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Multiple Forms of Phosphodeoxyribomutase from *Escherichia coli.* Physical and Chemical Characterization†

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ABSTRACT: Phosphodeoxyribomutase from *Escherichia coli* has been purified to homogeneity. Chromatography on DEAE-Sephadex revealed three peaks of enzyme activity, designated form I, form II, and form III. Form III could be further separated into form III-1 and form III-2 by polyacrylamide gel electrophoresis. The four different molecular forms of the enzyme thus isolated were shown not to be products of the column chromatography *per se.* The amino acid composition as well as the N-terminal amino acid were found to be identical for the different forms. Molecular weight determinations demonstrated that all four forms of the enzyme consist of a single polypeptide chain with a molecular weight of 45,000 ± 1000. Measurements of partial specific volumes, sedimentation coefficients, and absorption coefficients for form I and form II did not reveal any differences. It is concluded that the multiple forms of phosphodeoxyribomutase are caused by modifications of the polypeptide chain. Evidence is presented that form II is formed *in vitro* from form I by deamidation. It is probable that this deamidation occurs *in vivo* also. The different forms displayed only minor changes with respect to K_M for substrate and cofactor. Greater differences seem to exist among the four enzyme forms with respect to V_M and to cobalt binding.

Phosphodeoxyribomutase catalyzes the reversible transfer of a phosphate group between the C_1 and the C_5 carbon atoms of ribose and deoxyribose, respectively. This reaction has been demonstrated to occur both in bacteria and in mammal tissues and has in most cases been shown to be catalyzed by an enzyme different from the phosphoglucomutase. For further references see Kammen and Koo (1969), Hoffee and Robertson (1969), and Hammer-Jespersen and Munch-Petersen (1970).

The partial purification and some properties of phosphodeoxyribomutase from *E. coli* were reported earlier (Hammer-Jespersen and Munch-Petersen, 1970). This paper also reported the finding of three separate peaks of activity after hydroxylapatite chromatography.

The purpose of the present study was to investigate the polymorphism of the enzyme and to characterize the multiple forms of phosphodeoxyribomutase according to the subdivision of enzymes recommended by the IUPAC-IUB Commission on Biochemical Nomenclature ((1971), *Biochemistry 10,* 4825).

By use of genetic arguments some of the reasons for enzyme multiplicity could be excluded beforehand: mutants impaired in phosphodeoxyribomutase activity do arise with the same frequency as other mutations in single genes of *E. coli, e.g.,* mutations in *dra, tpp,* and *pup*[1] (K. Hammer-Jespersen, unpublished results). Thus the cause of the multiple forms of phosphodeoxyribomutase does not seem to be the existence of genetic independent proteins with phosphodeoxyribomutase activity.

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[1] Abbreviations used are: Dns dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; BAWP, 1-butanol–water–pyridine–glacial acetic acid (15:12:10:3); SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; dRib-P, deoxyribose 1-phosphate; Rib-P_2, ribose 1,5-diphosphate; TS 6.9 and TS 7.4, buffers containing 50 mM Tris, 50 mM succinic acid, and 1 mM EDTA, which are adjusted with solid sodium hydroxide to pH 6.9 or 7.4, respectively; MG-buffer, a 8.9 mM phosphate buffer adjusted to ionic strength 0.1 M with NaCl (pH 7.0) (Miller and Golder, 1950) (EDTA was added to a final concentration of 1 mM.); *dra, tpp, pup,* the genes coding for deoxyriboaldolase, thymidine phosphorylase, and purine nucleoside phosphorylase. Enzymes: Purine (deoxy)ribonucleoside phosphorylase or purine-nucleoside:orthophosphate (deoxy)ribosyltransferase (EC 2.4.2.1); thymidine phosphorylase or thymidine:orthophosphate deoxyribosyltransferase (EC 2.4.2.4); deoxyriboaldolase or 2-deoxy-D-ribose-5-phosphate acetaldehyde-lyase (EC 4.1.2.4); phosphoglucomutase or α-D-glucose-1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase (EC 2.7.5.1); phosphodeoxyribomutase or α-D-glucose-1,6-bisphosphate:deoxy-D-ribose-1-phosphate phosphotransferase (EC 2.7.5.6).

The following possibilities known to cause enzyme multiplicity were therefore investigated in the present study: (1) existence of heteropolymers (hybrids) of two or more polypeptide chains; (2) conjugation of the protein with nonprotein groups; (3) modifications of the same polypeptide chain; (4) different polymers of the same subunit; (5) conformationally different forms.

The molecular weight investigations of phosphodeoxyribomutase showed that the enzyme is not composed of subunits, thus excluding possibilities 1 and 4. For one of the forms (form II) positive evidence has been obtained that it may be produced from form I by deamidations.

Experimental Procedures

Materials

All chemicals employed were analytical grade. Dns-Cl was from Merck, Germany (zur Fluoreszenzmarkierung von Aminosäuren). Polyamide sheets were obtained from Cheng Chen Trading Co., Ltd., Hankow St. Taipei, Taiwan. Acrylamide and *N,N'*-methylenebisacrylamide were from Eastman, Kodak (for Electrophoresis quality). TPCK-treated trypsin (200 μ/mg) was obtained from Worthington Biochemical Corp. Thymidinephosphorylase was prepared according to Schwartz (1971) and deoxyriboaldolase according to Jensen *et al.* (1973). Bovine serum albumin, ovalbumin, cytochrome *c* (from horse heart, type III), and ribonuclease A were from Sigma Chemical Co. Catalase, chymotrypsinogen, and yeast alcohol dehydrogenase were Boehringer products. dRib-P was prepared as described by Hammer-Jespersen and Munch-Petersen (1970). The tetracyclohexylammonium salt of Rib-P_2 was a gift from H. Klenow, Biokemisk Institut B, University of Copenhagen.

Double glass-distilled water was used for the dialysis of samples for amino acid and fingerprint analysis. For all other purposes the water was glass distilled and then mixed-bed deionized on Amberlite IR 120/IRA 410.

All glassware was washed immediately before use with a 10-mg/l. solution of diphenylthiocarbazone in tetrachloromethane (Vallee and Gibson, 1948). Polystyrene tubes were found usable without further washing.

Sϕ 103, an *E. coli* K 12 strain, was used as source of enzyme (Hammer-Jespersen and Munch-Petersen, 1970).

Methods

Growth and Harvest of Bacteria. Cultures were grown from a single colony of Sϕ 103 in 1.5-l. batches at 37° with violent aeration. The growth medium contained per liter: 2 g of $(NH_4)_2SO_4$; 6 g of $Na_2HPO_4 \cdot 2H_2O$; 2 g of KH_2PO_4; 2 g of NaCl; 7 g of NH_4Cl; 407 mg of $MgCl_2 \cdot 6H_2O$; 15 mg of $CaCl_2 \cdot 2H_2O$; 11 mg of $FeCl_3 \cdot 6H_2O$; 20 g of D-(+)-glucose $\cdot H_2O$; 450 mg of DL-methionine; 10 g of bactotryptone; and 5 g of yeast extract. Restriction of the thymine content of the growth medium to what was contained in the bactotryptone and the yeast extract resulted in a 20-fold induction of phosphodeoxyribomutase (caused by thymine starvation). The cells were harvested in the early stationary phase. The yield was *ca.* 20 g of wet weight/l.

Assay. One enzyme unit is defined as the amount of enzyme which catalyzes the conversion of 1 μmol of substrate per min at 37° with saturating concentrations of substrate and cofactors present.

Phosphodeoxyribomutase was assayed spectrophotometrically by coupling the conversion of deoxyribose 1-phosphate to the oxidation of NADH and measuring the change in absorbancy at 366 nm (Hoffee and Robertson, 1969). An extinction coefficient of 3.3×10^3 l. mol^{-1} cm^{-1} was used.

Before assay the enzyme was activated by incubation in 10 mM Tris-HCl (pH 7.6) + 50 μM EDTA + 250 μM $CoCl_2$ at 0°. The reaction was started by adding 20 μl of Co^{2+}-activated enzyme to the assay mixture in a thermostated cuvet at 37°. The assay mixture contained 50 mM Tris-HCl (pH 8.3); 0.4 mM dRib-P; 0.25 mM NADH; 1.7 μM Rib-P_2; 3.2 U/ml of deoxyriboaldolase; 6 U/ml of alcohol dehydrogenase in a total volume of 400 μl. Phosphodeoxyribomutase was added to a final concentration of approximately 0.05 U/ml. The reaction velocity was independent of addition of $CoCl_2$ to the assay mixture. In some experiments the colorimetric assay described by Hammer-Jespersen and Munch-Petersen (1970) was employed.

Protein concentrations were determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. For bovine serum albumin dissolved in water $E_{1cm}(1\%)$ 6.3 at 280 nm was used (Kirschenbaum, 1971).

Polyacrylamide Gel Electrophoresis. ANALYTICAL GEL ELECTROPHORESIS. Slab electrophoresis was performed in an Ortec 4200 electrophoresis system equipped with an Ortec 4100 pulsed constant power supply using the following "continuous" system. Both upper and lower electrobuffer was 15 mM Hepes + 0.2 mM EDTA adjusted to pH 7.0 with Tris. The separation gel contained 7.5% acrylamide with 5% cross-linking *N,N'*-methylenebisacrylamide (T = 7.5%, C = 5% (Hjertén, 1962)) dissolved in 45 mM Hepes + 0.6 mM EDTA adjusted to pH 7.0 with Tris. For sample application a "well-former-gel" in which T = 3% and C = 5% in the same buffer was casted. The power supply was set on 250 V, 250 pulses/sec, and 0.5 mF. The gel was stained with Coomassie Brilliant Blue (Weber and Osborn, 1969). Samples containing 1–2 μg of each protein component were applied with 10% sucrose added and the starting zones were sharpened by keeping the conductivity of the sample approximately five times lower than that of the gel buffer (Hjertén *et al.*, 1965).

PREPARATIVE ELECTROPHORESIS. The described analytical electrophoresis system was adapted for preparative use in a Buchler preparative electrophoresis apparatus (Poly-Prep). The upper gel was omitted, but otherwise the composition of gel and electrode buffer was unchanged. To minimize pH changes during electrophoresis the electrode buffers were recirculated at a velocity of 10 l./hr. The membrane holder buffer was a tenfold concentrated electrode buffer. The electrophoresis was conducted at 250 V (constant voltage), and the temperature was kept at 4°. Elution was performed with a flow rate of 180 ml/hr and fractions of 3 min were collected.

AMINO ACID ANALYSIS was carried out according to Spackman *et al.* (1958) using a Durrum amino acid analyzer, Model 500. Hydrolysis using 6N redistilled HCl at 110° in evacuated, sealed tubes was carried out for three different periods, 25, 48, and 72 hr in order to allow extrapolation to zero (Thr, Ser) or infinite (Val, Ile) time of hydrolysis. Hydrolysate from about 0.1 nmol of protein was applied to the analyzer in each sample.

Cysteine plus cystine was determined as cysteic acid after performic acid oxidation as described by Moore (1963).

N-TERMINAL DETERMINATION. Samples containing about 10 nmol of protein were treated with 8 M urea and dansylated according to Narita (1970). Hydrolysis was carried out at 110° in 6 N redistilled HCl for two periods (19

Table I: Purification of Phosphodeoxyribomutase.

Treatment	Volume (ml)	Total Activity (units)	Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	2800	297,000	150,000	2.0	100	1.0
DEAE-Sephadex	5750	345,000	12,000	30	116	15
Sephadex G-100	835	304,000	2,100	143	102	73
DEAE-Sephadex						
Form I, tubes no. 147–175	290	85,000	336	251	28	128
Form II, tubes no. 176–215	400	104,000	488	214	35	109
Form III, tubes no. 216–245	300	16,000	231	67	5	34
Rechromatography						
Form I	185	51,000	163	310	17	158
Form II	272	62,000	218	284	21	145
Form III	123	5,200	66	78	2	40
Gel electrophoresis						
Form III-1, tubes no. 97–103	3.7	300	2.8	138	0.1	70
Form III-2, tubes no. 104–117	2.7	230	1.6	142	0.1	73

and 69 hr). The dansylated amino acids were identified by chromatography on polyamide sheets (Hartley, 1970).

FINGERPRINT ANALYSIS. Samples of about 2 mg of protein were dissolved in 1.5 ml 0.1 M *N*-ethylmorpholineacetate (pH 8.0) and boiled for 20 min; 20 μl of 1 mg/ml TPCK-treated trypsin dissolved in 1 mM HCl was added. The digestion was performed at 37° and after 1 hr another 20 μl of trypsin was added, and the incubation was continued for 2 more hr.

Paper electrophoresis in first dimension was carried out at about 50 V/cm in liquid-cooled tanks at pH 6.5. The neutral zone was then expanded at pH 1.9 (Ambler, 1963). For separation in second dimension BAWP chromatography was used (Waley and Watson, 1953). Dns-OH and Dns-arginine were used as internal markers. The separation was conducted on Whatman 3MM paper and the peptides were stained with ninhydrin-cadmium reagent (Heilman *et al.*, 1957).

MOLECULAR WEIGHT DETERMINATION. (1) Sephadex G-100 filtration was carried out as described by Hammer-Jespersen and Munch-Petersen (1970). (2) SDS electrophoresis was performed according to Weber and Osborn (1969). (3) Low-speed sedimentation equilibrium centrifugations were essentially performed as described by Richards *et al.* (1968). A Spinco Model E analytical ultracentrifuge equipped with interference optics was used. A mercury lamp equipped with a Wratten 77A filter was used as light source. The wavelength was 546 nm. The centrifugations were run at 20° in an An-D rotor using a double sector cell equipped with a 12-mm filled Epon centerpiece and quartz windows. A solution and solvent height of 1.5 mm was used. The initial concentration expressed in fringes was determined in a capillar type synthetic boundary cell. The speed of rotation was set to 11,575 rpm and the exact speed was determined from odometer readings. Before centrifugation the protein was dialyzed against MG-buffer. Dialysis buffer was used in the reference sector during the centrifugations. The calculations of the molecular weight were made from plots of the logarithm of the protein concentration (log c) against the square of the distance from the rotation axis (x^2).

Determination of Sedimentation Constants. Sedimentation centrifugations were carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. The rotation speed was set to 56,100 rpm and the exact speed was determined by odometer readings. A 12-mm single sector Kel-F centerpiece equipped with quartz windows was used. An An-D rotor was used and the temperature maintained at 20°. The sedimentation constants were calculated from plots of the logarithm of the axis distance of the concentration gradient maximum (log x) against time (t).

Partial Specific Volume. The partial specific volume ($\bar{\nu}$) in MG-buffer at 20° was determined from measurements of the density (δ) as a function of the weight per cent of protein on a Digital densimeter from Anton Paar, Graz, Austria (Bøje and Hvidt, 1971).

Absorption Coefficients of Pure Protein. The protein was desalted on a Sephadex G-25 column washed with water. From the desalted protein samples were taken for absorption measurements and other samples (7–9 mg) were evaporated to constant weight at 110°. The dried samples were weighed with an accuracy of ±0.01 mg.

Results

Purification of Phosphodeoxyribomutase

Preparation of Crude Extract. About 1000 g wet weight of bacteria was suspended in 0.1 M Tris-HCl –4 mM EDTA (pH 7.6). This and all subsequent steps were performed at 4°. The cells were disrupted with a Branson sonifier, and cell debris was then removed by centrifugation at 20,000g for 60 min. The supernatant constituted the crude extract (Table I).

Precipitation with Streptomycin and the First Chromatography on DEAE-Sephadex. Streptomycin was added to a final concentration of 10 mM (1.5%) and the suspension

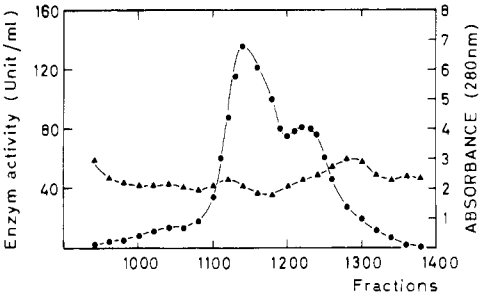

FIGURE 1: Chromatography upon the first DEAE-Sephadex A-50 column (10 × 60 cm). The column was eluted with a linear gradient of NaCl from 0.00 to 0.20 M in TS 7.4 (total volume 30 l.). Elution rate was 100 ml/hr and fractions of 25 ml were collected. Enzyme activity (●) and the absorbance at 280 nm (▲) are shown in the diagram. Tubes 1070–1310 were pooled.

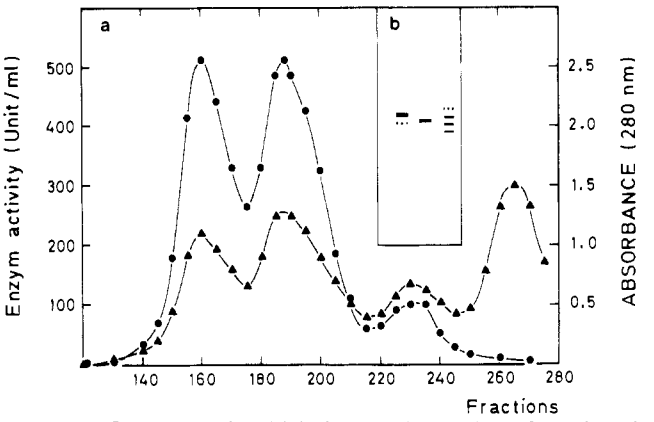

FIGURE 2: Separation of multiple forms. (a) The eluate from the gel filtration was applied to a 2.5 × 45 cm column of DEAE-Sephadex A-50. The column was eluted using a linear gradient ranging from 0.00 to 0.15 M sodium chloride in TS 6.9 (total volume 4 l.). The flow rate was 23 ml/hr and fractions of 10 ml were collected. Enzyme activity (●) and the absorbance at 280 nm (▲) are shown in the figure. Tubes 147–175, 176–215, and 216–245 were pooled to give form I, form II, and form III, respectively. (b) Polyacrylamide gel electrophoresis of 1 μg of the forms I, II, and III (from left to right). The cathode is at the top, and the anode at the bottom of the gel.

was left overnight with stirring. After centrifugation the supernatant was dialyzed against TS 7.4 and then absorbed on a column of DEAE-Sephadex A-50, equilibrated with TS 7.4. From the elution pattern shown in Figure 1 it is seen that two peaks of phosphodeoxyribomutase activity are partially resolved. The total activity peak was pooled (Table I).

Chromatography on Sephadex G-100. The eluate from the DEAE-Sephadex column was concentrated by ultrafiltration and then subjected to gel filtration on Sephadex G-100 equilibrated with TS 6.9. The top fractions were collected. The side fractions were rechromatographed and the fractions with maximal activity then combined with the previous top fractions (Table I).

Separation of Multiple Forms. Second DEAE-Sephadex Chromatography. The enzyme was next chromatographed on a column of DEAE-Sephadex A-50 equilibrated with TS 6.9. The elution profile shown in Figure 2a clearly demonstrates the three peaks of phosphodeoxyribomutase activity resolved by this procedure. Following the order of elution the tree peaks were named form I, form II, and form III (Table I).

Rechromatography of the Multiple Forms. Third DEAE-Sephadex Chromatography. Forms I, II, and III were dialyzed against TS 6.9 and rechromatographed separately on a DEAE-column using the same conditions as above for the separation of the three forms. Each form rechromatographed as a single peak except form I, which contained significant amounts of form II (20–25%). The rechromatographed forms I, II, and III were pooled from the fractions with maximal specific activity (Table I).

In forms I, II, and III altogether 40% of the initial activity was recovered.

Of the total amount of 118,000 units of activity in these three fractions 43% was due to form I, 53% to form II, and only 4% to form III.

Identification of the Multiple Forms by Polyacrylamide Gel Electrophoresis. Figure 2b shows the analytical gel electrophoresis of forms I, II, and III. Form I shows traces of a band of similar mobility as form II. This may be explained by the fact that the electrophoresis was carried out about 2 months after the isolation of form I by DEAE chromatography (see later; Interconversion between Form I and Form II). Only one band of protein is seen in form II. In contrast to this form III is seen to give rise to four separate bands.

Persulfate has been reported to give rise to artefacts (King, 1970). In the present case, however, this is excluded by the following experiments: (1) incorporation of 3% 2-mercaptoethanol in the gel or preelectrophoresis with 0.1 M thioglycollic acid adjusted to pH 7.0 with Tris did not change the electrophoretic pattern obtained in Figure 2b; (2) electrophoresis using 0.5% agarose gels gave the same results; (3) enzymatic activity could be extracted from the gels with a yield of 100%. It was therefore concluded that form III is not a homogeneous protein and a further purification of form III using preparative polyacrylamide gel electrophoresis in the Hepes–Tris system was attempted.

Fractionation of Form III by Gel Electrophoresis. The result of a preparative gel electrophoresis of about 20 mg of form III is shown in Figure 3. The elutions profile shows a fast migrating protein without activity, followed by an asymmetric peak of enzymatic activity. This might indicate partial resolution of at least two active forms of the enzyme. This notion was confirmed by analytical electrophoresis in Hepes–Tris and SDS electrophoresis, which showed that the inactive protein had a higher molecular weight than the two partially resolved proteins with phosphodeoxyribomutase activity. These three proteins corresponded to the three fastest moving bands in Figure 2b. The band with lowest electrophoretic mobility in Figure 2b was not seen, since it disappears after incubation with mercaptoethanol (see Interconversion section).

In order to remove any impurities from the acrylamide gel, form III-1 and form III-2 were subjected to a gel filtration on Sephadex G-100 and then concentrated by ultrafiltration (Table I).

The results of the purification are summarized in Table I. Using the absolute protein determinations (see later) the specific activities of form I and form II were 370 and 355 U/mg, respectively.

Molecular Weight Determinations. (1) Sephadex G-100 filtration of equal amounts of activity of forms I, II, and III gave a single symmetrical peak, and the molecular weight of 33,000 reported by Hammer-Jespersen and Munch-Petersen (1970) was confirmed.

(2) SDS electrophoresis of forms I, II, and III either separately or mixed two by two in the possible combinations gave only one band (except the impurity in form III) corre-

Table II: Molecular Weight of Forms I and II from Equilibrium Centrifugation.

Protein concn (mg/ml)	4.60	2.30	0.00
Form I	41,400	43,600	46,000
Form II	39,700	42,300	45,300

Table III: Density of Different Weight Per Cent Solutions of Forms I and II in MG-Buffer at 20°.

Form I		Form II	
Weight (%)	Density (g/cm^3)	Weight (%)	Density (g/cm^3)
0.685	1.00468	0.688	1.00473
0.457	1.00407	0.459	1.00412
0.229	1.00345	0.230	1.00349
0.114	1.00316	0.115	1.00315
0.000	1.00285	0.000	1.00283

sponding to a molecular weight of 43,000.

(3) Forms I and II were analyzed by equilibrium centrifugation each at two concentrations. Linear dependency of log c on x^2 was obtained indicating homogeneity of the proteins.

The molecular weight was calculated from the formula

$$M = \frac{2RT2.203\mathrm{d}\log c}{(1 - \bar{v}\rho)\omega^2 \mathrm{d}x^2}$$

where R is the gas constant, T the absolute temperature, and ω the speed of rotation in radians/sec. The determination of the density of the centrifuged solution and the partial specific volume of the protein are described below. The molecular weights of form I and II each at protein concentrations of 4.60 and 2.30 mg/ml are given in Table II together with the value obtained by extrapolation to zero concentration. The deviation between form I and form II is within the accuracy of the experiments. The conclusion of molecular weight determinations is that all forms of the enzyme consist of a single polypeptide chain with a molecular weight of 45000 ± 1000.

Partial Specific Volume. For forms I and II the time (T) elapsed after 40,000 vibrations in the digital densimeter was measured at five protein concentrations (0–7 mg/ml). The density of each solution is calculated from the formula $\rho_1 - \rho_2 = A(T_1^2 - T_2^2)$, where A is an apparatus constant which is determined from measurements on substances of known densities (here air and water). The results are quoted in Table III.

The reciprocal densities were plotted against weight per cent of protein and the partial specific volumes calculated by extrapolation to weight per cent = 100. The results were: form I; $\bar{v}$ = 0.731 ± 0.005 cm^3/g and form II: $\bar{v}$ = 0.722 ± 0.003 cm^3/g. Calculation of the partial specific volume according to Cohn and Edsall (1943) would give $\bar{v}$ = 0.73 cm^3/g.

Sedimentation Constants. Forms I and II were centrifuged at three different concentrations in MG-buffer. In each case the protein sedimented as a single symmetrical peak. During each sedimentation five photographs were taken over a period of approximately 2 hr. The sedimentation constant was calculated from the formula: s = 2.303d log $x/\omega^2 60\mathrm{d}t$. The calculated sedimentation constants were corrected to pure water as solvent ($s_{20,w}$) by means of the table given by Svedberg and Pedersen (1940). These corrected sedimentation constants were finally corrected for nonideality by extrapolation to infinite dilution to give $s_{20,w}^0$. For form I, $s_{20,w}^0$ = 3.75 (±0.06) × 10^{-13} sec, and for form II, $s_{20,w}^0$ = 3.71 (±0.06) × 10^{-13} sec.

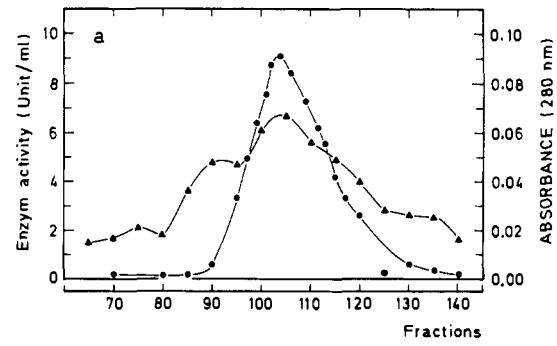

FIGURE 3: Preparative polyacrylamide gel electrophoresis of form III. The electrophoresis was performed on 20 mg of form III in 5 ml of 5 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 5 mM mercaptoethanol, and 10% sucrose. Fractions of 9 ml were collected. (●) Enzyme activity and (▲) absorbance at 280 mμ. Tubes 97–103 were pooled to constitute form III-1, and tubes 104–117 made up form III-2.

Absorption Measurement. The results of the absolute absorption coefficient determinations for forms I and II in 0.1 N NaOH was E_{260}(1 cm) 1.24 ± 0.01 (25°) for a 1.00-mg/ml solution of both forms. The absorbance of native protein in MG-buffer (25°) was E_{280}(1 cm) 0.95 for a 1.00-mg/ml solution of forms I and II. The possibility of a difference in the E_{280}/E_{260} ratio was checked on all four forms (prior to these measurements form III was resolved in III-1 and III-2), but no difference could be established within the accuracy of a Zeiss PMQ II spectrophotometer. In MG-buffer the ratio was 1.77 and in 0.1 N NaOH it was 0.76 for all forms.

Amino Acid Composition of Phosphodeoxyribomutase. The results of eight (forms I and II) and three (forms III-1 and III-2) independent amino acid hydrolysis are presented in Table IV. No significant differences in the amino acid composition of the different forms were revealed by these experiments. Furthermore the amount of amino acids found in the analyses was calculated to account for at least 98% of the dry weights of the samples, thus leaving only a small margin, if any, for nonprotein groups conjugated with the enzyme.

N-Terminal Determination. End group analyses were performed on form I, form II, and form III-1. The dansylated amino acids were identified by chromatography on polyamide layer sheets. Apart from *o*-Dns-Tyr and ε-Dns-Lys the chromatograms revealed two uv absorbing fluorescent spots after 19-hr hydrolysis. One spot corresponded to bis(Dns)-lysine while the other had a mobility corresponding to a Dns-dipeptide. After 69 hr of hydrolysis the second spot had disappeared, while the bis(Dns)-lysine spot was still present. Form I, form II, and form III-1 gave identical results after 19 hr and after 69 hr of hydrolysis, respectively. Thus the results point to lysine as the N-terminal amino acid of forms I, II, and III-1.

Interconversion of Multiple Forms of Phosphodeoxyribomutase. In order to determine whether interconversions between the different forms may occur, form I, form II, and form III (the latter containing both form III-1 and form

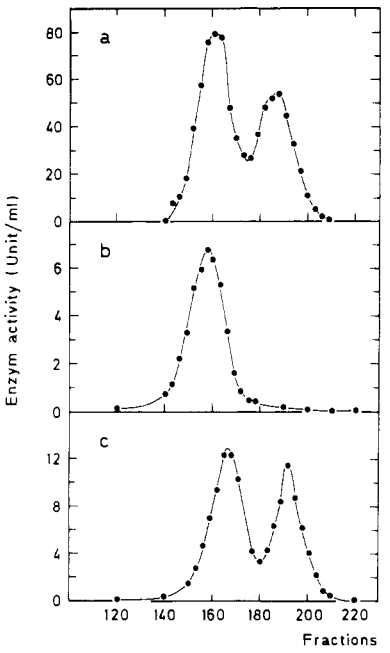

FIGURE 4: Conversion of form I. The column (1.6 × 29.5 cm) was eluted with a linear gradient ranging from 0.00 to 0.15 M sodium chloride in TS 6.9 (total volume 1 l.). The flow rate was 8.3 ml/hr and fractions of 2.5 ml were collected. (a) 6500 units of form I were applied to the column. Fractions 150–165 were pooled to constitute form I_{Re} (total activity = 2100 units). Fractions 183–195 were pooled to constitute form II_I (total activity = 1200 units). (b) The elution profile when 400 units of unmodified form I_{Re} was applied. (c) The elution profile when 1050 units of form I_{Re}, treated at pH 10, was applied. Fractions 187–205 were pooled to constitute form $II_{pH\ 10}$.

Table IV: Amino Acid Composition[a] of Multiple Forms of Phosphodeoxyribomutase.

	Form I	Form II	Form III-1	Form III-2
Lys	25	26	27	27
His	14	14	14	14
Arg	18	18	17	17
Asp	44	43	43	43
Thr	24	24	24	24
Ser	17	17	17	17
Glu	37	36	36	36
Pro	19	18	19	17
Gly	43	43	42	42
Ala	35	33	33	33
Half-Cys	6[b]	6[b]		
Val	20	21	22	21
Met	8	8	8	8
Ile	21	22	23	23
Leu	35	36	38	38
Tyr	11	12	12	13
Phe	20	20	20	20
Trp	5[c]	5[c]	5[c]	6[c]

[a] Residues per 45,000 daltons. [b] Determined after performic acid oxidation. [c] Determined by the method of Goodwin and Morton (1946).

III-2) were incubated as follows.

(a) 5 μM enzyme was incubated in 10 mM Tris-HCl (pH 7.6) with 0.5 mM dRib-P, 2.0 μM Rib-P_2, and 0.5 mM $CoCl_2$ either alone or in the various combinations for 5 min at 37°. These incubations did not change either the enzymatic activity or the electrophoretic patterns of the different forms. One exception was the addition of $CoCl_2$ which reduced the mobility of all the bands somewhat and blurred them as well. This change was counteracted by Rib-P_2.

In analogy to phosphoglucomutase (Ray and Peck, 1972) these incubations in the presence of dRib-P or Rib-P_2 might be expected to favor a conversion between phospho and dephospho forms of the enzyme, if such exists for phosphodeoxyribomutase. From the results obtained it is concluded that occurrence of phospho and dephospho forms of the enzyme does not seem to be the basis for the observed multiple forms of phosphodeoxyribomutase.

(b) 1.5 μM enzyme was incubated in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA and 50 mM mercaptoethanol for 30 min at 0°. Only one change in the electrophoretic patterns was observed: the slowest migrating band in form III disappeared. Thus this band seems to represent an oxidized form of either form III-1, form III-2, or the impurity (the fastest migrating band). No change in activity was found.

(c) 1.5 μM enzyme was incubated in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 50 mM mercaptoethanol, and 6 M guanidinium chloride for 30 min at 25°. The sample was then dialyzed against 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA prior to electrophoresis and activity measurements. (The same dialyses were performed after incubations d, e, and f.)

The treatment with 6 M guanidinium chloride is expected to unfold the protein fully. After refolding (during dialysis) the activity was regained, but there was no change in the electrophoretic patterns given by the different forms, except the change in form III which was also observed using 50 mM mercaptoethanol alone.

(d) 1.5 μM enzyme was incubated for 30 min at 0° in 2.5 M phosphate buffer (pH 7.7) containing 2 mM EDTA. (Under these conditions the protein forms a precipitate.)

(e) 10 μM enzyme was incubated with 0.25 M sodium acetate (pH 4.5) containing 5 mM EDTA, for 300 min at 25°.

(f) 10 μM enzyme was incubated with 0.35 M potassium phosphate (pH 10.0) and 5 mM EDTA for 300 min at 25°.

The conditions d, e, and f were tried since high ionic strength and extremes of pH are known to promote interconversions of multiple forms of a protein (Kaplan, 1968). This may be due to dissociation of groups, bound by ionic or polar bonds, or to an enhancement of conformational changes. Condition f has been reported to cause deamidation of asparagine residues in peptides (McKerrow and Robinson, 1971). The only effect observed resulted from condition f where the electrophoretic pattern indicated a conversion of form I to form II.

As further verification of the relationship between form I and form II the interconversion was studied using DEAE-Sephadex chromatography. For this purpose pure form I was reisolated from form I which had been stored for 6 months at 4°. The elution profile in Figure 4a showed that about half of form I had been converted to form II during the storage.

The reisolated form I (designated form I_{Re}) was split in two batches: one part was rechromatographed directly after dialysis against TS 6.9 (Figure 4b); the other was dialyzed for 320 min against 0.35 M phosphate (pH 10.0) at 25° followed by dialysis against TS 6.9 prior to rechromatography. The elution profile in Figure 4c shows that form II is produced by the incubation of form I_{Re} at pH 10. Moreover it was found that this treatment does not result in significant

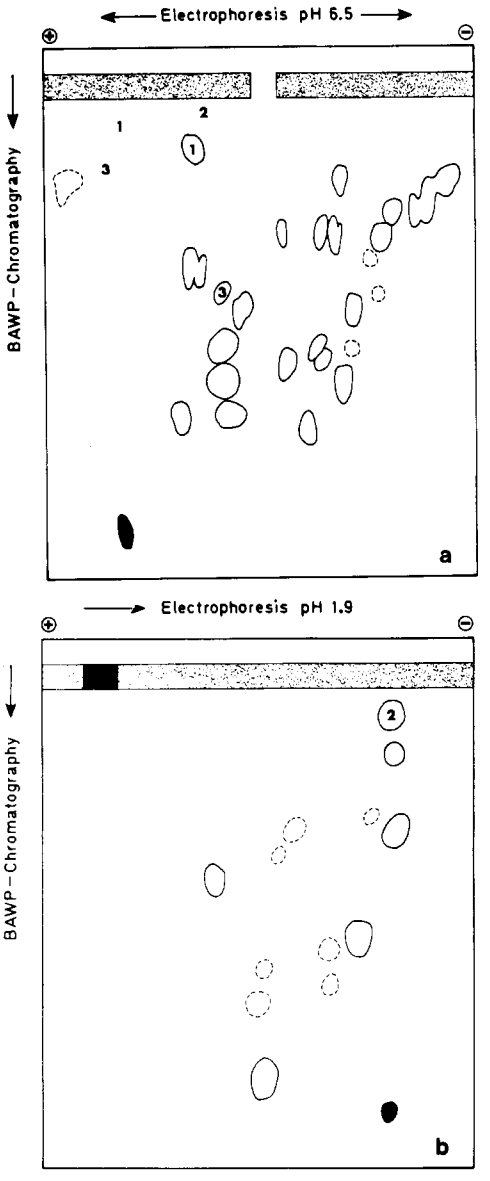

FIGURE 5: Fingerprints of form I. The numbered peptides are those missing in the different species of form II. The plain numbers indicate the positions of the corresponding new peptides from form II. The black spots indicate the dansyl markers. (a) Charged peptides. (b) neutral peptides (at pH 6.5).

loss of enzymatic activity.

From integration over the activity peaks in Figure 4c an apparent half-life of 7 hr for the conversion of form I to form II at pH 10 at 25° can be estimated. From Figure 4a a half-life of about 7 months was found when form I was kept at 4° in TS 6.9.

Tryptic Peptide Maps. If deamidation is involved in the production of form II, differences in the mobility of some of the tryptic peptides of form I and form II would be expected. Therefore fingerprint analyses of the tryptic peptides were performed on form I_{Re} (Figure 4a) and on the following species of form II: the enzyme originally isolated as form II (Table I), form II_I (Figure 4a), and form $II_{pH\ 10}$ (Figure 4c).

In Figure 5 the peptide maps of form I are shown as (a) representing the charged peptides and (b) the neutral peptides (at pH 6.5). The following differences between form I_{Re} and the different species of form II were observed. In all species of form II a yellow peptide (No. 1) was found at a position indicated by 1, and a neutral peptide in form I (No. 2) was found at position 2. For form $II_{pH\ 10}$ an additional change was observed namely the shift of peptide No. 3 to position 3. All the changes seen support the idea of deamidation since the new peptides are more acidic in the different species of form II than in form I. Thus we may conclude: form II and form II_I are identical and are produced from form I by the loss of at least two amides. This process seems to occur spontaneously, when form I is stored in TS 6.9 at 4°.

The accelerated conversion of form I in presence of 0.35 M phosphate at pH 10 seems to provoke one additional deamidation. Thus form $II_{pH\ 10}$ produced by this method does not seem to be exactly identical with form II and form II_I.

From Figure 5 it may also be seen that the tryptic fingerprint method has resolved about 32 peptides out of 44 possible tryptic peptides according to the amino acid composition of phosphodeoxyribomutase (Table IV).

Table V: Kinetic Constants for Phosphodeoxyribomutase.

	Form I	Form II	Form III
K_M^{app} for Rib-P_2[a] (μM)	0.24	0.27	0.29
K_M^{app} for dRib-P[b] (μM)	14	15	22
Half-life of cobalt-enzyme in 1 mM EDTA at 25°[c] (min)	15	31	2 and 38

[a] The activity was determined by the spectrophotometric assay using 0.4 mM dRib-P in the assay mixture. [b] The activity was determined by the colorimetric assay using 4.1 μM Rib-P_2 in the assay mixture. [c] The enzyme was preincubated for 25 min at 0° in 10 mM Tris-HCl (pH 7.3) containing 50 μM EDTA and 250 μM $CoCl_2$. At time zero it was diluted into 10 mM Tris-HCl containing 1 mM EDTA and incubated at 25°. At different times samples were removed for activity measurements. An exponential decrease in activity against time was seen. The half-lives of the measured enzyme activities are given in the table. Addition of a surplus of cobalt ions would in all cases restore the activities to the initial values, indicating that the enzyme is not unstable at the conditions used.

Kinetic Investigations of Phosphodeoxyribomutase K_M for Substrate and Cofactor. After the isolation of form I, form II, and form III kinetic investigations of the different forms were carried out in addition to the chemical and physical analysis described above. Table V gives the results. K_M toward Rib-P_2 was not significantly different for the three forms. Form III (consisting of a mixture of form III-1 and form III-2) did show a slightly higher K_M toward dRib-P than form I and form II.

Properties of Cobalt Activated Enzyme. It has been shown previously that the phosphodeoxyribomutase reaction is strictly dependent upon the presence of certain metal ions (Co^{2+}, Mn^{2+}, or Ni^{2+}) (Hammer-Jespersen and Munch-Petersen, 1970). The metal activation of the enzyme may be separated from the catalytic process: when the enzyme was preincubated at 0° for at least 5 min in buffer at pH 7.3 containing 250 μM $CoCl_2$ and 50 μM EDTA, it was possible to measure the enzymatic activity in the presence of 1 mM EDTA. Moreover, when the assay time was short (2 min), the measured activity was the same, whether 1 mM EDTA or 50 μM cobalt chloride was present during the assay. The removal of cobalt from the enzyme by EDTA could be measured as decrease in catalytic activity with time; it was found to be a first-order reaction.

These results indicate that a metal-enzyme complex constitutes the catalytic active enzyme.

A comparison of the metal-binding characteristics of the different forms of phosphodeoxyribomutase was desirable for the present study. For this purpose the kinetics of the removal of cobalt ions by EDTA from the different forms of the enzyme were studied. The half-lives of the cobalt–enzyme complexes at 25° in the presence of 1 mM EDTA are given in Table V. From the table it is seen that half-lives ranging from 2 to 28 min were found. The heterogeneity of form III (form III-1 and form III-2) was clearly revealed in these experiments. Thus in contrast to the experiments with forms I and II the removal of cobalt by EDTA in case of form III displayed a distinct biphasic curve. The two extrapolated half-lives are given in the table.

Discussion

The purification of phosphodeoxyribomutase from *E. coli* resulted in isolation of four separable forms of the enzyme. The main part of the enzyme activity was found to be present as form I and form II, and only about 10% of the activity was found in the forms III-1 and III-2.

From genetic evidence it was concluded that *E. coli* only contains one gene coding for an enzyme with phosphodeoxyribomutase activity. This conclusion was further supported by the finding that all four forms of the enzyme have the same amino acid composition (Table IV).

The molecular weight investigations showed that all forms of the enzyme have the same molecular weight, 45,000 ± 1000, and that they all consist of only one polypeptide chain. This last finding is in accordance with the occurrence of only one N-terminal amino acid (lysine) in forms I, II, and III-1.

These results leave open the following possibilities to explain the multiple forms of phosphodeoxyribomutase: (1) the enzyme may exist in different conformations; (2) the enzyme may be conjugated with different nonprotein groups; (3) the peptide chain may be differently modified, *e.g.*, by deamidation or proteolysis.

It has not been possible to demonstrate any significant difference in the physical parameters investigated: absorption coefficients, sedimentation coefficients and partial specific volumes. Therefore a major conformational difference between forms I and II is unlikely. Furthermore, studies of possible interconversions between the different forms did not give any positive evidence for (1) or (2).

The incubations with 0.35 M phosphate (pH 10.0) indicated that form II is formed from form I by deamidation. Tryptic fingerprints of form I and form II verified this hypothesis. The deamidation was shown also to occur when form I was stored in TS 6.9 at 4° (half-life 7 months).

With regard to proteolytic modifications of the enzyme only minor attacks from the C-terminal end would be in agreement with the molecular weight determinations, the amino acid analysis, and the N-terminal analysis. For Form III-2 a minor N-terminal digestion is not excluded by the present results.

Deamidation, limited proteolysis, as well as conjugation with nonprotein groups are all mechanisms known to accomplish modifications of proteins *in vivo* as well as *in vitro*.

After the demonstration by Flatmark and Sletten (1968) that cytochrome *c* is deamidated *in vivo* it was suggested by Robinson *et al.* (1970) that deamidation of proteins may play a central role as a molecular timer in living organisms.

Evidence has been presented that the deamidations occur nonenzymatically *in vivo*. The rate of deamidation is controlled by the sequence and may be also by certain metabolites (Robinson *et al.*, 1973a,b). Other reports on deamidation *in vivo* have appeared (Midelfort and Mehler, 1972).

In the case of phosphodeoxyribomutase multiple forms were revealed by DEAE-Sephadex chromatography of streptomycin treated crude extract 1 week after cell disruption. Much more form II was found than expected from the half-life of 7 months for form I in the Tris-succinate buffer. This indicates that form II is produced *in vivo*, unless a deamidating activity was present in the crude extract.

A biological function of the multiple forms of phosphodeoxyribomutase is not clear at all. No major differences in V_m or K_m for dRib-P and Rib-P_2 were found for the forms I, II, and III. The results indicated, however, that the multiple forms might show greater differences with respect to cobalt binding.

Multiple forms of enzymes represent distinct chemical molecules. Whether or not they occur *in vivo* they might display interesting features which could prove useful in the study of the structure and function of a particular protein. In the present case the differences in the binding of Co^{2+} might well give valuable information on the role of the metal ions in catalysis.

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Properties of the Stable Aerobic and Anaerobic Half-Reduced States of NADPH–Cytochrome *c* Reductase†

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ABSTRACT: The microsomal flavoprotein, NADPH-cytochrome *c* reductase, has been reexamined to determine: (1) the nature of the flavine bound to the enzyme and (2) the oxidation-reduction state of the "half-reduced" form of the flavoprotein. Iyanagi and Mason [Iyanagi, T., and Mason, H.S. (1973), *Biochemistry 12,* 2297] have recently proposed that NADPH-cytochrome *c* reductase contains both FAD and FMN as prosthetic groups in lieu of FAD as the sole constituent, as suggested by all previous studies of this enzyme. The data presented herein, utilizing the recently published fluorometric procedure of Faeder and Siegel [Faeder, E. J., and Siegel, L. M. (1973), *Anal. Biochem. 53,* 332] for the determination of FAD and FMN in mixtures, confirm the conclusions of Iyanagi and Mason for both rat and pig liver reductase preparations. Data for other flavoproteins are also presented. Iyanagi and Mason have also concluded that the air-stable "semiquinone" is a form of NADPH-cytochrome *c* reductase reduced by one electron per two flavines (F-FH). The present studies, however, do not agree with this conclusion, but instead support our previous results which indicate that *both* the aerobic and anaerobic half-reduced states of this flavoprotein exist in the two-electron reduced form (FH-FH). Removal of $NADP^+$ does not affect the spectrum of the air-stable half-reduced form of the flavoprotein, nor does it affect the back titration of this intermediate by potassium ferricyanide. The possible implications of these observations on the catalytic cycle of the flavines of NADPH-cytochrome *c* reductase are discussed.

The isolation of liver NADPH-cytochrome *c* reductase (EC 1.6.2.4) was first achieved by Horecker (1950) from pig liver acetone powder. The enzyme was subsequently identified as a microsomal constituent by Williams and Kamin (1962) and Phillips and Langdon (1962). Both groups purified the enzyme and studied its kinetic and physical properties. The studies of Horecker (1950), Williams and Kamin (1962), Phillips and Langdon (1962), Nishibayashi *et al.* (1963), and Omura and Takesue (1970) all indicated that FAD was the prosthetic group of NADPH-cytochrome *c* reductase. Masters *et al.* (1965b) suggested that the flavoprotein contained 2 mol of flavine with a minimal molecular weight of 35,000–40,000 g mol^{-1} while Masters and Ziegler (1971) reported that the homogeneous enzyme had two flavines per mol, and a molecular weight of 68,000 determined by sedimentation equilibrium. In the latter studies, however, only spectrophotometric determinations of flavine content were performed, since it was assumed on the basis of all of the previous data that FAD was the sole constituent of the flavoprotein.

Recently, Iyanagi and Mason (1973) reported that their preparations of NADPH-cytochrome *c* reductase from rat and pig liver microsomes contained both FAD and FMN in equimolar quantities, rather than FAD alone. We, therefore, reexamined our own available preparations of this enzyme, utilizing the recently described spectrofluorometric procedure of Faeder and Siegel (1973). The data to be presented show that our preparations do indeed contain both FAD and FMN in approximately equimolar quantities.

The studies of Masters *et al.* (1965a,b) and of Kamin *et al.* (1966) led to the proposal that the mechanism of catalysis by NADPH-cytochrome *c* reductase involved a redox cycle in which the two flavines acted cooperatively, cycling

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