the CD spectra were recorded at 60 °C, the mesophilic enzyme showed extensive denaturation, whereas the thermophilic one retained over 90% of its structure. The general aspect of the CD spectrum at 60 °C of the *E. coli* enzyme, showing a minimum near 225 nm and a crossover point from negative to positive at 213 nm, does not seem to correspond to a spectrum commonly attributed to the random coil (Greenfield and Fasman, 1969). Probably, the heat denatured protein more or less tends to stay in certain, albeit nonperiodic, conformations for longer than average periods of time. Actually, it has to be recalled that disagreement exists between the propositions of a standard CD spectrum for the random coil (Cortijo et al., 1973).

The emission maxima of tryptophan fluorescence are very dependent upon the chemical environment of tryptophan residues, so that measurement of their fluorescence should give information regarding aspects of protein structure (Brand and Witholt, 1967). Since the enzyme from both sources emits at 334 nm upon excitation at 295 nm, the tryptophan environment should be much the same in the two proteins and moderately hydrophobic, about the polarity of dioxane (Van Duuren, 1963). The thermal quenching of the fluorescence intensity (Gally and Edelman, 1962; Steiner and Edelhoch, 1962) can also be used to monitor conformational transitions occurring on heating. In fact, deviations from a monotonic decrease of intensity are indicative of changes of structure of the protein under study. The intensity of fluorescence measured at 334 nm gradually decreased up to about 50 °C with the *E. coli* enzyme and up to 65 °C with the *B. stearothermophilus* one (Figure 7). In conclusion, the fluorescence as well as the CD data are indicative that the mesophilic enzyme is conformationally less stable than the thermophilic one. In addition, the results obtained on the thermostability of the activity of the enzyme (Figure 3) parallel those of its conformation.

One distinct difference between the *E. coli* and *B. stearothermophilus* 6-phosphogluconate dehydrogenase is shown when the effect of temperature on the catalytic process of the enzyme is reported in the form of an Arrhenius plot. Whereas the mesophilic enzyme showed a linear relationship between the logarithm of velocity and the reciprocal absolute temperature, the thermophilic enzyme showed a broken plot with a point of discontinuity near 43 °C (Veronese et al., 1974; Pearse and Harris, 1973). Discontinuities in the Arrhenius plots have been noted previously with several enzymes (Levy et al., 1959; Dixon and Webb, 1964; Massey et al., 1966; Lumry and Biltonen, 1969) including thermostable ones (Muramatsu and Nosoh, 1971; Orengo and Saunders, 1972; Sando and Hogenkamp, 1973). Some authors (Levy et al., 1959) claimed conformational transitions occurring at the temperature where the break in the Arrhenius plot is observed and in some cases this fact has been confirmed (Massey et al., 1966). However, this change with the *B. stearothermophilus* enzyme was not detectable by CD and fluorescence measurements and the nature of the conformational change, if any, is not known. In act, the CD spectrum of the thermophilic enzyme does not show distinct differences in shape and $[\theta]$ values between 20–25 and 50–55 °C. The temperature quenching of fluorescence (Gally and Edelman, 1962; Steiner and Edelhoch, 1962; Brand and Witholt, 1967) of the thermophilic enzyme leads to a monotonic decrease of intensity up to about 65 °C, where heat denaturation occurs. However, it is worth recalling that interpretations of a nonlinear Arrhenius plot different from the conformational one are possible (Dixon and Webb, 1964).

Both the mesophilic and thermophilic 6-phosphogluconate dehydrogenases were exposed to the denaturing effect of urea of increasing concentrations. CD (Figure 5) and emission fluorescence measurements (Figure 8) clearly indicated that the secondary structure of the thermophilic enzyme is less sensitive to the denaturing action of urea, accounting for the high stability of the enzymatic activity (Figure 4). The kinetics of denaturation followed by the physical techniques do not parallel that obtained by measuring the residual activity after exposure to the action of denaturant.This apparent discrepancy was clearly related to a refolding of the denatured molecules in urea solution after dilution with buffer during the assay. In fact, a solution of the *E. coli* enzyme, which had been completely denatured in 8 M urea as evidenced by CD and fluorescence measurements, upon dilution with buffer showed a spectrum similar to that of the native enzyme, although the intensity of the dichroic signal was 40% less. In addition, the emission maximum of fluorescence, which was at 350 nm for the denatured enzyme, was shifted near 335 nm. Similar results have been obtained also with the *B. stearothermophilus* enzyme. Preliminary kinetic experiments also showed that the regain of structure, although not quantitative, was very rapid, whereas the regain of enzymatic activity was slower. These results parallel those obtained by Teipel and Koshland (1971) and others (Deal, 1969; Levy and Kaplan, 1971; Marangos and Costantinides, 1974) on the renaturation of multimeric enzymes.

In closing, we hope that the studies here reported on a bacterial mesophilic and thermophilic 6-phosphogluconate dehydrogenase will complement the body of information that is being accumulated on this important enzyme. In addition, the results of this comparative study on the properties of a meso-

philic and thermophilic enzyme indicate that a general similarity of the molecular and catalytic properties exists between the enzyme from the two sources. Our results, together with those reported by other authors (Suzuki and Imahori, 1973; Fujita and Imahori, 1975; Singleton and Amelunxen, 1973; Matthews et al., 1974), clearly indicate that mesophilic and thermophilic proteins are also structurally very similar, and that no gross alteration of the architecture of a protein is necessary in order to confer thermostability. The physicochemical measurements on 6-phosphogluconate dehydrogenase both from *E. coli* and *B. stearothermophilus* here reported clearly indicate that thermostability arises from very subtle differences in the structure of the protein molecule, contrary to what was once suspected (Brock, 1967).

Acknowledgments

The authors thank Dr. B. Salvato (Centro di Studio sulle Emocianine del C.N.R., Padova) for valuable guidance in the ultracentrifugation studies. Some experiments here mentioned have been carried out by Dr. C. Grandi. The excellent technical assistance of Mr. M. Zambonin, S. Fioretto Da Rin, and F. Miozzo is also gratefully acknowledged.

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